Abiotic Stresses in Plants

Abiotic Stresses in Plants

Edited by

Luigi Sanità di Toppi
University of Parma,
Parma, Italy

and

Barbara Pawlik-Skowrońska
Polish Academy of Sciences,
Lublin, Poland

SPRINGER-SCIENCE+BUSINESS MEDIA, B.V.

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN 978-90-481-6434-9 ISBN 978-94-017-0255-3 (eBook)
DOI 10.1007/978-94-017-0255-3

Printed on acid-free paper

Originally published by Kluwer Academic Publishers in 2003
Softcover reprint of the hardcover 1st edition 2003

To Stefano & Lorenzo Sanità di Toppi

Contents

Preface

Much of Europe has been complaining recently of unseasonal weather - disastrous floods in Eastern Europe, temperatures reaching over 40°C in Central Europe, no decent rain for months in parts of the Balkans, coupled with unusually long and severe frosts in winter. Indeed, wheat yields in Serbia for 2003 are expected to be reduced by over 30% because of the combination of a long frost during winter with insufficient protective snow cover, very low rainfall in the spring months and sudden high temperatures reaching over 30°C at the time of flowering. So, with this background, it is very timely that this volume on Abiotic Stresses in Plants has been put together.

Each of the eight chapters focuses on a different aspect of abiotic stress, presenting reviews of recent advances in the subject. Rather than summarise the contents of each chapter, I'll focus on some of the advances in technologies presented here for elucidating the molecular, genetic and biochemical mechanisms that regulate plant responses to stresses and which also provide opportunities for improving plant performance under abiotic stresses. The last 20 years has seen a revolution in the availability of technologies for this, starting with the development of transformation technologies to study the role of an individual gene, then came molecular marker technologies to study the genetic control of stress responses, and in recent years the '-omics' (genomics, proteomics and metabolomics) have been developed to create an integrated picture of how the plant responds to a particular stress.

So, whereas it was a major achievement 15 to 20 years ago to identify and describe the expression of a single stress-induced gene, and even more of a challenge to sequence it, today the challenge is to decide which of the dozens of up-regulated and sequenced genes you find on your micro-array chip are the ones that should be studied in detail. Part of the answer to this question comes from the relatively new science of bioinformatics which provides tools to interrogate databases of sequence information to help assign a function to each stress-responsive gene. Another part of the answer comes from comparative genetics using information from model plant species whose DNA has now been completely sequenced (*Arabidopsis thaliana* for the dicots and *Oryza sativa* for the monocots).

These are tremendously valuable resources at the DNA level, allowing us to assign functions to genes relatively quickly. However, the challenge nowadays is more in identifying how we use this wealth of information at the level of DNA transcription and translation to improve plant responses under

stress conditions in the field. There was early excitement when an improved response to an abiotic stress was achieved by transforming tobacco with a stress-induced gene constitutively expressed in all tissues with the 35S promoter and torturing the transformants under artificial cabinet stress treatments. This has been replaced by a realisation that a plant's response to abiotic stresses is an extremely complex process and that over-expression of a single gene will rarely manifest itself as an improved phenotype in a crop plant growing in the field.

Another problem has arisen with attempts to extrapolate information collected from model crop species, particularly *Arabidopsis*, to crop species. As described by Maggio *et al.* in Chapter 3, *Arabidopsis* genes don't always have an obvious counterpart in crop plants. Add to this the diversity amongst species in molecular, biochemical, physiological, developmental and morphological mechanisms available for coping with a particular stress, and it becomes apparent that identifying the genes involved in a stress response is a long way from understanding how we can actually make plants cope with the stress better.

Nevertheless, help is at hand for this task in the form of QTL (quantitative trait locus) analysis. Over the last ten years or so, this marker-based technology has provided many new and powerful tools to associate the genotype with the phenotype. Thus, as demonstrated by Tuberosa *et al.* in Chapter 4, QTL analysis can provide a handle on the genes that are important in determining plant responses to a particular stress. The chromosomal location of more and more genes of known function is becoming available. Therefore, it is increasingly possible to link the phenotype for a particular trait with a specific gene by comparing sequence variation for candidate genes mapping to the same chromosomal location as a QTL for variation in the trait.

So, whereas the study of plant responses to abiotic stresses 20 years ago was largely the preserve of physiologists and biochemists, and then molecular biologists, today there is just as important a role for the geneticist, bioinformaticist and breeder in helping to elucidate the mechanisms of plant responses to abiotic stresses and manipulating these responses in crop plants more effectively.

Abiotic stresses are serious limitations to the continued expansion in food production needed to keep pace with the extra mouths to feed around the world. New technologies are providing improved understanding of the ways in which crop plants respond to these stresses. Application of the knowledge through breeding to create new varieties more productive under abiotic

stresses will help to keep pace with the growing demand for food. This book provides a valuable insight into how the area of plant adaptation to abiotic stresses has progressed through the application of the new technologies.

Steve A. Quarrie
University of Belgrade

Contributors

Editors

L. Sanità di Toppi
Department of Evolutionary and Functional Biology, Section of Plant Biology, University of Parma, Viale delle Scienze 11/A, 43100, Parma, Italy

B. Pawlik-Skowrońska
Centre for Ecological Research, Polish Academy of Sciences, Experimental Station, Niecała 18/3, Lublin, Poland

Chapter Contributors

A. Alpi
Department of Crop Plant Biology, University of Pisa, Via Mariscoglio 34, Pisa, Italy

M. Bracale
DBSF, Università of Insubria, via J.H. Dunant 3, 21100, Varese, Italy

R.A. Bressan
Center for Plant Environmental Stress Physiology, 1165 Horticulture Building, Purdue University, West Lafayette, IN 47907-1165, USA

C.S. Cobbett
Department of Genetics, University of Melbourne, Australia

I. Coraggio
IBBA, CNR, via Bassini 15, 20133, Milano, Italy

R.P. Ellis
Scottish Crop Research Institute, Dundee, UK

P. Gremigni
CSIRO Plant Industry, Centre for Mediterranean Agricultural Research, Wembley, Australia

S. Grillo
Institute of Plant Genetics (CNR-IGV), Research Division of Portici, Portici (NA), Italy

L. Helyes
Department of Botany and Plant Physiology, Faculty of Agricultural and Environmental Sciences, Szent Istvan University, 2103 Gödöllõ, Hungary

G.M. Lanini
CNR-IBIMET, Piazzale delle Cascine 18, 50100 Florence, Italy

A. Leone
Department of Pharmaceutical Sciences, University of Salerno, Fisciano (SA), Italy

G. Lorenzini
Dipartimento Giovanni Scaramuzzi, Università di Pisa, Via del Borghetto, 80 – 56124 Pisa, Italy

E. Loreti
Institute of Biology and Agricultural Biotechnology, CNR, via del Borghetto 80, 56124 Pisa, Italy

A. Maggio
Department of Agricultural Engineering and Agronomy, University of Naples Federico II, Via Università 100, Portici (NA), Italy

B. Maresca
Department of Pharmaceutical Sciences, University of Salerno, Fisciano (SA), Italy

Z. Nagy
Department of Botany and Plant Physiology, Faculty of Agricultural and Environmental Sciences, Szent Istvan University, 2103 Gödöllõ, Hungary

B. Pawlik-Skowrońska
Centre for Ecological Research, Polish Academy of Sciences, Experimental Station, Niecała 18/3, Lublin, Poland

P. Perata
Department of Agricultural Sciences, University of Modena and Reggio Emilia, Via Kennedy 17, 42100 Reggio Emilia, Italy

C. Perrotta
Department of Biological and Environmental Sciences and Technologies, University of Lecce, Lecce, Italy

M.N.V. Prasad
Department of Plant Sciences, School of Life Sciences, University of Hyderabad, India

A. Raschi
CNR-IBIMET, Piazzale delle Cascine 18, 50100 Florence, Italy

C. Ruggiero
Department of Agricultural Engineering and Agronomy, University of Naples Federico II, Via Università 100, Portici (NA), Italy

C. Saitanis
Laboratory of Ecology and Environmental Sciences, Agricultural University of Athens, Iera Odos 75 – Botanikos, 11855, Athens, Greece

L. Sanità di Toppi
Department of Evolutionary and Functional Biology, Section of Plant Biology, University of Parma, viale delle Scienze 11/A, 43100, Parma, Italy

Z. Tuba
Department of Botany and Plant Physiology, Faculty of Agricultural and Environmental Sciences, Szent Istvan University, 2103 Gödöllõ, Hungary

R. Tuberosa
Department of Agroenvironmental Science and Technology, University of Bologna, Italy

D. Vodnik
Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1001, Ljubljana, Slovenia

L. Xiong
Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132, USA

Language Reviser

Anila Scott-Monkhouse
University of Parma, Viale delle Scienze, 45/A, Parma, Italy

Acknowledgements

The assistance in the preparation of the book of Jacco Flipsen, Noeline Gibson, Riccardo Percudani and Zulema Vattuone is greatly acknowledged. A particular thank is due to Tommaso Ganino for his accurate and skilful help in the camera ready make-up of the chapters.

CHAPTER 1

PLANT TOLERANCE TO HEAT STRESS: CURRENT STRATEGIES AND NEW EMERGENT INSIGHTS

A. LEONE[a], C. PERROTTA[b] AND B. MARESCA[a]

[a] *Department of Pharmaceutical Sciences, University of Salerno, Fisciano (SA), Italy*
[b] *Department of Biological and Environmental Sciences and Technologies, University of Lecce, Lecce (Italy)*

Abstract. Temperatures above the optimal temperature range for plant growth and reproduction cause deleterious cellular damage, which in turn affects plant productivity. To relieve these effects, plants adapt to high temperature by activating a series of physiological and biochemical changes necessary to re-establish a new cellular homeostasis compatible with the increase in temperature. The genetic control of the heat shock (HS) response is quite complex and requires the activation of a network of genes, involved in the perception and transduction of the HS signal, which, in turn, trigger the up-regulation of other target genes. The induced genes code for proteins and enzymes (HS proteins, active oxygen detoxifying enzymes), playing a direct role in the protection of cellular and subcellular organelles or genes encoding enzymes involved in the biosynthesis of protective compatible compounds (sugars, polyols, betaines). Membrane lipid instauration, controlled by desaturase genes, is also a critical component of thermotolerance. This chapter covers the principal aspects of the plant HS response and the role of different class of genes in the acquisition of thermotolerance. The molecular breeding strategies currently available to alter genetically the level of the protective proteins, enzymes and molecules, that may ameliorate plant tolerance and productivity under high temperature stress, will be also discussed.

1. INTRODUCTION

High temperature is an important process affecting plant growth and development even for relatively short lengths of time, such as temperature changes during the day. About 23% of the earth' land has an annual mean air temperature above 40°C, which means that, under strong irradiance, leaf temperatures may reach values around over 50°C. This situation is worsened by the reported increase in temperature, caused by the excess of carbon dioxide and other gas emission [1].

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 1-22.

Although the impact of this last phenomenon on the world food production has not been accurately estimated, improvement of heat tolerance remains one of the primary goals of the current breeding programs of many economically important crops.

Plants have an optimal temperature range for growth and reproduction. Since plants, unlike homeothermic animals, are incapable of maintaining a temperature optimal for their growth, a slight increase in temperature, even transiently, may affect physiological and biochemical processes crucial for plant growth. In general, plants are able to withstand temperatures 5-10°C above the optimal temperature without being stressed. At temperature 12-15°C higher than this optimal range, plants suffer from heat stress. A sudden increase in temperatures of 15°C or more above the optimal temperature range may affect seriously the plant growth and development, depending upon duration of the heat stress. Lethality results from a combination of cellular changes that the heat induces and the inability to restore normal cellular function afterwards. HS events provoke irreversible damage during both vegetative and reproductive stages in many crop plants, causing low photosynthetic activity, poor floral development, pollen sterility, which affect seed and fruit set and quality [2]. High temperature impairs many physiological activities associated with seedling growth and vigour, root growth, nutrient uptake, water relations of cells, solute transport, photosynthesis, respiration, general metabolisms, fertilization and maturation of fruits. Prominent HS-induced ultrastructural changes in plants have been reported for the nucleus, endoplasmic reticulum, mitochondria and plastids. The rate of photosynthesis in most species declines at about 35°C which is ascribed to protein denaturation, loss of membrane integrity, photoinhibition and ion imbalance. High temperature affects chloroplast biogenesis and senescence, causes disintegration of chloroplast grana, brings about disruption of the structure of membrane proteins, influences protein-lipid interactions, affects electron transport activity and substantially decreases the activity of Rubisco enzyme [3,4].

To alleviate these effects, plants adapt to high temperature by activating a series of physiological and biochemical changes necessary to re-establish a new cellular homeostasis compatible with the increase in temperature. The adaptive response of plants to high temperature stress is a typical polygenic trait, controlled by a network of genes, from what it is possible to envisage the difficulty in improving plant heat tolerance, by both conventional and innovative breeding approaches. The main question to be addressed is to establish which gene(s) would be fundamental for genetic improvement of crops against high temperature stress.

Without entering into the details of the complex metabolic changes that plants adopt to cope with high temperature stress, ultimately associated to thermotolerance (covered exhaustively by excellent reviews, as reported in the web site www.plantstress.com), we will focus on plant genes and gene products, proved thus far to play a role in plant thermotolerance. The genetic control of adaptive mechanisms to temperature stress, such as smaller leaf size, enabling more conventional cooling, or reduced inclination and increased reflectance, which reduce energy load, or other morphological traits that might improve thermotolerance

through indirect effects (presence of wax, trichomes etc), will not be covered in this chapter.

2. SPECIFICITY AND CROSS-TALK OF PLANT STRESS RESPONSE

As a consequence of their sessile and poikilothermic nature, plants are exposed to a daily multi-stress challenge, which explain why a multiplicity of partially overlapping stress response system has evolved in plants. The integrated and highly flexible stress network is characterized by a number of multivalent or even general stress metabolites and proteins. The genes for these proteins and compounds are indeed triggered by high temperature, but also by several other environmental stressors. This explains also why exposure of plants to a previous sub-lethal stress is able to cross-protect plants towards several other environmental stresses.

It is well known that plants, as other organisms, are able to sense variation in the external surrounding environment and change gene expression accordingly to cope with the stress conditions. Based on current information, models of signalling external stimuli should incorporate a membrane receptor/sensor and a series of other proteins and molecules with the role of amplifying intra-cellularly the signal and to activate genes, which may contribute to the adaptation of the plant to stress. Although there are few recent papers describing attempts to identify plant receptors able to monitor in a specific fashion changes in the environment, it is, however, evident that different stresses share entirely or partially signal transduction pathways, such as the well known MAP-kinase cascade, highly conserved in all organisms [5,6].

The HS transduction cascade hits down-stream genes, that fall in the following categories: i) genes encoding HS proteins (HSPs); ii) genes improving membrane stability under stress conditions through changes in lipid composition; iii) genes encoding enzymes necessary to preserve cellular and subcellular organelle structure, such as those encoding antioxidant enzymes of the ROS pathway, or genes involved in the synthesis of protective molecules, such betaines and polyols.

The role and function of this general stress pathway is unquestionably fundamental for the plant cell survival and recovery from heat stress, contributing to the overall plant heat tolerance. Nevertheless, in plants there are specific metabolic functions, such as the complex photosynthetic machinery, that need to be protected by heat stress damages. Photosystem II (PSII) is well known to be sensitive to high temperature, and it is often cited as the most heat-sensitive component of photosynthesis [7,8]. It follows that some stabilizing and/or repair mechanisms have to be specific for protecting PSII and preserving its functionality under not optimal temperature conditions.

3. SENSORS FOR TEMPERATURE STRESS

As reported below, the molecular effects of temperature shocks on living systems, including plants, have been explored, so far, mainly for the roles that HSPs have in the cell and for their mode of transcriptional regulation. Among others, HSPs have been found to function *in vivo* as chaperones, while heat-shock effects leading either to cell death or to repair/recovery have been essentially overlooked.

One of the models, proposed to reveal the transcriptional regulation of HS genes, suggests that accumulation of denatured proteins, occurring under heat stress, induces activation of the stress genes [9,10]. However, this model does not take into proper consideration the fact that all plants and warm-acclimated animals, that constitute the vast majority of all living species on Earth, do not induce HSPs when their physiological temperature increases during seasonal acclimation. In addition, it is well known that HSPs are present in abnormal levels in a variety of human degenerative diseases, in spite of the unchanged accumulation of denatured proteins during HS. Furthermore, during the aging process, when denatured proteins accumulate at higher level, there is no evidence of increase in accumulation of HSPs, rather a decrease in the HS response. Thus, protein denaturation may represent one but not the exclusive mechanism to accumulate HSPs.

In the last few years, several laboratories, based on the studies of temperature acclimation of plants, cold blooded animals as well as mammals and microorganisms, have focused their attention on the decisive role of membranes as primary targets of HS and have proposed a new model that associates the interactions of lipid/protein membrane to the transcriptional regulation of HS genes. Such molecular associations have been shown to be involved critically in the conversion of physical and chemical factors with sequential processes occurring inside the cell, from the membrane to the nucleus, culminating in transcriptional activation of stress genes [11,12].

In addition, certain stress proteins have been shown to interact with specific membranes domains remodelling the pre-existing membrane physical order [13]. It has been proposed that the specificity of HS or cold shock gene expression is obtained by the irregular distribution of membrane lipid/protein domains that can recognize precisely biological and environmental signals such as different forms of stresses. This model is based on the hypothesis that lipid composition and the pre-existing physical state of membranes are crucial components in the processes of perception and transduction of temperature shock into an appropriate biological signal that elicit the transcriptional activation of HS genes. The membrane composition and physical state existing prior to temperature shock, and that is determined by the environmental temperature (e.g. specific phospholipid classes) is responsible of the damage induced to the membranes by heat or cold stresses. Further, a transient association of specific HSPs with membranes re-establishes the lateral packing order (membrane fluidity), bilayer stability and membrane

permeability, thus restoring membrane functionality during and after heat stress [14, 15]. Therefore, some HSPs assist membranes during the recovery process that follows a rapid temperature shock re-establishing the physical state of membrane present prior to the stress state. This association, in turn, determines inactivation of the external signal that perturbed membrane, thus switching HS gene synthesis off in a feedback loop.

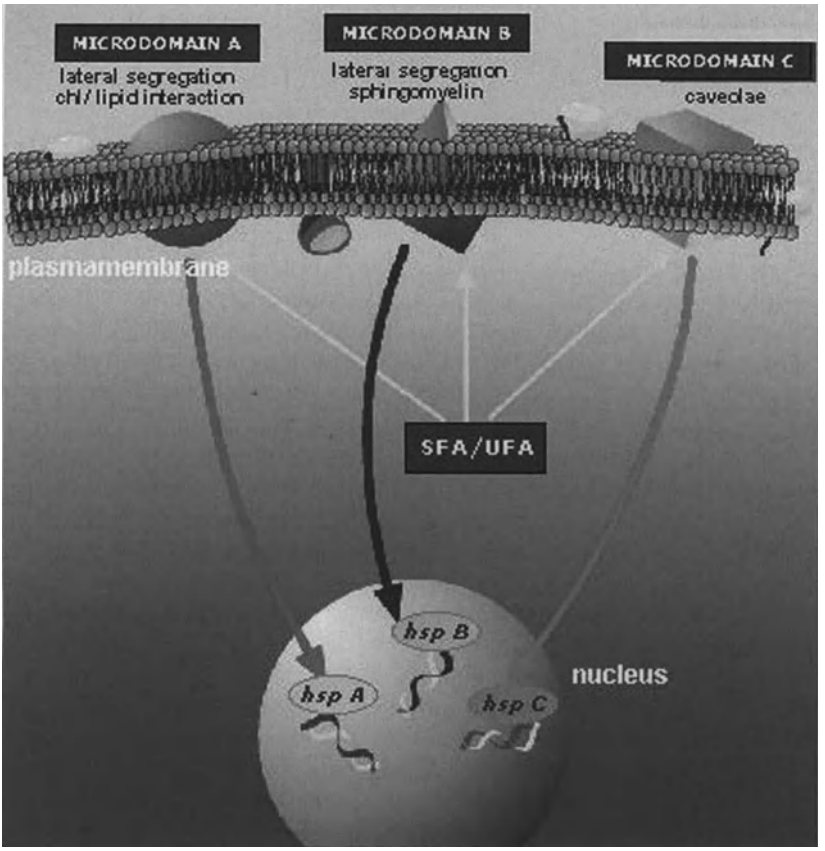

Figure 1. A model for changes in membrane lipid composition, physical state, lipid/protein ratio, and associated induction of hsps. Chl, cholesterol; SFA/UFA, saturated fatty acids/unsaturated fatty acids.

A decrease in the extent of instauration of fatty acids in the plasma membrane of the cyanobacterium *Synechocystis* PCC 6803, obtained by catalytic lipid hydrogenation under isothermal conditions *in vivo* [16,17] induces the expression of an acyl-lipid desaturase, that is otherwise inducible only by low-temperature shifts

[11]. Further, genetic modification of lipid instauration and membrane fluid state in the yeast *Saccharomyces cerevisiae* resets the optimal temperature of HS response [12]. Therefore, changes of membrane fluid state plays a primary role in the perception of temperature change causing transcriptional induction of desaturases and a reset of the optimal temperature of HS gene transcription. Murata and his co-workers showed that cold shock induces an abrupt change in membrane fluidity in *Synechocystis* and, simultaneously, a signal is transduced from the membrane to the chromosomes. These authors identified by selective gene knockouts two membrane histidine kinases together with a response regulator as key components of the signal cascade in cold shock conditions [18].

It has been shown that the physical state of the thylakoid membrane of *Synechocystis* [19] and those of *Salmonella typhimurium* and *Mycobacterium marinum* [20] modify the temperature threshold expression of HS genes. Furthermore, the physical order of thylakoids - or of cytoplasmic membrane - is reduced in response to either a downshift of the growth temperature or administration of benzyl alcohol (BA, a membrane fluidising agent) that was paralleled, in both models, by an enhanced thermosensitivity of the photosynthetic and cytoplasmic membranes [19, 21]. Therefore, under physiological temperatures, membrane fluidity, regulated by the environmental temperature, determines the temperature threshold at which HS and cold inducible genes are transcribed. Genetic manipulation of the UFA/SFA ratio, obtained by over-expression of an exogenously added Δ^9-desaturase gene or treatment with BA, had, in *S. cerevisiae,* a significant change of the expression of the *Hsp70* and *Hsp82* genes [12].

In general, the higher is the growth temperature, the lower is the ratio of UFA/SFA. This capacity to synthesize different classes of phospholipids allows cell membranes to have an almost identical fluidity at any given growth temperature and it has been termed homeoviscous adaptation [22]. Such an adjustment of membrane fluidity to variable environmental temperatures is a general phenomenon in plants and poikilothermic organism. Abrupt changes in temperature cause an almost immediate, non-equilibrium state in the lipid order within the membrane. Virtually all membranes contain considerable amounts of lipids that do not spontaneously form bilayers but, rather, have a strong preference to form non-lamellar structures, most commonly the inverted hexagonal phase, H_{II} [23]. Presence of non-bilayer lipid phase has been demonstrated in heat-stressed pea thylakoids membranes [24]. On the other hand, formation of transient and local non-lamellar structures paradoxically seems to be critically important to several processes, such as membrane fusion, cell division, activation of membrane enzymes, and the *trans*-bilayer movement of lipids and proteins [25].

Cells tend to reorganize membranes immediately after an abrupt and temporary change of temperature (or exposure to membrane perturbing agents) so that the physical properties compensate for the new temperature conditions. During a rapid heat stress, there is no sufficient time for either remodelling the lipid head-groups or a significant elevation of the level of more saturated lipid species. Only desaturase enzymes are present in the cells that act in response to cold, while saturases, which might, in theory, be required during heat stress, have not been described. Thus, to

stabilize the membrane during heat (or possibly) cold stress, a specific subset of HSPs associates transiently with the membranes. This macromolecular association represents a powerful *escamotage* with which cells can cope with rapidly fluctuating temperature conditions (or particular forms of stress) to achieve a temporary restructuring of the membrane physical state with the consequent preservation of membrane architecture and functionality. This form of molecular protection is particularly important in plant cells, in which chloroplasts and photosynthetic membranes are particularly sensitive to temperature shocks.

Several authors have suggested that during cold exposure membrane rigidify and that such rigidification can be mimicked by addition of DMSO or by membrane lipid hydrogenation or can be prevented by treatment with BA [11, 26]. In addition, a treatment with membrane fluidifiers blocks all tested responses to cold, implying that membrane rigidification is a necessary condition for cold signaling in plants [27]. Further, the rigidification of membrane at isothermal conditions induces in alfalfa cells [27] and in *Brassica napus* [28] the expression of cold inducible genes.

It has also been suggested [29] that the cold-induced membrane rigidification may be coupled to the opening of mechanosensitive Ca^{2+} channels. Using alfalfa cells, these authors have shown that rearrangements of cytoskeleton may mediate the transduction of the cold signal from the rigidified membrane to the Ca^{2+} channels [27]. These authors have also shown that cold triggered Ca^{2+} influx is inhibited by membrane rigidification. Thus, changes of the cytoskeleton probably transduce physical stresses into appropriate biochemical signals [30]. Cytoskeleton components are attached to the plasma membrane and ion channels [31] and destabilization of microfilaments and microtubules causes Ca^{2+} influx in plant cells [32], whereas a stabilization of microfilaments inhibits gene expression and freezing tolerance in alfalfa cells [27].

Furthermore, it has been reported that a HS-activated MAPK (HAMP), immunologically related to ERK (Extracellular signal-Regulated Kinase) is activated by high temperature through membrane fluidisation [33].

4. SMALL HEAT SHOCK PROTEINS: SOMETHING PECULIAR TO PLANTS

A sudden elevation in temperature triggers a stress response found in all organisms that brings about a global transition in gene expression. Typically, the expression of most genes is shut down or greatly attenuated, while a specific group of genes, called HS genes, is rapidly induced to high level [34]. Protein encoded by HS genes enable cells to survive to harmful effects of heat by preventing irreversible protein damage and helping cellular recovery after stress. The HS response is transient in nature, usually peaking 1 to 2 hr after onset, providing protection from acute episodes of thermal stress.

HSPs represent a remarkable example of ancient and highly conserved proteins: they are present in every species and the level of amino-acid identity between prokaryotic and eukaryotic proteins can reach 50% [9]. These proteins belong to

highly conserved protein families classified on the basis of their sequence homology and typical molecular weight: HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP10 and small HSPs (smHSPs) families [35]. In eukaryotes many families comprise multiple members differing in function, inducibility and cellular localization. The many diverse functions of the major HSPs belonging to these classes have been extensively investigated revealing that HSPs not only have been highly conserved at the amino-acid sequence level but they also appear to have quite similar functions. Some members of the different HSP families are constitutive, while others are specifically induced by stress. This highlights the double role of these proteins in both normal cellular physiology, and in cell protection against stress conditions. In fact, although they were first discovered as proteins induced by stress, in the last few decades their important role during normal cell-cycle has been assessed.

Several exhaustive reviews are available about the structure and functions of HSPs in both prokaryotes and eukaryotes with particular interest in their chaperone function and in their biochemistry [9, 34, 36].

In this chapter, we will focus on the general properties and functions of the most representative class of inducible plant HSPs. Most major classes of HSPs are present in plants and include HSP60, HSP70 (and a cognate protein HSC70), HSP90 and HSP100 [34]. However, the robust synthesis of the numerous small HSPs (smHPSs) in the HS response differentiates plants from other eukaryotes, such as *Drosophila* or humans, in which expression of HSP70 dominates the response. Moreover, the smHSPs, besides to be peculiar of plant HS response, appear to play a particularly important role in plant response to HS.

The smHSP family comprises a diverse group of proteins ranging from 12 to 42 kDa in monomer size [37], found in bacteria, archaea, and eukaryotes. Peculiar to these proteins is the presence of a so called "α–crystallin" domain, about 100 aminoacids long, in the carboxy-terminal region, in common with the α-crystallin proteins, first identified by Ingolia and Craig [38] and confirmed by several subsequent studies [39, 40, 41]. This domain is preceded by an N-terminal region of variable size and sequence and is followed by a non-conserved C-terminal sequence.

In prokaryotes the smHSPs are cytosolic proteins and some homologues have become structural components of the spore coat as in *Bacillus subtilis* or associate with membranes. The search for *HSP* sequences in 15 bacteria and 4 archaea has led to the identification of smHSPs in 7 bacterial genomes studied; no HSP sequences were found in *Chlamydia pneumoniae, Haemophilus influenzae, Helicobacter, etc.* In other bacterial genomes the number varies from one to three (two in *E. coli* and *M. tuberculosis*). Of the four archaea, three have only one smHSP gene while the fourth has two. The size of the proteins coded by all these smHSP genes varies from 13.6 to 20.4 kDa [42].

In eukaryotes smHSP genes have been reported in yeasts, fungi, *Drosophila*, vertebrates and plants. Apart from plants, other eukaryotes have a low number of smHSPs ranging from only one in mammals (HSP26), to two in *Saccharomyces cerevisiae* (HSP26 and HSP43), to four in *Drosophila.* No evidence of organelle-localized smHSPs was seen in any of these organisms.

In contrast with most other eukaryotes, plants are characterized by an extreme diversification of smHSPs; in fact they have at least 20 different smHSPs, and in some species as many as 40 smHSPs have been detected [34]. This is probably due to the need for plants to have a peculiar kind of adaptation to stress because they are sessile organisms. Plant smHSPs are encoded by a large multigene family, producing proteins that are targeted to different cellular compartments classified into 5 classes according to their cellular localization in cytosolic (class I and II), chloroplastic, mitochondrial and smHSPs associated with the endoplasmic reticulum [34, 43]. A sixth class of smHSP was reported in *Glycine max*, GmHSP23, characterized by the presence of a signal pepetide at the N-end, that is probably localized in a membrane compartment. Sequence similarities are higher between smHSPs from different species but belonging to the same class, than between smHSPs from the same organism but of different classes. In general sequence identity between smHSPs is quite low. This is true not only for comparisons between smHSPs from different species but also for comparison between different smHSP classes. The similarity among class I cytoplasmic smHSPs including pea HSP18.1, soybean HSP17.3 and HSP17.5, barley HSP17, sunflower HSP17.9 and other, ranges from 80.1 to 92.9% (identity 68.2-85.1%), interestingly highest identity is between pea and soybean since they belong to the same taxonomic family [34, 44]. Comparison of genes of the same species, belonging to different classes, exhibit lower similarity values. For example pea HSP18.1 and pea HSP17.7 share only 59.7% similarity, with identity lower than 50%; the similarity value decreases when nucleic acid sequences are considered. These data are confirmed for the class II and chloroplast-localized smHSPs. The only region exhibiting a high level of identity is limited to the "HS domain" at the C-terminus that spans about 100 aminoacids. This region can be divided into two sub-domains separated by a hydrophilic region of variable length. Although the two sub-domains share similar hydropathy profiles and secondary structure, their importance in determining smHSP function has not been established. On the contrary the N-terminal domains of smHSPs are quite divergent; for instance in organelle-localized smHSPs typical targeting peptides are present at the N-ter, while the other smHSPs exhibit a consensus domain typical for each class [45]. The presence of highly conserved N-ter domains, specific for the different classes of smHSPs indicates a very important role for these consensus sequences and for their involvement in determining the specific function of smHSPs. Several smHSP genes from other eukaryotes contain introns including human *HSP27* [46] and two smHSP genes from *Caenorhabditis elegans*. Usually plant smHSP genes do not contain introns, with few exceptions, such as soybean *HSP27* gene [47] or the chloroplast-localised *HSP21* from *Arabidopsis,* for which a more efficient splicing of intron during high temperature stress is present [48], as it has been reported for other plant HSPs [47, 49]. This accounts for the evolution in plants of molecular mechanisms aimed at a more efficient response to heat stress.

Another structural characteristic unique to HS response is the presence of a poly (A) tail of variable length in HSP transcripts. As shown for the *Ath21* transcript, the poly (A) tail is longer during an abrupt stress than during gradual stress. This

phenomenon was observed also for other plant HSP genes and is considered to be related to thermotolerance [48]. The same increase in poly(A) tail length in response to abrupt stress was reported also for *Drosophila* smHSP transcripts [50].

After separation by non-denaturing gel electrophoresis smHSPs are found as high molecular weight aggregates ranging in size from 150 to 800 kDa composed of oligomers of 9-32 subunits that sometimes appear also as a dynamic quaternary structure that varies continuously [37, 51, 52, 53, 54]. In same cases however, they form only dimers or tetramers [42]. The aggregates appear to be formed of homo-oligomers in human HSP27, avian HSP25 and murine HSP25. As determined by native protein electrophoresis, plants class I cytoplasmic, chloroplast and mitochondrial smHSPs are also found in homo-oligomers of 200-300 kDa. The presence of homo-oligomers has been studied in detail for class I and class II cytoplasmic smHSPs because they both accumulate in the cytoplasm; *in vivo* and *in vitro* data indicate that they do not form hetero-oligomers [41]. Furthermore dissociation of recombinant class I and class II oligomers by urea or guanidine and reassembling on dialysis, results in homo-oligomers formation, even when the two different proteins are mixed together [55].

The crystal structure has been determined only for HSP16.5 from *Methanococcus jannaschii* [37]. The crystal is composed of 24 subunits arranged in octahedral symmetry and the general structure is a hollow sphere that has an outer diameter of 120A and an inner diameter of 65A. Subsequent studies by cryo-electron microscopy have indicated that the quaternary structure is variable due to dynamic subunit exchange. These data have been reported not only for the *M. jannaschii* HSP16.5 but also for human αB-crystallin, human HSP27, bovine α-crystallin, and unfolded α–lactalbumin. All of these smHSP complexes appear to have some degree of structural variability in solution indicating a plasticity in their quaternary structure. An explanation for this phenomenon is the chaperone function of smHSPs related to their need to recognize and bind proteins that are diverse and characterized by conformation flexibility [54].

Mammalian smHSPs are phosphorylated through a MAP kinase cascade, phosphorylation results in oligomer size reduction accompanied by change in functions [56, 57, 58, 59]. The significance of phosphorylation is not clear, some reports indicate that it is necessary for smHSPs involvement in thermotolerance, others indicate that it is not required [56, 58, 60, 61]. Phosphorylation represents a further difference between plant and animal smHSPs, it has been reported in fact that smHSP phosphorylation does not occur in tomato cells [62].

A characteristic of heat stress response is the formation of very large molecular aggregates, although their structure and significance has not yet been completely elucidated. In fact, all smHSPs including the organelle-localised forms, under certain especially severe stress conditions, form insoluble structures named "HS granules" that reach very high molecular weight (greater than 1 MDa). These are probably aggregates of smHSPs and their substrates, the latter being denatured proteins and/or untranslated mRNAs [36]. Formation of these large structures is reversible.

Although a small number of smHSPs are specifically expressed during different phases of the cell cycle or stages of growth and development, the majority of small HSPs are induced by heat stress, demonstrating their crucial role in the HS response. This is supported by findings related to their accumulation during the heat stress response. In fact smHSPs accumulation during heat stress is proportional to temperature, furthermore this response is very rapid and related also to stress duration. Many data suggest that the maximum synthesis of smHSPs is induced by temperatures just below lethal temperatures. The most abundant smHSPs induced are class I that can amount to over 1% of total leaf or root cell protein [63, 64]. Other smHSPs, such as the chloroplast-localised ones, account for only 0.02% of total leaf protein [65]. In prokaryotes smHSPs expression can reach very high levels, 22% of total proteins, as reported for HSP16.4 of *Streptococcus thermophilus*. This protein is plasmid-encoded, like other small prokaryotic HSP, explaining therefore its high level of expression [42].

The evolutionary mechanisms that gave rise to smHSPs is unknown, but it is clear that these proteins are considerably more divergent than other HSP groups such as HSP60 and HSP70.

In prokaryotes, the diversity of smHSPs is greater than in plants or animals [42], which arose as monophyletic groups from different ancestral prokaryotic smHSPs. From evolutionary studies the different classes of smHSPs appear to have originated by gene duplications before the divergence of the major angiosperm groups more than 150 million years ago [41]. However the presence of a cytosolic class I gene in a gymnosperm indicates that these families are probably older, but no information is available on earlier plant groups that might help in defining when duplications occurred. The only algal smHSP known has been identified in *Chlamydomonas* (HSP22), but this protein does not group with the angiosperm smHSP classes identified by Waters and co-workers [41] Comparison between plant and other eukaryotes smHSPs reveals that plant and yeast smHSPs share a greater similarity with respect to human ones.

5. FUNCTIONS OF HSPs AND GENETIC EVIDENCE OF THEIR INVOLVEMENT IN PLANT THERMOTOLERANCE.

While the function of the several other classes of HSPs has been well investigated, the function of smHSPs has not been so well studied. Genetic analysis of smHSPs function has yielded different results in mammals and yeast. In hamster and mouse cells, data were obtained on their involvement in determining thermotolerance. Conversely, in yeast, overexpression of HSP26 provides only very slight increase in thermotolerance [9]. In mammals, data obtained show that enhanced expression of smHSPs is associated with increased thermotolerance related to the stabilization of actin and cytoskeleton [61, 66]. *In vitro* experiments demonstrated that both smHSP and α-crystallin selectively recognize and stabilize non-native proteins acting as molecular chaperones. This is the second cellular role established for smHSPs in both prokaryotes and eukaryotes. Molecular chaperones are proteins capable of

assisting in stabilization of native protein conformations, protein folding, formation and stabilization of oligomers, protein translocation and protection of proteins from denaturation by heat and other stresses.

The molecular mechanism is not yet well established, but there is strong evidence that smHSPs act in conjunction with other chaperones. In particular, multichaperone network with HSP60 and HSP70 has been well established not only in *E. coli* but also in other prokaryotes [42].

Chaperone activity has been established *in vitro* for mammalian smHSPs, as well as for α-crystallin. They assist in preventing thermal aggregation of other proteins, recognizing and stabilizing a variety of non-native proteins. Although the specific mechanism of action is unknown, the data available from prokaryotes multi-chaperone complexes allow the hypothesis of hydrophobic interactions between non-native substrates and smHSPs [67]. In plants, *in vitro* chaperone activity for smHSPs has been reported. Lee and co-workers [53] demonstrated that recombinant pea HSP18.1 can act as an ATP-independent chaperone, like it has been reported for mammalian smHSPs [68]. The same group reported in subsequent studies that HSP18.1 *in vitro* can selectively and stably bind to non-native proteins, to form high molecular weight complexes. Denatured substrates form large aggregate, coating the HSP18.1 dodecamers. Usually upon lowering the temperature, bound substrates do not dissociate, however it has been shown that sometimes, in conjunction with ATP-dependent molecular chaperones, bound substrates can be refolded [67]. Chaperone activity for organelle-localized smHSPs has not yet been demonstrated.

The *in vitro* chaperone activity of both class I and class II smHSPs has also been confirmed *in vivo* by studies performed on transformed *Arabidopsis* cells [69, 70]. The chaperone function of smHSPs appears to be specifically related to stress and developmental stages and is not required for normal cellular functions.

A chaperone-like function has been well established for most prokaryotic smHSPs. The smHSPs associated with membranes such as *Mycobacterium tuberculosis* HSP16.3 stabilize and rigidify membranes under HS. A link between smHSPs and thermotolerance was established for HSP17 of *Synechocystis*. In particular Török et al [13] demonstrated that the interaction between HSP and membranes can protect the latter from thermal damage by increasing their stability. The mechanism involved seems to be a modulation of membrane fluidity and permeability, thus preserving its structure and function.

Several genetic approaches have proved the role of HSPs in plant heat tolerance, including identification and physiological analysis of plant mutants that fail to adapt to high temperature or through the genetic "gain and loss" approach of specific HS genes. Massive screening of EMS mutagenized M_2 seeds of *Arabidospis thaliana* have defined four separate genetic loci, *hot1-4*, required for thermotolerance [71]. These are the first mutants defective in thermotolerance that have been isolated in any higher plant. *hot1* was found to have a mutation in the *Hsp101* gene, which can be complemented by the wild type gene. The thermosensitivity of *hot1* mutant provide a direct involvement of HSP101 protein in acquired plant thermotolerance, as found in bacteria and yeast. Another *Arabidopsis* mutant lacking the ability of acquiring thermotolerance was found, with the

mutation affecting mainly the level of a 27 kD HSP [72]. A more direct evidence of the implication of HSPs in heat tolerance comes from suppression or over-expression of specific HSP genes. Expression of a rice *Hsp16.9* gene in *Escherichia coli* resulted in increased bacterial thermotolerance [73], as reported also for the chestnut gene *Hsp17.5* [74]. Analysis of thermotolerance of transgenic cells and regenerated plants in which the carrot Hsp17.7 was silenced or over-expressed provided evidence that this gene is able to both increase and decrease thermotolerance [75]. Additional evidence of the crucial role of HSP101 in plant thermotolerance have been reported by analysing transgenic plants expressing less than usual amounts of the HSP101 protein, a result of either antisense or cosuppression [76]. These under-expressing HSP101 plants had a severely diminished capacity to acquire heat tolerance after mild conditioning pre-treatment. Conversely, plants over-expressing HSP101 tolerated sudden shifts to extreme temperatures better than wild type plants. The fact that over- and under-expression of HSP101 does not affect normal plant growth and development makes this protein an especially attractive target for engineered expression.

Some of the induced smHPSs are targeted to chloroplasts, suggesting a specific function in protecting the photosynthetic machinery. There are several data proving the correlation between the production of the chloroplast smHSPs and PSII thermotolerance [77, 78, 79]. Functional disruption of the small, methionine-rich chloroplast HSP, using anti-hsp antibodies, have clearly demonstrated that this hsp protects the thermolabile PSII and accounts completely for heat acclimation of electron transport in pre-heat-stressed plants [79].

Finally, over-expression of certain transcriptional regulator of HSP expression, HSF1 and HSF3, causes plants to constitutively express at least some HSPs and produces somewhat higher basal thermotolerance, but not increases acquired thermotolerance [80, 81].

6. CHANGES IN MEMBRANE LIPID COMPOSITION ARE CRITICAL FOR PLANT THERMOTOLERANCE

Beside their crucial role as temperature sensors, changes in membrane lipid composition are crucial for plant thermotolerance [82]. Variation in fatty acid saturation is *per se* one of the best characterized mechanisms of acclimation of higher plants to temperature stress [83]. Cis-unsaturated double bonds affect profoundly the T_m of fatty acids, which in turn results in a considerable modulation of the temperature at which the membrane gel to liquid-crystalline phase transition occurs. Though the plant lipids are quite complex, as a rough indication, C18:0 has phase separation at temperatures around 70°C, while one centrally positioned cis-double bond in C18:1 effectively decreases the phase transition temperature to approximately -5°C [24]. It is believed that such changes in the fatty acid instauration level are required to preserve the membrane particular physical state so that its function and stability is preserved under changing temperature conditions. This adaptive phenomenon has been widely described at physiological and

biochemical levels in many prokaryotic and eukaryotic organisms [84]. In plants, the role of membrane saturated fatty acids in thermotolerance have been clearly elucidated by analyzing the response to high temperature of plants with an altered membrane composition, achieved by different approaches. It has been demonstrated that increasing fatty acid saturation of isolated chloroplast stabilizes the PSI complex of pea chloroplasts at high temperature [85]. Chloroplast membrane of *fadB* and *fadC Arabidopsis* mutants, with reduced level of fatty acid poly-instauration, are more thermally stable compared to the wild type chloroplast membranes [86, 87]. As expected, the same mutants are more sensitive to low temperature, supporting the general model of regulation of membrane lipid composition in the response to upward and downward temperature shift.

At molecular level, a still open question is whether or not the variation in the level of fatty acid saturation/instauration upon temperature shift is controlled through regulation of transcription of desaturase genes. There are some evidences for transcriptional regulation of desaturase genes in response to cold in low organisms [88] and in plants [89, 90, 91], though not found in all plant species [92, 93]. As far as high temperature, the most striking evidence of the involvement of regulatory mechanisms of desaturase genes has come from the recent results on down-regulation of *fad7* gene, encoding a ω–3 fatty acid desaturase enzyme, in tobacco plants [94]. Silencing by cosuppression of this chloroplast gene in tobacco plants caused a lower level of trienoic acid than in wild type plants, with a remarkable increase in thermotolerance. Differences in growth rate were noted at 36°C, and transgenic plants survived for 2h at 47°C, a treatment lethal for the wild type plants. This work proved that thermotolerance is a function of the lipid profile of photosynthetic membranes.

7. ACCUMULATION OF COMPATIBLE COMPOUNDS IS ALSO IMPORTANT FOR PLANT HEAT THERMOTOLERANCE

One of the complex changes in the plant caused by heat stress, as well as by other stressors, is the accumulation of low-molecular compounds (glycine betaine, sugars, polyols, amino acids), which is important for maintaining vital cellular function. Though most of the beneficial effects of betaine accumulation have been reported for cold and drought stressed plants [95, 96], evidence has been obtained *in vitro* that glycine betaine enhance also tolerance to high temperature. For example, Paleg et al [97] found that betaine protects some enzymes against heat-induced inactivation. Betaine is particularly effective in protecting highly complex proteins, such as the oxygen-evolving PSII complex proteins, in the photosynthetic machinery, against heat-induced inactivation [98, 99]. Therefore, it has been postulated that the accumulation of betaine *in vivo* is related to the ability of plants to tolerate high temperatures. A major role of betaines might be to protect membranes and macromolecules from the damaging effects of the stress. Concentration of betaine at certain cellular level might provide substantial protection even at very level of accumulation, as found in leaves of halophyte plants [100]

sites, supporting the notion of their primary role as protective molecules rather than controlling osmotic adjustment. Although no direct evidence for such a relationship has been reported, Murata and co-workers [101] have transferred genes for choline oxidase from *Arthrobacter globiformis* in *A. thaliana* plants. This enzyme converts choline into betaine in one-step reaction. Plants over-expressing this enzyme do indeed over-produce this compound. Physiological tests showed that the transgenic plants developed a significantly higher stress tolerance during germination and growth than control plants. Transgenic plants also synthesized lower level of HSP70. Altogether, these results prove that betaine might have protected intracellular proteins from high temperature-induced damage, and that HSPs were not the cause of the observed tolerance.

Although mainly associated to desiccation and cold tolerance, accumulation of trehalose, a non-reducing disaccharide consisting of two glucose units, has been also related to acquisition of tolerance to high temperature in yeast [102, 103, 104]. Trehalose also enhances the thermal stability of cytoplasmatic yeast enzymes *in vitro* [105]. Plants are unable to accumulate trehalose in response to environmental stress, although they have genes involved in the synthesis of trehalose, mainly because of the high activity of the degrading trehalose activity in plant tissues. It is thought that sucrose, another non-reducing disaccharide, replaces trehalose in plants [106]. Since much higher amount of sucrose than of trehalose are needed for similar protective effects, plants have been genetically engineered to accumulate trehalose by introducing bacterial and yeast genes for trehalose synthesis [107, 108]. Drought tolerance was only slightly affected in transgenic tobacco plants, which, however, were not tested for a putative increase in thermotolerance.

8. ACTIVE OXYGEN DETOXYFYING ENZYMES CONTRIBUTE TO PLANT THERMOTOLERANCE

A common plant response to many abiotic and biotic stress, such as heat, chilling, excessive light, drought, ozone exposure, UV-B irradiation, osmotic stress and biotic stress is the accelerated generation or accumulation of oxidative signals, including hydrogen peroxide (H_2O_2) [109 and other chapters in the present book]. High dosage of H_2O_2 results in a hypersensitive cell death, [110, 111], while low levels block cell cycle progression [112]. Plants respond to the primary or secondary oxidative stress with an increase in the production of antioxidant enzymes, including glutathione S-transferases (GSTs), peroxidases, superoxide dismutases and catalases, as well as the activation of protective genes encoding HSPs or pathogenesis-related proteins [113]. Transgenic plants over-expressing some of the genes encoding this class of genes have shown a variable increase in tolerance to oxidative stress and cold tolerance [114, 115], while tolerance to heat stress was not tested. However, a paper from Sheen's group [113] has shown that transgenic *Arabidopsis* plants with high levels of GST, due to over-expression of the mitogen-activated protein kinase kinase kinase, ANP1, which mimics the H_2O_2 effect and initiates the MAPK cascade, are more heat tolerant. Exposure to 48°C killed all the wild-type plants, while

independent ANP1 transformants survived at various extents, according to the level of expression of the transgenes. The increased thermotolerance was due to both GST and HSPs accumulation.

9. HEAT TOLERANT PLANTS: CURRENT STRATEGIES, BOTTLENECKS AND FUTURE PERSPECTIVES

Identification of crucial genes involved in tolerance to heat stress is fundamental to define the possible strategies that might be employed for producing high temperature-tolerant plants. Transgenic plants reported to have enhanced heat tolerance, obtained by manipulation of a single gene, are summarized in Table 1. Though the pioneer work in model plants has been fundamental to uncover the role of single genes in heat tolerance, most of heat tolerant transgenic plants over-expressing individual target genes gain minor protection to limited stress conditions. Moreover, possible negative pleiotropic effects of the genetic manipulation on the plant phenotype, due to the intercrossing of the signal transduction pathway and other metabolic pathways, have been completely under-estimated. Overcoming the above mentioned bottlenecks is of one of the most propelling priorities that scientists have to face to move from model plants to realistic heat tolerant crops in the field [116].

To ameliorate the small effects on heat tolerance produced by manipulation of individual genes, it is necessary to identify regulatory genes, able to control the whole battery of genes crucial for heat tolerance. The manipulation of specific genes of signal perception and transduction has proved indeed useful in controlling expression of multiple genes through single gene transfer. As an example, tobacco plants over-expressing the ANP1 gene were able to tolerate better heat stress, as well as freezing, drought and high salt condition [113]. The multiple stress tolerance is due to the fact that ANP1 codes for a protein kinase, activated by H_2O_2, which initiates a phosphorylation cascade involving mitogen activated protein kinases (MAPKs). The final result is the activation of many stress-responsive genes, including genes for HSPs and detoxification enzymes. Furthermore, several works have shown that by changing levels of transcription factors, it is possible to activate many down-stream stress-responsive genes [80, 117, 118].

Secondly, the potential negative phenotypic effects linked to the constitutive expression of genes associated to thermotolerance might be avoided by using HS inducible promoters, as already demonstrated for drought-responsive genes by Kasuga et al [119]. In this way, tolerance genes are activated only when the heat event occurs, minimizing the negative pleiotropic side effects.

Manipulation of membrane lipid composition is another promising approach for heat tolerance [94], also in the view of the emerging role of membrane lipids in regulating changes in gene expression upon heat stress.

Many down-stream HS-induced genes have been thus far identified and characterized for their involvement in thermotolerance in different plants. However,

most of the genetic complexity of the HS response still waits to be fully exploited, at least as far as genes encoding regulatory genes, acting up-stream in the perception and transduction of the HS signal. New and more extensive methods of analysis are required, aimed at the simultaneous identification of the many modifications in gene activity as a consequence of HS. In the last 10 years an ever increasing number of gene sequences are becoming available from the genome sequencing program for many organisms that are in progress, in some cases already completed, such as the ones for *S. cerevisiae*, human, *Arabidopsis*, rice. The projects under way are also producing a huge number of EST sequences that gave a fundamental contribution to the understanding of the expressed genes [120, 121, 122, 123].

The fundamental strategy of functional genomics is to expand the study of biological systems from the role of single genes to the study of a large number of genes (ideally all) of an organism, to provide simultaneously information on the functions of multiple genes. This is particularly useful in the case of modification of the expression profile due to exposition to environmental stresses. The many projects that are now under way (on *Arabidopsis*, barley, wheat etc.) promise the rapid identification, and perhaps isolation, of all the genes involved in this response. Many sites are available for searching on-line such as the Stress Functional Genomic Consortium website (http://stress-genomic.org/). This approach will allow the systematic and quantitative analysis of gene expression and the identification of novel genes, by differential screening of cDNA libraries deriving from different tissues, tissues treated with different stressors or with different hormones. Several reports of microarray analyses have been published, that analyse plant response to different environmental stresses [109, 123, 124, 125, 126, 127], but a systematic analysis of the HS response in plants has not been undertaken yet. With the aim to identify novel genes responding to HS and protein kinase C activation, Schena and co-workers [128] reported the use of microarray to study 1046 anonymous cDNAs from human cells, hybridised with probes originating from both control and HS stressed tissues.

One interesting application of large scale analysis of genes for thermotolerance through micro-array analysis is the possibility of comparing heat sensitive and heat tolerant genotypes, as is currently done for salt stress in rice [129]. The analysis of the output data would provide a global vision of the genetic differences in the regulation of heat-responsive genes in the two contrasting genotypes. This scientific approach will shed light on the endless controversy of physiologists and molecular biologists on methodologies to be used to breed crop plants for enhanced tolerance to environmental stresses.

With the current available technology and knowledge, it will be soon possible to approach, at molecular level, the mechanisms of heat tolerance linked to morphological traits. Genes controlling plant form and architecture traits are very well described [130]. Many of these genes act as regulatory proteins of the plant cell cycle [131] and appear to play a role in plant growth and development not only under normal environmental conditions but also under stress [132].

10. CONCLUSIONS

The genes thoroughly described in this chapter have been proved to be fundamental for plant survival, but their real contribution to the maintenance of crop productivity under heat stress has not been fully investigated. The current challenge in producing heat tolerant crop plants is to identify genes associated to thermotolerance that, beside survival, ensure that under heat stress plants still retain a high portion of their yield potential. The best candidate genes for engineering plant heat tolerance are those involved in sensing changes in external temperature and able, in turn, to trigger rapidly the expression of a set of genes driving the cell towards a new cellular homeostasis, compatible with active plant growth and development and, ultimately, yield.

Table 1. Plants engineered for heat tolerance

Introduced gene	*Source of the transgene*	*Host plant*	*Remarks*	*Reference*
Heat shock transcription factor (*AthHSF1* and *AthHSF3*)	Plant	*A. thaliana*	Over-expression enhances basal heat tolerance	[80]
Heat shock protein (*Hsp70*)	Plant	*A. thaliana*	Over-expression enhances heat tolerance	[134]
Small heat shock protein (*Hsp 17.7*)	Plant	Carrot	Over-expression enhances heat tolerance	[114]
Heat shock protein (*Hsp101*)	Plant	*A. thaliana*	Over-expression enhances heat tolerance Silencing decreases heat tolerance	[76]
Betaine aldehyde dehydrogenase	Bacterial	Rice	Over-expression enhances heat tolerance	[133]
Choline oxidase	Bacterial	*A. thaliana*	Over-expression enhances heat tolerance	[101]
FAD7 desaturase	Plant	*A. thaliana*	Silencing increases heat tolerance	[94]

Table 1. Continued

Introduced gene	*Source of the transgene*	*Host plant*	*Remarks*	*Reference*
Mitogen-activated protein kinase, ANP1	Plant	Tobacco	Over-expression increases heat tolerance	[113]

REFERENCES

1. Minorsky, P.V. (2002). *Plant Physiol.* 129, 1419.
2. McKersie, B. R. (2001). *Crop Sci.*, 41, 1988.
3. Crafts-Brandner, S.J., and Salvuci, E. (2002). *Plant Physiol.* 129, 1773.
4. Law, R.D., and Craft-Brandner, S.J. (1999). *Plant Physiol.* 120, 173.
5. Tena, G., Asai, T., Chiu, W.L., and Sheen, J. (2000). *Curr. Opin. Plant Biol.*, 4 392.
6. Mizoguchi, T., Ichimura, K., Yoshida , R. and Shinozaki, K. 2000. *Results Probl. Cell Differ.* 27, 29.
7. Berry, J.A., and Bijorkman C. (1980). *Annu. Rev. Plant Physiol.* 31, 491.
8. Havaux, M. (1993). *Plant Cell and Environ.* 16, 461.
9. Parsell, D.A., and Lindquist, J. (1993). *Annu. Rev. Genet.* 27, 437.
10. Roussou, I., Nguyen, T., Pagoulatos, G.N. and Bensaude, O. (2000). *Cell Stress Chaperones.* 5, 8.
11. Vígh, L., Los, D.A., Horváth, I. and Murata, N. (1993). *Proc. Natl. Acad. Sci. USA.* 90, 9090.
12. Carratù, L., Franceschelli, S., Pardini, C.L., Kobayashi, G.S., Horváth, I., Vígh, L. and Maresca, B. (1996). *Proc. Natl. Acad. Sci. USA.* 93, 3870.
13. Török, Z., Goloubinoff, P., Horváth, I., Tsvetkova, N.M., Glatz, A., Balogh, G., Varvasovszki, V., Los, D.A., Vierling, E., Crowe, J.H. and Vígh, L. (2001). *Proc. Natl. Acad. Sci. USA* . 98, 3098.
14. Török, Z., Horváth, I., Goloubinoff, P., Kovács, E., Glatz, A., Balogh, G. and Vígh, L. (1997). *Proc. Natl. Acad. Sci. USA.* 94, 2192.
15. Vigh, L., and Maresca, B. (2002). Cell and Molecular Responses to Stresses, KB Storey and JM Storey, Eds Elsevier Science (in press)
16. Vígh, L., and Joó, F. (1983). *Febs Lett.* 162, 423.
17. Joó, F., Balogh, N., Horváth, L.I., Filep, Gy., Horváth, I., and Vígh, L. (1991). *Anal. Biochem.* 194, 34.
18. Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K., and Murata, N. (2000). *EMBO J.* 19, 1327.
19. Horváth, I., Glatz, A., Varvasovszki, V., Török, Zs., Páli, T., Balogh, G., Kovács, E., Nádasdi, L., Benk, S., Joó, F. and Vígh, L. (1998). *Proc. Natl. Acad. Sci. USA.* 95, 3513.
20. Colonna-Romano, S., Török, Z., Vigh, L., Maresca, B. (2002). *Mol. Microbiol.*, submitted.
21. Glatz, A., Horváth, I., Varvasovszki, V., Kovács, E., Török, T. and Vígh, L. (1997). *Biochem. Biophys. Res. Commun.* 239, 291.
22. Sinensky, M. (1974). *Proc. Natl. Acad. Sci. USA.* 71, 522.
23. Cullis, P.R. and de Kruijff, B. (1979). *Biochim. Biophys. Acta.* 559, 399.
24. Quinn, P.J., Joó, F. and Vígh, L. (1989). *Prog. Biophys. Mol. Biol.* 53, 71.
25. Rietveld, A.G., Killian, J.A., Dowhan, W. and de Kruijff, B. (1993). *J. Biol. Chem.* 268, 12427.
26. Murata, N. and Los, D.A. (1997). *Plant Physiol.* 115, 875.
27. Orvar, B.L., Sangwan, V., Omann, F. and Dhindsa, R.S. (2000). *Plant J.* 23, 785.
28. Sangwan,V., Foulds, I., Singh, J. and Dhindsa, R.S. (2001). *Plant J.* 27, 1.
29. Monroy, A.F. and Dhindsa, R.S. (1995). *Plant Cell.* 7, 321.
30. Schmidt, A. and Hall, M.N. (1998). *Annu. Rev. Cell Dev. Biol.* 14, 305.
31. Trewavas, A.J. and Malho, R. (1998). *Curr. Opin. Plant Biol.* 1, 428.
32. Mazars, C., Thion, L., Thuleau, P., Graziana, A., Knight, M.R., Moreau, M. and Ranjeva, R. (1997). *Cell Calcium.* 22, 413.

33. Sangwan, V., Örvar, B. L., Beyerly, J., Hirt, H. and Dhindsa, R.S. (2002). *Plant J.* 31, 629.
34. Vierling, E. (1991). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42, 579.
35. Feder, M.E. and Hofmann, G.E. (1999). *Annu. Rev. Physiol.* 61, 243.
36. Nover, L. (1991). Heat Shock Response, Boca Raton FL, CRC Press.
37. Kim, K.K., Kim, R. and Kim, S.H. (1998). *Nature*. 394, 595.
38. Ingolia, T.D. and Craig E.A. (1982). *Proc. Natl. Acad. Sci USA*. 79, 2360.
39. Plesofsky-Vig, N., Vig, J. and Brambl, R. (1992). *J. Mol. Evol*. 35, 537.
40. Caspers, G.J., Leunissen, J. A-M. and de Jong, W.W. (1995). *J. Mol. Evol*. 40, 238.
41. Waters, E.R., Lee, G.J. and Vierling, E. (1996). *J. Exp. Bot*. 47, 325.
42. Kappé, G., Leunissen, J. A-M. and de Jong, W.W. (2002). Progress in Molecular and Subcellular Biology, vol. 28, A-P Arrigo, W.E.G. Muller (Eds), Springer-Verlag, Berlin Heidelberg.
43. Lenne, C. (1995). *Biochem. J.* 311, 805.
44. Treglia, A.S., Gulli, M. and Perrotta, C. (2001). *DNA Sequence*. 12, 397.
45. Waters, E.R. (1995). *Genetics*. 141, 785.
46. Bond, U. (1988). *EMBO J.* 7, 3509.
47. Czarnecka, E., Nagao, R.T., Key, J.L. and Gurley, W.B. (1988). *Mol. Cell. Biol*. 8, 1113.
48. Osteryoung, K.W., Sundberg, H. and Vierling E. (1993). *Mol. Gen. Genet.* 239, 323.
49. Winter, J., Wright, R., Duck, N., Gasser, C., Fraley, R. and Shah, D. (1988). *Mol. Gen. Genet*. 211, 315.
50. Berger, E.M., Vitek, M.P. and Morganelli, C.M. (1985). *J. Mol. Biol.* 186, 137.
51. Chang, Z., Primm, T.P., Jakana, J., Lee, I.H., Serysheva, I., Chin, W., Gilbert, H.F. and Quiocho, F.A. (1996). *J. Biol. Chem*. 271, 7218.
52. Groenen, P.J., Merk, K.B., de Jong, W.W. and Bloemendal, H. (1994). *Eur. J. Biochem*. 225, 1.
53. Lee, G.J., Pokala, N. and Vierling, E. (1995). *J. Biol. Chem*. 270, 10432.
54. Haley, D.A., Bova, M.P., Huang, Q-L., Mchaourab, H.S. and Stewart, P.L. (2000). *J. Mol. Biol*. 298, 261.
55. Helm, K.W., Lee, G.J. and Vierling, E. (1997). *Plant Physiol*. 114, 1477.
56. Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J. and Lutsch, G. (1994). *J. Biol. Chem*. 269, 20780.
57. Freshney, N.W., Rawlison, L., Guesdon, F., Jones, E., Cowle, S., Hsuan, J. and Saklatvala, J. (1994). *Cell*. 78, 1039.
58. Knauf, U., Jacob, U., Engel, K., Buchner, J. and Gaestel, M. (1994). *EMBO J.* 13, 54.
59. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A.R. (1994). *Cell*. 78, 1027.
60. Kato, K., Hasegawa, K., Goto, S. and Inaguma, Y. (1994). *J. Biol. Chem*. 269, 11274.
61. Lavoie, J.N., Lamert, H., Hickey, E., Weber, L.A. and Landry, J. *(1995). Mol. Cell. Biol*. 15, 505.
62. Nover, L. and Scharf, K. (1984). *Eur. J. Biochem*. 139, 303.
63. De Rocher, A.E., Helm, K.W., Lauzon, L.M. and Vierling, E. (1991). *Plant Physiol*. 96, 1038.
64. Hsieh, M-H., Chen, J-T., Jinn, T-L., Chen, Y-M. and Lyn, C-Y. (1992). *Plant Physiol*. 99, 1279.
65. Chen, Q., Lauzon, L.M., De Rocher, A.E. and Vierling, E. (1990). *J. Cell. Biol.* 110, 1873.
66. Lavoie, J.N., Hickey, E., Weber, L.A. and Landry, J. (1993). *J. Biol. Chem.* 268, 24210.
67. Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997). *EMBO J.* 16, 659.
68. Jacob, U. and Buchner, J. (1994) *Trends Biochem. Sci.* 19, 205.
69. Forreiter, C., Kirschner, M. and Nover, L. (1997). *Plant Cell*. 7, 2171.
70. Löw, D., Brändle, K., Nover, L. and Forreiter, C. (2000). *Planta*, 211, 575.
71. Hong, S.-W. and Vierlieng, E. (2000). *Proc. Natl. Acad. Sci., USA*. 97, 4392.
72. Burke, J.J., O'Mahony, P.J. and Oliver, M.J. (2000). *Plant Physiol*. 123, 575.
73. Yeh, C.H., Chang, P.L., Yeh, K.W., Lin,W.C., Chen, Y.M and Lin, C.Y. (1997). *Proc Natl Acad Sci USA*. 94: 10967.
74. Soto, A., Allona, I., Collada, C., Guevara, M.A., Casaso, R., Rogriguez Cerezo, R., Aragoncillo, C. and Gomez, L. (1999). *Plant Physiol*. 120, 521.
75. Malik, M. K., Slovin, J. P., Hwang, C.I. and Zimmerman, J.L. (1999). *Plant J*. 20, 89.
76. Queitsch, C., Hong, S.-W., Vierling, E. and Lindquist, S. (2000). *Plant Cell*. 12, 479.
77. Stapel, D., Kruse, E. and Kloppstech, K. (1993). *J. Photochem. Photobiol.* B21, 211.
78. Clarke, A.K. and Critchley, C. (1994). *Physiol. Plant*. 92, 118.
79. Heckathorn, S.A., Downs, CA, Sharkey, T.D. and Coleman, J.S. (1998). *Plant Physiol*.116, 439.
80. Lee , J.H., Hubel, A. and Schöffl, F. (1995). *Plant J*. 8, 603.

81. Prändl, R. Hinderhofer, K., Eggers-Schumacher, G. and Schöffl, F. (1998). *Mol. Gen. Genet.* 256, 269.
82. Iba, K. (2002). *Annu. Rev. Plant Biol.* 53, 225.
83. Harwood, J.L. (1988). *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 39, 101.
84. Hazel, J.R. and Williams ,E.E. (1990). *Prog. Lipid Res.* 29, 167.
85. Vigh, L., Gombos, Z., Horvarth, I. and Joo, F. (1989). *Biochim. Biophys. Acta.* 979, 361.
86. Hugly, S., Kunst , L., Browse, J. and Somerville, C. (1989). *Plant Physiol.* 90, 1134.
87. Miquel, M., James Jr., D., Dooner, H., Browse, J. (1993). *Proc. Natl. Acad. Sci. USA.* 90, 6208.
88. Murata, N. and Wada, H. (1995). *Biochem. J.* 308, 1.
89. Gibson, S., Arondel, V., Iba, K. and Somerville, C. (1994). *Plant Physiol.* 106, 1615.
90. Fukuchi-Mizutani, M., Tasaka, Y., Tanaka, Y., Toshihiko, A., Kusumi, T. and Murata, N. (1998). *Plant Cell Physiol.* 39, 247.
91. Berberich, T., Harada, M., Kazuyuki, S., Kodama, H., Iba, K. and Kusano, T. (1998). *Plant Mol. Biol.* 36, 297.
92. Okuley, J., Lightnen, J., Feldmann, K., Yadav, N., Lark, E. and Browse, J. (1994). *Plant Cell.* 6, 147.
93. Heppard, E.P., Kinney, A.J., Stecca, K.L. and Miao, G-H. (1996). *Plant Physiol.* 110, 311.
94. Murakami, Y., Tsuyaama, M., Kobayashi, Y., Kodama, H. and Iba, K. (2000). *Science.* 287, 476.
95. Sakamoto, A. and Murata, N. (2000). *J. Exp. Bot.* 342, 81.
96. Chen, T.H. and Murata, N. (2002). *Curr. Opin. Plant Biol.* 5, 250.
97. Paleg, L.G., Douglas, T.J., van Daal, A. and Keech, D.B. 1981. *Aust. J. Plant Physiol.* 8, 107.
98. Allkhverdiev, S.I., Feyziev, Y.M., Ahmed, A., Hayashi, H., Aliev, J.A., Kimlov, V.V., Murata, N. and Carpentier, R.. (1996). *J. Photochem. Photobiol.* 34, 149.
99. Mamedov, M., Hayashi, H. and Murata, N. (1993). *Biochim. Biophys. Acta.* 1142, 1.
100. Matoh, T., Watanabe, J., Takahashi, E. (1987). *Plant Physiol.* 84, 173.
101. Alia, Hayashi, H., Sakamoto, A, and Murata, N. (1998). *Plant J.* 16, 155.
102. Hottiger, T, Boller, T. and Wiemken, A. (1987)a. *FEBS Lett.* 225, 249.
103. Hottiger, T, Boller, T. and Wiemken, A. (1987)b. *J. Bacteriol.* 169, 5518.
104. Gadd, G, Chalmes, K. and Reed, R. (1987). *FEMS Microbiol. Lett.* 48, 249-254.
105. Hottiger, T., De Virgilio, M, Hall, T., Boller, T. and Wiemken, A. (1994). *Eur. J. Biochem.* 219, 187.
106. Muller, J., Boller, T. and Wienlen, A. (1995). *Plant Sci.* 112, 1.
107. Romero, C., Belles, J.M., Vaya, J.L., Serrano, R. and Culiáñez-Macià, F.A. (1997). *Planta.* 201, 293.
108. Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T.E. and Welin, B. (2000). *J. Exp. Bot.* 51, 177.
109. Vranová, E., Inzé, D., and Van Breusegem, F. (2002). *J. Exp. Bot.* 53, 1227.
110. Lamb, C. and Dixon, R. A. (1997). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251.
111. Alvarez, M.E., Pennell, R.I:, Meijer, P. J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998). *Cell.* 92, 773.
112. Reichheld, J.-P., Vernoux, T., Lardon, F., Montagu, M.V. and Inzè, D. (1999). *Plant J.* 17, 647.
113. Kovtun,Y., Chiu W-L., Tena, G. and Sheen, J. (2000). *Proc. Natl. Acad. Sci. USA.* 97, 2940.
114. McKersie, B. D., Chen, Y., de Beus, M., Bowley, S. R., Bowler C., Inzé, D., D'Halluin, K. and Botterman, J. (1993). *Plant Physiol.* 103, 1155.
115. Van Camp, W., Capiau, K.,Van Montagu, M., Inzé, D. and Slooten, L. (1996). *Plant Physiol.* 112, 1703.
116. Leone, A., Costa, A,, Consiglio, F., Massarelli, I., Dragonetti, E., De Palma, M .and Grillo, S.(1999). *Potato Res.* 42, 333.
117. Jagglo-Ottosen, R., Gilmour, S.J., Zarkr, D.G., Schabenberger, M. F. and Thomashow, M. (1998). *Science.* 280, 104.
118. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Setsuko, M., Yamaguchi-Shinozaki, K. and. Shinozaki, K. (1998). *Plant Cell.* 10, 1391.
119. Kasuga, K., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999). *Nature Biotechnol.* 17, 287.
120. Höfte, H., Desprez, T., Amselem, J., Chiapello, H., Caboche, M., Moisan, A., Jourjon, M-F., Charpenteau, J-L., Berthomieu, P., Guerrier, D., Giraudat, J., Quigley, F., Thomas, F, Yu, D-Y., Mache, R., Raynal, M., Cooke, R., Grellet, F., Delseny, M., Parmentier, Y., Marcillac, G., Gigot, C.,

Fleck, J., Philipps, G., Axelos, M., Bardet, C., Tremousaygue, D. and Lescure, B. (1993). *Plant J.* 4, 1051.
121. Newman, T., Debruijn, F.J., Green, P., Keegstra, K., Kende, H., Mcintosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Tomashow, M., Retzel, E. and Somerville, C. (1994). Plant Physiol. 106, 1241.
122. Cooke, R., Raynal, M., Laudie, M., Grellet, F., Delseny, M., Morris, P.C., Guerrier, D., Giraudat, J., Quigley, F., Clabault, G., Li, Y.F., Mache, R., Krivitzky, M., Gy, I.J., Kreis, M., Lecharny, A., Parmentier, Y., Marbach, J., Fleck, J., Clement, B., Philipps, G., Herve, C., Bardet, C., Tremousaygue, D., Hofte, H. (1996). *Plant J.* 9, 101.
123. Asamizu, E., Nakamura, Y., Sato, S. and Tabata, S. (2000). *Plant Cell.* 12, 647.
124. Ruan, Y., Gilmore, J. and Conner, T. (1998). *Plant J.* 15, 821.
125. Aharoni, A., Keizer, L.C., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blaas, J., van Houwelingen, A.M., De Vos, R.C., van der Voet, H., Jansen, R.C., Guis, M., Mol. J., Davis, R.W., Schena, M., van Tunen, A.J., O'Connell, A.P. (2000). *Plant Cell.* 12, 647.
126. Reymond, P., Weber, H., Damond, M. and Farmer, E.E. (2000). *Plant Cell.* 12, 707.
127. Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguki-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001). *Plant Cell.* 13, 61.
128. Schena, M., Shalon, D,, Heller, R., Chai, A., Brown, P.O. and Davis, R.W. (1996). *Proc. Natl. Acad. Sci. USA.* 93, 10614.
129. Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D, and Bohnert, H. J. (2001). *Plant Cell.* 13, 889.
130. Nakajima, K. and Benfey, P.N. (2002). *Plant Cell.*14, Suppl., 265.
131. den Boer, B.G.W., Murray, J. A. H. (2000). *Curr. Opin. Biotech.* 11, 138.
132. Stals H, Inzé D. (2001). *Trends Plant Sci.* 6, 359.
133. Kishitani, S., Takanami, T., Suzuki, M., Oikawa, M., Yokoi S. (2000). *Plant Cell Environ.* 23, 107.
134. Lee, J.H. and Schöffl, F. (1996). *Mol. Gen.* 252, 11.

CHAPTER 2

CHILLING AND FREEZING STRESSES IN PLANTS: CELLULAR RESPONSES AND MOLECULAR STRATEGIES FOR ADAPTATION

M. BRACALE[a] AND I. CORAGGIO[b]

[a] *DBSF, Università of Insubria, via J.H. Dunant 3, 21100, Varese, Italy*
[b] *IBBA, CNR, via Bassini 15, 20133, Milano, Italy*

Abstract. Cold affects agronomic yield and product quality. The mechanisms by which plants translate cold perception into specific gene expression are not yet completely understood; the available evidence is not yet arranged into an overall coherent picture. Nevertheless: 1) signal transduction pathways are being elucidated; 2) evidence is accumulating on control of cold-related gene expression, with the identification of *cis*- and *trans*-acting elements; 3) a large number of gene products, putatively involved in cold tolerance, have been characterised; 4) transgenic plants are contributing to the understanding of the function of specific genes. These efforts are of substantial interest. Success in the production of crop varieties with improved cold tolerance will have great beneficial economic and ecological impact, meeting the philosophy of a sustainable (intensive versus extensive) agriculture. This chapter discusses recent progress in knowledge on the molecular and cellular mechanisms underlying cold tolerance in higher plants.

1. INTRODUCTION

Plants can grow only in species-specific temperature intervals. Below the minimum temperature their performance is reduced by cold stress, one of the most serious abiotic environmental stress that plants have to cope with throughout their life cycle.

In the next years, "a range of credible scenarios of greenhouse gas emission could increase radiative forcing to cause a 3-6°C increase in mean land surface temperature at high and temperate latitudes" [1]. Thus, the mean temperature will rise. Nevertheless, an increase in damages due to cold stress induced for example by late frost events in early springs, sudden lowering in temperature during mild winter, low night temperature after a mild day and low snowfalls in winter are expected. Moreover, a long period with mild temperature in autumn will prevent plants acclimation towards the winter frost period.

Cold stress has strong limiting effect with regard to:

1. the geographic distribution of wild and crop species hindering the human necessity to grow some crops outside the limits imposed by their natural distribution. In fact, many crops and horticultural species are tropical and

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 23-51.

subtropical in origin and then they are only marginally cold-adapted in temperate earth regions, so that they hardly withstand to cold stress.

2. the agronomic yield. Cold events reduce the plant growth with negative and unforeseeable effects on the biomass and cause a large gap between potential and actual yield. Steponkus [2] has quoted in 10-100 millions dollars the losses due to freezing damage and Wilson reported that the loss in USA for cotton was 60 million dollars in 1980, a year characterised by unseasonably low temperatures [3].

3. the product quality. Cold reduces synthesis, accumulation and storage of proteins and polysaccharides. Also fruit ripening is affected by low temperatures, with consequences on nutritional and taste profiles of the product.

Complexity characterises the study of cold tolerance: first of all, different plant species and, within a species, different cultivars display different degrees of sensibility to cold. Second, cold tolerance depends not only on the severity and duration of the stress, but also on the rate of cooling, on seasonal and diurnal plant activities, on the concomitant presence of other environmental conditions as, for example, air humidity, water soil availability, wind presence (causing dehydration), light intensity (causing photoinhibition).

Third, cold tolerance is related to the stage of plant development and different plant organs exhibit different cold tolerance. For instance, seeds are very tolerant due to their extreme dehydration. On the other hand, roots, rhizomes and bulbs are very sensitive to cold, but, due to the moderating effect of the soil, these tissues seldom experience severe low temperatures.

In spite of the complexity of the matter, research on cellular responses and molecular strategies for the adaptation to cold stress in plants has recently experienced a burst of knowledge. Genes, signal transduction factors, proteins and enzymes related to drought tolerance are continuously being identified. Different approaches (genetics, biochemistry, physiology, molecular biology, genetic engineering) and analysis tools contribute towards a global vision of this phenomenon and a comprehension of the basic mechanisms involved in cold tolerance. The production and the study of transgenic plants help to shed light on the importance and the role of the specific genes in cold tolerance. In fact, by overexpressing specific genes in transgenic plants or by knocking out their expression with antisense RNA, their individual contribution to cold tolerance may be verified.

A satisfactory and general comprehension of the plant adaptive molecular response to cold stress will possibly materialise in the next decade.

However, until now, a number of relevant questions remain to be answered:

- which are the key elements involved in the cold signal perception and transduction?
- which gene products do actually play important roles in cold tolerance?
- how do these stress-inducible gene products cooperate to achieve a synergistic response to cold?

Success along these lines will not only be of scientific value, but their applications to important crop plants will bring great economic and ecological

benefits. These stress tolerant plants will meet the philosophy of a sustainable (intensive versus extensive) agriculture, allowing to obtain the same level of product output with a lower energy input. Moreover, as a consequence, stabilisation of the productivity during successive years and management costs reduction (costs for greenhouses, for heating, etc.) may be expected.

This chapter intends to give an overview of the recent knowledge developments on the complex molecular and cellular mechanisms underlying cold tolerance in higher plants.

2. COLD, FREEZING AND ACCLIMATION

Both chilling and freezing stresses, share some injuries due to the direct effect of low temperature on cellular processes such as enzymatic activity lowering and fatty acid fluidity. Nevertheless, they differ on several important aspects. While cold stress acts directly on damaging the cell components, freezing has in addition important indirect damage effects owing to ice crystal formation and their expansion in the extra-cellular compartments. Ice formation can damage plants, acting directly by swelling and tearing tissues apart and by brooking intercellular connections, as well as indirectly, by drawing large fluxes of water out of the cell across the plasmalemma. The water freezing in the extra-cellular spaces results in the solute concentration increasing; this induces a water flux out of the cells with subsequent cell dehydration and shrinkage. It is for these common injuries induced by freezing and dehydration, that these two stresses share some adaptive responses, such as those mediated by the ABA hormone.

In cold susceptible plant germination, growth and development are possible up to temperatures around to 12°C. On the contrary, cold tolerant plant species can survive up to temperatures below 12°C and freezing-tolerant plants can survive also at temperatures below the freezing limit. However, non-tolerant plants can also survive to a freezing stress, if previously subjected to sub-lethal stress conditions. This mechanism is referred to as acclimation.

As an example, non acclimated rye is not able to tolerate temperatures around -5°C, but, it can however, survive to temperatures down to -30°C if it has been previously exposed to a low, non-freezing temperature. In *Arabidopsis,* enhanced freezing tolerance up to a -12°C temperature has been observed after 3 days of acclimation at 1°C with long photoperiods [4]. Due to the above mentioned common adaptive responses, osmotic stress, dehydration, salt and exogenous applications of ABA can also enhance freezing tolerance [5,6].

Cold adaptation depends on changes in the expression levels of specific genes, whose products are able to confer greater tolerance to stress conditions. In this sense "acclimation is the expression of the genetic potential under inductive conditions" [7].

2.1. Responses and damages induced by cold stress in plants

Owing to the great complexity of plant response to cold, it should be emphasised that it is often difficult to distinguish clearly between cold damages and adaptive responses. This means that for many of the stress-induced gene products, it is not clear if they are involved in cold tolerance or if they merely accumulate as a consequence of cell damages.

At the whole organism level, cold induces both structural (i.e., an increase of leaf thickness) and developmental changes (i.e., in normal fruit ripening). One of the most cold-sensitive processes during the life cycle of cold-sensitive species is pollen maturation [8]. Other consequences of cold stress are a general loss of plant vigour and a reduction of plant development and growth rates: plants may remain stunted also after rewarming. However, growth reduction due to cold action can also be evaluated as an adaptive response to stress in order to limit the transpiration rate.

A number of physiological responses occur in cold stress conditions: the respiration rate decreases, the enzyme activities are altered, the levels of growth regulators change, with an increase of ABA and a decrease of gibberellin levels [9-11].

However, the most important cold effect is perhaps at photosynthetic level.

When the electron transport chain is impaired by cold and chloroplasts are exposed to an excess of excitation energy, oxygen photoreduction occurs with concomitant production of reactive oxygen intermediates, such as superoxides and peroxides. The uncontrolled increase of free radicals cooperates with cold in damaging membranes (causing lipid peroxidation), enzymes and macromolecules, particularly in chloroplasts and mitochondria. The cellular ability to contrast cold-induced oxidative injuries is an important component of cold tolerance. The relationship between abiotic stress tolerance and a functional antioxidant system has been found in transgenic alfalfa plants overexpressing a superoxide dismutase gene [12].

At the cellular and molecular level, cold slows down all metabolic reactions and affects several molecular and sovramolecular structures. The major structural damage is at membrane level, due to the changes in fatty acid fluidity, freeze-mediated dehydration and production of reactive oxygen species. The freeze-mediated cellular dehydration affects more specifically the plasma membrane, while reactive oxygen species damages principally the organelle membranes.

Membrane lipids are usually in a fluid (liquid-crystalline) phase, but, with lowering temperatures, as well as with lowering of the water content, a transition to a more rigid gel phase is induced, and the membranes become leaky or dysfunctional. This results in injuries in carrier-mediated transport and loss in the activity of membrane-bound enzymes and receptors. Under extensive cold events, loss of water through membranes leads to an irreversible damage [13]. Expansion-induced-lysis is elicited, as well as lamellar-to-hexagonal-II phase transition and fracture jump lesions [14,15]. The loss of cellular membrane integrity leads to a leakage of cellular solutes into apoplastic compartments.

Therefore, membrane lipid composition plays an important role in the control of water and solutes permeability. An adaptive response to hinder the cold-induced membrane stiffness, is the increase in polyunsaturated acyl chains of membrane phospholipids [16]. In fact, transgenic plants with higher instauration degree of membrane lipids [17], or with a lower concentration of saturated species of phosphoglycerol [18], are more tolerant to chilling, due to a lowering of the membrane melting temperature. Recent data suggest that also the protein fraction and the lipid composition asymmetry of transmembrane bilayer could be involved in cold adaptation [19]. For instance, cold-induced changes have been reported in the lipid-protein ratio of thylakoid membranes, in the activity of plasma membrane H^+-ATPase and in tonoplast enzymes [20-22]. At genomic level, stable genomic changes are inducible by environmental stresses. For example, in *Mesembryanthemum crystallinum*, salt, drought and low temperature change the ploidy number and in *Brassica nigra*, temperature stress results in stable changes of the rDNA copy number [23,24]. However, the mechanisms that lead to these changes and their adaptive significance are not understood to date.

3. ANALYSIS OF THE COLD-RESPONSIVE SIGNAL TRASDUCTION PATHWAY

In the cold acclimation transduction pathway, plants perceive the low non-freezing temperatures and activate an acclimation response able to increase tolerance also at freezing temperature (Figure 1). Recent studies suggest that changes in membrane fluidity, cytoscheleton rearrangement and calcium influx are the earliest events of this signal transduction pathway. Cytoplasmatic calcium level increase is required for both ABA mediated and ABA independent cold acclimation and, although little is known between calcium influx and gene expression, involvement of phosphates and kinase have been demonstrated. Up to now, three classes of transcriptional factors (CBF, Myb4 and SCOF-1) involved in cold acclimation have been described, two of them acting in the ABA independent and the third in the ABA mediated pathways. Several mutants affecting cold response have been isolated such as *eskimo*, showing a constitutive acclimated phenotype, *sfra* [25-30] sensitive to freezing and *Hos1* and *Hos2*, with enhanced expression of cold responsive genes and a very sensitive phenotype.

3.1. Hunting for temperature sensor

A well developed model for temperature signalling has emerged from studies on the cyanobacterium *Synecocystis* PCC6803 [25, 26]. When *Synecocystis* is shifted from 34 to 22°C, transcription of three (*desA*, *desB* and *desD*) of the four fatty acid desaturase-encoding genes is induced approximately ten times [25]. The increased expression of desaturase genes, modulating the degree of fatty acid desaturation and thus the membrane fluidity, represents an adaptive response to the low temperature. It has been demonstrated that *desA* transcription can be induced also at 34 °C, by reducing the membrane fluidity chemically [26].

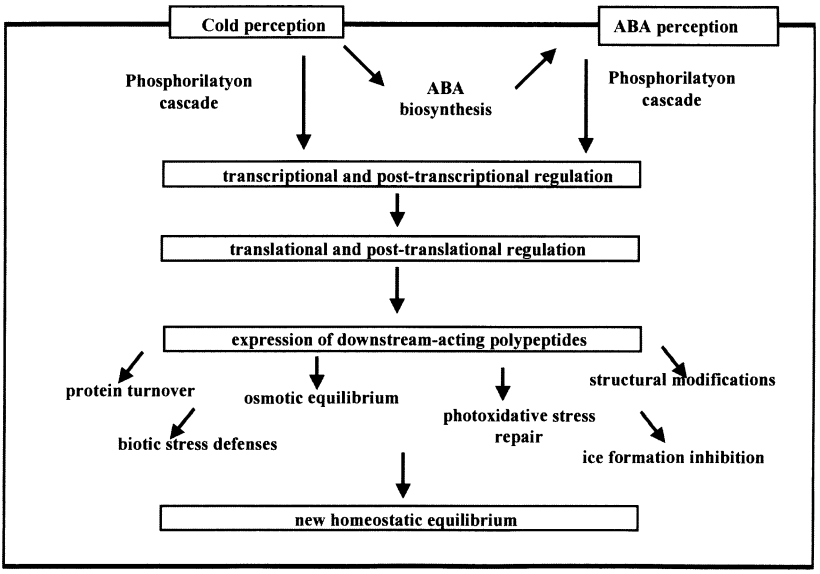

Figure 1. Scheme of the cold acclimation transduction pathway.

Using both target insertional mutagenesis and random insertional mutagenesis, three genes involved in cold sensing have been isolated, encoding for two histidine kinases, Hik33 and Hik19 and for a response regulator, Rer1. According to the Susuki working model, Hik33 activation by change in membrane fluidity, allows its autophosphorylation and subsequent phosphate transfer to Hik19. Mutations in Hik33 and Hik19 impair the cold-induced transcription of *desB*, *desD* and *crh*, however, only the *desB* transcription is mediated by the response regulator Rer1. The *desA* cold induction is not mediated by Hik33 and Hik19, suggesting the presence of two independent sensors [27]. This hypothesis is supported by microarray data showing that on 70 genes transcriptionally regulated by change in temperature, in *hik33* mutant, 14 are temperature insensible, 35 show a lower effect of temperature on the expression and 21 of them show the same level of induction or repression as in wild type These data suggest the presence of at least two sensors, each regulating one set of genes and sharing the regulation of a third set of genes [28].

Two component regulators and hybrid histidin kinase have been described as osmotic sensor in *E. coli* and yeast [29, 30].

In plants, both two component sensors and hybrid histidin kinases have been described in the pathways of response to ethylene and cytokinines; it is likely that histidin kinases are also the basis of the osmotic response [31 and references therein]. Although *hik33* and *hik19* homologous genes have not been found in

plants, cold-induced response regulators have been described in *Arabidopsis*, suggesting that higher plants could employ histidin kinase cold sensors [32].

Although demonstrated only at a crude physiological level, the concept of a "biological thermometer" as a sensor detecting changes in membrane fluidity is accepted also for higher plants [33]. Cultured alfalfa cells, grown at 25 °C, were treated with DMSO (reducing membrane fluidity) or benzyl alcohol (increasing membrane fluidity) before being subjected to cold stress. In DMSO treated cells there was an increase of both the freezing tolerance and the level of cold-induced *cas30* gene transcription; conversely, benzyl alcohol pre-treatment reduces both *cas30* expression and the ability to fully cold acclimate [34]. The authors suggest that changes in membrane fluidity result in cold acclimation through changes in cytoskeleton organization and in calcium influx.

Other cold sensor candidates are the redox state of the PSII and the soluble carbohydrate level [35-37].

3.2. Low temperature signal transduction pathway

The involvement of change in cytoskeleton organization and in calcium influx as early steps in cold transduction pathway have been well documented.

It has been reported that plants react to cold-shock by an immediate rise in cytosolic Ca^{2+}, due largely to Ca^{2+} influx from extracellular storage and also to Ca^{2+} release from internal stores [38-42].

The importance of Ca^{2+} as second messenger in cold acclimation has been demonstrated through the use of chemical and pharmacological reagents affecting its concentration [38, 39, 43, 44]. It has been shown that Ca^{2+} increase is needed both for cold induction of at least some cold-regulated genes and for freezing tolerance. For instance, in alfalfa it has been shown that Ca^{2+} chelators or Ca^{2+} channel blockers both inhibit the Ca^{2+} influx and negatively affect both the expression of the cold-inducible *cas15* gene and the plant ability to cold acclimate. The same authors also reported that *cas15* expression may be induced at 25°C, stimulating the Ca^{2+} influx with the ionophore A23187 [39, 43].

To be noted the presence, in onion, of a mechano-sensitive calcium-selective cation channel activated in response to low temperature [45]. A working model on how membrane fluidity may regulate ion channel activity, suggests a key role for the actine cytoskeleton. Mazars et al. report that disruption of microtubules and actine microfilaments stimulates the cold-induced Ca^{2+} influx in tobacco protoplasts [46].

Similar results were reported on K^+ channel activity in stomatal opening and in mechanical stress signalling [47, 48]. The presence in the aminoacid sequence of AKT1, putatively encoding a K^+ channel, of an ankyrin-like repeat supports the idea of a direct interaction between ion channel and cytoskeleton [49]. More recently, Orvar et al. have shown that *cas30* expression and Ca^{2+} influx at 4°C are prevented by an actine microfilament stabilizer (jasplakinolide) and induced by an actine microfilaments destabilizer (cytochalasin D). They also found that jasplakinolide action prevents the *cas30* expression induced by membrane rigidity, but not by Ca^{2+} influx. These results point out the cytoscheleton reorganization as an integral

component in cold signal transduction acting as a link between changes in membrane fluidity and Ca^{2+} influx [50].

Little is known about the steps between Ca^{2+} influx and cold-activated gene expression, but it appears that protein phosphorylation is involved [51-54]. For instance, Monroy et al. reported that transcript levels of the cold induced *cas15* gene increase at normal growth temperatures in plants treated with the protein phosphatase inhibitor okaidic acid. Accordingly, they fail to accumulate upon low temperature treatment in the presence of the protein kinase inhibitor, staurosporine. The same authors also reported that in alfalfa cold treatment induce a rapid and dramatic decrease in protein phosphatase 2A activity, dependent on calcium influx. Their results, taken together, suggest that low temperature, lead to an influx of calcium that inhibit phosphatase 2A activity and, as a direct or indirect consequence, to the phosphorylation of one or more proteins involved in the *cas15* expression and in cold acclimation [51].

Up to now, the protein kinase(s) responsible for cold-induced gene expression and for freezing tolerance induction has not been identified. However, several interesting candidates have been described. In alfalfa Map kinase ($p44^{MMK4}$) specifically cold activated within ten minutes of low temperature exposition has been described. Although the protein level seems to be unaffected, a rapid (twenty minutes) increase of its mRNA level in response to low temperature has also been reported [55].

Also for other kinases, an increase in the transcript levels in response to low temperature has been reported. Genes encoding for several kinase (such as a MAP kinase kinase kinase, a S6 ribosomal protein kinase, a MAP kinase) induced both by cold and by other abiotic stresses have been identified in *Arabidopsis* [56]. In the same plant, the cold-induced accumulation of the transcripts for a calcium dependent protein kinase (CDPK), a receptor-like protein kinase and two component response regulator-like proteins have also been shown [57, 43, 44]. The cold induction of a CDPK transcript has been reported also in alfalfa [39].

The gene encoding the rice CDPK7 is induced by both cold and salt stresses. Transgenic plants overexpressing CDPK7 shows increased tolerance to both stresses; the authors suggest that CDPK7 kinase activity is post-transcriptionally regulated [58, 59].

More recently, it has been reported that the kinase and autophosphorylating activity of membrane bound rice CDPK increases after several hours of low temperature treatment. Owing to the constant level of protein amount, the increased activity appears to be due to post transcriptional activation [60].

4. COLD MEDIATED TRANSCRIPTION REGULATION

Many lines of evidence indicate that multiple mechanisms are involved in cold acclimation response. Parallel and cross-talking signalling pathways constitute a network of molecular events that co-operate to determine chilling and freezing tolerance. Cold response involves in fact both transcriptional and post-transcriptional processes; moreover, many adaptive changes, as lipid composition

and sugar accumulation, may derive at least in part on post-translational activation of pre-existing enzymes. Studies on *COR* genes indicate the co-presence and the cross-talk between ABA-dependent and ABA-independent pathways. A different pathway involves the *eskimo1* gene, having a dramatic role in cold and freezing tolerance unrelated to the *CORs* expression.

Analysis of the freezing sensitive mutants (*sfr1-6*), supports a complex network model for cold acclimation: most of them retain a 50% acclimation ability in respect of the wild type suggesting that each mutation blocks one signalling pathway and is still able to partially acclimate through pathways unaffected by mutation.

4.1. The CBF/DREB1 regulatory pathway

Functional analyses have demonstrated the transcriptional cold induction of the several *COR* gene promoters (COR15a, COR6.6 and COR78 of Arabidopsis). Yamaghuchi-Shinozaki and Shinozaki identify the cis-regulatory sequence responsible for cold-induced transcriptional regulation consisting of a nine base pair consensus containing a 5 bp core sequence (CCGAC) named C-repeat (CRT). These elements present in all the COR promoter regions, are named either Drought Responsive Elements (DREs) or Low Temperature Responsive Elements (LTREs). DRE/LTREs stimulates gene expression in response to cold, high salinity and drought, but not in response to the exogenous application of ABA [61-66].

Using the yeast one hybrid system, the DRE/ CRT elements have been used as baits to isolate DRE/CTR binding proteins. The five different isolated DRE binding proteins have been grouped in two classes: DREB1 and DREB2 [67]. All of them bind specifically to the DRE/CRT and transcriptionally activate the expression of the *COR* genes (Figure 2).

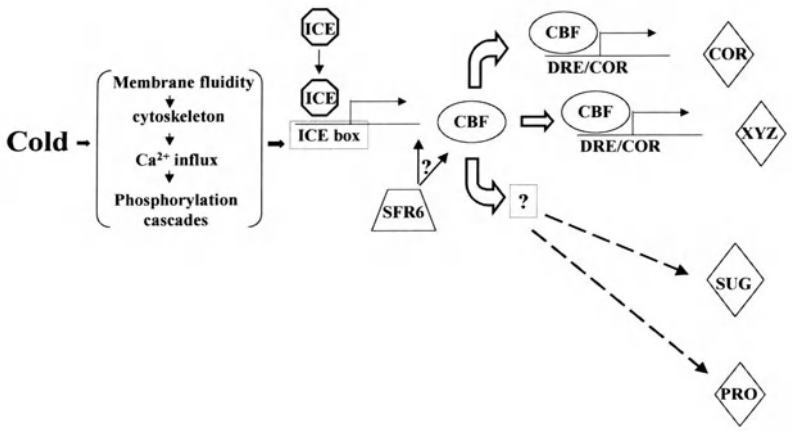

Figure 2. Cold signal transduction pathway and COR regulon induction.

The DREBs aminoacid sequences show a conserved 60 aa DNA binding motif, AP2 domain, present in a large number of plant transcriptional factors (such as Apetala2, Aintegumenta, Tiny, EREBP and several others). It has been suggested that the AP2 containing proteins constitute a super family of DNA binding proteins that recognize a family of cis-acting elements, sharing the common core CCG. Differences in the sequences around the CCG core determine the specific AP2 member(s) able to bind and activate specific downstream genes.

The DREB1 proteins are also called CBF (CRT Binding Factor); the three DREB1/CBF encoding genes are present in tandem on Arabidopsis chromosome 4, in the order DREB1B (CBF1), DREB1A (CBF3) and DREB1C (CBF2); their expression is specifically cold induced [68].

The expression of the genes encoding the two DREB2 proteins, DREB2A and DREB2B, are specifically drought induced and seem to be post-translationally regulated [67].

Overexpression of CBFI/DREB1b or CBF3/DREB1a in transgenic *Arabidopsis* plants induces the expression of the entire battery of COR genes at warm temperatures [69-71]. Moreover, freezing tolerance in absence of cold acclimation is strongly improved.

In wild type *Arabidopsis* plants, CBF3 transcripts are present at very low levels also after several days of cold acclimation, whereas transgenic plants, expressing CBF3 under the CaMV35S constitutive promoter, display high levels of CBF3 transcript. As a consequence, this results in a constitutive over induction of up to 5 fold COR polypeptides with respect to fully acclimated wild type plants. The authors report that CBF3 overexpression in non acclimated plants causes an improvement of the EL_{50} value (temperature causing a 50% ion leakage) of approximately 3.5 °C. Untransformed plants in fact show an EL_{50} value of approximately -4.5, whereas transformed plants had an EL_{50} value of approximately -8. Moreover, after seven days of cold acclimated at 5°C, wild type and CBF3 transgenic plants show an EL_{50} value of -6 and -11 °C (or lower), respectively.

Overexpression of CBF3 transcription factor in transgenic *Arabidopsis* plants results in multiple biochemical changes associated with cold acclimation, namely COR expression induction as well as increased proline and soluble sugars contents. These results suggest a role of CBF3 role in the integration and activation of multiple components of the cold acclimation response.

However, the ability of transgenic plants to develop greater levels of cold tolerance after acclimation suggests that CBF3 activates actually a subset of the total cold acclimation response [69, 71].

Plants overexpressing CBF3/DREB1a, and consequently the *COR* genes, are more tolerant also to dehydration stress caused by either salt and drought. The biological ratio of these results is evident. As temperature drops below freezing point, ice formation in the extracellular spaces causes movement of the water from inside the cell to outside, owing to the low ice chemical potential with respect to the water. Water continues to flow from the cells to the extracellular space up to the equilibrium of chemical potential. For instance, at the freezing temperature of -10°C

more than the 90% of the osmotically active water will flow out of the cells, resulting in severe osmotic and dehydration stresses [67, 71].

Actually, the CBF/DREB1 genes are expressed specifically under low temperature conditions and are not responsive to dehydration; nevertheless the *COR* genes are highly expressed also in response to dehydration caused by salt and drought. As reported above, in this case the *CORs* activation is mediated by the DREB2 responsive to these stresses.

Thus by overexpressing a single gene, it is possible to improve tolerance to several stresses at an extent well beyond that achieved naturally by acclimation. This raises the question of why in plants *CBF* is not a housekeeping gene or at least why acclimation does not lead to the maximum tolerance physiologically possible. The more likely answer is that cold and freezing tolerance (as all stress tolerance) mechanisms have evolved pushed by two different plant needs: to survive in stress conditions and be competitive enough also in favourable growth conditions. Consistent with this hypothesis, transgenic plants overexpressing CBF3/DREB1A have a severely compromised growth and development even in the optimal growth conditions of the experimentally controlled environments [67, 71].

This negative effect would make an applicative use of CBF on crops impracticable. However, Shinozaki's group overcame, at least partially, this problem: they took advantage of a cold inducible COR gene promoter and obtained transformed *Arabidopsis* plants expressing CBF3/DREB1A coding region under the *rd29A* promoter (rd29A::DREB1A). rd29A::DREB1A transformed plants, although retaining a slight growth retardation compared to the untransformed wild type plants, have a greatly improved appearance under experimentally benign control conditions [69, 72].

Although the mechanism whereby the CBF/DREB1 genes are activated by low temperature is not yet known, it appears clear that it involves cold-responsive promoters, not subject to autoregulation.

The transcript levels for the three *CBF* genes increase in only 15 minutes of low temperature treatment and reach their maximum in two hours [67, 73]. This induction, at least in part, is caused by transcriptional activation as demonstrated by the induction, at low temperature, of hybrid genes containing the CBF/DREB1 promoter fused to reporter genes [74]. The absence in the CBF/DREB1 promoter of the DRE/CTR sequence and the lack of induction of CBF3 transcripts in CBF1 transformed plants pooled out a possible transcriptional autoregulation of the CBF/DREB1 genes.

Taking into account all these data, Gilmour et al. [73] named ICE (Inducer of CBF Expression) the putative transcriptional factor responsible of their transcriptional activation. They suggest ICE to be constitutively expressed and kept at warm temperature in an inactive state, either because it is sequestered in the cytoplasm or is unable to bind DNA and/or activate transcription. Thus the *COR* genes induction would involve a two steps cascade of transcriptional activators. Upon exposition to low temperature, modification of either ICE or of an associate protein, would activate ICE, and thus allow induction of CBF/DREB1 expression. In turn, CBF/DREB1 will transcriptionally activate the *COR* genes.

As noted by Gilmour et al., ICE may regulate as well the expression of other genes involved in cold acclimation and unrelated to the CBF/DREB1 regulon [73].

4.2. Conservation of the CBF/DREB1 regulatory pathway

Up to now, most of the experimental research on the cold signal transduction pathway has been performed in *A. thaliana* and few data on the presence or absence of CBF pathway in other species are available. Nevertheless, they support the idea that the CBF/DREB1 regulatory pathway is conserved. In particular, Singh et al. [63] reported that the cold induction of the *BN115* gene (an ortholog of Arabidopsis *COR15a*) is mediated by the presence of a CRT/DRE sequence in its promoter. Similar results have been reported by Sarhan et al. on the *wcs120* cold inducible gene of wheat [75, 76]. These authors also showed that the *wsc120* promoter is cold inducible in both several monocotyledonous (barley, rye and rice) as well as dicotyledonous (alfalfa, Brassica and cucumber) plants. For unknown reasons, it is not cold inducible in tomato and pepper [75].

Recently, it has been reported that the expression of *A. thaliana* CBF1 in tomato, is able to drive tolerance to cold, drought and oxidative stress but not to freezing. Tomato is a subtropical plant that does not cold acclimate and tomato COR homologous genes have not been reported. Authors suggest that the cold tolerance of transgenic tomato plants depends on the high level of proline and catalase [77].

4.3. A Myb transcription factor acting in the ABA-independent cold-acclimation pathway

Another transcriptional factor encoding a gene involved in cold acclimation is the rice *Osmyb4* gene. Its expression, detected at low level in rice seedlings grown for three days at 29°C, is strongly induced by treatments at 4°C [78].

The induction of *Osmyb4* expression at not-freezing temperatures (from 15 to 4°C) and its ability to transactivate, in transient expression experiments, the specifically cold-induced *ScD9 SAD* promoter of *S. commersonii* (encoding for the D9 desaturase) suggest its involvement in cold acclimation.

According to an *Osmyb4* involvement in acclimation process, transgenic overexpressing plants showed increase in freezing tolerance, both as PSII and membrane stability.

It may be supposed that the effect of Osmyb4 overexpression on membrane and PSII stability depends on the direct transactivation of genes encoding for desaturase and, as a consequence, on a different fatty acid composition of cellular and organelle membranes.

Transgenic plants overexpressing Myb4 showed a strongly increased cold and freezing tolerance also as whole plants. In fact, while wild type plants in soil were severely damaged after ten days a 10 °C and did not survive at 24 hours of freezing conditions (-6 °C), transgenic plants were undamaged by both cold and freezing treatments.

Myb4 expression in transgenic *Arabidopsis* plants results in multiple biochemical changes, commonly observed in plants during cold acclimation. In particular, we found constitutive changes in the level of COR15a, COR78, PAL2 and proline [79]. Expression of COR genes and proline has been shown to be associated with freezing tolerance in *Arabidopsis* [67, 69, 70]. So the enhancement of stress tolerance in *Osmyb4* transgenics may be at least partially due to the constitutive expression of these genes. Cold stress induces also genes that are linked to pathogen resistance as the *PAL2* gene [80]. Myb4 is able to induce a constitutive expression of the *PAL2* gene; experiments of transient expression in tobacco protoplasts suggest that this effect is due to direct transactivation. The involvement of *Osmyb4* in the regulation of genes acting in different aspects of cold tolerance leads to propose that: a) *Osmyb4* integrates the activation of multiple components of the cold stress response, b) a heterologous transcriptional factor from rice is able to induce a strong freezing tolerance in *Arabidopsis.* This an interesting result because rice is a chilling-sensitive species and does not acclimate like *Arabidopsis thaliana.* As above mentioned, it has been reported that the expression of *A. thaliana* CBF in the chilling sensitive tomato is able to drive chilling, but not freezing tolerance [77]. Altogether these data suggest that the cold signaling pathway is at least in part conserved between hardy and not-hardy species. However, the degree of tolerance seems to depend on the expression of downstream genes which specifically evolved in the different species.

4.4. The ABA role

In several plant species, both a transient increase in the ABA levels in response to low temperatures as well as enhanced freezing tolerance mediated by application of exogenous ABA have been reported [81 and references therein]. Moreover, Chen and colleagues [82] report that ABA levels increase transiently in *Solanum commersonii* able to cold acclimate, but not in *Solanum tuberosum*, which is unable to acclimate. The inability to cold acclimate of Arabidopsis mutants affecting the synthesis of (*aba1*) or the sensitivity to (*abi1*) the ABA has also been reported. All these results led to suppose that ABA has a key role in activating cold acclimation [83-85]. However, in *Arabidopsis*, the ABA levels transiently increase, peak at 24 hours and return to normal levels in two days, while freezing tolerance increases with longer cold acclimation (up to seven days) and remains elevated for several weeks [86]. Moreover, the *aba1* and *abi1* mutations have pleiotropic effects, display a wilted phenotype and have reduced vigour, suggesting a different interpretation of their inability to cold acclimate. Decreased freezing tolerance might be an indirect effect due to the ABA key roles in plants growth and development, instead of a direct consequence due to a fundamental role of ABA in acclimation.

However, exogenous application of ABA does activate cold acclimation. These results may be explained by the above mentioned role of CRT/DRE regulon expression in cold and freezing tolerance. Significantly, the COR gene family (and possibly other CBF-regulated genes not yet identified) are highly expressed in response to exogenous ABA. It is to be noted that *aba* and *abi* mutations abolish the

ABA-mediated induction of the COR genes expression, but do not affect their expression at low temperatures. Actually, in the COR genes promoters an ABRE consensus is present, permitting their transcriptional activation by b-ZIPs, involved in the ABA-mediated pathway of stress response [83, 87].

Some *Arabidopsis* mutants affect COR78 expression (positively or negatively) in response to both cold and ABA, suggesting that the 'ABA-dependent' and the 'ABA-independent' pathways of cold acclimation are not completely independent, but "cross-talk" in some steps [88].

According to this model, in soybean, a gene has been identified, *scof1*, whose expression is specifically induced by cold and ABA, but not by drought or high salinity, encoding a zinc finger transcriptional factor (Figure 3; [89]).

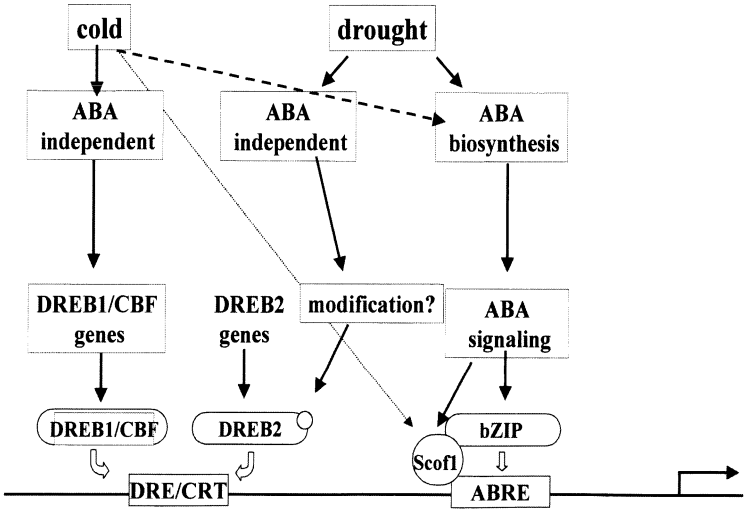

Figure 3. Multiple signalling pathway mediate COR transcription induction.

Constitutive expression of Scof1 induces both COR genes expression and enhances cold tolerance in non-acclimated Arabidopsis and tobacco transgenic plants. Nevertheless, Scof1 does not bind directly neither the DRE nor the ABRE elements. However, it greatly increases the ability of a soybean bZip (SGBF1) to bind *in vitro* the ABRE element and to transactivate transiently an ABRE containing promoter. Kim et al., taking into account as well the ability of SCOF1 to interact physically with SGBF1 (demonstrated with the two hybrid system), suggest that it may function as a positive regulator of the ABRE-mediated COR genes expression [89].

The cold regulated expression of some genes as *Rab18* and *LTI65*, according to the presence in their promoter of ABRE, but not DRE elements, is severely impaired in *aba2* or *abi1* mutants and appears to be directly mediated by ABA, through the

action of bZip factors [87, 90-93]. However, the induction of both *Rab18* and *LTI65* in response to low temperature is very weak if compared with that induced by drought or exogenous ABA addition. These results are not surprising, according to the much higher level of ABA induced by drought with respect to that induced by cold stress [86].

In conclusion, up to now, although the available evidences suggest that ABA has a relatively minor role in cold acclimation, a definitive response to the question of the direct importance of ABA in cold acclimation is unresolved.

4.5. Other genes affecting cold acclimation

Warren et al., using chemical mutagenesis, identified seven Arabidopsis mutants, corresponding to five genes, unable to cold acclimation. Due to their consequent sensitivity to freezing, they were designed *SFR1, 2, 4, 5-1, 5-2* and *6* (Sensitivity to Freezing; 94, 95). These mutations have no obvious negative effects, neither under warm control conditions, nor during the cold treatment.

Instead, all the mutations affect the freezing tolerance measured after a 24 h treatment at -6°C, following two weeks of cold-acclimation at 4°C. In particular, *sfr1* affects only the young leaves, *sfr2, 4 and 5* affect all leaves at same extent, and *sfr6* has its most strict effect on young leaves, but affects also the other leaves. As indicated by the ion leakage experiments, all mutations impair the cryostability of the plasma membrane and for all, but not for *sfr2*, the severity of the freezing damages in the whole plants correlate with the results of the electrolyte leakage test. *Sfr* genes have not yet been isolated, nevertheless, some physiological characterizations have been performed.

For instance, *Sfr4* results both in reduced accumulation of sucrose, glucose and anthocyanin and lowered levels of unsaturated fatty acids (18/1 and 18:2). Given the role of sucrose and compatible solutes as osmolytes/osmoprotectants and that of fatty acid composition in the membrane stability, it is reasonable to deduce that the *sfr4* freezing sensitivity depends on these alterations. To date, the molecular mechanism that subtends to the pleiotropic *sfr4* phenotype is unknown [94, 95].

Sfr1, 2, 4, 5 mutations do not affect the COR genes expression after acclimation, while for *sfr6*, a role has been described in regulating the expression of the CBF regulon [96]. *Sfr6* plants are deficient in cold inducible expression of several, if not all, cold induced genes containing a CRT/DRE motif in their promoters (Kin1, cor15a, Lt178). *sfr6* plants are unable to induce expression of these genes also in response to ABA or osmotic stress.

In contrast, *sfr6* mutation does not affect the cold inducible expression of genes lacking the DRE/CRT motif (such as *CBF1, 2, 3* and *ATP5CS1*). It has been suggested that *sfr6* may act somewhere between the *CBFs* transcription and the induction of *CBF* regulon. However, the finding that expression of *COR* genes is affected also in response to osmotic stress and exogenous ABA addiction, suggests a more general role: Sfr6 may be involved in post-transcriptional and/or post-translation activation of several transcription factors, or it may code for a co-activator involved in *COR* genes transcription [96].

Between the mutants affecting freezing tolerance, *Hos1* and *Hos2* are of particular interest [97, 98]. Both of them show a super induction of cold responsive genes induced specifically by cold treatment, while responses to both high salinity and ABA are not affected. They also share an unexpected freezing sensitivity when compared to wild type plants. Nevertheless, *Hos1* plants, after two days of cold acclimation, acquired the same grade of freezing tolerance as do the wild type, while *Hos2* mutants are less capable of acclimation. They also differ for the constitutive vernalized phenotype of *Hos1* and the normal vernalization response of *Hos2*. Both *Hos1* and *Hos2* have been proposed to have a role as negative regulators of cold signal transduction and vernalization (*Hos1*) or acclimation (*Hos2*) [97, 98].

Hos1 has been cloned and encodes a RING finger protein that localizes in the nucleus in response to low temperature [99].

Xin and Browse pay their attention to constitutively freezing tolerant Arabidopsis mutants; i.e. mutants that were more freezing tolerant than wild type without cold acclimation. This resulted in the identification of *eskimo1*, a gene with a major effect on freezing tolerance [100]. *Eskimo1* increases freezing tolerance of both non acclimated Arabidopsis plants (LT50 -10.6 °C compared to -5.5 °C of the wild type) and cold acclimated (LT50 -14,8 °C compared to -12.6 °C of the w.t). The basis of this strong tolerance seems to depend on a constitutive increase in proline and sugar contents (30 and 3 fold higher than in wild type, respectively) and in expression of Rab18 and LeaII. The *eskimo1* mutation does not seem to affect the expression of the COR genes, suggesting that it acts in a different pathway. The fact that two independently isolated mutations are recessive suggests that Eskimo1 may act as a negative regulator (Figure 4).

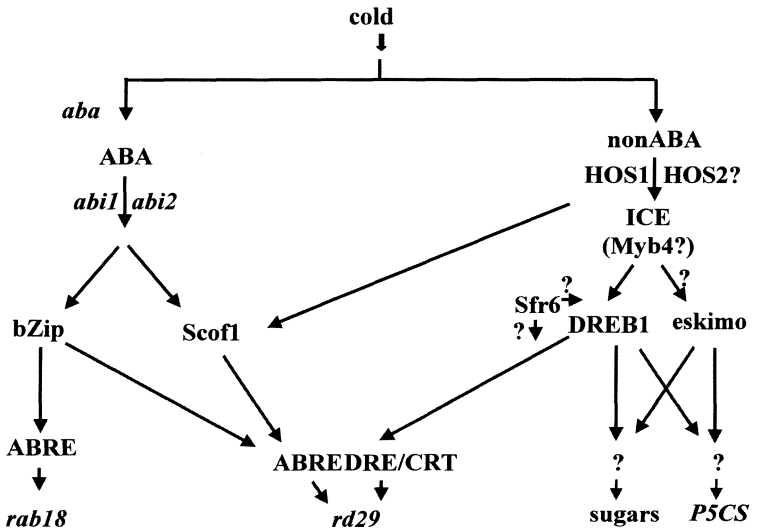

Figure 4. A schematic outline of genes affecting cold tolerance.

4.6. Trascriptome changes in response to cold stress

A first microarray analysis, to compare changes in transcript levels in response to dehydration, cold and CBF3 overexpression, was performed on 1300 Arabidopsis EST. Of the 19 cold induced genes identified, 12 are CBF3 inducible. On the basis of these results it may be hypothesized that almost 60% of cold induced genes are transcriptionally regulated by CBF1; actually, these data demonstrate that one or more additional pathways determining cold response do exist [72]. More recently, several microarray-based studies, in *A. thaliana*, demonstrated the highly complex cross-talk between different biotic and abiotic stress signalling pathways. Hundreds of potentially important genes up and down regulated upon exposure of plants to stress have been identified and genes belonging to overlapping or specific responses have been described [72, 80, 101-103].

In particular, with a 8000 genes microarray analysis, the effect of cold stress on wild type plants was compared with that of the CBF1, CBF2 or CBF3 constitutive expression. About 300 genes have been identified as being cold responsive, 48 of these encode known or putative transcription factors. Only the 12% of the genes identified are surely regulated by CBFs and about 30% of them are definitely CBF independent. Fifteen of them encode for transcriptional factors suggesting that several independent pathway play an integral role in cold acclimation [80]. CBFs repress expression of some cold repressed genes, indicating that in addition to gene induction also gene repression may act a significant role in cold response.

Statistical analysis of the promoter regions of cold induced genes show unambiguous enrichment of known conserved transcription factor binding sites, such us DRE- and ABRE- like elements [101].

Finally, it have been observed that the expression of hundreds of the known circadian controlled genes is cold affected, supporting the idea that any important function of the circadian clock is to anticipate predictable stresses, such us the nocturnal cold [103].

Up to now, there is not enough information to understand if the multibranched pathways respond to a single cold sensor or if different sensors activate different parallel pathways.

5. POST-TRANSCRIPTIONAL REGULATION

As mentioned above, cold, as other stresses, may regulate gene expression at several levels: transcriptional, post-transcriptional, translational and post-translational. The presence of a post transcriptional mechanism affecting the abundance of specific mRNAs has been reported in several plant species. For instance, nuclear run-on analysis of nine barley cold-induced genes showed that three of them are primarily post-transcriptionally regulated [104, 105]. The involvement of post-transcriptional cold-induced regulation on the specific mRNAs' amounts has been reported in several other species, such as Arabidopsis and alfalfa [106, 107].

The isolation of genes encoding RNA-binding proteins suggests that regulated splicing may be one of the post-transcriptional mechanisms involved in cold

acclimation [108, 109]. These genes encode proteins of 17000 M_r nearly, containing in their amino-terminal portion a RNA-binding domain of about 80 amino acids, found in many RNA binding proteins localized in nucleus, cytoplasm and organelles [110]. The carboxy-terminal region also contains a conserved domain consisting of repeating glycine residues interspersed with tyrosines and arginines [108, 109].

Genes encoding glycine rich-RNA binding proteins (GR-RNPs) induced by stresses and ABA have been isolated from several plants [111-114]. The two-domain structure of plant GR-RNPs is similar to that of the group A1 and A2/B1 group of animal proteins binding nuclear heterogeneous RNA, (hnRNP). These animal proteins are localized in the nucleus and are implicated in regulated pre-RNA splicing [110].

6. EXPRESSION OF SPECIFIC GENES AND ACHIEVEMENT OF A NEW HOMEOSTATIC CELLULAR CONDITION

Under cold stress conditions, a number of specific genes have been found to be up-regulated. Many of them have been isolated and characterised in a wide range of plant species and by a variety of different approaches. Although, as reported above, some of them are involved in the cold signal transduction pathway, the most encode polypeptides constituting the final target of stress induced molecular events and have a direct role in the cold and freezing tolerance. Most of them are not unique to cold, but are also induced by drought, salt and ABA-treatment. The biological meaning of the partial common response to different stresses lies in the common consequences induced by the different abiotic stresses.

Following the initial period in which research was essentially concerned about the identification and characterisation of stress responsive genes, now focus is moving towards unravelling the functions of their gene products. Nevertheless most of these polypeptides still have unknown function, and some important questions remains unravelled:

- are any of these genes more relevant than others in warranting the adaptive response?
- where can we put limits between proteins that are related to cold adaptation and those that have merely accumulated as a consequence of this stress?

Although for many of the downstream acting polypeptides induced by cold stress the cellular function is not yet clear, nevertheless it is possible to identify four main functions induced by acclimation: avoidance, protection, reparation, protection against other stresses. Each of these functions is achieved by the concerted action of several classes of polypeptides (Figure 5).

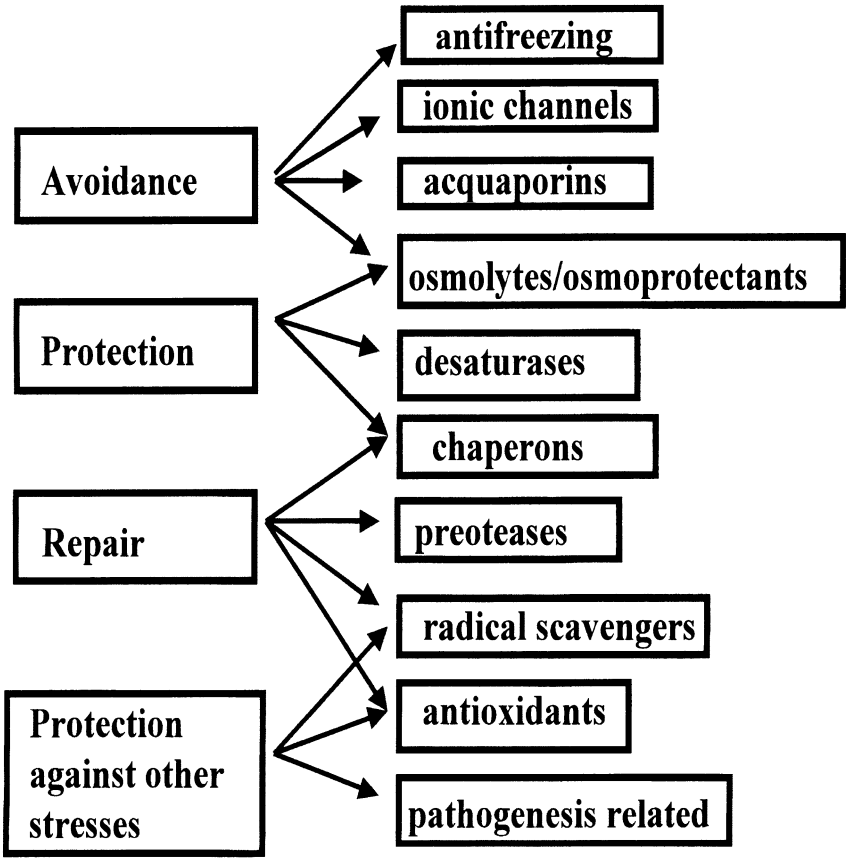

Figure 5. Main functions induced by cold acclimation.

A number of genes proposed as relevant in several of the above mentioned functions encode the so called Late Embryogenesis Abundant (LEA) proteins that accumulate also in response to cellular dehydration and by ABA exogenous-treatments. These proteins are highly conserved across distantly related organisms ranging from green algae [115] to higher plants [116] (Table 1).

Table 1. Late Embryogenesis Abundant (LEA) proteins in distantly related organisms ranging from green algae to higher plants

Plant	Gene name	gene product	reference
Arabidopsis thaliana	COR15a	LEA	117
Arabidopsis thaliana	COR6.6kin1	LEA	118,119
Arabidopsis thaliana	COR 78RD29A	LEA	62, 90
Arabidopsis thaliana	kin2	LEA	118
Arabidopsis thaliana	LTI140	LEA	120
Arabidopsis thaliana	LT130 XERO2	LEA	121
Arabidopsis thaliana	RC12A	LEA	122
Arabidopsis thaliana	ERD14	LEA	123
Brassica napus	BN115	LEA	124
Brassica napus	BN28	LEA	125
Hordeum vulgare	Dhn5	LEA	126
Hordeum vulgare	HVA1	LEA	127
Hordeum vulgare	COR14	LEA	128
Hordeum vulgare	blt101	LEA	129
Hordeum vulgare	blt14	LEA	105
Hordeum vulgare	paf93	LEA	130
Hordeum vulgare	COR75		131
Medicago sativa	CORa	LEA	132
Medicago sativa	ESIPa	LEA	133
Prunus persica	PCA60	LEA	134
Solanum tuberosum	ci7	LEA	135
Spinaci oleracea	CAP85	LEA	136
Triticum aestivum	wcs120	LEA	137,138
Triticum aestivum	wcor410	LEA	139
Triticum aestivum	COR39	LEA	140
Triticum aestivum	COR47	LEA	141,142

It has been proposed that they may act as chaperones by binding, through their polar groups, to macromolecules, and to polar membrane head groups. In this way, they will replace the hydration shell and provide the hydrophilic interactions necessary for stability [143].

For one of the LEA protein families, dehydrins, some cellular functions have been reported. Cryoprotective activity on enzyme function has been demonstrated with the *Prunus persica* dehydrin [144] and the birch (*Betula pubescens*) RAB-16-like dehydrins [145]. Evidence in support of a structural role comes from Danyluk et al. [146] who showed that during freezing acclimation in wheat the acidic dehydrins accumulate as peripheral proteins near the plasma membrane in the vascular transition area, a region where freeze-induced dehydration is particularly severe. An antifreeze activity has been reported for the peach (*Prunus persica*) PCA60 dehydrin

that is able to alter the shape of the ice crystals, which could aid in reducing the freezing damage to the cells [144].

A highly studied LEA cold induced gene family is the COR family. The appearance of COR polypeptides coincides with the onset of freezing tolerance, their synthesis remain high during all stress periods and then declines after rewarming. One of the better studied genes of this group is the stromal polypeptide COR15am, involved in stabilisation of chloroplast membrane [147]. Its constitutive expression in transgenic *Arabidopsis* plants enhances the freezing tolerance both of chloroplasts and protoplasts, but is not sufficient to enhance the freezing tolerance of the whole plant. Only the multiple expression of the entire battery of COR genes, accomplished by overexpressing the transcriptional activator CBF1, is able to induce an increased freezing tolerance in *Arabidopsis* plants [148]. These results underline the more effective action of coordinated expression of several cold induced genes. Moreover, they suggest that the more reliable way to obtain stress tolerant plants is the overexpression of the upstream acting genes, such as transcriptional factors involved in the tolerance achievement.

Cold also induces synthesis and accumulation of other downstream acting polypeptides potentially involved in tolerance, but structurally unrelated to LEA proteins (Tab. 2). Under cold stress conditions, plants maintain a proper physical state and fluidity of cellular and subcellular membranes with changes in lipid composition towards an increase in unsaturated fatty acids in membrane lipids. These changes are catalysed by fatty acid desaturases, that modifying the membrane structure, protect the cell against the cold induced damages. Cold-induced fatty acid desaturases are for instance the *Arabidopsis* FAD8 [155] and FAD7 (a chloroplast ω-3 desaturase); synthesis *de novo* of the D12oleoyl- desaturase in response to low temperatures has been demonstrated in cold-acclimated *Solanum commersonii*, a cold tolerant wild potato but not in *Solanum tuberosum*, the cultivated species known to be unable to cold-acclimate. This finding was consistent with the increase in linoleic acid (18:2) of plasma membrane reported for *S. commersonii* upon cold-acclimation [176]. The importance of the membrane fatty acid composition in cold acclimation has been clearly demonstrated by the finding that the Arabidopsis FAD7 gene overexpression enhances cold tolerance in tobacco transgenic plants [177]. Another class of cold-induced polypeptides, involved in structural changes, is that responsible for cell wall modifications, as a xyloglucan endotransglycosylase [158]. Probably the induction of these proteins is due to the necessity of cold stressed plants to resist to the cell collapse induced by water loss.

Table 2. Induced polypeptides potentially involved in cold tolerance

Plant	Gene name	Gene product	reference
Arabidopsis thaliana	ADH	alcohol dehydrogenase	149
Arabidopsis thaliana		b-tubulin	150
Arabidopsis thaliana	CCR1	RNA-binding protein	151
Arabidopsis thaliana	ERD6	Sugar transporter	152

Table 2. Continued

Plant	Gene name	Gene product	reference
Arabidopsis thaliana		Ascorbate peroxidase	153
Arabidopsis thaliana		galactinol synthase	154
Arabidopsis thaliana	FAD8	Fatty acid desaturase	155
Arabidopsis thaliana	ALA1	aminophospholipid translocase	156
Arabidopsis thaliana	PLC1	Phosphatidylinositol- specific phospholipase C	157
Arabidopsis thaliana	TCH4	Xyloglucan endotransglycosylase	42,158
Arabidopsis thaliana	TCH2	Calmodulin related protein	159
Arabidopsis thaliana	TCH3	Calmodulin related protein	42
Hordeum vulgare	blt4	non specific Lipid transfer protein	160
Hordeum vulgare	blt4.1	Lipid transfer protein	161
Maize		non-specific lipid transfer protein	162
Maize		w-3 fatty acid desaturase	163
Maize		elongation factor 1a	164
Medicago sativa	CIC	Cell wall protein	165
Spinacia oleracea	HS704		166
Spinacia oleracea	Hsc70-12	Heat shock protein	167
Solanum tuberosum		Sucrose phosphate synthase	168
Spinacia oleracea		Sucrose phosphate synthase	169
Spinacia oleracea	antifreeze proteins		170-172
Brassica napus	Hsp90	Molecular chaperon	173
Brassica napus	BN59	ATP-ase	125
Brassica napus	BnPEPCK	PEP carboxykinase	174
Brassica napus	btg-26	Aldehyde dehydrogenase	175

A way to avoid one of the most dramatic freezing injuries is to neutralize ice nucleators and so inhibit ice crystals growth and recrystalization. This is achieved by an overspread class of proteins called Anti Freezing Proteins (AFPs). Although at least 30 species of angiosperms are capable of antifreeze AFPs-mediated activity after acclimation to low temperature, most of agricultural crops do not do so. Plant AFPs are similar to those found within the animal kingdom and appear to behave similarly at freezing temperatures. Between these genes, for instance, there are spinach hsp70 [166] and *Brassica napus* hsp90 genes [173]. Intriguingly, some of these AFPs have strong sequence homology to the pathogenesis-related proteins [170]. On the other hand, a number of stress related genes are better known as

pathogenesis related genes. This is for instance the case of osmotin and of non-specific lipid transfer proteins. This is a further protective mechanism, taking into account the increased susceptibility to pathogens driven by a stress condition.

On the other hand, the sequence homology may not correspond to a function identity as showed by the data on *Brassica oleracea* cryoprotectin, a cold inducible protein with cryoprotecting properties. Sequencing of cryoprotectin showed homology to the WAX9 proteins, belonging to the class of aspecific lipid transfer proteins. However, cryoprotectin is structurally and functionally different from WAX9, and, while WAX9 has lipid transfer activity for phosphatydilcholine, but no cryoprotective activity, cryoprotectin has cryoprotective, but no lipid transfer activity [178]. Low temperatures lead to oxidative stress in plants through freeze-induced production of reactive oxygen species that contribute to cellular damage. Therefore, it is important that the activities of antioxidant enzymes (as ascorbate peroxidase, glutathione reductase, superoxide dismutase, etc.) taking part in the scavenging of free radicals, as well as the levels of antioxidant compounds, are induced by cold [179,180].

As mentioned above, one of the consequences of freezing is cellular dehydration and because one of the most effective responses to drought is solute accumulation, it is not surprising that in cold stress a class of enzymes that contribute to solute accumulation is a up-regulated. Among them, sucrose phosphate synthase, galactinol synthase and Δ(1) pyrroline-5-carboxylate synthase have been well characterized [181].

Cold-induced chaperonines, stabilizing proteins against denaturation, have both protective and repair functions. Molecular chaperons are for instance the spinach HSP70 and the *Brassica napus* HSP80. A very interesting member of this class is the strongly cold up-regulated cyclophilin that repair *trans* and *cis* isomerisation of peptidylpropyl bonds [182].

Many other cold induced genes produce proteins with a still unknown function.

In general the overall picture emerging from data on cold-stimulated downstream acting proteins is:

- Cold induces a lot of different stress-related functions in the plant cell.
- These functions are similar in different plant species and accomplished by the same proteins: this indicates a general uniformity of cold-induced responses amongst unrelated plant species.
- Up to now, particular functional genes responsible for cold tolerance, present in cold-tolerant species and absent in non-sensitive species, have not be identified.
- Some stress-responsive polypeptides, but not all, are not unique to cold, but are also induced by other stresses. Nevertheless, different mechanisms regulate their expression in different stresses.

7. COLD INDUCED OSMOLITES/OSMOPROTECTANTS

Plants, in response to cold as well as to other osmotic stresses, accumulate a range of compatible solutes, such as cerebrosides, free sterols, sterols glucosides, acylated

sterol, glucosides, raffinose, arabinoxylans, sugars. The oligosaccharides raffinose and stachyose are especially associated with cold hardiness, low temperature and dormancy, but sucrose also enhances cold hardiness and desiccation tolerance of buds in woody plants [183].

In addition, plants accumulate other solutes, such as proline [184] or glutamic acid [185], when exposed to low temperatures or during natural hardening.

These compounds, which are subsequently degraded after stress relief, are collectively referred to as osmolytes, osmoprotectants or compatible solutes, the latter because their accumulation at high concentration in plant cells does not apparently disturb biochemical functions.

The role of osmolytes in stressed plants has been essentially attributed to antifreezing properties and to maintaining turgor through osmotic retention. In fact, osmolytes have been shown to mediate several aspects of stress tolerance: a) increasing the osmotic cellular concentration, depress the freezing point of the tissue; b) some of them, as arabynoxylans, reduce ice formation and expansion; c) they preserve membrane integrity, retarding membrane fusion, phase transition and phase separation, in such a way that the physical properties of cold-stressed membranes resemble those of normal membranes; d) through the enhancement of water binding to biopolymers, they also preserve the integrity of macromolecules, protein folding and enzyme activities.

In addition to these primary osmotic functions, osmolytes have been proposed to play further and more subtle functions, as antioxidant and hydroxyradical scavenging functions [186].

Other recent results suggest that osmolytes may act also as regulatory molecules. For example, myo-inositol may: 1) act as signal to the root of the photosynthetic activity in leaves; 2) sustain membrane biosynthesis; 3) facilitate long-distance sodium transport [187]. In this context, osmolyte flux (as metabolic signals among tissues) could be more important than cellular osmolyte accumulation.

At present, research on osmolytes is presently aimed at clarifying their role and mechanism of action and understanding the control of genes involved in their biosynthesis and catabolism.

8. CONCLUSION AND PERSPECTIVES

Plant physiologists and plant molecular biologists have always been interested in mechanisms involved in plant tolerance to cold and other stresses, for both the theoretical interests and the applicative importance of this subject. Nevertheless, up to less then 10 years ago, most of data regarded the downstream acting genes and no information on the upstream events of the cascade of molecular events following the cold perception and resulting in cold acclimation were available. In the last years, many steps of the transduction pathway have been investigated: several mutants affecting early events in cold acclimation have been identified and isolated, the regions in the promoter of cold induced genes responsible for their regulated expression have been identified and transcriptional factors belonging to three

different classes involved in cold-responsive transcriptional regulation have been isolated.

Despite the massive quantity of information, these have not yet led to a comprehensive scheme of this multi-step event: only some steps of the complex multibranched and cross-talking pathway have been clarified; although very attractive candidates have been proposed, there is not yet a sure identification of one cold sensor; for many of the downstream acting genes no clear demonstration of the cellular function has been obtained, etc. However, the powerful new proteomics and genomics approaches will permit in the next years to compare at the same time complex differences between plants. In particular, it will be possible to compare the broad spectrum of molecular differences of wild type plants with respect to mutants in genes affecting cold tolerance or to transgenic plants overexpressing a transcriptional factors inducing a great extent of tolerance. It may be supposed that in a few years these approaches will clarify most of the unidentified steps in cold acclimation. This will also allow for the rational design of plants with increased cold and freezing tolerance.

REFERENCES

1. Saxa, H., Cannell, M.G.R., Johnson, O., Ryan, M.G., and Vourtilis, G. (2001). *New Phytol.*, 149, 369.
2. Steponkus, P.L. (1992). Adv. Low-Temp. Biol., 1, IX-XI.
3. Wilson, J.H. (1984). *Outlook Agric.*, 14, 197.
4. Wanner, L.A., and Junttila, O. (1999). *Plant Physiol.*, 120, 391.
5. Mantyla, E., Lang, V., and Palva, E.T. (1995). *Plant Physiol.*, 107, 141.
6. Ryu, S.B., Costa, A., Xin, X., and Li, P.H. (1995). *Plant Cell Physiol.*, 36, 1245.
7. Pearce, R.S. (1999). *Plant Growth Reg.*, 29, 47.
8. Patterson, B.D., Mutton, L., Paull, R.E., and Nguyen, V.Q. (1987). *Plant Cell Environ.*, 10, 363.
9. Zeevart, J.A.D., and Creelman, R.A. (1988). *Plant Mol. Biol.*, 39, 439.
10. Skriver, K. and Mundy, J. (1990). *Plant Cell*, 2, 503.
11. Chandler, P.M., and Robertson, M. (1994). *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 45, 113.
12. Mc Kersie, B.D., Bowley S.R., Harjauto E., and Leprince O. (1996). *Plant Physiol.*, 111, 1177.
13. Crowe, J.H., Hoekstra F.A., and Crowe L.M. (1992). *Annu. Rev. Physiol.*, 54, 579.
14. Uemura, M., and Steponkus, P.L. (1997). Plant Cold Hardiness. Molecular Biology, Biochemistry and Physiology, Plenum, New York.
15. McKersie, B.D., and Bowley, S.R. (1997) Plant cold Hardiness. Molecular Biology, Biochemistry and Physiology, Plenum, New York.
16. Nishida, I., and Murata, N. (1996). *Annu Rev. Plant Physiol. Plant Mol. Biol.*, 47: 541-568.
17. Ishizaki-Nishizawa, O., Fujii, T., Azuma, M., Sekuguchi, K., Murata, N., Ohtani, T., and Toguri, T. (1996). *Nat. Biotechnol.*, 14, 1003.
18. Wolter, F.P., Schmidt, R., and Heinz, E. (1992). *EMBO J.*, 11, 4685.
19. Gomès E., Jacobsen M.K., Axelsen K.B., Geisler M., and Palgren, M.G. (2000). *Plant Cell*, 12, 2441.
20. Vogg, G., Heim, R., Gotschy, B., Beck, E., and Hansen, J. (1998). *Planta*, 204, 201.
21. Hellergren, J., Widell, S., Lundborg, T., and Kylin, A. (1983) *Physiol. Plant.*, 58, 7.
22. Yoshida, S., Hotsubo, K., Kawamura, Y., Murai, M., Arakawa, K., and Takezawa, D. (1999). *J. Plant Res.*, 112, 225.
23. Bonhert, H.J., Nelson, D., and Jensen, R.G. (1995). *Plant Cell*, 7,1099.
24. Walters, E.R., and Schaal, B.A., (1996). *Proc. Natl. Acad. Sci. USA*, 93, 1449.
25. Los, D.A, Ray, M.K., and Murata, N. (1997). *Mol. Microbiol.*, 25, 1167.
26. Vigh, L., Los, D.A., Horvath, I., and Murata, N. (1993). *Proc. Natl. Acad. Sci. USA*, 90, 9090.
27. Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K., and Murata, N. (2000). *EMBO J.*, 19, 1327.

28. Suzuki, Kanesaki, Y., Mikami, K., Kanehisa, M., and Murata, N. (2001). *Mol. Microbiol.*, 40, 235.
29. Parkinson, J.S., and Kofoid, E.C. (1992). *Annu. Rev. Genet.*, 26, 71.
30. Wurgler-Murphy, S.M., and Saito, H. (1997). *Trends Biochem. Sci.*, 22, 172.
31. Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000). *Trends Plant Sci.*, 5, 67.
32. Thomason, P.A., Traynor, D., Cavet, G., Chang, W-T., Harwood, A.J. & Kay, R.R. (1998). *EMBO J.*, 17, 2838.
33. Murata, N., and Los, D.A. (1997). *Plant Physiol.*, 115, 875.
34. Örvar, B.L., Sangwan, V., Omann, F., and Dhindsa, R. (2000). *Plant J.*, 23, 785.
35. Gray, G.R., Chauvin, L.P., Sarhan, F., and Huner, N.P.A. (1997). *Plant Physiol.*, 114, 467.
36. Huner, N.P.A., Oquist, G., and Sarhan, F. (1998). *Trends Plant Sci.*, 3, 224.
37. Guy, C.L., Yelenosky, G., and Sweet, H.C. (1980). *Florida Sci.*, 43, 265.
38. Knight, H. Trewavas, A.J., and Knight, M.R. (1996). *Plant Cell*, 8, 489.
39. Monroy, A.F., and Dhindsa, R.S. (1995). *Plant Cell*, 7, 321.
40. Plieth, C., Hansen, U.P., Knight, H., and Knight, M.R. (1999). *Plant J.*, 18, 491.
41. Kiegle, E., Moore, C.A., Haseloff, J., Tester, M.A., and Knight, M.R. (2000). *Plant J.*, 23, 267.
42. Polisensky, D.H., and Braam, J. (1996). *Plant Physiol.*, 111, 1271.
43. Monroy, A.F., Sarhan, F., and Dhindsa, R.S. (1993). *Plant Physiol.*, 102, 1127.
44. Tahtiharju, S., Sangwan, V., Monroy, A.F., Dhindsa, R.S., and Borg, M. (1997). *Planta*, 203, 442.
45. Ding, J.P., and Pickard, B.G. (1993). *Plant J.*, 3, 713.
46. Mazars, C., Thion, L., Thuleau, P., Graziana, A., Knight, M.R., Moreau, M., and Ranjeva, R. (1997). *Cell Calcium*, 22, 413.
47. Hwang, J.-U., Suh, S., Hanju, Y., Kim, J., and Lee, Y. (1997). *Plant Physiol.*, 115, 335.
48. Morelli, J.K., Zhou, W., Yu, J., Lu, C., and Vayda, M.E. (1998). *Plant Physiol.*, 116, 1227.
49. Sentenac, H., Bonneaud, N., Minet, M., Lacrout, F., Salmon, J-M., Gaymard, F., and Grignon, C. (1992). *Science*, 256, 663.
50. Örvar, B.L., Sangwan, V., Omann, F., and Dhindsa, R.S. (2000). *Plant J.*, 23, 785.
51. Monroy, A.F., Sangwan, V., and Dhindsa, R.S. (1998). *Plant J.*, 13, 653.
52. Holoppa, L.D., and Walker,-Simmons, M.K. (1995). *Plant Physiol.*, 108, 1203.
53. Hong, S.W., Jon, J.H., Kwak, J.M., and Nam, H.G. (1997). *Plant Physiol.*, 113, 1203.
54. Jarillo, J.A., Capel, J., Leyva, A., Martinez-Zapater, J.M., and Salinas, J. (1994). *Plant. Mol. Biol.*, 25, 693.
55. Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S., and Hirt, H. (1996). *Proc. Natl. Acad. Sci. USA*, 93, 11274.
56. Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., (1996). *Proc. Natl. Acad. Sci. USA*, 93, 765.
57. Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). *FEBS Lett.*, 427, 175-178.
58. Saijo, Y., Hato, S., and Izui, K, (1998). *Plant Cell Physiol.* Suppl., 39, 127.
59. Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., and Izui, K. (2000). *Plant J.*, 23, 319.
60. Martín, M.L., and Busconi, L. (2001). *Plant Physiol.*, 125, 1442.
61. Baker, S.S., Wilhelm, K.S., and Thomashow, M.F., (1994). *Plant Mol. Biol.*, 24, 701.
62. Horvath, D.P., McLarney, B.K., and Thomashow, M.F. (1993). *Plant Physiol.* 103, 1074.
63. Jiang, C., Iu, B., and Singh, J. (1996). *Plant Mol. Biol.*, 30, 679.
64. Neven, L.G., Haskell, D.W., Hofig, A., Li, Q.B., and Guy, C.L. (1993). *Plant Mol. Biol.*, 21, 641.
65. Wang, H., Datla, R., Georges, F., Loewen, M., and Cuttler, A.J. (1995). *Plant Mol. Biol.,* 28, 605.
66. Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). *Mol. Gen. Genet.*, 236, 331.
67. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). *Plant Cell*, 10, 1391.
68. Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997). *Proc. Natl. Acad. Sci. USA*, 94, 1035.
69. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). *Nature Biotechnol.*, 17, 287.
70. Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schbenberger, O., and Thomashow, M.F. (1998). *Science*, 280, 104.
71. Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000). *Plant Physiol.*, 124, 1854.
72. Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001). *Plant Cell*, 13, 61.

73. Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Hounghton, J.M., and Thomashow, M.F. (1998). *Plant J.*, 16, 433.
74. Shinawari, Z.K., Nakashima, K., Miura, S., Kasuga, M., Seki, M.. (1998). *Biochem. Biophys. Res. Commun.*, 250, 161.
75. Oullet, F., Vasquez-Tello, A., and Sarhan, F. (1998). *FEBS Lett.*, 423, 324.
76. Vasquez-Tello, A., Oullet, F., and Sarhan, F. (1998). *Mol. Gen. Genet.*, 257, 157.
77. Hsieh, T.H., Lee, J.T., Yang, P.T., Chiu, L.H., Charng, Y.y., Wang, Y.C. and Chan, M.T. (2002). *Plant Physiol.*, 129, 1086.
78. Pandolfi, D., Solinas, G., Valle, G., and Coraggio, I. (1997). *Plant Physiol.*, 114, 747.
79. Vannini C., Locatelli F., Bracale M., Magnani M., Marsoni M., Osnato M., Mattana M. and Coraggio I. (2003) Submitted to Plant Journal.
80. Fowler, S. and Thomashow, M.F. (2002). *Plant Cell*, 14, 1675.
81. Thomashow, M.F. (1999). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 50, 571.
82. Chen, H-H, Li, P.H., and Brenner, M.L. (1983). *Plant Physiol.*, 71, 362.
83. Gilmour, SJ, and Thomashow, M.F. (1991). *Plant Mol. Biol.*, 17, 1233.
84. Mäntylä, E., Lâng, V., and Palva, E.T., (1995). *Plant Physiol.*, 107, 141.
85. Heino, P., Sandman, G., Lâng, V., Nordin, K., and Palva, E.T., (1990). *Theor. Appl. Genet.*, 79, 801.
86. Lâng, V., Mäntylä, E., Welin, B., Sundberg, B., and Palva, E.T., (1994). *Plant Physiol.*, 104, 1341.
87. Guiltinan, M.J., Marcotte, W.R. Jr., and Quatrano, R.S., (1990). *Science*, 250, 267.
88. Ishitani, M., Xiong, L;, Stevenson, B., and Zhu, J.-K; (1997). *Plant Cell*, 9, 1935.
89. Kim, J.C., Lee, S.H., Cheong, Y.H., Yoo, C-M., Lee, I. S. Chun, H.J., Yun, D.-J., Hong, J.C., Lee, S.Y., Lim, C.O., and Cho, M. J. (2001). *Plant J.*, 25, 247.
90. Nordin, K., Vahala, T., and Palva, E.T., (1993). *Plant Mol. Biol.*, 21, 641.
91. Welin, B.V., Olson, A., Nylander, M., and Palva, E.T., (1994). *Plant Mol. Biol.*, 26, 131.
92. Lâng, V., and Palva, E.T. (1992). *Plant Mol. Biol.*, 20, 951.
93. Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). *Plant Cell*, 6, 251.
94. McKersie, B.D., Kuroki, G., and Warren, G. (1996). *J. Exp. Bot.*, 47, 1919.
95. Warren, G., McKown, R., Marin, A., and Teutonico, R. (1996). *Plant Physiol.*, 111, 1011.
96. Knight, H., Veale, E.L., Warren, G.J., and Knight, M.R. (1999). *Plant Cell*, 11, 875.
97. Ishitani, M., Xiong, L., Lee, H., Stevenson, B., and Zhu, J.-K. (2000). *Plant Cell*, 10, 1151.
98. Lee, H., Xiong, L., Ishitani, M., Stevenson, B., and Zhu, J.-K. (1999). *Plant J.*, 17, 301.
99. Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. and Zhu, J.K. Genes Dev. (2001) 15: 912-924.
100. Xin, Z., and Browse, J. (1998). *Proc. Natl. Acad. Sci. USA*, 95, 7799.
101. Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X., and Zhu, T. (2002). *Plant Cell*, 14, 559.
102. Seki, M., Narusaka, M., Ishida, J., Nanjo, T. Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A, Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y. and Shinozaki, K. (2002). *Plant J.*, 31, 279
103. Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X. and Harper, J. (2002). *Plant Physiol.*, 130, 2129.
104. Dunn, M.A., Goddard, N.J., Zhang, L., Pearce, R.S., and Hughes, M.A. (1994). *Plant Mol. Biol.*, 24, 879.
105. Phillips, J.R., Dunn, M.A., and Hughes, M.A. (1997). *Plant Mol. Biol.*, 33, 1013.
106. Hajela, R.K., Horvath, D.P., Gilmour, S.J., and Thomashow, M.F. (1990). *Plant Physiol.*, 93, 1246.
107. Wolfraim, L.A., Langis, R., Tyson, H., and Dhindsa, R.A. (1993). *Plant Physiol.*, 101, 1275.
108. Carpenter, C.D., Krebs, J.A., and Simon, A.E. (1994). *Plant Physiol.*, 104, 1015.
109. Dunn, M.A., Brown, K., Lightowlers, R., and Hughes, M.A. (1996). *Plant Mol. Biol.*, 30, 947.
110. Dreyfuss, G., Matunis, M.J., Piñol-Roma, S., and Burd, C.G. (1993). *Annu. Rev. Biochem.*, 62, 289.
111. Ludevil, M.D., Freire, M.A., Gomez, J., Burd, C.G., Albemcio, F., Giralt, E., Dreyfuss, G., and Pagès, M. (1992). *Plant J.*, 2, 999.
112. Sturm, A. (1992). *Plant Physiol.*, 99, 1689.
113. Didierjean, L., Frendo, P., and Burkard, G. (1992). *Plant Mol. Biol.*, 18, 847.
114. Bergeron, D., Beauseigle, D., and Bellemare, G. (1993). *Biochem. Biophys. ACTA*, 1216, 123.

115. Joh, T., Honjoh, K., Yoshimoto, M., Funabashi, J., Miyamoto, T., and Hatano, S. (1995). *Plant Cell Physiol.*, 36, 85.
116. Skriver, K., and Mundy, J., (1990). *Plant Cell*, 2, 503.
117. Lin, C., and Thomashow, M.F. (1992). *Plant Physiol.*, 99, 519.
118. Kurkela, S., and Borg-Franck, M. (1990). *Plant Mol. Biol.*, 15,137.
119. Wang, H. Georges, F., Pelcher, L.E., Saleem, M., and Cutler, A.J. (1994). *Plant Physiol.*, 4, 291.
120. Nordin, K., Heino, P., and Palva, E.T. (1991). *Plant Mol. Biol.*, 16, 1061.
121. Welin, B.V., Olson, A., Nylander, M., and Palva, E.T. (1995). *Plant Mol. Biol.*, 29, 391.
122. Capel, J., Jarilo, J.A., Salinas, J., and Martinez-Zapater J.M. (1997). *Plant Physiol.*, 115, 569.
123. Kiyouse, T., Abe, H., Yamaguchi-Shinizaki, K., and Shinozaki, K. (1998). *Biochim Biophys. Acta*, 1370,187.
124. Weretilnyk, E., Orr, W., White, T.C., Iu, B., and Singh, J. (1993). *Plant Physiol.*, 101, 171.
125. Orr, W., White, T.C., Iu, B., Robert, L., and Singh, J. (1995). *Plant Mol. Biol.*, 28, 943.
126. Van Zee, K., Chen, F.Q., Hayes, P.M., Close, T.J., and Chen, T.H.H. (1995). *Plant Physiol.*, 108, 1233.
127. Hong, B., Barg, R., and Ho, T-H.D. (1992). *Plant Mol. Biol.*, 18, 663.
128. Crosatti, C., Soncini, C., Stanca, A.M., and Cattivelli, L. (1995). *Planta*, 196, 458.
129. Goddard, N.J., Dunn, M.A., Zhang, L., White, A.J., Jack, P.L., and Hughes, M.A. (1993). *Plant Mol. Biol.*, 23, 871.
130. Grossi, M., Cattivelli, L., Terzi, V., and Stanca, A.M. (1992). *Plant Physiol. Biochem.*, 30, 97.
131. Rizza, F., Crosatti, C., Stanca, A.M., Cattivelli, L. (1994). *Euphytica*, 75, 131.
132. Laberge, S., Castonguay, Y., and Vezina, L.P. (1993). *Plant Physiol.*, 101, 1411.
133. Luo, M., Liu, J., Mohapatra, S., Hill, R.D., and Mohapatra, S.S. (1992). *J. Biol. Chem.*, 267, 15367.
134. Artlip, T.S., Callahan, A.M., Bassett, C.L., and Wisniewski, M.E. (1997). *Plant Mol. Biol.*, 33, 61.
135. Kirch, H.H., vanBerkel, J., Glaczinski, H., Salamini, F., and Gebhardt, C. (1997). *Plant Mol. Biol.*, 33, 897.
136. Neven, L.G., Haskell, D.W., Hofig, A., Li, Q.B., and Guy, C.L. (1993). *Plant Mol. Biol.*, 21, 291.
137. Houde, M., Dhindsa, RS., and Sarhan, F. (1992). *Mol. Gen. Genet.*, 234, 43.
138. Sarhan, F., Ouellet, F., Vazquez-Tello, A. (1997). *Physiol. Plant.*, 101, 439.
139. Danyluk, J., Houde, M., Rassart, E., and Sarhan, F. (1994). *FEBS Lett.*, 344, 20.
140. Guo, W., Ward, R.W., and Thomashow, M.F. (1992). *Plant Physiol.*, 100, 915.
141. Gilmour, S.J., Artus, N.N., and Thomashow, M.F. (1992). *Plant Mol. Biol.*, 18, 13.
142. Welin, B.V., Olson, A., Nylander, M., and Palva, E.T. (1994), *Plant Mol. Biol.*, 26, 131.
143. Close, T. J. (1996). *Physiol. Plant.*, 97, 795.
144. Wisniewski, M., Webb, R., Balsamo, R., Close, T.J., Yu, X.M., and Griffith, M. (1999). *Physiol Plant.*, 105, 600.
145. Rinne, P.L.H., Kaikurantam P.L.M., van der Plas, L.H.W., and van der Schoot, C. (1999). *Planta*, 209, 377.
146. Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N., and Sarhan, F. (1998). *Plant Cell*, 10, 623.
147. Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin C.T., and Thomashow M.F. (1996). *Proc. Natl. Acad. Sci. USA*, 93, 13404.
148. Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., and Thomashow, M. F. (1998). *Science*, 280, 104.
149. Jarillo, J.A., Leyva, A., Salinas, J., and Martinez-Zapater, J.M. (1993). *Plant Physiol.*, 101, 833.
150. Chu B., Snusted, P., and Carter, J.V. (1993). *Plant Physiol.*, 103, 371.
151. Carpenter, C.D., Krebs, J.A., and Simon, A.E. (1994). *Plant Physiol.*, 104, 1015.
152. Kiyose, T., Abe, H., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). *Biochim. Biophys. Acta*, 1370,189.
153. Zhang, H., Wang, J., Nickel, U., Allen, R.D., and Goodman, H.M. (1997). *Plant Mol. Biol.*, 34, 967.
154. Liu, J.J., Galvez, A.F., Krenz, D.C., and de Lumen, B.O. (1997). *Plant Physiol.*, 114, 600.
155. Gibson, S., Aronde, V., Iba, K., and Somerville, C. (1994). *Plant Physiol.*, 106, 1615.
156. Gomès, E., Kied Jacobsen, M., Axelsen K.B., Geisler, M., and Palgren M.G. (2000). *Plant Cell*, 12, 2441.
157. Hirayama, T., Ohto, C., Mizoguchi, T., and Shinozaki, K. (1995). *Proc. Natl. Acad. Sci. USA*, 92, 3903.
158. Xu, W., Cambell, P., Vargheese, A.K., and Braam, J. (1996). *Plant J.*, 9, 879.

159. Braam, J. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 3213.
160. Dunn, M.A., Morris, A., Jack, P.L., and Hughes, M.A. (1993). *Plant Mol. Biol.*, 23, 221.
161. Dunn, M.A., Hughes, M.A., Zhang, L., Pearce, R.S., Quigley, A.S., and Jack, P.L. (1991). *Mol. Gen. Genet.*, 229, 389.
162. Hughes, M.A., Dunn, M.A., Pearce, R.S., White, A.J., and Zhang, L. (1992). *Plant Cell Envir.*, 15, 861.
163. Berberich, T., Harda, M., Sugawara, K., Kodama, H., Iba, K., and Kusano, T. (1998). *Plant Mol. Biol.*, 36, 297.
164. Berberich, T., Suguwara, K., Harada, M., and Kusano, T. (1995). *Plant Mol. Biol.*, 29, 611.
165. Castonguay, Y., Laberge, S., Nadeau, P., and Vezina, L.P. (1994). *Plant Mol. Biol.*, 24, 799.
166. Anderson, J.V., Li, Q.B., Haskell, D.W., and Guy, C.L. (1994). *Plant Physiol.*, 104, 1359.
167. Guy, C.L., and Li, Q.B. (1998). *Plant Cell*, 10, 539.
168. Reimholz, R., Geiger, M., Haake, V., Deiting, U., Krause, K.P., Sonnewald, U., and Stitt, M. (1997). *Plant Cell Envir.*, 20, 291.
169. Guy, C.L., Huber, J.L.A., and Huber, S.C. (1992). *Plant Physiol.*, 100, 502.
170. Antikainen, M., and Griffith, M. (1997). *Physiol. Plant.*, 99, 423.
171. Griffith, M., Antikainen, M., Hon, W-C., Pihakaski-Maunsbach, K., and Yu, X-M. (1997). *Physiol. Plant.*, 100, 327.
172. Worrall, D., Elias, L., Ashford, D., Smallwood, M., Sidebottom, C., Lillford, P., Telford, I., Holf, C., and Bowles, D. (1998). *Science*, 282, 115.
173. Krishna, P., Sacco, M., Cherutti, J.F., and Hill, S. (1995). *Plant Physiol.*, 107, 915.
174. Saez-Vasquez, J., Raynal, M., and Delseny, M. (1995). *Plant Physiol.*, 109, 611.
175. Stroeher, V.L., Boothe, J.G., and Good, A.G. (1995). *Plant Mol. Biol.*, 27, 541.
176. Grillo, S., Consiglio, F., Costa, A., and Leone, A. (1998). In Proceedings of XV Eucarpia "Genetics and breeding for crop quality and resistance". Viterbo, September 20-25, p 100.
177. Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M., and Iba, K. (1994). *Plant Plysiol.*, 105, 601.
178. Hincha, D.K., Meins, F., and Schmitt, J.M. (1997). *Plant Physiol.*, 114, 1077.
179. Tao, D.L., Öquist, G., and Wingsle, G. (1998). *Cryobiology*, 37, 38.
180. Taulavuori, E., Taulavuori, K., and Laine, K. (1999). *Plant Biol.*, 1, 187
181. Igarashi, Y., Yoshiba, Y., Sanada, Y., Yamaguchi-Shinozaki, K, Wada, K., and Shinozaki, K. (1997). *Plant Mol. Biol.*, 33, 857.
182. Droual, A.M., Maaroufi, H., Creche, J., Chenieux, J.C., Rideau, M., and Hamdi, S. (1997). *J. Plant Physiol.*, 151, 142.
183. Stushnoff, C., Seufferheld, M.J., and Creegan, T. (1997). Plant Cold Hardiness. Molecular biology, Biochemistry, and Physiology. Plenum Press, New York.
184. Jouve, L., Frank, T., Gaspar, T., Cattivelli, L., and Hausman, J.F. (2000). *J. Plant Physiol.*, 157, 117.
185. Pietilä, M., Lähdesmäki, P., Pietiläinen, P., Ferm, A., Hytönen, J., and Pätilä, A. (1991). *Environ. Pollut.*, 72,103.
186. Shen, B., Jensen, R.G., and Bonhert, H.J. (1997). *Plant Physiol.*, 113, 1177.
187. Nelson, D.E., Rammesmayer, G., and Bonhert, H.J. (1998). *Plant Cell*, 10, 753.

CHAPTER 3

SALT TOLERANCE: PLACING ADVANCES IN MOLECULAR GENETICS INTO A PHYSIOLOGICAL AND AGRONOMIC CONTEXT

A. MAGGIO[a], R.A. BRESSAN[b], C. RUGGIERO[a], L. XIONG[c] AND S. GRILLO[d]

[a]*Department of Agricultural Engineering and Agronomy, University of Naples Federico II, Via Università 100, Portici (NA), Italy*
[b]*Center for Plant Environmental Stress Physiology, 1165 Horticulture Building,Purdue University, West Lafayette, IN 47907-1165, USA*
[c]*Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132, USA*
[d]*Institute of Plant Genetics (CNR-IGV), Research Division of Portici, Portici (NA), Italy*

Abstract. **In recent years the study of the functional biology of salinity tolerance in plants has largely focused on six major areas of research: ion homeostasis, water homeostasis, osmolyte production, ROS scavenging, growth regulatory processes, and signal transduction. Genetic engineering and mutation analyses have both significantly contributed to advance our understanding of the fundamental biology underlying salt stress adaptation. Many biochemical and metabolic components of salinity tolerance have been thoroughly described and directly and/or indirectly implicated in salt stress adaptation at cellular level. In contrast, morphological and anatomical features that also may have important functions during whole plant stress adaptation have been overlooked. Mutants with altered responses to salinity in agriculturally important species are increasingly, and represent an important resource for plant physiologists and agronomists that are interested in understanding salinity tolerance at a whole plant level. The results obtained so far suggest that a greater connection between molecular genetic and physiological analyses would greatly benefit the functional assessment of plant salt stress tolerance.**

1. INTRODUCTION

In the last ten years, our understanding of the molecular basis of plant salt tolerance has considerably improved. Mutation analysis and transgene technologies have been essential to test basic hypotheses derived from physiological studies [1] and to identify potential targets for genetic engineering [2]. Over the last few years, dissecting the sequence of events that follow stress perception and signal transduction leading to adaptation has been a primary goal of many investigators. As a result, various

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 53-69.

models have been proposed to explain the complexity of plant responses to salinity and to uncover interactions between metabolic pathways and the relationships between responses to different stresses [3]. The practical outcome of these efforts has been the establishment of a list of determinants that are involved in salt stress adaptation. However, we still do not have a complete comprehension of which determinants we should genetically engineer to obtain a salt tolerant plant. It is worth noting that virtually all the research developed in this field in the last decade has focused mainly on biochemical and metabolic components of salinity tolerance that are largely manifested at the cellular level, whereas morphological and anatomical features that also have critical functions during stress acclimation, have been largely overlooked [4, 5]. In addition, it is also becoming clearer that many functional components that have been identified as being specifically involved in salt tolerance in Arabidopsis do not always have an obvious counterpart in agriculturally important species, suggesting either that alternative adaptation mechanisms within different glycophytes exist or that Arabidopsis genes may have diverged significantly from their counterparts in other species so as to be unrecognisable with present technology [6]. Therefore, 1) identifying regulatory molecules, able to simultaneously activate several stress adaptive responses; 2) framing salt tolerance in an agronomic and environment-specific context; 3) and extending mutation analyses to model systems other than Arabidopsis are approaches that require further development in salinity research.

In this chapter we will summarize the current literature on salt stress in plants and the known adaptation mechanisms as bases for possible strategies to pursue genetic improvement of salt tolerance of crop plants.

2. MOLECULAR BIOLOGY OF SALT STRESS TOLERANCE

The known and most studied physiological mechanisms for salt tolerance in plants can be grouped into four major categories: osmolyte production, ion homeostasis, Radical Oxygen Species (ROS) scavenging and growth regulatory processes [7, 8]. The regulation of water transport via the aquaporin pathway may also play a fundamental role during salt stress [9]. However, an unequivocal link between the function of aquaporins and water homeostasis in stress environments remains to be established.

2.1. Osmolyte production

During osmotic stress, cells accumulate solutes to prevent water loss by decreasing osmotic potential and thereby maintain cellular turgor that is necessary for growth [10]. Solutes that accumulate in saline environments include ions such as K^+, Na^+, Cl^- and organic molecules, including quaternary ammonium compounds (glycine betaine), some amino acids (proline), polyols (inositol and mannitol) and sugars

(sucrose, fructans and trehalose) [11]. Na^+ and most of the Cl^- are compartmentalized into the vacuole since they interfere with cellular functions, whereas organic solutes (compatible solutes or osmolytes) are compatible with normal cellular metabolism and can be accumulated to high levels in the cytosol [11, 12].

A number of genes encoding enzymes involved in the metabolism of osmolytes have been isolated and several of these have been used to generate transgenic plants . .glycine-betaine level has been obtained by over-expressing genes involved in choline oxidation from *E. coli* [13], *Arthrobacter* spp. [14, 15] and spinach [12] in transgenic plants. More recently, Mc Neil et al. [16] have reported that overexpression of the gene encoding phospho-ethanolamine-N-methyltransferase (PEAMT), the enzyme controlling the limiting step in the choline biosynthetic pathway, allowed a 30fold increase of the glycine betaine level. The accumulation of proline has been the target of intense research [17], also. Kishor et al. [18] reported that overexpression of the gene encoding the moth bean Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in transgenic tobacco results in a 10-18fold accumulation of proline and better plant growth under osmotic stress. In addition, recent reports have shown that expression of antisense P5CS inhibits proline production and results in hypersensitivity to osmotic stress, suggesting that proline may contribute to stress tolerance both as a compatible solute and a free radical scavenger [19, 20]. Although these and other reports indicate that there is a positive correlation between proline accumulation and salt tolerance, the role of proline in plant osmotolerance remains controversial [21]. For instance, the *s*alt-*o*verly-*s*ensitive *sos1* Arabidopsis mutant surprisingly was found to accumulate more proline than wild-type plants [22].

One of the first metabolic pathways to be engineered with the aim of developing salt tolerant plants was the biosynthesis of polyols, which include sugar alcohols such as glycerol, sorbitol, mannitol as well as cyclictols (pinitol, D-ononitol). Overexpression in transgenic tobacco and Arabidopsis of the gene encoding mannitol-1-phosphate dehydrogenase from *E. coli* resulted in mannitol production and increased salt tolerance [23, 24]. Further studies indicated that mannitol accumulation contributed only marginally to osmotic adjustment, whereas it had some protective effects against stress-induced ROS damage [25, 26]. Transgenic plants accumulating high levels of mannitol or sorbitol showed growth retardation and necrotic lesions respectively [27, 28], indicating that the physiological consequences of diverting a precursor of organic molecules away from primary metabolism may have some deleterious effects on plant growth and should be carefully considered in metabolic engineering of salt stress tolerance [28, 29].

Although less studied, overproduction of several other metabolites has been shown to ameliorate plant response to salinity. Ectoine, a zwitter-ionic tetrahydropyrimidine from a halophytic eubacterium [30] has been reported to have strong protective effects on enzyme activity in the presence of sodium. Multiple engineering of enzymes involved in ectoine biosynthesis led to ectoine accumulation and increased hyperosmotic stress tolerance in transgenic tobacco BY2 cells [31]. Similarly to threalose and fructan [32], ectoine could function in preserving the

functionality and permeability of cell membranes rather than decreasing the cellular osmotic potential [31].

Overall genetic engineering for osmolyte accumulation has marginally contributed to increase plant stress tolerance. The results so far obtained clearly indicate that the osmotic contribution of these osmolytes to stress tolerance may not account completely for their function and that accumulation of different osmolytes may not have always the same effects on the specific process [21, 29].

In view of these variable functions (membrane stabilization, protection by ROS damage, osmotic adjustment) of different compatible solutes, a multiple engineering approach has been recently proposed [29]. Clearly, the simultaneous accumulation of different stress protecting molecules by reiterative engineering or genetic crosses has to be guided by a thorough analysis of metabolite fluxes and pool sizes using emerging technologies such as *in vivo* NMR spectroscopy [12].

2.2. Ion homeostasis

Most research in salinity tolerance has been developed to understand the basis for Na^+/K^+ homeostasis since the ability of plants to select K^+ vs. Na^+ uptake and to compartmentalize Na^+ (and Cl^-) into the vacuole have been considered potential targets for genetic engineering of salt tolerance. To date, it is broadly accepted that K^+ and Na^+ enter into the plant via common uptake systems [33, 34, 35, 36, 37]. Although Na^+ seems to enter the cell mainly via electrophoretic flux, high and low affinity K^+ uptake systems may significantly contribute to Na^+ influx also [1, 38]. The existence of a Na^+ influx system in plant roots, which negatively regulates high affinity K^+ transport has been reported, also [39]. The molecular mechanisms of Na^+ and K^+ uptake in plants have been recently summarized by Mäser et al. [40]. Compared to the overexpression of compatible solutes, there are only a few examples of the genetic engineering of Na^+/K^+ homeostasis machinery. Overexpression of a cDNA encoding a Na^+/H^+ antiporter in transgenic Arabidopsis [41] and tomato [42] moderately increased plant salt tolerance. Interestingly enough, higher K^+ leaf (and/or root) concentration has never been shown to be associated to enhanced salt stress tolerance in Arabidopsis, whereas it has been functionally associated to salt tolerance in wheat [43] and tomato [44], indicating that different species may adjust to salt stress through somewhat different physiological mechanisms [6, 40].

The role of Ca^{2+} is pivotal in signalling and activating stress responses in saline environments [45, 46]. It is well established that Ca^{2+} can facilitate higher K^+/H^+ selectivity [47, 48]. Nevertheless, Ca^{2+} homeostasis is thought to have a more complex role in the regulation of Na^+ and K^+ fluxes because it is involved in many cellular mechanisms, including response and adaptation to biotic stresses [49]. Much research has been developed on Ca^{2+}-ATPases since these are likely to mediate Ca^{2+} transport from the cytosol into the apoplast or intracellular compartments [50, 51].

Genetic engineering of Ca^{2+}-ATPase and Ca^{2+}/H^{+} antiport aimed to maintain low cytosolic Ca^{2+} have been marginally successful [52] or unsuccessful [53] to improve plant salt tolerance, however.

Little is known about the molecular biology of Cl^{-} homeostasis [54] and the possibility of genetically engineering Cl^{-} homeostasis to improve salt tolerance via manipulation of Cl^{-}/H^{+} symporters or other transport components remains mostly speculative [55].

2.3. ROS scavenging

Environmental stresses enhance the generation of reactive oxygen species (ROS) [56]. Since ROS are associated with several forms of cellular damage, the identification of genes encoding for the enzymes involved in ROS detoxification has been a primary goal in plant salt stress research [57, 58, 59, 25, 26]. Scavenging of hydroxyl radicals and other toxic oxygen derivatives in plants is primarily under the control of the ascorbate/gluthathione cycle [60]. Tocopherols, carotenoids and other chemicals able to act as reductants also neutralize free radicals to non-reactive forms and provide protection against oxidative damage [61]. However, since ROS are involved in the control of gene expression and signal transduction, the regulation of ROS production and detoxification implicates that the function of antioxidants is beyond just limiting the risk of hydroxyl radical formation. Recently, activation of the ascorbate cycle has been shown to mediate the ABA-induced stomatal closure in the early response to hyperosmotic stress [62]. Furthermore, the general function of ascorbate as a co-substrate of many dioxygenases has been recently discussed [63] and should be considered to function as a general stress response mechanism.

2.4. Water transport

During stress adaptation, water and ion homeostasis have to be regulated in order to avoid water deficit and/or ion toxicity. After the discovery of plant aquaporins [64, 65, 66] the possibility of metabolic control of water movement across plants membranes became part of the mechanism thought to allow adaptation to salinity or drought. Aquaporins belong to the family of the MIP (Major Intrinsic Protein) involved in water movement in many organisms. Aquaporins are membrane proteins that have been localized at both the plasma membrane (PIP) and tonoplast (TIP) [67, 68]. The two halves of the polypeptide spanning the lipid bilayer are arranged in a typical hourglass symmetric structure, forming a narrow channel were water molecules selectively cross the membrane [69]. Plant aquaporins allow water movement following osmotic gradients and do not actively transport water. It remains to be established whether and how these proteins are directly involved in regulating water homeostasis under stress.

Changes in root hydraulic conductivity have been documented in many species exposed to salinity and they appear to be associated with possible mechanisms

regulating opening/closure of water channels [70]. In line with this view, reduced phosphorylation in spinach plasma membrane PM28A has been shown to be induced by a low water potential [71]. However, at the present, conclusive proof of the existence of a gating mechanism of water channels is not yet been established [9]. Conceivably, a mechanism of regulation of plant aquaporins would occur at the endodermis level and possibly also in guard cells, two critical check points for controlling the rate of water movement in plants. Another important role for plant aquaporins during stress acclimation may be the redistribution of water between symplast/apoplastic and cellular compartments or even different plant organs upon stress [72, 73, 74].

2.5. Regulatory systems

Many genes encoding for enzymes involved in osmolytes biosynthesis and ion homeostasis are responsive to ABA [1], which also controls other stress-induced responses, including stomatal closure [75] and plant hydraulic conductivity properties [76]. The increase in free radical levels following drought or saline stress is under the control of the ascorbate-gluthatione detoxifying system [60, 77, 78], which is possibly activated by H_2O_2 production [79]. Several compatible solutes have been shown to act as free radical scavengers, also [26]. Therefore the ROS detoxifying system may directly and/or indirectly be under hormonal control. In addition, evidence for a cross-talk between the ascorbate cycle, H_2O_2 and ABA mode of action has recently been documented [79, 62]. The least characterized regulatory system at the molecular level is perhaps the one controlling aquaporin synthesis, function and mode of action, whereas the best characterized is the ion homeostasis system, largely because it shares similarities with the better characterized yeast ion homeostasis system [80, 45]. Arabidopsis salt sensitive mutants have been selected and utilized to dissect the signal transduction pathway controlling responses to salt stress [3]. *sos* mutants (salt overly sensitive) have been isolated by screening approximately 250,000 EMS, fast neutron- or T-DNA mutagenized seedlings. Five complementation groups (sos1-5) have been identified. SOS3 encodes a Ca^{2+}-binding protein whose gene product shares similarities to the B-subunit of calcineurin [81, 82]. SOS3 is required for plant survival under K^+ starvation and it has been proposed to mediate the favourable effect of Ca^{2+} under salt stress [48]. SOS2 has also been cloned [83]. The SOS2 gene product is a Ser/Thr protein kinase with similarities to the yeast SNF1 (Sucrose non fermenting 1) and mammalian AMP-activated protein kinase (AMPK), although the functions of these genes are quite different [84]. SOS2 regulates Na^+ and K^+ homeostasis and Na^+ tolerance. SOS1 encodes a putative Na^+/H^+ antiporter that may function at the plasma membrane level by controlling loading/unloading of Na^+ into/from the xylem [85, 86]. The most recent hypothesis at this regard is that the SOS2 regulatory domain interacts with SOS3, which in turn triggers the kinase activity of SOS2. Genetic evidence has been provided in support of SOS2 and SOS3 being positive regulators

of salt tolerance acting in the same pathway [87], in which Ca^{2+} plays a pivotal role. Based on the analysis of *sos2* and *sos3* mutants, it has been concluded that the upregulation of *sos1* and therefore of the rate of Na^{+} transport across the plasma membrane, is activated by the *sos2-sos3* signal transduction pathway [3]. The SOS pathway will be further discussed in the following section because of its broad involvement in salt stress adaptation.

3. LOSS-OF-FUNCTION MUTANTS REVEAL IMPORTANT GENES FOR SALT TOLERANCE

Abiotic stress tolerance, including salinity tolerance, have always been considered to be complex traits involving several genetic factors [1]. Recent projects (http://www.stress-genomics.org/) utilizing a genomic approach to this problem have confirmed that numerous genetic loci contribute to salinity tolerance or sensitivity. Consistent with the tolerance mechanisms that we have just described, functional genetic screens have resulted in the identification of genetic components whose mechanistic functions fall within the previously mentioned categories including ion homeostasis (Table 1). Moreover, these mutant screens have identified several important proteins, including transcription factors, protein kinases and phosphatases [3]. The SOS pathway is the most characterized response system for salinity adaptation in plants and clearly participates in ion homeostasis through the mediation of signal transduction via a Ca^{2+} binding protein (SOS3) and a protein kinase (SOS2). However, several other genes involved in adaptation to salinity have recently been identified by mutation analysis that implicate the involvement of expected mechanisms such as ABA biosynthesis and perception, protein phosphorilation and ion transport. However, unanticipated processes such as RNA metabolism, co-factor functions, vesicular trafficking and protein glycosylation have also been found to participate in salinity tolerance through mutation screening [3]. Thus, knowledge of genes responsible for salinity tolerance has been advanced greatly by the isolation of Arabidopsis mutants with altered salinity tolerance [88]. It is clear that adaptation to salinity involves the activation of genes by signal transduction involving the SOS pathway, as we previously described. However, besides the SOS signal pathway genes (SOS2, SOS3), some genes encoding proteins directly affecting ion homeostasis such as the HKT1 transporter protein have been shown recently to participate in Na^{+} entry into plants thereby controlling sensitivity to NaCl [39]. Genes encoding proteins involved in ABA biosynthesis (ABA1/LOS6, ABA3/LOS5) also result in significant changes in tolerance to salinity when mutated [89, 90]. The mutation of *nced3,* which encodes the ABA biosynthetic enzyme 9-cis-epoxicarotenoid dioxygenase results in impaired accumulation of ABA after stress exposure. More rapid germination and plant growth during NaCl stress occurs in the mutants, in which the ethylene level does not decrease upon exposure to NaCl (Ruggiero et al., submitted). Mutation of the *SOS4* gene which encodes a biosynthetic enzyme for pyridoxil phosphate cofactor of the ethylene biosynthesis

gene ACC synthase also alters NaCl sensitivity, further implicating ethylene in the growth response to NaCl stress [91]. Both ABI1 and ABI2, which are required for full responsiveness to ABA also alter salinity tolerance after mutation [92, 93]. The FRY1 gene that encodes a catabolic enzyme for the signal molecule IP3 mediates salinity tolerance through at least partly the control of IP3 induced changes in gene expression [94]. The SAD1 gene encoding a Lsm5-like SnRNP protein is an example of a mutation that leads to enhanced expression of several osmotically induced genes but results in salt sensitivity presumably because an important function of SAD1 for salt tolerance is lost resulting in increased sensitivity, and this in turn results in an increase in salinity responsive gene expression. Alternatively, a separate function of SAD1 when lost may lead to an enhanced expression of some stress tolerance gene separate from a normal tolerance response [89].

Surprisingly some mutations in genes encoding proteins that affect what otherwise would appear to be normal "housekeeping" metabolic function such as mRNA synthesis and processing revealed significant changes in salinity tolerance. The SST3 gene (H. Koiwa, unpublished) also affects specifically salinity tolerance when mutated. The SST3 protein is involved in the glycosylation process. Mutation of the *OSM1/SYP61* gene affects both, tolerance to salinity and soil desiccation. *OSM1/SYP61* encodes a member of the syntaxin gene family, which is required for proper vesicular targeting and fusion to target cell membranes. The *OSM1/SYP61* mutation simultaneously controls both stomatal function and root growth in response to salinity stress [95].

At this time it is clear that numerous genes must function in the adaptive response of plants to salinity stress. Indeed, although the functional genetic components of salt tolerance so far identified are a modest number, extensive screens of mutant collections indicate the participation of many more (http://www.stress-genomics.org/). Several important challenges remain for the future. Paramount among them will be to complete the identification of the genetic participants so that classification of genes within functions and their relative importance to function can be ascertained. Only then can the true minimal number of genes responsible for adaptability be determined. Also a higher order of understanding of the complexity of the relationship between plant genotype and phenotype and environmental fitness can finally be gained.

It is also not a minor consideration to point out that most efforts to determine the functional relationship between genes and stress tolerance have utilized ectopic expression of candidate tolerance genes using constitutive or stress-induced foreign promoters in transgenic plants or in yeast complementation experiments. Far fewer genes have been identified by loss of function mutation screening so far. It is with considerably uncertainty that the natural function of an ectopically expressed gene be assigned to function in controlling the observed phenotypic effect that altered expression produces. It has been pointed out previously that ectopic expression can often lead to protein interactions that do not occur naturally. In fact, all ectopically expressed genes that produce an altered phenotype should be examined also in loss

of function (knock-out experiments as well) [1]. We will need to be much more comprehensive in mutation screening in the future. It may be assumed that another higher stage of endeavor to control stress tolerance will result from attempts to produce more effective signal responses to stress, by the identification of superior alleles of the most crucial genes. This process may be carried out through different approaches including: 1) in vitro mutagenesis followed by selection for altered phenotypes in model selection systems with very high transformation efficiencies such as yeast (when gene function is applicable to yeast) or even in Arabidopsis; 2) by site-directed mutagenesis when sufficient information is available for structure /function assessment, or 3) by searching for superior orthologs in plant species that are naturally salt tolerant (halophytes). This latter approach may offer great promise since recently the relationship between gene sequences of halophytic and non-halophytic types of closely related species has been found to be highly similar [96]. This indicates that especially effective halophytic versions of genes may be easily identified and isolated by bioinformatics and PCR approaches.

4. PHYSIOLOGY OF SALT STRESS TOLERANCE AT WHOLE PLANT LEVEL

4. 1. Water movement and salt loading to the shoot

The physiological response to salt stress in the whole plant system has been extensively studied in terms of water and salt movement in the soil-plant-air continuum [97]. Although environmental variables may simultaneously affect plant growth and salt tolerance, little attention has been put on the regulation of salt loading to the shoot relatively to the growth rate of the plant [98]. This is not a trivial issue since a dilution of the cellular ion content resulting from an increased cellular growth rate may increase the salinity tolerance threshold, without affecting any specific salt tolerance determinant other than growth response. Since the growth rate is universally inhibited upon salt stress, then it is apparent that growth rate and salt stress adaptation are somehow linked [21]. Considering that plant growth can be affected by both variable salinity of the root zone and sub-optimal environmental conditions, Dalton et al. [98, 99, 100] have recently highlighted the intrinsic limitations of assessing plant salt tolerance based only on root-zone-salinity. They proposed an alternative index (Salinity Stress Index) to assess plan salt tolerance, which is based on the accumulated shoot chloride vs. total shoot biomass. In contrast to the root-zone salinity index, the SSI provides an environmentally invariant measure of plant salt tolerance because it integrates biochemical and variable physical parameters in the soil-plant-air-continuum, which control salt loading to the shoot and also simultaneously affect transpiration and growth [99, 101]. Comparison of plant salt tolerance based on the traditional root zone salinity index and the recently proposed SSI at varying environmental parameters revealed that whereas the

apparent salinity tolerance (i.e. the biomass produced at increasing soil salinity) may increase upon variation of environmental factors such as root temperature, photosynthetic photon flux density (PPFD) or atmospheric CO_2, the shoot Cl^- concentration is a rather stable parameter and, consequently, a better interpretation of the intrinsic plant salt tolerance properties (Table 2). The SSI does not allow us to assess the relative partitioning (cytoplasm vs. vacuole) within the shoot of the specific ion considered (Cl^-). However, it indicates that salt loading relative to the shoot growth rate is a parameter that may be altered by manipulating the root/shoot ratio and/or the function of root/shoot in water and ion transport [98, 99]. Variation in root architecture has been documented and in some instances has been linked to adaptation to salinity and drought stress tolerance [102, 103, 104, 105]. The co-ordination of root development with the pattern of seasonal fluctuations of soil salinity also may affect plant adaptation to hyperosmotic environments [104, 105].

The possibility of testing the effect of different root morphologies in saline environments is greatly facilitated by the availability of single gene mutations responsible for changes in root morphology in isogenic backgrounds and has been discussed in more detail in Maggio et al. [4]. This may be a useful approach to identify specific genes that function in salt tolerance and to identify their counterparts in agriculturally important crops.

4.2. Role of aquaporins

Most attention has been focused on the role of aquaporins in roots for several reasons. First, the root system is the organ responsible for water (and solute) uptake and distribution into the plant and roots are directly exposed to variations in soil water potential. Second, the variability of root hydraulics has been known for many years, but no clear connections have ever been demonstrated between such variability and stress adaptation. Third, the first *in vivo* evidence for aquaporin function was documented in roots [106]. Water flow through the roots occurs via both apoplastic and cell-to-cell pathways (symplastic, i.e. through plasmodesmata plus transcellular, across cell membranes). Aquaporins may affect the symplastic pathway and directly regulate the rate of both inter- and intracellular water transport [68, 107]. The function of water channels has been associated with several physiological circumstances in which the regulation of water movement is assumed to be critical, including drought and salt stresses [73], circadian rhythms of water fluxes [108, 109], diurnal adjustment to transitory stresses (midday water stress), reverse gradients of water potentials (night) and re-equilibrium of the vacuole/cytoplasm water potentials. Current models that are used to explain the role of plant aquaporins are based on the existence of fine and coarse regulation of water movement. According to the composite transport model [73] transient responses to changes in the external water potentials (non-steady state conditions) may be under the regulation of water channel activity. In contrast, at steady state water fluxes (actively transpiring plants) the function and regulation of aquaporins may be less

critical [73]. Developmental regulation of water fluxes and the relative contribution of the apoplastic vs. symplastic pathway during the growth cycle have to be considered, also [74].

The elucidation of the aquaporin role at cell, tissue, organ and whole-plant levels are limited by the absence of "good" aquaporin mutants. The abundance and complexity of TIPs and PIPs (23 MIPS in Arabidopsis thaliana) renders the generation of knock-out lines very difficult and even the reverse genetic approach has been so far unfruitful in studying aquaporin function in plants. Alternative strategies may aim to identify guard cell and endodermis expressed PIPs (or TIPs) using guard cell (KAT) and endodermis (SCARECROW) specific promoters fused to the cytogenic marker green fluorescent protein (GFP) [110,111]. Plants transformed with this gene fusion can be used to isolate nuclei or protoplasts from guard cell or endodermis by flow cytometry [112]. A cDNA library can then be constructed from the sorted cells or nuclei, which can be subsequently screened using PIP consensus sequence probes. This can be used to target specific knock-out lines for guard cells and endodermis-expressed PIPs.

5. CONCLUSIVE REMARKS

Functional assessment of plant salt tolerance is increasingly invoking a greater connection between molecular genetic and physiological analyses (Figure 1). The increasing availability of mutants with altered responses to salinity in Arabidopsis and other agriculturally important species is generating an unprecedented important resource for plant physiologists and agronomists that are now called to transfer this genetic knowledge and resource into a better understanding of salinity tolerance at a whole plant level, and more importantly to an ecological level that explains the dramatic difference in salt tolerance between plant species (halophyte versus glycophyte). This approach will generate a new level of information to identify novel strategies to improve plant salt tolerance. We will possibly be surprised to find out that physiological mechanisms, which may have been recognized in the past as fundamental *dogmas* in salt stress adaptation may eventually be understood to have only a limited function in salinity tolerance in a field context.

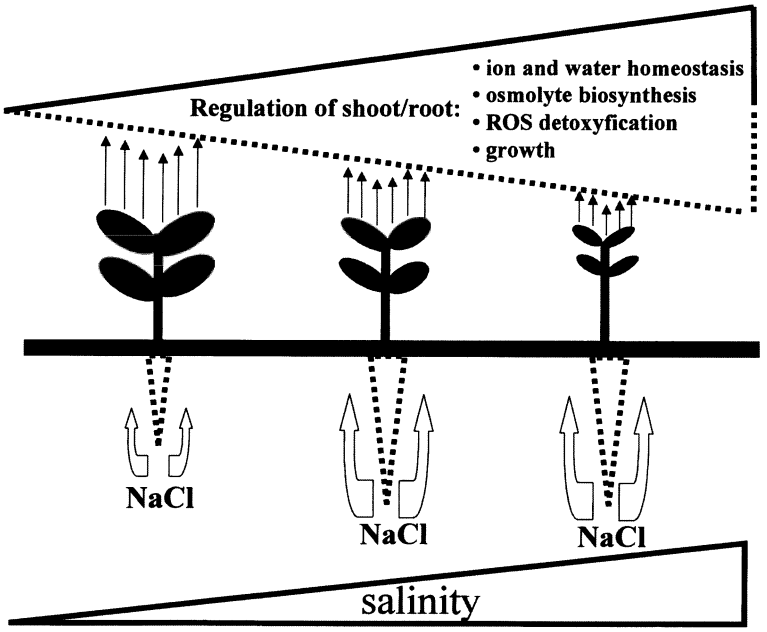

Figure 1. Plant response to soil salinity: Increasing concentration of NaCl in the solution in contact with the roots initiates adaptive responses, which involve activation of specific metabolic pathways and growth regulatory processes. Overtime, the control of water transport and ion homeostasis/detoxification at cellular level has to be tightly linked to plant's ability to modulate the flux of toxic ions to the shoot relatively to its growth rate.

Table 1. Arabidopsis genetic loci identified by loss-of-function screening that affect salt stress tolerance

Locus symbol	*Encoded product*	*GeneBank accession*	*TAIR accession*
HKT1	Na^+ transporter	AF237672	At4g10310
SOS1	Na^+/H^+ antiporter	AF256224	At2g01980
SOS2	Ser/thr protein kinase	AF237670	At5g35410
SOS3	Ca^{2+} sensor	AF060553	At5g24270
SOS4	pyridoxal 5-kinase	AF400125	At5g37850
ABA1/LOS6	zeaxanthin epoxidase	AY093145	At5g67030
ABA3/LOS5	molybdenum cofactor sulfurase	AY034895	At1g16540
ABI1	Type 2C protein phosphatase	X77116	At4g26080
ABI2	Type 2C protein phosphatase	Y08965	At5g57050
FRY1	Inositol polyphosphate 1-phosphatase/3'(2')5'-bisphosphate nucleotidase	AY034894	At5g63980
SAD1	Lsm5-like snRNP	AY034896	At5g48870
OSM1/SYP61	syntaxin	AAL59937	At1g28490
SST3	Glycosil transferase	AY056191	

*Table 2. Tomato root zone salinity and SSI thresholds in response to environmental variables**

Environmental parameter	*Root zone salinity (Maas-Hoffman threshold) [mM Cl⁻]*	*Leaf Cl⁻ concentration (SSI threshold) [mmol gDW^{-1}]*
Root Temperature (18°C)	33	1.10
Root Temperature (25°C)	64	1.19
PPFD (400 mmol m^2 sec^{-1})	28	0.97
PPFD (600 mmol m^2 sec^{-1})	28	0.97
Atmospheric CO_2 (700 ppm)	51	1.0

* (*After Ref. 98-101*)

ACKNOWLEDGEMENTS

Contribution No. 19 of Institute of Plant Genetics (CNR-IGV), Research Division of Portici, Italy. The assistance of Ms. Arianna Da Ros in the preparation of the manuscript is greatly acknowledged.

REFERENCES

1. Hasegawa, P. M., Bressan, R. A., Zhu, J. K., and Bohnert, H. J. (2000). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51, 463.
2. Maggio, A., Mastumoto, T. K., Hasegawa, P. M., Pardo, J. M., and Bressan, R. A. (2002). In: *Lauchli A and Luttge U (eds) Salinity: Environment - Plants - Molecules, Kluwer Academic Publisher, Netherlands*, 505.
3. Zhu, J.K. (2002). *Annu. Rev. Plant. Biol.*, 53, 247.
4. Maggio, A., Bressan, R. A., Hasegawa, P. M., Consiglio, M. F., and Joly, R. J. (2001). *Aust. J. Plant Physiol.*, 28, 999.
5. Maggio, A., Joly, R. J., Hasegawa, P. M., and Bressan, R. A. (2003). In*: Rains B (ed), Crop Production Under Saline Agriculture*. In press.
6. Bressan, R. A., Zhang, C. Q., Zhang, H., Hasegawa, P. M., Bohnert, H. J., and Zhu, J. K. (2001). *Plant Physiol.*, 127, 1354.
7. Xiong, L., and Zhu, J. K. (2002). *Plant, Cell, Environ*, 25, 131.

8. Xiong, L., and Zhu, J. K. (2002). In: *Scheel D and Wasternack C (eds) Plant Signal Transduction. Frontiers in Molecular Biology Series, Oxford University Press*, 165.
9. Tyerman, S. D., Bohnert, H. J., Maurel, C., Steudle, E., and Smith, J. A. C. (1999). *J. Exp. Bot.*, 50, 1055.
10. Morgan, J. M. (1984). *Ann. Rev. Plant Physiol.*, 35, 299.
11. Yancey, P. H. (1994). In*: K. Strange (ed) Cellular ad molecular physiology of cell volume regulation, CRC press,* 347.
12. Nuccio, M. L., Rhodes, D., McNeil, S. D., and Hanson, A. D. (1999). *Curr. Opin. Plant Biol.*, 2, 128.
13. Lilius, G., Holmberg, N., and Bulow, L. (1996). *Biotechnology*, 14, 177.
14. Hayashi, H., Alia Mustardy, L., Deshnium, P., Ida, M., and Murata, N. (1997). *Plant J.*, 12, 133.
15. Hayashi, H., Alia, Sakamoto, A., Mustardy, L., Deshium, P., Ida, M., and Murata, N. (1998). *J. Plant Research*, 111, 357.
16. McNeil, S. D., Nuccio, M. L., Ziemak, M. J., and Hanson, A. D. (2001). *Proc. Natl. Acad. Sc.i*, 98, 10001.
17. Delauney, A. J. and Verma, D. P. S. (1993). *Plant J.*, 4, 215.
18. Kishor, P. B. K., Hang, Z., Miao, G. H., Hu, C. A., and Verma, D. P. S. (1995). *Plant Physiol.*, 108, 1387.
19. Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukuya, H., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). *Plant J.*, 18, 185, 193.
20. Hong, Z. L., Lakkineni, K., Zhang, Z. M., and Verma, D. P. S. (2000). *Plant Physiology*, 122, 1129.
21. Maggio, A., Miyazaki, S., Veronese, P., Fujita, T., Ibeas, J. I., Damsz, B., Narasimhan, M. L., Hasegawa, P. M., Joly, R. J., and Bressan, R. A. (2002). *Plant J.*, 31, 699.
22. Liu, J., and Zhu, J. K. (1997). *Plant Physiol.*, 114, 591.
23. Tarczynski, M. C., Jensen, R. G., and Bohnert, H. J. (1993). *Science,* 259, 508.
24. Thomas, J. C., Sepahi, M., Arendall, B., and Bohnert, H. J. (1995). *Plant Cell Environ.*, 18, 801.
25. Shen, B., Jensen, R. G., and Bohnert, H. (1997). *Plant Physiol.*, 115, 527.
26. Shen, B., Jensen, R. G., and Bohnert, H. J. (1997). *Plant Physiol.*, 113, 1177.
27. Karakas, B., Ozias Akins, P., Stushnoff, C., Suefferheld, M., and Rieger, M. (1997). *Plant Cell Environ.*, 20, 609.
28. Sheveleva, E., Marquez, S., and Zegeer, A. (1998). *Plant Physiol.*, 117, 831.
29. Bohnert, H. J., and Shen, B. (1999). *Scientia Hort.*, 78, 237.
30. Galinski, E. A., Pfeiffer, H. P., and Truper, H. C. (1985). *Eur J Biochem*, 149, 135.
31. Nakayama, H., Yoshida, K., Ono, H., Murooka, Y., and Shinmyo, A. (2000). *Plant Physiol.*, 122, 1239.
32. Demel, R. A., Dorrepaal, E., Ebskamp, M. J. M., Smeekens, J. C. M., and de Kruijff, B. (1998). *Biochim. Biophys. Acta*, 1375, 36.
33. Schachtman, D., and Liu, W. (1999). *Trends Plant Sci.*, 4, 281.
34. Schachtman, D., and Schroeder, J. I. (1994). *Nature*, 370, 655.
35. Santa-Maria, G. E., Rubio, F., Dubcovsky, J., and Rodriguez-Navarro, A. (1997). *Plant Cell,* 9, 2281.
36. White, P. J. (1999). *Trends Plant Sci.*, 4, 245.
37. Blumwald, E., Aharon, G., and Apse, M. P. (2000). *Biophy. Acta*, 1465, 140.
38. Maathuis, F. J. M. and Sanders, D. (1999). *Curr. Opin. Plant Biol.*, 2, 236.
39. Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B. H., Matsumoto, T. K., Koiwa, H., Zhu, J. K., Bressan, R. A., and Hasegawa, P.M. (2001). *Proc. Natl. Acad. Sci. USA*, 98, 14150.
40. Maser, P., Gierth, M., and Schroeder, J. I. (2002). *Plant and Soil*, 247, 43.
41. Apse, M. P., Aharon, G. S., Snedden, W. A., and Blumwald, E. (1999). *Science*, 285, 1256.
42. Zhang, H. X. and Blumwald, E. (2001). *Nature Biotechnology*, 19, 765.
43. Rascio, A., Russo, M., Mazzucco, L., Platani, C., Nicastro, G., and Di Fonzo, N. (2001). *Plant Science*, 160, 441.
44. Rus, A. M., Estan, M. T., Gisbert, C., Garcia-Sogo, B., Serrano, R., Caro, M., Moreno, V., and Bolarin, M. C. (2001). *Plant, Cell Environ.*, 24, 875

45. Bressan, R. A., Hasegawa, P. M., and Pardo, J. M. (1998). *Trends Plant Sci.*, 3, 411.
46. Sanders, D., Brownlee, C., and Harper, J. (1999). *Plant Cell,* 11, 691.
47. Liu, J., and Zhu, J. K. (1997). *Proc. Natl. Acad. Sci. USA*, 94, 14960.
48. Liu, J., and Zhu, J. K. (1998). *Science*, 280, 1943.
49. Nurnberger, T., and Scheel, D. (2001). *Trends Plant Sci.*, 8, 372.
50. Perez-Prat, E., Narasimhan, M. L., Binzel, M. L., Rotella, M. A., Chen, Z., Valpuesta, V., Bressan, R. A., and Hasegawa, P. M. (1992). *Plant Physiol.*, 100, 1471.
51. Wimmers, L. E., Ewing, N. N., and Bennett, A. B. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 9205.
52. Geisler, M., Frange, N., Gomes, E., Martinoia, E., and Palmgren, M. G. (2000). *Plant Physiol.*, 124, 1814.
53. Hirschi, K. D. (1999). *Plant Cell*, 11, 2113.
54. Tyerman, S. D. and Skerrett, I. M. (1999). *Sci. Hort.*, 78, 175.
55. Hedrich, R. (1994). *Curr Topics Membranes*, 42, 1.
56. Smirnoff, N. (1998). *Curr. Opin. Biotechnol.*, 9, 214.
57. Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., and Inze, D. (1991). *EMBO J.,* 10, 1723.
58. Mc Kersie, B. D., Bowley, S. R., Harjanto, E., and Leprince, O. (1996). *Plant Physiol.*, 111, 1177.
59. Roxas, V. P., Smith, R. H. Jr., Allen, E. R., and Allen, R. D. (1997). *Nature Biotechnol.*, 15, 988.
60. Noctor, G., and Foyer, C. H. (1998). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 49, 249.
61. Davison, P. A., Hunter, C. N., and Horton, P. (2002). *Nature*, 418, 203.
62. Maggio, A., McCully, M.G., Kerdnaimongkol, K., Bressan, R. A., Hasegawa, P. M., and Joly, R. J. (2002). *Functional Plant Biology*, 29, 845.
63. Arrigoni, O., and De Tullio M. C. (2002). *Biochim. Biophy. Acta*, 1569, 1.
64. Chrispeels, M. J., Crawford, N. M., and Schroeder, J. I. (1999). *Plant Cell*, 11, 661.
65. Maurel, C., Javot, H., Lauvergeat, V., Gerbeau, P., Tournaire, C., Santoni, V., and Heyes, J. (2002). *International Review Of Cytology - A Survey Of Cell Biology*, 215, 105.
66. Baiges, I., Schaffner, A. R., Affenzeller, M. J., and Mas, A. (2002). *Physiologia Plant.*, 115, 175.
67. Schäffner, A. R. (1998). *Planta*, 204, 131.
68. Kjellbom, P., Larrson, C., Johannson, I., Karlsson, M., and Johanson, U. (1999). *Trends Plant Sci.,* 4, 308.
69. Tyerman, S. D., Niemietz, C. M., and Bramley, H. (2002). *Plant Cell Environ.*, 25, 173.
70. Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. (1996). *Plant Cell*, 8, 1181.
71. Johansson, I., Karlsson, M., Shukla, V. K., Chrispeels, M. J., Larsson, C., and Kjellbom, P. (1998). *Plant Cell*, 10, 451.
72. Steudle, E. (2000). *Plant and Soil.*, 226, 45.
73. Steudle, E. (2000). *J. Exp. Bot.*, 51, 1531.
74. Ruggiero, C., De Pascale, S., Angelino, G., and Maggio, A. (2003). *Plant and Soil,* 250, 121.
75. Luan, S. (2002). *Signalling drought in guard cells. Plant Cell Environ.*, 25, 229.
76. Hose, E., Steudle, E., and Hartung, W. (2000). *Planta,* 211, 874.
77. Niyogi, K. (1999). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 50, 333.
78. Kuzniak, E. (2002). *Acta Physiologiae Plant.*, 24, 97.
79. Pei, Z. M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G. J., Grill, E., and Schroeder, J. I. (2000). *Nature*, 406, 731.
80. Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). *Microbiol. Mol. Biol. Rev.*, 62, 1264.
81. Mendoza, I., Rubio, F., Rodriguez-Navarro, A., and Pardo, J. M. (1994). *J. Biol. Chem.*, 269, 8792.
82. Pardo, J. M., Reddy, M. P., Yang, S., Maggio, A., Huh, G. H., Matsumoto, T., Coca, M. A., Paino-D'Urzo, M., Koiwa, H., Yun, D. J., Watad, A. A., Bressan, R. A., and Hasegawa, P. M. (1998). *Proc. Natl. Acad. Sci. USA USA*, 95, 9681.
83. Liu, J., Ishitani, M., Halfter, U., Kim, C. S., and Zhu, J. K. (2000). *Proc. Natl. Acad. Sci. USA USA,* 97, 3730.

84. Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., and Zhu, J. K. (2002). *Proc. Natl. Acad. Sci. USA USA*, 99, 8436.
85. Shi, H., Ishitani, M., Kim, C., and Zhu, J. K. (2000). *Proc. Natl. Acad. Sci. USA*, 97, 6896.
86. Shi, H., Quintero, F. J., Pardo, J. M., and Zhu, J. K. (2002). *Plant Cell*, 14, 465.
87. Halfter, U., Ishitani, M., and Zhu, J. K. (2000). *Proc. Natl. Acad. Sci. USA*, 97, 3735.
88. Zhu, J. K. (2001). *Curr. Opinion Plant Biol.*, 4, 401.
89. Xiong, L., Gong, Z., Rock, C. D., Subramanian, S., Guo, Y., Xu, W., Galbraith, D., and Zhu, J. K. (2001). *Dev. Cell*, 1, 771.
90. Xiong, L., Ishitani, M., Lee, H., and Zhu, J. K. (2001). *Plant Cell*, 13, 2063.
91. Shi, H., Xiong, L., Stevenson, B., Lu, T., and Zhu, J. K. (2002). *Plant Cell*, 14, 575.
92. Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). *Proc. Natl. Acad. Sci. USA*, 97, 11632.
93. Xiong, L., and Zhu, J. K. (2001). *Physiol. Plant.*, 112, 152.
94. Xiong, L., Lee, B. H., Ishitani, M., Lee, H., Zhang, C., and Zhu J. K. (2001).*Genes Dev.,* 15, 1971.
95. Zhu, J. H., Gong, Z., Zhang, C., Song, C. P., Damsz, B., Inan, G., Koiwa, H., Zhu, J. K., Hasegawa, P. M., and Bressan, R. A. (2002). *Plant Cell,* 14, 3009.
96. Zhu, J. K. (2001). *Trends Plant Sci.*, 6, 66.
97. Kramer, P. J. and Boyer, J. S. (1995). *Water Relations of Plants and Soils. Academic Press, San Diego,* 495pp.
98. Dalton, F. N., Maggio, A., and Piccinni, G. (1997). *Plant and Soil*, 192, 307
99. Dalton, F. N., Maggio, A., and Piccinni, G. (2000). *Plant and Soil*, 219, 1.
100. Dalton, F. N., Maggio, A., and Piccinni, G. (2001). *Plant and Soil*, 229, 189.
101. Maggio, A., Dalton, F. N., and Piccinni, G. (2002). *European J. Agronomy*, 16, 197.
102. Derera, N. F., Marshall, D. R., and Balaam, L. N. (1969). *Experimental Agriculture*, 5, 327.
103. Kummerow, J. (1980). In*: Adaptation of Plants to Water and High Temperature Stress. NC Turner and PJ Kramer (eds), John Wiley & Sons Publishers, New York,* 57.
104. Passioura, J. B. (1988). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39, 245.
105. Passioura, J. B. (1994). In: *KJ Boote, JM Bennett, TR Sinclair and GM Paulsen (eds) Physiology and Determination of Crop Yield, American Society of Agronomy Crop Science Society of America Soil Science Society of America, Madison WI USA*, 343.
106. Maggio, A., and Joly, R. J. (1995). *Plant Physiol.*, 109, 331.
107. Steudle, E. and Peterson, C. A. (1998). *J. Exp. Bot.*, 49, 775.
108. Maggio, A. and Joly, R. J. (2000). In*: S Hohmann and S Nielsen (eds), Molecular biology and physiology of water and solute transport, Kluwer Academic Press*, 309.
109. Henzler, T., Waterhouse, R. N., Smyth, A. J., Carvajal, M., Cooke, D. T., Schaffner, A. R., Steudle, E., and Clarkson, D. T. (1999). *Planta*, 210, 50.
110. Nakamura, R. L., Mckendree, W. L., Hirsch, R. E., Sedbrook, J. C., Gaber, R. F., and Sussman, M. R. (1995). *Plant Physiol.*, 109, 371.
111. Malamy, J. E. and Benfey, P. N. (1997). *Trends Plant Sci.*, 2, 390.
112. Galbraith, D. W. (1994). In: *D.W. Galbraith, D.P. Bourque, H.J. Bohnert (eds), Methods in Cell Biology, Academic Press,* 539.

CHAPTER 4

UNRAVELLING THE GENETIC BASIS OF DROUGHT TOLERANCE IN CROPS

R. TUBEROSA[a], S. GRILLO[b] AND R.P. ELLIS[c]

[a] *Department of Agroenvironmental Science and Technology, University of Bologna, Italy*

[b] *Institute of Plant Genetics (CNR-IGV), Research Division of Portici, Portici (NA), Italy*

[c] *Scottish Crop Research Institute, Dundee, UK*

Abstract. Drought is the most important abiotic stress curtailing crops' yield world-wide. This chapter provides indications on how conventional and non-conventional approaches can contribute in furthering our understanding of the bases of the adaptive response of plants to drought and how such knowledge can be exploited to improve the yield and sustainability of crops under conditions of water deficit. The identification of the appropriate morpho-physiological and/or biochemical traits to indirectly improve crops' yield and its stability under drought is a difficult task. Among the available options, traits directly related to yield (e.g., yield components) or traits integrating information on metabolic processes throughout the plant life cycle (e.g., stable carbon isotope discrimination) are particularly valuable. Traits related to the phenology of the crop (e.g., early vigour, flowering time, etc.) have been successfully exploited to indirectly improve yield under drought conditions. Other biochemical traits (e.g., accumulation of drought-induced proteins) improving the survival of the plant under severe conditions of dehydration may not be of great relevance in the majority of the field conditions experienced by crops, when escape and avoidance mechanisms play a more pivotal role. As to the role that the novel approaches will play in integrating conventional breeding practices, marker-assisted selection is likely to provide meaningful contributions for improving yield and its stability under drought conditions, while it remains to be ascertained to what extent genetic engineering may also contribute in such direction. Although very encouraging results have been reported with genetically engineered plants grown under controlled conditions, scanty evidence indicates a similar benefit under field conditions. A better understanding of the intricate signalling pathways involved in the adaptive response to drought coupled with the targeted manipulation of the regulatory genes controlling the expression of large suites of drought-regulated genes will provide better opportunities to more effectively tailor drought-resistant crops.

1. INTRODUCTION

The serious challenges posed by the effects of drought at all levels of human society have long been recognized [1]. In most of the less-developed countries (LDCs), the major staple crops, particularly cereals, are grown under rainfed conditions in

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 71-122.

environments often characterized by relatively low and erratic rainfall. While intermittent precipitation causes large yearly fluctuations in cereal production, terminal drought and heat usually represent the most critical and common constraints to cereal production in LDCs. In maize grown in the tropics it has been estimated that ca. 24 million tons of grain are lost on a yearly basis because of insufficient water, with losses reaching up to 60% of well-watered production [2]. Additionally, drought stress also limits the uptake of nitrogen and other nutrients, thus impairing kernel development and negatively affecting its nutritional value. The advancing of desertification and/or unsanitary conditions due to lack of water plague vast areas in Sub-Saharan Africa and many periurban areas of the tropics. It is estimated that of the total water available world-wide, ca. 80% is used for irrigated agriculture. Clearly, in view of the fast increasing demand for water uses not related to agriculture, a higher proportion of the available water will be diverted to stakeholders other than farmers, thus calling greater attention to the release of cultivars with an improved water-use efficiency (WUE).

The deployment of biotechnological tools and strategies (e.g., marker-assisted selection, genetic engineering, microarrays, etc.) that can contribute to breed for more drought-resilient crops and improve their WUE is not a complete answer to the development of a more sustainable agricultural production. Similarly, traditional technologies alone, such as conventional breeding and irrigation, cannot provide in the foreseeable future the solutions required for increasing as necessary the agricultural outputs in LDCs while preserving long-term sustainability. Irrigation is often economically infeasible not least as water needed for irrigation is unavailable in the immediate area. Furthermore, in regions that are becoming more arid (e.g., the Mediterranean basin), irrigation unavoidably increases the vulnerability of the environment, particularly soil erosion [3] and salinization [4]. It has been estimated that ca. 20% of irrigated soils is under risk of salinization, while each year ca. 1% of all irrigated soils is lost consequent to an excessive accumulation of salt. In addition, irrigation can also increase the risk of nutrient leaching, groundwater spoilage and ground water contamination, all of which reduce quality of life.

Therefore, the predicted effects of climate change [5] emphasize the need to urgently explore and develop new methods to more effectively improve the long-term sustainability of agricultural production. This includes the screening and use of available genetic resources and studies on crop management methods to increase the efficiency of water and nutrient use. The effects of climate change on water availability differ from region to region with, for example, Finland becoming wetter and Spain becoming drier. Studies are needed to understand the effect of environmental factors on crop water relations, to identify the genes controlling water economy of crops exposed to drought and to devise effective breeding strategies to improve WUE. While these studies are more urgently needed in the crops more important for feeding mankind, current progress in understanding and comparing the genetic make-up of cereals means that it is particularly convenient to examine progress in these species. Additionally, studies in *Arabidopsis*, a model species for genetic analysis [6], have permitted the identification of genes underlying important physiological processes. Thus, in turn, it may be possible to use DNA sequences

from *Arabidopsis* to monitor and manipulate gene expression in crop species. Analogously, the availability of the complete genome sequence in rice [7] is a valuable and unparalleled resource for gene discovery in other cereals and grasses. Water shortages in LDCs consequent to climatic changes have the potential to decrease social coherency and may result in an unequal basis for rural development in association with a reduced employment in the agribusiness sector. In the long term, the ability to foster the cultural heritage is decreased especially in areas suffering most severely from water shortages induced by climate changes. Furthermore, sustaining agricultural landscape and its natural habitats is important from an environmental, social and economic perspective. The development of crops using less water per unit of produced biomass, hence having a better capacity to tolerate and cope with water shortages, is thus highly desirable. An increased WUE concomitantly improves uptake and utilization of nutrients which is important for preserving the environment while enhancing the profitability of the agricultural system and farmers' livelihoods.

2. DEFINING YIELD COMPONENTS UNDER WATER-LIMITED CONDITIONS

Yield results from many developmental and physiological processes such as apical differentiation, seedling growth, water and mineral uptake, carbon fixation, dry matter translocation and maturation. In turn, each one of these processes results from biochemical pathways that are catalysed by enzymes, whose activity is influenced by environmental factors. In cereals, grain yield is related to more simple components which, as such, are more suitable for genetic analysis:

$$GY = NEm^{-2} \times NK \times KW$$

where GY = grain yield; NEm^{-2} = number of ears per m^2; NK = number of kernels per ear; KW = kernel weight.

In water-stressed environments, a more meaningful formula was suggested by Passioura [8]:

$$GY = W \times WUE \times HI$$

where W = total amount of water transpired by the crop; WUE = water-use efficiency, i.e. the amount of biomass produced per unit of transpired water; HI = harvest index, i.e. the ratio between GY and total biomass. In this formula the importance of total biomass (W x WUE) and its partitioning (HI) are emphasized and the critical nature of WUE becomes obvious. In low-stress environments, HI has been improved particularly by the use of dwarfing genes [9]. Biomass production has been extensively studied and useful levels of genetic control have been identified [10]. The interdependence of individual traits was underlined in a study of droughted pearl millet conducted by Yadav et al. [11]. They concluded that the quantitative

trait locus (QTL) alleles that contributed to an increased drought tolerance did so either through better than average biomass productivity or HI.

As compared to the formula suggested by Passioura, the formula suggested by Edmeades et al. [2] incorporates greater physiological complexity:

GY = RAD x %RI x GLD x RUE x HI

where RAD = incident solar radiation received per day; %RI = percent intercepted radiation over crop life cycle; GLD = green leaf duration; RUE = radiation use efficiency over the life of the crop; HI = harvest index. Few studies have investigated radiation interception [12] and because of the difficulty in taking measurements they have been confined to cultivars rather than populations suitable for QTL detection.

Yield and its components, as well as all other quantitative traits, are subject to environmental influences that lower their heritability (i.e. the percentage of the phenotypic variation under genetic rather than environmental control). High heritability values indicate a better chance of reliably detecting QTLs with effects large enough to warrant the use of marker-assisted selection. It is thus important to obtain unbiased estimates of heritability, which is possible only when a rather large number of separate experiments are considered. In the case of simple GY components in cereals, heritability is higher than that of the composite trait, i.e. GY. This is often not the case for many of the physiological traits that have been considered in studies aimed at identifying reliable and useful predictors of GY in water-limited conditions [13]. In fact, one important prerequisite for selecting a physiological trait to improve yield is that its heritability is higher than that of yield itself. Other desired prerequisites include a high genetic variance of the physiological trait coupled with the possibility to perform a reliable, early screening at low cost in different environments and/or experimental conditions.

3. MECHANISMS CONFERRING TOLERANCE TO DROUGHT

Severe drought is an environmental stress that can lead to plant death. Different mechanisms allow crops to escape, avoid and/or tolerate the deleterious effects of drought [14-18]. Crops in mainstream agriculture also experience transient drought episodes which reduce yield but do not usually cause a complete crop loss. The environmental conditions of these two situations overlap because of the world-wide variability of rainfall and evapotranspirative demand of each crop. Strategies for developing new cultivars tolerant to drought stress range from the analytical almost "gene-by-gene" approach [19-22] to the development of tolerance by breeding approaches [23, 24] possibly followed by a retrospective analysis of related changes in traits other than yield [2, 25]. Ludlow and Muchow [16] distinguished traits related to drought escape and drought resistance, with the latter further subdivided into those conferring dehydration avoidance or dehydration tolerance. Dehydration avoidance depends on maintenance of turgor through an increase in water uptake

and/or reduction in water loss. Dehydration tolerance depends on biochemical mechanisms allowing the cell to tolerate water loss. A number of reviews [15, 18, 23, 24, 26-30] have extended these concepts to a wide range of crops.

Drought escape is typified by early flowering in barley [31], rice [32] and wheat [33] where "stored" water is exploited before the onset of seasonal drought. In crops of the Mediterranean and temperate regions, photoperiod sensitivity is a widespread mechanism that allows flowering to match the period of maximum solar radiation. An appropriate match between crop phenology and the prevailing environmental conditions will maximize seedling biomass production. Biomass components include structural and metabolic elements, which have often been modelled to investigate sink-source relationships [34, 35]. Unless early growth and development provide the appropriate plant structure, yield potential cannot be realized by the re-mobilization of soluble carbohydrates, as reported for dwarf sorghum [36]. A striking difference between early and late maturing genotypes is often seen in differences in leaf area duration and the development of crop biomass [34]. Leaf area duration is important in stressed environments because early leaf senescence may reduce photosynthesis. During maturation, delayed leaf senescence (stay-green) is a major component of an increased carbon export from leaves in *Lolium temulentum* and, consequently, an improved productivity [37]. "Stay-green" traits have been described in many other species such as tomato [39], *Capsicum annuum* [39], *Pisum sativum* [40], sorghum [41-43] and maize [44] and a number of underlying mechanisms have been identified [37].

Dehydration avoidance involves mechanisms that allow the plant to maintain a relatively higher water status, thus avoiding the deleterious effects of dehydration. This strategy implies a higher capacity to extract water from the soil layers (e.g., a deep root system [45]) and/or a lower water loss from the canopy (e.g., leaf rolling). In rice, Fukai and Cooper [46] suggested that root traits allowing access to additional water played a more important role than traits reducing water loss. Other mechanisms can contribute to dehydration avoidance. In *Phaseolus acutifolius*, high yield in drought conditions was associated with deep root penetration and a higher stomatal sensitivity to dehydration [47]. The importance of a deep root system has also been repeatedly emphasized in rainfed rice [48, 49]. In sorghum, a thicker epicuticular wax layer contributed to dehydration avoidance [50].

Dehydration tolerance depends on osmotic adjustment [15, 51] or "stay-green" traits [15, 52]. In *Poa bulbosa*, dehydration tolerance was associated with, but not fully explained by, the expression of specific dehydrin proteins [53]. Osmotic adjustment has received considerable attention as a key mechanism for drought tolerance since the accumulation of solutes within the cell maintains turgor [15]. Osmotic adjustment is not a constitutively inherited trait but is induced by drought; interestingly, some cereal cultivars capable of high osmotic adjustment produce longer roots and higher root biomass [51, 54]. Scavenging of free radicals may also play an important role in mitigating the damage to the biochemical machinery of the cell consequent to an excessive redox potential that usually occurs during drought episodes [55, 56]. This is an obvious area for future investigations attempting to

integrate the information from studies on the interaction between genotype and environment and the integration of response signals within the plant.

3.1. Examples in cereal crops

Herein, we briefly summarize some case studies in barley, maize and rice, three major crops that have been extensively investigated for the secondary traits and morpho-physiological mechanisms conferring drought resistance as well as for the identification of the corresponding genes and/or QTLs. The use of QTL mapping to dissect the genetic basis of grain yield (GY) and other secondary traits under drought conditions is more extensively examined in section *4.1.* As to wheat, another major cereal crop that is often grown under water-limited environments, the relevance of secondary traits in determining drought resistance has been extensively discussed in a number of recent articles [57-61]. Additional studies on other crops have recently been reported in a special issue edited by Griffiths and Parry [62].

3.1.1. Rice

In rainfed ecosystems, water stress is the abiotic factor most severely curtailing rice production [63, 64]. The magnitude and relevance of this problem is evident in consideration that rainfed rice represents ca. 45% of the total area planted with rice. A comprehensive special issue edited by Wade on the improvement of tolerance to abiotic stresses in rainfed lowland rice has recently appeared [63]. These studies and the progress in the development of new breeding strategies [48, 49] compare starkly with the review of rice breeding in India between 1965 and 1975 [65]. Although drought was recognized as a factor that limited GY, no crosses were made to improve tolerance as programmes mainly concentrated on the use of dwarfing genes to increase lodging resistance and GY. This omission has now been repaired, since in the past decade the genetic control in drought-stressed rice of root length, root weight, stomatal frequency and other drought-related traits has received an increasing attention [32, 48, 49, 66, 67].

Rice landraces have been the subject of similar studies [68] to identify novel genes for drought tolerance. Wade and colleagues [63, 64] reviewed genotype x environment interaction in rice over the entire Asian region. They classified a sample of rice cultivars as drought susceptible, stable over all environments, specifically adapted to recovery after drought or adapted to a late-occurring drought. This study identified a panel of cultivars that could be used to categorize specific environments.

In rainfed rice breeding, there are no obvious target traits such as reducing the anthesis-silking interval in maize [2] or improving "stay-green" in sorghum [69, 70] that result in an increased drought tolerance [32, 71]. Hence, research has primarily focused on identifying traits that are thought to contribute to drought resistance such

as leaf rolling, stomatal response, cuticular waxes, WUE, osmotic adjustment, membrane stability and photoinhibition resistance.

3.1.2. Maize

It is interesting to compare the trait-analysis approach used in rice with the review of Bruce et al. [72] in maize. Maize productivity has improved rapidly since the introduction of single-cross hybrids and modern hybrids show improved yield and yield stability under drought [73]. Selection for a shorter anthesis to silking interval (ASI) has been a major factor in improving drought tolerance, particularly in the breeding program carried out at CIMMYT [2, 74]. If ASI is prolonged, then pollen shed may occur too late for fertilization, thus causing an increased ear sterility and lower GY [2]. A drought episode coinciding with flowering can reduce GY two to three times more when compared to drought episodes of similar intensity occurring at other growth stages. The particularly high sensitivity of maize to drought at flowering has been related to its peculiar floral structure, characterized by separate male and female organs and the almost synchronous development of florets on a single ear [2]. Under this respect, ASI represents an easily scorable indicator of assimilate partitioning to the ear, and possibly also of the water status of the plant.

A large-scale breeding programme at CIMMYT, starting with a single population in 1975 and later extended to five additional populations, resulted in a GY increase of more than 130 kg/ha/year [72]. This followed after eight cycles of recurrent selection and was consistent over a wide range of test sites. GY was the target trait and all other traits were considered to be secondary and not given high priority in selection. However, an effect of selection for higher GY was associated with changes in other characters such as reduced barrenness, slightly earlier anthesis, reduced number of spikelets, more rapid ear growth, reduced ASI and reduced root biomass in the top 50 cm of the soil [25, 75, 76]. It was suggested that the decrease of root biomass to selection for higher GY under drought was due to the assimilate cost of a sustained growth of the root at flowering, i.e. when the availability of assimilates limits sink size. Furthermore, it was particularly striking that no change was seen in any trait more directly associated with water stress, such as plant water status, osmotic adjustment and canopy temperature. This does not imply that these traits have no adaptive value; simply, they were not associated with an advance in GY under drought conditions in that particular environment. Bruce et al. [72] concluded that when the GY components are set at different stages, such as in cereals, it is helpful to consider changes in plant life cycle. It is evident that the molecular genetics of kernel development will become the main focus for research in GY and its stability under drought conditions.

3.1.3. Barley

A primary aim of a breeding program is to match the life cycle of a crop to the local environment, hence the possibility of sowing spring barley in Scotland in March and

the same cultivars being sown in the autumn in Spain. The seasonal shift allows the drought and heat typical of Mediterranean climates to be avoided in part, although not completely as indicated by changes in grain biochemistry [77]. The underlying phenomenon in both scenarios is that the time from sowing to flowering matches the availability of rain or stored soil water. This allows for a vigorous seedling growth, with some evidence in barley of genetic loci that are independent of dwarfing genes [78]. The plant cannot develop an optimal structure unless it has a functional root system. Genetic variation for root length has been reported in wild barley [79]. Therefore, domestication of barley may have resulted in changes in size and/or architecture of root systems; more work is required to establish the extent of the modification.

The effects of drought on the physiology of the barley plant relate to the life cycle of the crop. In barley, plant development and growth begins with germination and reactivation of the meristems. Stem apex development critically depends on temperature and daylength [80] while the processes of root initiation are less well understood. Tiller emergence and survival are fixed by the rate of growth and the time of canopy closure. GY is limited by the absorption of solar radiation, the efficiency of conversion to dry matter and leaf senescence.

Germination begins when the dry grain imbibes water. In normal field conditions there is sufficient water to initiate germination soon after the crop is drilled. Germination is inhibited by drought, as the top 4-5 cm of soil can often be completely dry. When the soil is dampened by light, intermittent rainfall, germination can be followed by death of the seedlings. This indicates that germination and crop establishment constitute a vulnerable stage of crop growth. Until the roots are deep enough to contact soil moisture in the sub-soil, the seedling will be threatened by drought.

The life cycle of a barley crop after seedling emergence is a series of discrete phases: apical differentiation, tiller emergence, stem extension, ear growth, anthesis, grain filling and maturity [81]. The physiology of plant development and growth have been analysed in a number of studies [80, 82, 83] that focus on the need for greater partitioning of dry matter into the apex. Small changes in apex size, through either larger embryonic meristems or through a reduction in main-stem apical dominance, can increase yield potential in drought-stressed environments. Before this potential can be achieved, the photosynthates fixed by the canopy have to be remobilised into the grain by a series of complex metabolic pathways.

Time of heading is of vital importance as, by this point, main-stem extension is complete, apical primordium differentiation ceases and tiller growth stops. Anthesis can occur near the date of heading but is difficult to record in closed-flower accessions where inflorescences need to be dissected for precise observation. Late heading results in a crop that is difficult to harvest in wet environments while early heading can result in a profusion of secondary tillers that ripen too late. Time of heading is affected by many genes such as those controlling response to vernalization and daylength as well as earliness *per se* [84-88].

Early flowering, conditioned by a daylength response and low vernalization requirement, is appropriate for Mediterranean latitudes. Typically, in dry, rainfed

situations early flowering is important to exploit winter rainfall and escape drought stress associated with high temperatures in mid-summer [31]. In contrast, the highest spring barley yields occur in moist, cool environments in which long days allow slow plant development [80, 89-91]. In contrast to heading date and like anthesis, apical primordium differentiation is difficult to observe and there are no recent reports of the genetic control of the rate of apical differentiation. Kirby [83] suggested a developmental template that could be used to relate apical development to the rate of leaf appearance. In barley, six QTLs have been identified to control the number of leaves of the main culm [78, 92]. The initiation of tiller bud growth, emergence of the tiller from the subtending leaf sheath and the development of a fertile ear depend on the availability of water, nutrients and light. When all the resources are abundant, high yields result from an increased ear density, consequent to an increased tiller survival [92-94]. Tiller emergence can be observed as readily as leaf emergence [78] but careful records are needed to ensure that later seedling growth is recorded correctly. By the time of heading (ear emergence) a number of main-stem leaves will have senesced and may no longer be visible in dissected seedlings. The common effects of abiotic stresses are to restrict the accumulation of dry matter [91] and to increase rates of leaf senescence.

In addition to morphological features, abiotic stresses modify biochemical pathways within the barley plant. These effects are even less accessible than apical development and are difficult to analyse in mapping populations. This is all the more so given the complexity of abiotic stress tolerance; furthermore, it is important to distinguish between the direct effects of genes that confer tolerance and their pleiotropic effects or epistatic interactions. Genetic mapping and the measurement of discrimination for the natural carbon isotopes (^{12}C and ^{13}C) to integrate the stress response have been suggested as a possible approach [95]. Carbon isotope discrimination ($\delta^{13}C$) values are interpretable in terms of a well-established physiological model [96]. Conditions which induce stomatal closure (e.g., water stress, either directly or indirectly through salinity and freezing) restrict CO_2 supply to carboxylation sites, thus reducing $\delta^{13}C$. Shoot $\delta^{13}C$ was more heritable than other seedling traits [78] and this seems to be a general property for all the *Triticeae* as similar data were found in wheat [97]. The implication is that a reasonable proportion of the phenotypic variation in plant $\delta^{13}C$ can be genetically manipulated. However, the relationship between $\delta^{13}C$ and barley yield is complex and early attempts to develop $\delta^{13}C$ as a direct assay for yield in barley were not successful [98]. The reasons for this are suggested by the results of Ellis et al. [78]: only one of the three primary QTLs for GY was associated with a primary QTL for stem $\delta^{13}C$.

4. MOLECULAR APPROACHES FOR DISSECTING THE MOLECULAR BASIS OF DROUGHT TOLERANCE

Improving GY under drought would greatly benefit from a more accurate dissection of the molecular processes involved in sensing changes in water status and signalling

such changes to drought-inducible genes capable of mitigating the effects of dehydration either through an accumulation of specific proteins/metabolites and/or through adaptive changes in plant architecture and its metabolism. While traits related to the function of a single gene can be handled in a fairly straightforward manner, more sophisticated approaches are required to deal with the traits determining GY under drought conditions, due to the their low heritability [16, 18, 99]. However, as suggested by Blum [27], the complexity of drought tolerance can be greatly reduced if two major points are considered: 1) a number of plant traits crucial for the control of plant water status and yield under drought are constitutive and not stress adaptive; 2) plant water status, more than plant function, controls crops performance under drought. In the following paragraphs we will describe how a number of molecular approaches can help us to unravel the complexity of drought tolerance in crops.

4.1. Applications of QTL analysis

The identification of QTLs provides excellent opportunities for an interdisciplinary approach among molecular geneticists, physiologists and breeders. One of the advantages is that genetically complex (quantitative) traits can be resolved into single components (i.e. the QTLs) and the corresponding chromosome regions manipulated at will and investigated in much greater detail by using near isogenic lines at target QTLs. This, in turn, will allow us to elaborate models for predicting the variation in phenotype due to particular alleles and to acquire a more refined mechanistic understanding of phenotypes corresponding to particular genotypes [100, 101]. Because obtaining a mapping population suitable for identifying QTLs is no trivial undertaking in terms of resources and time required to complete the study, particular attention should be devoted to the choice of the target traits and the parental lines of the mapping population. Among the drought-related traits that have been the subject of QTL analysis, the current understanding of their breeding value is only good for the anthesis-silking interval in maize [44, 74, 75, 102] and is otherwise poor (e.g., root traits) or even controversial, as in the case of abscisic acid [15, 17, 101, 103-108].

To a certain extent, QTL analysis allows us to determine whether the genetic association between a particular trait and yield is more likely to be caused by to pleiotropy or linkage. The interpretation of the results obtained with these studies is easier with traits whose effects on yield, on a physiological basis, are preferentially unidirectional. An example is provided by anthesis-silking interval (ASI) in maize, where the most critical stage in terms of deleterious effects of drought on GY is just prior to and during flowering [109-111], thus leading to a negative association of ASI and GY under drought conditions [25, 75, 112]. In this case, the relative additive effects of the QTL for the two traits will have opposite signs. For each trait, the relative additive effect of a QTL is computed according to the formula (A1A1 -

A2A2)/2, where A1A1 and A2A2 represent the mean phenotypic values of the two groups of families homozygous for the parental QTL alleles (A1 and A2).

More in general, variations in the temporal expression patterns of the gene/s underlying a QTL may have contrasting pleiotropic effects on final yield according to the growth stage of the plant and/or the prevailing environmental conditions that, according to the crop, limit relocation of assimilates to and accumulation in the grain or other storage organs (e.g., bulbs, tubers, seeds, fruits, etc.).

4.2. Identification of QTLs for yield and related traits under water-limited conditions

The large number (ca. 80) of studies reporting QTLs under drought conditions confirms the current interest in this approach. The vast majority of these reports have been conducted in cereals, in view of the availability of several mapping populations for these species. The extensive synteny (i.e., conservation of the linear order of genes on the chromosomes of different species) existing among closely related species allows for a comparative analysis of QTL data in cereals, thus augmenting the value of these studies and their interest for possible applications (e.g., marker-assisted selection, cloning, etc.). However, recent results based on sequence identity to determine the correspondence between the rice chromosomes and those of the other cereals [7] have revealed a picture considerably more complex than that previously obtained through comparative mapping [113].

Among the studies reporting QTLs for drought-related traits, only a limited number have been considered herein. With only a few exceptions, all these studies have been conducted adopting mapping populations derived from parents belonging to the same species. We will first summarize the results obtained with this approach and then analyse the results obtained with mapping populations exploring inter-specific variation through advanced backcross QTL analysis as advocated by Tanksley and Nelson [114].

4.3. Identification of QTLs in mapping populations derived from intra-specific crosses

Herein, we provide an overview of the main results of the cereal crops that have been most intensely investigated for drought-related QTLs, namely rice, sorghum, maize and barley. Although GY in wheat is also heavily curtailed by drought on a global scale, only preliminary results have so far been reported for drought-related QTLs [115, 116], mainly due to the limited number of well-developed linkage maps available for this important crop.

4.3.1. Rice

Rice is one of the first crops for which a map based on molecular markers was constructed [117] and QTLs were identified [118]. Improving the drought resistance of high yielding rice varieties for upland areas is an important goal, since upland rice relies exclusively on rainfall and, consequently, is generally low yielding. Drought resistance is also considered the most complex trait required for rice cultivars grown in rainfed lowland [119]. Among all cereals, rice is by far the species which is exposed to the widest range of growing conditions in terms of water availability (e.g., submerged rice, paddy rice and upland rice) and, consequently, also in terms of soil structure and related characteristics. A deep, thick root system has been shown to have a positive effect on the yield of upland rice under water stress conditions [48]. Due to the difficulty of scoring root traits, marker-assisted selection (MAS) could be used for the improvement of root morphology [71, 103]. Therefore, several rice studies focused on QTLs controlling root architecture and related traits governing the adaptation to a wide range of environmental conditions. For the sake of conciseness, the results of only a limited number of these reports have been considered.

Champoux et al. [120] investigated the overlap of QTLs associated with root morphology and QTLs associated with drought avoidance/tolerance in the field. Root thickness, maximum root length, root-to-shoot ratio, root dry weight/tiller and deep root dry weight/tiller were measured in 203 recombinant inbred lines (RILs) derived from a cross between Co39 an *indica* cultivar of lowland adaptation and Moroberekan, a traditional *japonica* upland cultivar. The RILs were also grown in three field experiments where they were drought-stressed at the seedling, early vegetative and late-vegetative growth stages, and assigned a visual rating (based on leaf rolling) for their degree of drought avoidance/tolerance. Correlations of root parameters measured in greenhouse experiments with field drought avoidance/tolerance were significant but not highly predictive. Twelve of the 14 chromosomal regions containing putative QTLs associated with field drought avoidance/tolerance also contained QTLs associated with root morphology. Thus, selecting for Moroberekan alleles at marker loci associated with the putative root QTLs was advocated as an effective strategy for altering the root phenotype towards that commonly associated with drought-resistant cultivars.

A sample of 52 lines derived from Co39 x Moroberekan were also investigated for the presence of QTLs associated with dehydration tolerance and osmotic adjustment [121]. The measurements obtained and the QTLs identified were compared to those of root traits and leaf rolling scores measured on the same lines. One major QTL for osmotic adjustment was suggested to be homologous with a single gene previously identified for the same trait in wheat. The putative osmotic adjustment locus and two of the five QTLs influencing dehydration tolerance were close to chromosomal regions affecting root morphology. In this population, osmotic adjustment and dehydration tolerance were negatively correlated with root morphological characters associated with drought avoidance. High osmotic adjustment and dehydration tolerance were associated with Co39 alleles, while a larger root system was associated with Moroberekan alleles.

The mapping population that has been most extensively investigated for root characteristics and other drought-related traits has been derived from Bala x Azucena, two drought resistant rice varieties. Azucena has root traits that contribute to drought resistance, while Bala has a number of shoot-related mechanisms that make it adapted to drought-prone environments. In a number of studies, Price and co-workers have reported and compared a vast number of QTLs governing root characteristics and other drought-related traits (e.g., leaf rolling, leaf drying, leaf relative water content, etc.) under a wide range of experimental conditions [32, 66, 67, 71, 122, 123].

Results on root QTLs obtained with other mapping populations and their utilization to improve drought tolerance have been discussed in a number of other studies [119, 124-126]. This extensive body of data on different mapping populations profiled with a number of common RFLP (Restriction Fragment Length Polymorphism) and SSR (Simple Sequence Repeat) markers has allowed a detailed comparison of the QTL position for root traits across populations, thus leading to the identification of a number of key QTL regions with a more substantial and consistent effect in controlling variation in roots [123, 127-129] and other traits [71, 123, 129, 130]. Based on the results of this comparative analysis a number of NILs and backcross-derived lines (BDLs) at target QTLs have been derived using MAS. These congenic strains have allowed for a more accurate evaluation of the effects of the single QTLs and for ascertaining the presence of epistatic interactions [123, 127].

A trait that has received particular attention in upland rice is rooting depth [71]. In the same study, QTLs for leaf rolling and leaf drying were also identified but did not map to the same locations as those for root traits as would be expected if the traits all contributed to drought resistance. A number of reasons for this lack of co-location were suggested, including the difficulty in collecting precise data from field trials because of variability in soils and rainfall.

Finally, a comparative analysis based on syntenic relationships between maize and rice presented by Quarrie [103] indicated that at least five QTL regions for root traits in Polj17 x F2 correspond to regions in rice that regulate root characteristics [120]. The most notable coincidence was again between the region near *umc11* on chromosome 1 in maize and the region between *RG104A* and *RG227* on chromosome 3 in rice. This chromosome region of rice also influenced root penetration ability [67] and root pulling force [129]. When this approach was applied to analyse the correspondence of QTLs for root traits in seven rice and four maize mapping populations, a number of syntenic regions showed the presence of QTLs in both species [131], thus providing useful information to prioritise the choice of QTLs for future efforts aimed at their positional cloning.

A comprehensive review on QTL mapping in rice, including QTLs for drought resistance, has recently been presented by Li and co-workers[132-134]. An interesting finding of this study is that most QTLs appear to be epistatic and complimentary interaction appears to be the most common form of epistasis. Based on these findings, a new strategy of molecular breeding has been developed to facilitate simultaneous QTL identification and introgression [134].

4.3.2. Sorghum

Drought is a major constraint in sorghum production world-wide. Sorghum is well-adapted to hot, dry environments and regarded as a model for studying drought resistance among the grasses, particularly maize, due to the extensive synteny between the genomes of these two species [113] as well as to the small size of its genome. Notably, significant progress in genome mapping of this crop has been recently reported [135].

In sorghum exposed to drought, rapid and premature leaf death usually occurs during grain filling. Premature leaf senescence, in turn, leads to charcoal rot, stalk lodging and significant yield losses. More than 80% of commercial sorghum hybrids in the U.S. is grown under non-irrigated conditions and although most of them show pre-flowering drought resistance, many do not have any significant post-flowering drought resistance. Breeding for improving post-flowering drought tolerance in sorghum hybrids remains an important priority. Because evaluation of stay-green is difficult and unreliable under field conditions due to the unpredictability of moisture stress and large environmental interactions, progress in improving stay-green in sorghum by conventional breeding methods has been slow. Consequently, increasing attention has been devoted to the study of QTLs for stay-green, a post-flowering drought resistance trait that contributes to normal grain filling and reduces the incidence of charcoal rot disease and stalk lodging.

Tuinstra et al. [69] evaluated 98 RILs developed from a cross between TX7078 (pre-flowering tolerant but post-flowering susceptible) and B35 (pre-flowering susceptible but post-flowering tolerant). In total, 13 QTLs were associated with one or more measures of post-flowering drought tolerance. Two QTLs were identified with major effects on GY and stay-green under post-flowering drought. More importantly, these QTLs also affected GY under fully irrigated conditions. QTLs for both rate and duration of grain development were also identified. High rate and short duration of grain development were generally associated with larger seed size, but only two of these loci were associated with differences in stability of performance under drought. Following these encouraging results, NILs were developed to test the phenotypic effects of three major QTLs affecting agronomic performance in drought and/or well-watered environments [70]. In most cases, NILs contrasting for a specific QTL allele differed in phenotype as predicted by the previous QTL study. NILs contrasting at QTL marker *tM5/75* indicated large differences in GY across a range of environments. Further analysis indicated that differences in agronomic performance may be associated with a drought tolerance mechanism that also influences heat tolerance. Additionally, NILs contrasting at QTL marker *tH19/50* differed in GY under both water regimes. The analysis of these NILs indicated that these differences may be influenced by a drought tolerance mechanism that conditions plant water status and the expression of stay-green. NILs contrasting at QTL marker *t329/132* differed in GY and seed weight. In this case, differences appeared to be caused by two QTLs that are closely linked in repulsion phase.

Crasta et al. [41] evaluated in four environments a set of RILs derived from the cross B35 x Tx430 for post-flowering drought resistance and maturity. Three major stay-green QTLs (*SGA*, *SGD* and *SGG*) accounted for 42% of the phenotypic

variability and four minor QTLs (*SGB*, *SGI.1*, *SGI.2* and *SGJ*) contributed an additional 25% of the phenotypic variability in stay-green ratings. As to maturity, one QTL (*DFB*) alone contributed 40% of the phenotypic variability, while a second QTL (*DFG*) contributed an additional 17%. Although stay-green ratings were significantly correlated ($r = 0.22$, P 0.05) with maturity, six of the seven stay-green QTLs were independent of the QTLs influencing maturity.

Stay-green was also investigated in two parallel studies conducted on a RIL-mapping population developed from the cross B35 x Tx7000 and tested in seven environments [42, 43]. Both studies evidenced four stay-green QTLs (*Stg1*, *Stg2*, *Stg3* and *Stg4*). Additionally, the comparison of the location of these QTLs with those of earlier reports indicated their consistency across different genetic backgrounds. A significant epistatic interaction for stay-green and chlorophyll content involving *Stg2* and a region on linkage group C was also identified, leading Subudhi et al. [43] to conclude that *Stg2* is the most important QTL for stay-green. The same authors suggested targeting the *Stg2* QTL region for gene discovery in order to improve the basic understanding of stay-green.

In the case of post-flowering drought stress, lodging can severely curtail GY in mechanized agriculture. Kebede et al. [136] searched for QTLs controlling pre-flowering drought tolerance, post-flowering drought tolerance (stay-green) and lodging tolerance using a RIL population derived from the cross SC56 x Tx7000. The RILs, along with their parents, were evaluated for the above traits in multiple environments. Nine QTLs were detected for stay-green in several environments. Comparison of the location of these QTLs with those uncovered in B35 x Tx7000 [43, 137] indicated that three QTLs on linkage groups A, G and J were consistent. It should be noted that line SC56 was derived from a source different from that of B35. More importantly, comparative mapping showed that two of these sorghum stay-green QTLs corresponded to stay-green QTLs in maize. Additionally, these genomic regions were also reported to be congruent with other drought-related agronomic and physiological traits in rice and maize, which led Kebede et al. [136] to suggest that these syntenic regions might be hosting a cluster of genes influencing drought tolerance mechanisms in these grass species. In addition, three and four major QTLs responsible for lodging tolerance and pre-flowering drought tolerance, respectively, were detected.

Recently, Sanchez et al. [138] presented a comprehensive review on QTLs for stay-green, discussed progress towards their physical mapping and critically analysed the possible roles and functions of drought-induced genes. Their conclusion was that the molecular genetic dissection of the stay-green QTLs, through the evaluation of NILs, will provide further opportunities to elucidate the underlying physiological mechanisms involved in drought resistance in sorghum and other grasses.

4.3.3. Maize

In maize, cross-referring QTL studies is facilitated by the fact that the main reference genetic map (UMC map) has been subdivided into 100 sectors (bins) of

comparable length (ca. 17 cM) and flanked by reliable RFLP markers [139]. The bin framework is useful for comparison of QTL positions across maize experiments [140] and facilitates the comparison of QTL positions across closely-related species, such as maize and rice [103, 131]. In maize, the average genetic length of bins is roughly similar to the average chromosome interval supporting a QTL peak. Additionally, the UMC map allows us to compare the map position of mutants with that of QTLs, thus contributing relevant information for the identification of possible candidate genes for a particular trait. This provides the opportunity for testing Robertson's hypothesis [141], namely that a mutant phenotype at a particular locus may be caused by an allele whose effect is more drastic than that of the QTL alleles at the same locus.

QTLs for abscisic acid concentration. An increase in abscisic acid (ABA) concentration is a universal response of plants subjected to drought and other abiotic stresses [20, 142-144]. ABA modulates the expression of genes whose products may protect the cell from the harmful effects of excessive dehydration [20, 145], a condition which in tropical maize occurs more frequently during seedling establishment. In maize seedlings subjected to artificially-induced conditions of water deficit [144], an increased ABA concentration improved the root-to-shoot ratio, an adaptive change which at a later stage can be beneficial for avoiding dehydration when water is available in deeper layers of the soil profile. The role of ABA in sustaining root cell elongation at low water potential involves an interaction with ethylene production [146, 147]. It has also been shown that ABA facilitates water uptake into roots as the soil begins to dry, particularly under non-transpiring conditions, when the apoplastic path of water transport is largely excluded [148].

In maize, results for QTLs for leaf ABA concentration (L-ABA) have been reported in three mapping populations [100, 149, 150]. Of the 16 QTLs identified in the Os420 x IABO78 background [150], the most important and consistent QTL mapped on bin 2.04 near *csu133*. In the same region, a QTL for L-ABA has also been described in Polj17 x F2 [100]. The QTL for L-ABA near *csu133* has been validated by a molecular analysis [151] applied to F4 families derived following two cycles of divergent selection for L-ABA in field-grown plants derived from 480 (Os420 x IABO78) F2 plants [106, 152]. These results prompted the derivation of backcross derived lines (BDLs) differing for the parental alleles (Os420 or IABO78) at this QTL [153, 154]. A preliminary field evaluation under well-watered and water-stressed conditions indicated that the BDLs differ significantly for L-ABA as well as root lodging, the number of kernels/plant and GY, particularly under drought conditions [154].

For a more targeted manipulation of the QTLs controlling ABA concentration it would be interesting to dissect their biochemical and physiological bases. To this end, Tuberosa et al. [150] verified whether mapped mutants affecting ABA biosynthesis might be possible candidates for the QTLs controlling L-ABA. Using RFLP markers common to the reference UMC map and the (Os420 x IABO78) linkage map, it was shown that the map position of mutants impaired in ABA biosynthesis was always outside the support intervals of the QTLs influencing L-ABA. These results leave the question open as to what sort of genes may underlie

these QTLs. Feasible candidates could be the genes influencing the intensity of the transduction signal associated with turgor loss following dehydration, a major determinant in the regulation of ABA concentration [155] and/or genes controlling morpho-physiological traits (e.g., leaf area, leaf angle, root size and architecture, osmotic adjustment, etc.) affecting the water balance of the plant and, hence, its turgor. Indeed, a fairly extensive overlap among QTLs for L-ABA and QTLs for leaf RWC was found in Os420 x IABO78: of the 16 QTLs which significantly affected L-ABA, seven concomitantly also influenced leaf RWC [101]. At all QTLs but one, the corresponding relative additive effects for leaf RWC and L-ABA were antagonistic (i.e, +/- or -/+), a result which suggests that in this case L-ABA mainly represented an indicator of the severity of drought stress experienced by the plant at the time of sampling. Quarrie et al. [156] reported that recurrent selection for GY under drought conditions significantly changed allele frequencies at *csu133* in two populations ("Tuxpeño Sequia" and "Drought Tolerant Population") developed at CIMMYT [25, 73]. Collectively, these results further substantiate the importance of this QTL region in controlling drought-related traits and GY in maize.

A high number of QTLs were also found to influence ABA concentration in xylem sap and leaf samples collected from drought-stressed plants in the cross Polj17 x F2 [100]. All chromosomes, with the exception of chromosome 8, harboured QTLs influencing the concentration of ABA. Analogously to what was reported in Os420 x IABO78 [150], also Lebreton et al. [100] identified a major QTL affecting L-ABA on bin 2.04 near *csu133*.

QTLs for root traits. Lebreton et al. [100] searched for QTLs governing root traits in the Polj17 x F2 population, which was also investigated for ABA concentration in the leaf and xylem sap. Significant QTLs were detected for the number of both seminal roots and roots at the base of the stem, and for root pulling force (RPF). Because QTL data were also available for ABA concentration, the relationships between root traits and ABA concentration were analysed. In this case, the findings supported the hypothesis that ABA concentration was more likely to regulate RPF than vice-versa, an interpretation consistent with the positive relationship between the endogenous ABA concentration and primary root growth in maize seedlings subjected to artificial conditions of drought stress [157, 158].

As an alternative to field studies, hydroponics offers a number of advantages for investigating root characteristics. A major disadvantage of hydroponics is the very unnatural environment in which roots grow. Despite this, if QTLs governing root traits in hydroponics also regulate root growth in the field, it may be possible to identify among such QTLs those with an associated effect on GY under drought conditions, provided of course that variability in root traits affects GY. In maize, the most comprehensive study for QTLs for root traits in hydroponics was carried out in a mapping population derived from Lo964 x Lo1016 [158]. In total, eleven, seven, nine and ten QTLs (LOD > 2.5) influenced primary root length (R1L), primary root diameter (R1D), primary root weight (R1W) and the weight of the adventitious seminal roots (R2W), respectively. The QTL region with the most sizeable effects (LOD values of 14.7, 6.4 and 8.3 for R1D, R1L and R2W, respectively) was found on chromosome 1 (bin 1.06). In order to verify whether some of the QTL regions

influencing root traits in hydroponics also modulate root growth in the field, the same mapping population was tested for RPF in three field experiments [159]. QTLs were assigned to 19 bins, 11 of which also harboured a QTL for one or more root traits in hydroponics. The most noticeable overlap for QTLs influencing root traits in hydroponics and RPF in the field occurred on bin 1.06, which also harboured QTLs for root traits in hydroponics in Ac7729 x Ac7643/TZSRW [160] and for RPF in Polj17 x F2 [100].

QTLs for anthesis-silking interval and grain yield under drought conditions. A number of QTLs for anthesis-silking interval (ASI) and GY at three water regimes were reported by Ribaut and co-workers [161, 162], whose main interest was to elucidate the effects of ASI on GY. The results highlighted the presence of QTLs with fairly stable effects on ASI across the two drought-stress treatments: of the seven QTLs evidenced altogether, five were common to both water regimes.

The collocation between QTLs for ASI under drought conditions and those for GY and L-ABA at stem elongation and tassel appearance was reported in a two-year study by Sanguineti et al. [101]. With only one exception, at all the other QTLs a high L-ABA was associated with a longer ASI. Overlap of QTLs for L-ABA and GY occurred at two of the four QTLs for GY in 1994 and four of the six QTLs for GY in 1995. In general, the results reported in Sanguineti et al. [101] suggested that L-ABA mainly represented an indicator of the severity of drought stress experienced by plants at sampling. This, in turn, was related to the fact that plants grown under drought field conditions and differing in traits influencing their water status will differ also in L-ABA, in part independently from their capacity to accumulate ABA at a similar level of drought stress. Accordingly, when populations divergently selected for L-ABA in the field starting from Os420 x IABO78 and from B88 x Mo17 were evaluated under drought conditions, a negative association was detected between L-ABA and GY [106].

QTLs for GY under well-watered (WW) and water-stressed (WS) conditions, together with QTLs for root traits in hydroponics, were reported in Lo964 x Lo1016 [158]. Several overlaps occurred between the QTLs for root traits and QTLs affecting GY. Among the root traits investigated (see previous section), R2W most frequently and consistently overlapped with QTLs for GY-WW and GY-WS. In particular, at four QTL regions (bins 1.06, 1.08, 10.04 and 10.07), an increase in R2W was positively associated with GY. A possible interpretation of these results is that a higher value of R2W indicates a more rapid and vigorous growth of the root system, a condition which, if present also under field conditions, could allow the plant to extract more water from the soil.

QTLs for other drought-related traits. At the biochemical level, interesting results were reported on QTLs for invertase activity in a maize population (F2 x Io) subjected to drought stress [163]. In this case, water shortage produced an early and large stimulation of acid-soluble invertase activity in adult leaves whereas cell-wall invertase activity remained constant. This response was closely related to the mRNA level for only one (*ivr2*) of the invertase genes. Interestingly, the number of QTLs for invertase activity detected under drought (nine in total) was more than twice the

number detected under well-watered conditions (four in total), an indirect indication of the importance of this enzyme under drought conditions. One QTL common to both treatments was located near *Ivr2* on bin 5.03. A number of QTLs for invertase activity were mapped in close proximity to carbohydrate QTLs with the two main clusters located on bins 1.03 and 5.03. In drought-stressed maize, it has been suggested that the observed reduction in acid invertase activity under drought could impair sink strength because photosynthates cannot be converted rapidly to starch [2, 164, 165]. Accordingly, reproductive failure of maize plants exposed to low water potentials at anthesis was partially prevented with a stem infusion of sucrose, thus indicating that also the source activity plays a pivotal role in kernel abortion [166].

An important category of traits for which no QTL information is presently available and whose knowledge would help in interpreting the adaptive response to drought, particularly in terms of modulating the plasticity of organ development, is the sensitivity of different organs to growth regulators. Sensitivity to ABA is an interesting target in consideration of the sharp increase in ABA concentration under drought conditions and its crucial role in the regulation of root/shoot elongation, stomatal conductance, ear fertility and grain filling [142, 143]. In maize, significant variability among maize lines has been detected for stomatal sensitivity to ABA concentration of detached leaves [167] and for pollen tube growth at different ABA levels [168]. These preliminary findings indicate the feasibility of identifying suitable maize lines for a QTL study aimed at dissecting sensitivity to ABA in this species.

4.3.4. Barley

In barley, the most extensive set of data for yield QTLs under drought has been obtained using a population of 167 RILs developed by ICARDA and CIMMYT from the cross Tadmor and Er/Apm. Tadmor is a two-rowed line selected from a Syrian landrace, which is characterized by high yield stability in droughted conditions of the southern and eastern Mediterranean rim. Er/Apm is an ICARDA selection adapted to moderate water deficit conditions, but is drought susceptible in terms of yield stability. The parental lines contrast for traits associated with drought tolerance (e.g., plant architecture, osmotic adjustment, growth habit and chlorophyll content, etc.), and the RILs have been used in QTL analyses to genetically map these traits [94, 169, 170]. Teulat et al. [94] reported the results of four years of trials (1995, 1996, 1997 and 1999) in Mediterranean countries; because in 1997 and 1999 two different water regimes were applied, data were collected for a total of six different environments. Despite the heterogeneity between environments, numerous QTLs were common to several environments, particularly for plant height and kernel weight. Major QTLs which explained the largest part of the phenotypic variation among RILs were obtained for plant height on chromosomes 3 (3H) and 6 (6H). The multiple-environment analysis indicated the presence of 24 QTLs, 11 of which showed a significant main effect, seven presented "QTL x environment" interaction and six showed both effects. In addition, 18 of these QTLs were common to other published work and six seemed specific to this study. This led Tealut et al. [94] to

suggest that these six specific QTLs could be involved in specific adaptation to Mediterranean conditions or could be specific to the genetic background of the RIL population. Finally, when the rainfed and the irrigated environments were considered separately, a total of 16 QTLs showing main effects over the two water conditions were identified, whereas five QTLs seemed dependent on the water conditions.

The same population was also tested in a growth chamber [170] under two water regimes to identify QTLs influencing traits related to plant water status and osmotic adjustment (OA). Relative water content (RWC), leaf osmotic potential and the water-soluble carbohydrate concentration were measured at 100 and 14% of the field capacity. In a previous evaluation of the same materials, 12 QTLs were identified for RWC, leaf osmotic potential and OA with an incomplete genetic map [92]. In this new evaluation, Telaut et al. [170] used an improved map and performed the QTL analysis using adjusted means. Compared to previous results [92], eight additional regions carrying 22 new QTLs were identified, increasing to 13 the total number of chromosomal regions (with a total of 32 QTLs) controlling traits related to plant water status and/or OA in this barley genetic background.

4.4. Identification of QTLs in mapping populations derived from inter-specific crosses

The genetic bottleneck caused by domestication has strongly reduced allelic biodiversity within each cultivated species, thus limiting the possibility of detecting QTLs. To overcome this limitation, advanced backcross quantitative trait analysis (ABQA) has been devised. ABQA offers the opportunity to quickly discover and exploit beneficial QTL alleles identified in wild germplasm [114]. The strategy relies on the evaluation of backcross (BC) families between an elite variety used as recurrent parent and a donor accession, more commonly a wild species sexually-compatible with the cultivated species. QTL analysis is usually delayed until the BC2 generation after selecting in BC1 against characteristics with a negative effect on the agronomic performance (e.g., ear shattering in barley). Although ABQA has already proven its validity for the exploitation of exotic germplasm in tomato [171, 172] and rice [173], limited work has so far been carried out to search alleles conferring resistance to drought.

In barley, genetic diversity studies have demonstrated that domestication greatly reduced the overall level of genetic diversity [174], suggesting that improvements in this crop can be pursued by gene introgression from *Hordeum spontaneum*, the wild progenitor species. Wild barley can thus be considered as a source of useful variation for stress tolerance [79]. Indeed, applying an ABQA strategy [175], a number of beneficial QTL alleles for GY under drought conditions have been uncovered in *H. spontaneum* [176].

In rice, an ABQA strategy was used by Moncada et al. [177] to identify QTLs for eight agronomic traits in 274 BC2F2 families derived from an interspecific cross

between Caiapo, an upland *Oryza sativa* subsp. *japonica* variety from Brazil, and an accession of *Oryza rufipogon* from Malaysia. Caiapo is one of the most-widely grown dryland cultivars in Latin America and may be planted as a monoculture or in a multicropping system with pastures. Two objectives of this study were to detect trait-enhancing QTL alleles from *O. rufipogon* in the BC2F2 families grown under the drought prone, acid soil conditions to which Caiapo is adapted and to compare the identified QTL regions with those previously reported. In total, two putative *O. rufipogon*-derived QTLs were detected for GY, 13 for GY components, four for maturity and six for plant height. It is noteworthy that *O. rufipogon* contributed 56% of the trait-enhancing QTL alleles.

In maize, two breeding programs based on ABQA are in progress to identify exotic favourable alleles in teosinte populations which may improve drought tolerance [74; A. Charcosset, personal communication].

5. IDENTIFICATION OF CANDIDATE GENES FOR DROUGHT-RELATED TRAITS AND YIELD

A number of functional genomics approaches provide additional opportunities for furthering our understanding of the molecular and biochemical basis of yield under drought and to identify candidate genes accounting for QTL effects [178].

5.1. Transcriptome analysis

The spectacular progress in the mass-scale profiling of mRNAs through microarray analysis [21, 179- 184] offers the possibility of investigating the response to drought of thousands of genes or, when the entire genome sequence is available like in rice [7], of all the annotated genes. Microarrays are now commercially available for *Arabidopsis* and a number of major crops (rice, maize and barley). A microarray-based analysis performed using mRNA extracted from the lines of a mapping population will identify mRNA-QTLs for the surveyed transcripts. This information, coupled with that on the map position of the surveyed genes, may in turn allow for the identification of candidates for the QTLs of target traits. An example is reported in Figure 1 where the coincidence between the map position of a particular cDNA with the peaks of (i) a QTL for a target trait and (ii) a QTL for the level of expression of the same cDNA, identifies a possible candidate gene influencing the target trait, particularly when a plausible cause-effect relationship can be established between the variability for the product of the candidate gene (e.g., an enzyme influencing nitrogen assimilation) and the trait (e.g., yield under low nitrogen). In this case, the mRNA-QTL can be caused by (i) polymorphism in the promoter region of the ORF and/or in tightly linked cis-acting elements influencing the

expression level and/or (ii) polymorphism in the ORF influencing the final level of mRNA and/or its

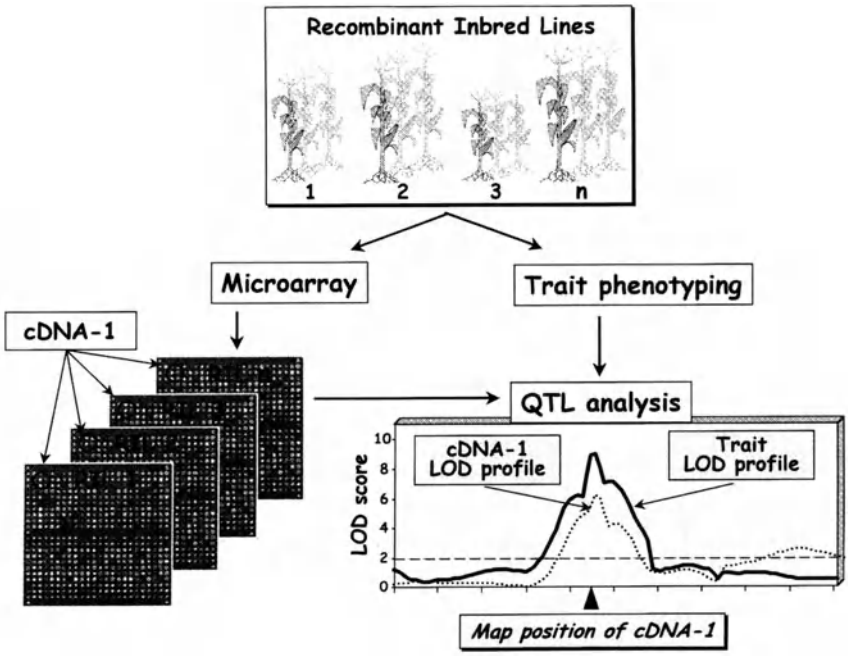

Fig. 1. Identification of mRNA-QTLs and candidate genes using microarray analysis. For each one of the N recombinant inbred lines of a mapping population, the mRNA is extracted and the level of expression of all the genes surveyed with the microarray is determined. Phenotypic data are also collected for the target trait. All data are then subjected to QTL analysis. The coincidence between the map position of a particular cDNA (e.g., cDNA-1) with the peaks of a QTL for the level of expression of the same gene (e.g., cDNA-1; dotted LOD profile) and a QTL for the target trait (solid LOD profile) indicates a possible candidate gene, particularly when the candidate gene and the trait are linked by a plausible cause-effect relationship (see text for further details).

stability. However, when the gene encoding for the mRNA is loosely associated with the mRNA-QTL peak or segregates independently, the mRNA-QTL is likely caused by a functional polymorphism in a regulatory sequence encoding for a trans-acting factor influencing, transcriptionally or post-transcriptionally, the level of the surveyed mRNA. However, the cost to implement the microarray profiling of hundreds of RNA samples from a mapping population is still too high to conceive its routine utilization and to date no results are available on the utilization of this approach. Instead, microarrays are well-suited for detailed studies involving a limited number of genotypes, such as in the case of transgenics, NILs and parental

lines of a mapping population, i.e. whenever a small number of genotypes are considered.

Besides the high costs, other factors limit a more widespread utilization of microarrays in drought-related studies: (i) biological and sampling variation is difficult to control, (ii) the correlation between the level of mRNAs and their biological effect can be low, due to translational and post-translational modifications, (iii) low-abundant mRNAs may not be represented by the array (except of course when the array includes all the genes of one particular species) and/or detected upon hybridisation, and (iv) it is difficult to profile gene expression in small samples. In alternative to close-ended methods such as microarrays, a number of open-ended methods for gene expression profiling are available: differential display [185], cDNA-AFLP [186], SAGE (Serial Analysis of Gene Expression [187, 188]), GeneCalling [189] and MPSS (Massively Parallel Signature Sequencing [190]). The advantage of these methods is that they allow for a genome-wide scanning, thus representing an ideal tool to investigate the response of the plant to environmental cues. Monitoring large-scale changes in transcript profiles under drought may eventually lead to the identification of transcript networks partially accounting for "genotype x environment" interactions.

Profiling studies unveil the complexity of the signalling network governing the response to stresses. Importantly, the role for each transcription factor (TF) revealed through transcriptome analysis can be tested rapidly and more extensively with other techniques (e.g., RT-PCR). Furthermore, circumstantial evidence on the role of each TF in controlling variability in drought tolerance in a mapping population can be obtained by comparing the map position of QTLs for yield with the map position of the genes encoding for TFs. More compelling evidence on the role of the TF gene can then be obtained through the analysis of mutants and/or by altering (down- or up-regulating) its expression level by genetic engineering.

5.1.1. Case studies in crops

Of interest, for better understanding the response of maize to drought, is a study profiling the changes in RNA expression of root tissue of two lines characterized by contrasting root-related traits [191]. Among ca. 13,500 cDNA fragments which were analysed at two growth stages, 69 showed a two-fold or greater difference between the lines at both samplings, suggesting a relationship between these genes and root anchorage traits These genes may represent possible candidates for QTLs regulating the response to drought.

In maize, Zinselmeier et al. [184] applied a targeted and a non-targeted gene expression profiling to dissect the stress sensitivity of reproductive development following a reduction in the source of photosynthates to the ear, a condition typical of plants exposed to drought in the field. This reduction in photosynthates to the ear was obtained with artificial shading or by withholding water. A four- to six-day-long water deficit reduces maize photosynthesis to near zero and can disrupt kernel growth, thus causing a significant reduction in GY [109, 165, 192]. In the targeted approach, the microarray included 384 maize genes representing four metabolic

pathways important for kernel growth, such as ABA signalling and starch biosynthesis. The results indicated that the genes of the starch biosynthetic pathway are co-ordinately regulated under stress and the decreased expression of these genes is related to the loss of starch. As to the ABA-signalling pathway, the increased expression of ABA-related genes mirrored the increase in endogenous ABA levels. Interestingly, the profiles of the genes involved in this pathway were largely unaltered after one day of shading, which led the authors to suggest that if an early stress-sensing pathway is activated, it was not detected in their experiment. The non-targeted approach relied on the use of a microarray representing 1,502 maize genes, including over 300 unknown ESTs, that were annotated into 27 unique metabolic pathways. Samples were collected at two stages of ear and kernel development. This experiment revealed a set of genes that were affected by water stress regardless of tissue type, although in some tissues gene expression is more responsive to stress than other tissues; additionally, a set of genes unknown to respond to water stress were identified. The authors concluded addressing a number of important issues related to the challenges that still remain unsolved before gene expression profiling can be a adopted as a selection tool.

In barley, Ozturk et al. [183] focused on dehydrated leaf and root samples of young barley plants using a microarray containing 1,463 transcripts derived from cDNA libraries of leaves and roots of water-stressed barley plants. In the same study, the response to salinity was also monitored, thus allowing for a comparison of the molecular events elicited by these two abiotic stresses. Even though the collection of transcripts used in this study only represented a fraction of the whole genome, a number of interesting conclusions were drawn. Drought and salinity stresses affected largely different sets of transcripts. The differences were often in isoforms of transcripts for similar functions, an indication that the same function seems to be required by the plant to adapt to more than one abiotic stress. It was suggested that the differences should lie in different activation circuits either through alternative signal transduction, separate transcription factors, and/or altered promoter structures. When focusing on the drought-induced transcripts, Ozturk et al. [183] listed ca. 100 strongly up-regulated sequences, half of which were functionally unknown; furthermore, a number of these transcripts have not been reported as drought-inducible. These results indicate the power of microarray analysis in unveiling the regulatory circuitry responding to water stress. From an applicative standpoint, Ozturk et al. [183] questioned whether arrays of size similar to that used in their study are sufficient for providing meaningful information from a breeding standpoint. Additionally, because the administered dehydration-shock treatment is not comparable to the much slower water loss experienced by barley plants in the field, these results might be of only partial value for crop physiologists and breeders. A more recent study has indeed shown that the correlation between the fold-change variation in gene expression under conditions of shock-treatment and more naturally occurring dehydration, although significant and positive, is usually low [193]. This set of genes responding to both types of experimental conditions deserves further attention, also in view of the fact that it may be possible to investigate their functions

under controlled conditions. A number of these genes have been mapped and their map position compared with that of QTLs for drought-related traits [193].

5.2. Proteomics and metabolomics

Among other emerging approaches, increasing attention is being devoted to proteomics [194, 195]. The rapid improvement and automation in the techniques required to quantify proteins allows for the quantification of up to ca. 2,000-2,500 proteins in a single sample. If the technique is applied to the individuals of a mapping population, it allows for the application of QTL analysis for mapping genes influencing protein quantity (PQL, Protein Quantity Locus [196-201]). Co-localization of a PQL with its protein-coding locus would indicate that allelic differences at that locus influence the expression level of the protein, while co-localization between a PQL and a QTL for a different trait allow us to infer an association between the "candidate protein" and trait variability. This strategy has been described in details in maize in order to identify suitable candidate genes for QTLs influencing drought resistance [163, 198].

Another promising avenue is the metabolic profiling of tissue samples collected from organs playing a key role in determining yield under drought. This rapidly progressing technology allows for the identification of up to ca. 2,000 metabolites in a single sample. Analogously to transcriptome and proteome analysis, metabolome profiling can be used to monitor the metabolic changes occurring during a drought episode in one or more genotypes and, when applied to a mapping population, to identify the QTLs regulating the level of a particular metabolite and verify its coincidence with QTLs for yield. In maize, the changes occurring during a drought episode have been described for the level of a limited number of key metabolites such as sugars and starch in the reproductive organs and in the growing kernel [165, 192].

6. APPLICATIONS OF GENOMIC APPROACHES FOR IMPROVING DROUGHT TOLERANCE

6.1. Marker-assisted selection

Improving and/or stabilizing yield and its quality under drought should bear no detrimental effects on productivity when sufficient rainfall occurs. Therefore, studies for identifying QTLs influencing yield under drought conditions should preferably be conducted considering at least two water regimes (e.g., well-watered and water-stressed), thus allowing one to distinguish between the constitutive (*per se*) and adaptive nature of QTL effects. In other words, testing over a wider range of

environmental conditions will provide the opportunity to sort out QTLs with a more limited interaction with the environment. On the same line, the QTLs influencing secondary traits with a more important role for the survival of the plant under severe drought should receive greater attention in regions where such extreme conditions occur more frequently. Collectively, this information would be of great value for applying marker-assisted selection (MAS) more effectively according to the level of drought stress expected in each target environment. The effectiveness of MAS compared to direct selection for drought tolerance in rice has been critically evaluated and discussed by Atlin and Lafitte [13].

In principle, once QTLs have been identified, introgression of the favourable alleles and their pyramiding into elite germplasm (e.g., parental lines, populations, etc.) becomes possible through MAS using the information at the markers flanking the chromosome regions of interest [103, 202, 203]. The application of MAS does not require any *a priori* knowledge and/or assumption concerning the physiological mechanisms imparting drought tolerance. Additional advantages of MAS are the possibility of selecting at an early stage the plants carrying the most favourable allelic combination at the target QTLs as well as the possibility of selecting under non-target conditions (e.g., winter nurseries) and in absence of drought, thus reducing the number of individuals to be considered and increasing the response to selection/year. As the technology is still relatively expensive, it is unlikely that MAS will be applied routinely in the large populations that breeders normally handle. Instead, MAS will be particularly appropriate for improving the efficiency of selection for specific objectives, such as resistance to environmental stresses. PCR- and non-gel-based diagnostic screening tests will greatly facilitate large-scale applications of MAS [204].

As to MAS applied to the improvement of drought resistance, large-scale efforts are in progress at CIMMYT in maize [74], ICRISAT in pearl millet and sorghum [205] and IRRI in rice [13]. Due to the key role played under rainfed conditions by roots in determining rice yield, MAS for root depth has been deployed at IRRI to more specifically tailor new varieties to the range of environments present in rice growing areas [119]. We report in more details the results obtained in maize [74], where QTLs for GY and key morpho-physiological traits (e.g., ASI, etc.) involved in drought-tolerance mechanisms have been identified in a population developed from the cross Ac7643 x Ac7729/TZSRW [161, 162]. A backcross marker-assisted selection (BC-MAS) project based on the manipulation of five QTLs for ASI was started in 1994 [74, 102]. The line Ac7643 was the drought-tolerant donor and CML247 was used as the recurrent parent. CML247, an elite line with high yield *per se* under well-watered conditions, is drought susceptible and shows long ASI under drought. The chromosome regions harbouring QTL alleles for short ASI were transferred through MAS from Ac7643 into CML247. A number of lines (ca. 70) were derived and crossed with two testers. These hybrids and the selected lines were evaluated for three years under different water regimes. Under severe stress conditions reducing GY of at least 80%, the mean of the selected lines outyielded the unselected control. This advantage, however, decreased at a lower stress intensity, and disappeared for a stress reducing GY less than 40%. Across the water-limited

trials, a few genotypes consistently outyielded the controls. Interestingly, under well-watered conditions the selected lines did not show any yield reduction when compared to the control lines. Notwithstanding the success of this BC-MAS experiment, Ribaut et al. [74] pointed out that QTL manipulation to improve germplasm for polygenic traits has a number of limitations, the most distinct being the inability to predict the phenotype of any given genotype based on its allelic composition. This constraint is particularly valid when epistatic interactions influence the expression of the target trait. Another clear limitation of MAS pertains to the high costs associated to QTL discovery, their validation and the release of superior lines. New strategies to overcome these limitations have been developed at CIMMYT that are aimed at improving the cost effectiveness of MAS and delivering new germplasm instead of improved versions of existing lines which, as compared to the former, carry a more limited value [206]. These strategies include (i) the construction of a consensus map that combines information related to QTL characterization and gene expression and (ii) the identification through functional genomics of a set of key genes/pathways involved in maize drought response that will be used as selection tools in breeding programs [74].

7. POSITIONAL CLONING OF QTLS

One of the difficulties in interpreting the results of QTL analysis is ascertaining whether the simultaneous effects of a chromosome region on two or more traits are caused by linkage or pleiotropy. Fine mapping of the QTL and, eventually, the cloning of the gene/s influencing the investigated traits will reveal the genetic basis of the association. However, the complete dissection of a QTL requires considerable efforts even when the entire genome sequence is available.

Because of its low resolution power, QTL analysis cannot provide us with a complete molecular dissection of the genetic basis underlying a QTL. As an example, several hundreds genes are expected within the support interval of a QTL which often spans 20-30 cM. A sizeable and parallel increase in the size of the segregating population and the number of informative markers at the region of interest greatly improve the level of map resolution; in some cases, this can lead to resolving a single QTL into multiple tightly linked loci of smaller effect [207-209]. Eventually, if the level of genetic resolution is high enough, the positional cloning of the QTL can be attempted [114]. Among the seven QTLs which have been cloned thus far in crop plants, those controlling transition from the vegetative to the reproductive stage in rice [210, 211] are of possible interest for improving drought resistance via a manipulation of flowering time. A similar approach is well-advanced in maize for cloning a QTL (*Vgt1*) on bin 8.06 near *umc89a* controlling flowering time [207, 212, 213]. The availability of the rice sequence and the extensive synteny between rice and the other cereals [7] will facilitate the isolation of drought-related QTLs through comparative positional cloning in these important crops [131].

8. MOLECULAR MECHANISMS OF ADAPTATION TO DROUGHT

As sessile organisms, plants have developed a wide variety of adaptive strategies to cope with environmental stresses; accordingly, plant cells have evolved signalling pathways to perceive and integrate different signals from their surroundings and to respond by modulating the expression of the appropriate genes [214].

Water stress tolerance is the result of the coordination of biochemical and physiological alterations at the cellular and molecular levels such as the increase in ABA, the accumulation of various osmolytes and proteins coupled with an efficient antioxidant system. Many of these mechanisms have been characterized and have been found to exist in both drought tolerant and non-tolerant plants [19]. It is now clear that the difference between tolerant and non-tolerant crops at the molecular level involves a large number of genes. Based on the results of recent microarray experiments, it has been estimated that the response to a stressful environment involves not less than 2,000 genes, most of which are up-regulated upon stress [215-217; see also section IV.3.1]. However, it is still unclear how many and which genes are directly involved in the activation of adaptive mechanisms. In fact, up-regulated genes do not necessarily have a role in adaptation: some might be induced because of stress-caused cell injury [20, 218]. In addition, the genes responding to dehydration showed marked differences in time-scale response. The early responsive genes may provide initial protection and amplification of signals, while the genes that respond later may be involved in adaptation to stress conditions [219].

8.1. Genes and genes products involved in drought adaptation and tolerance

Three approaches have been used to dissect the complex molecular and biochemical mechanisms underlying plant stress response, identify the genes involved and establish their contribution to stress tolerance: 1) identification of plant genes whose expression is modulated in response to stress that can be triggered in any plant species, regardless of their degree of tolerance; 2) comparison of gene expression between halophytes and xerophytes with glycophytes, to identify mechanisms of stress tolerance absent or not appropriately regulated in glycophytes; 3) discovery of plant genes by complementation of stress sensitive mutants or by over-expression of plant cDNAs in other model eukaryotes based on fundamental homology in cellular response to stress [220, 221]. Application of these interconnected strategies has permitted the identification of several genes associated to stress response and/or tolerance, some of which have shown to be common to different environmental stresses (e.g., water deficit, salt and freezing stress) sharing a physiological osmotic component as determinant of the stress signal [222-224]. ABA, a growth regulator largely reported to accumulate in plant tissues in response to drought and other abiotic stresses [142], has been shown to regulate the expression of several stress-induced genes, although parallel ABA-independent stress-signal pathways are also operative [222]. The induced genes are thought to function not only in protecting cells from water deficit by the production of some effector molecules (e.g.,

metabolites, proteins or components of biochemical pathways) but also in the activation of regulatory circuitries controlling the amount and timing of the effectors in response to water stress. The genes involved in the response to water stress can be divided into two groups: functional genes and regulatory genes.

8.2. Functional genes

A variety of genes are directly involved in the different mechanisms enabling the cell to cope with drought-related effects. The products of these gene are proteins or enzymes with vital roles in reducing water loss, protecting the cellular machinery, repairing cellular damage and restoring a new cellular homeostasis compatible with persistent stressful conditions [225].

Cellular homeostasis and transport. Upon osmotic stress, cells accumulate solutes to raise osmotic pressure thus preventing water loss and maintaining cellular turgor. These solutes include ions such as K^+, Na^+, Cl^- and organic solutes such as quaternary ammonium compounds (e.g., glycine betaine), some amino acids (e.g., proline), polyols (e.g., inositol and mannitol) and sugars (e.g., sucrose, fructans and trehalose). These compounds are also known as compatible osmolytes since their accumulation does not interfere with normal cellular metabolism. Genes coding for crucial steps in the biosynthesis of osmolytes have been isolated from plants and microorganisms which share some osmoprotective mechanisms with plants [226]. In the last decade, the accumulation of osmolytes through metabolic engineering of biosynthetic pathways has been the target of intense research recently reviewed [182, 227]. The mechanism of protection provided by the osmolytes is still under debate. Often, the accumulation is insufficient to account for the observed level of drought tolerance. Therefore, it is reasonable to speculate that besides osmotic adjustment, the osmolytes have additional functions, such as scavenging reactive oxygen species (ROS [228]).

Another important class of proteins with a pivotal role in water deficit-avoidance and osmoregulation, are the transporter proteins. This class, which acts to regulate ion homeostasis and facilitate water movement across membranes, includes water channel proteins (aquaporins) and ion pumps such as H^+ vacuolar and plasmalemma ATPase, Na^+ antiporter and high affinity K^+ transporters [229, 230]. Aquaporins are a complex family of channel proteins that facilitate the transport of water along transmembrane water potential gradients regulating the hydraulic conductivity of membranes [231]. Several genes encoding aquaporins are up-regulated by dehydration; for example *rd28* from *Arabidopsis* [232] or the tomato-ripening-associated membrane protein [233].

The maintenance of ion homeostasis, through cellular ion uptake, sequestration and export as well as long-distance transport, is also critical to overcome high osmotic stress [229, 230]. Much progress has been recently made in the identification and molecular characterization of plant ion transporters using yeast as genetic model system (functional complementation of transport deficient yeast mutants [234]). The complete set of transport proteins involved in Na^+, K^+ and Ca^{2+}

homeostasis will emerge shortly with the current application of high throughput genomic approaches and the availability of complete plant genomes sequences [7]. Less understood is the physiological function of these transport systems in osmotic stress adaptation. The availability of wide collections of *Arabidopsis* mutants has recently allowed the functional characterization of the AKT1 potassium channel transporter [235, 236], SOS1 plasma membrane Na^+/H^+ antiporter [237] and HKT1 high-affinity K^+ transporter [238], defining their crucial functional role in stress adaptation.

Protection of cellular structures. Under stress, a diverse array of gene products accumulates to protect cell structures and important metabolic functions. The osmotic stress response most actively studied in plants is that leading to the synthesis of a group of proteins with no clear biochemical function and no significant sequence similarity to other well-characterized proteins. These proteins, known as LEA (Late Embryogenesis Abundant)-like proteins have a biased amino acid composition, are highly hydrophilic, glycine-rich and remain soluble even when boiled [239, 240]. LEA-like proteins accumulate in vegetative tissues in both monocot and dicot species in response to abiotic stress [19, 20]. Many LEA-like genes have been cloned and most of them are regulated by osmotic stress (e.g., drought, salinity and/or cold) as well as by ABA [241, 242]. Interestingly, LEA-like proteins are also found in bacteria [243] and in yeast [244]. The observation that these proteins likely exist in all organisms and are mostly induced by osmotic stress [244], suggests that they may underlie a common adaptation strategy to osmotic stress and may have similar functions in diverse organisms. For example, a tomato LEA gene when expressed in yeast increases salt and freezing tolerance of the yeast cells [245]. The function of LEA-like proteins has also been explored by over-expression studies in transgenic plants. Overexpression of transcription factors that regulate the LEA-like genes significantly improved plant tolerance to various abiotic stresses under controlled conditions [246, 247]. LEA proteins have been predicted to play various physiological roles: maintenance of protein and membrane structure, sequestration of ions and binding of water [20, 29, 240]. The current hypothesis is that they act as chaperones to prevent misfolding or denaturation of proteins [240, 248]. One interesting analysis with the LEA group of proteins suggests that some of them have features of RNA-interacting ribosomal proteins [244], consistently with their over-representation of charged amino acid residues that facilitate nucleic acid binding.

In desiccation-tolerant plants (e.g., resurrection plants), protecting the integrity of the photosynthetic apparatus has been found to be crucial for survival under stress. The photosynthetic machinery is very sensitive and liable to injury and needs to be maintained or quickly repaired upon rehydration [249]. In *Craterostigma plantagineum*, genes coding for chloroplast-localized proteins were found to be expressed preferentially upon desiccation [250]. These proteins were proposed to play a role in the maintenance of chloroplast structures. Furthermore, it was shown that the synthesis of one of these proteins (DSP22) depends on the extent of photoinhibitory damage [251]. This mechanism of protection seems to be operative also in non-tolerant species that accumulate drought-induced proteins in thylakoids

[252]. Rey et al. [253] have characterized a dehydration-induced stromal thioredoxin-like protein (CDSP32) from potato, whose expression is independent of ABA. In animal cells, thioredoxin was shown to be involved in repair or protection mechanisms by regenerating proteins inactivated by oxidative stress [254]. Rey et al. [253] hypothesized that CDSP32 might play a role in the preservation of native protein structures by reducing intermolecular disulfide bonds.

Damage limitation and repair. Many genes coding for proteins involved in intracellular damage prevention and repair, as well as in the removal of toxic compounds, are induced by water stress. Protein synthesis is one of the cellular processes most sensitive to osmotic stress [255]. However, it remains unclear how the protein synthesis machinery copes with osmotic stress. One essential component of protein synthesis, elongation factor1-alpha, accumulates dramatically in plant cells adapted to salt and drought stress [256, 257]. This may indicate a mechanism of osmotic adaptation to protect protein synthesis. Also, proteases and ubiquitin, functioning in degrading proteins irreparably damaged by the effects of drought, were found to be induced by water deficit [258]. Counteracting these degrading mechanisms are chaperones and protease inhibitors (Kunitz-type) that are also induced by stress [259]. Whereas the production of protease inhibitors seems to protect proteins from protease released after drought-induced membrane disruption, the chaperones and chaperonins are directly involved in protein repair [20]. A ubiquitous class of chaperonins is the heat shock proteins (HSPs), which accumulate rapidly during and after heat shock [260]. Mammalian HSPs have been demonstrated to function as molecular chaperones assisting in the recovery of native protein conformation [261]. Some low molecular weight HSPs were also found to be induced by osmotic stress in sunflower, tobacco and potato [262-264] and are thought to function similarly.

Dehydration, like other environmental stresses, leads to oxidative stress and to the accumulation of ROS which, in turn, can damage cellular structures [265]. ROS include hydrogen peroxide, hydroxyl radicals and superoxide anions. The capacity to scavenge ROS and to reduce their damaging effects on macromolecules, such as proteins and DNA, is an important stress-tolerance trait. For example, the *Arabidopsis* mutant *pst1* exhibits a higher tolerance to osmotic stress coupled with an increased capacity to scavenge ROS [266]. Elimination of ROS is mainly achieved by antioxidant compounds such as ascorbic acid, glutathione, thioredoxin and carotenoids as well as by ROS scavenging enzymes (e.g., superoxide dismutase, glutathione peroxidase and catalase). The expression of genes encoding this class of enzymes was found to be regulated by different stresses as well as by ABA [267]. Under drought, the activity of the enzymes that participate in ROS scavenging increases and a higher scavenging activity may correlate with enhanced drought tolerance [268]. ROS generated by osmotic stress may also damage DNA. Cellular responses to DNA damage include activation of stress signalling pathways, delay in cell cycle progression and the initiation of DNA damage repair. In yeast, the *HOG1* (High Osmolarity Glycerol 1) pathway also activates *DDR2* (DNA damage-responsive gene 2), which may be involved in DNA damage recognition and repair

[269]. In *Arabidopsis*, the connection between genotoxic damage repair and stress sensitivity is suggested by the *uvs66* mutant, which was identified as hypersensitive to UV-C and to DNA damage. Interestingly, the *uvs66* mutant is also sensitive to salt stress and ABA. Expression of the osmotic stress-responsive *RAB18* gene (a LEA gene) is also altered in the mutant [270]. Recent results suggest that pathways leading to the accumulation of stress-induced proteins could be activated in response to stress damage and that some components could be intermediate for damage-repairing pathways [223, 248]. In the case of activation of HSP synthesis, it is well-recognized that damaged/denatured proteins are the signals that trigger the expression of HSP genes [271]. Similarly, proline accumulation may reflect cell damage [272, 273].

8.3. Regulatory genes

Many dehydration-responsive genes [20, 29, 222] have only minimal effects on conferring tolerance [274]. The identification of the molecular switches and regulatory genes controlling their expression would improve our understanding of the molecular bases of drought tolerance and would provide better opportunities to develop more effective stress improvement strategies. Therefore, research in stress molecular biology has recently focused on identifying regulatory genes able to control the whole battery of genes crucial for stress tolerance. With this objective as a priority, studies were focused on understanding how a stress signal can be transduced into plant cells and transmitted into the nucleus by cellular components, to induce the appropriate terminal events, i.e. the stress response and, possibly, tolerance.

Progress was recently made in the identification of crucial components of the signal transduction cascade (i.e. ABA signalling factors, second messengers like Ca^{2+} and phospholipids, kinase/phosphatase enzymes, etc.) and transcription factors controlling the expression of downstream genes. The complexity of the signalling pathways and the contribution of the different components in signal amplification and relay have been exhaustively reviewed [214, 221, 224, 248].

Sensors and transducers of the stress signal. In the case of water stress, signals that can be recognized by the cell are a reduced water potential, a decrease in turgor pressure, the different concentration of small molecules, changes in cell volume and/or alterations in conformation of cellular macromolecules [14].

At present, the primary site for sensing water stress is unknown, but it is supposed that plants have sensing mechanisms similar to yeast, where osmosensors have been described and characterized. Osmosensors in yeast, and *E. coli* as well, are two component systems containing an histidine kinase as sensor and a response regulator that relays the phosphorylation signal and leads to downstream gene activation [275]. Recently, a cDNA encoding a novel hybrid-type histidine kinase (AtHK1 [276]) with structural similarity to the yeast osmosensor SLN1 has been isolated from dehydrated *Arabidopsis* plants [277]. The ability of AtHK1 to complement the *sln1* yeast mutant defective in osmosensitivity indicates that AtHK1

may function as an osmosensor in plants. As with a number of other potential regulatory genes, AtHK1 transcript level is up-regulated by osmotic stress. In addition, in *Arabidopsis* the expression of *RPK1,* a receptor-like kinase gene, was induced by ABA, dehydration, high salt and cold treatments [278]. However, the functional significance in osmotic stress responses of the up-regulation of these transcripts remains obscure.

In contrast to the scanty data on the identification of the primary sensor of the stress signal, a rapidly increasing number of genes encoding factors and enzymes in the further steps of the signal transduction have been demonstrated to be transcriptionally induced by different environmental stresses. Changes in protein phosphorylation were observed when plants were exposed to water deficit, suggesting reversible protein phosphorylation as a regulator [279]. Numerous protein kinases with close sequence similarities to MAPKs (Mitogen Activated Protein Kinases) and other kinases belonging to the MAPK cascade have been identified in plants in response to dehydration/ABA [280, 281]. Transcript levels for a number of protein kinases including a two-component histidine kinase, MAPKKK, MAPKK and MAPK increase in response to osmotic and other stress treatments [282]. It is unclear whether the protein, or more importantly, the activity levels of these kinases change upon osmotic stress treatment. It is clearly vital to identify the signal factors (input) activating the kinases activity as well as the signals activated downstream (output) through the kinases activity. The input signal could be the osmotic stress (e.g., turgor changes) or derived from osmotic stress injury. The output could be the osmolyte accumulation that helps re-establish osmotic homeostasis, stress damage protection and/or repair mechanisms (e.g., induction of LEA/dehydrin-type stress genes [224]).

Another important event of the signalling pathway activated by water deficit is the elevation of intracellular concentration of calcium. Calcium signalling acts via Ca^{2+} regulated effector proteins including calmodulins, calcium-dependent protein kinases (CDPKs) and calcium regulated phosphatases [283, 284]. About 40 different CDPKs are present in the genome of *Arabidopsis*. Transient expression studies indicate that there are specific CDPKs isoforms for different stress signalling pathways [285], e.g. AtCDPK1 and AtCDPK2 seem to be involved in the response to osmotic stress due to salt and drought [285, 286]. Another group of proteins interacting with Ca^{2+} and affecting the signalling and cellular response includes serine/threonine phosphatases. In *Arabidopsis*, the gene *AtCBL1* (*Arabidopsis thaliana* calcineurin B-like protein) coding a type 2C protein phosphatase, was induced in response to drought, wounding and cold stress [287]. Calcinuerin B-like proteins are involved in a variety of signalling pathways in animals [288] and in adaptation to salt stress in yeast and plants [289-291].

Abscisic acid signalling. One of the major signals operating during drought stress is provided by ABA, a phytohormone involved in the regulation of many stress-induced genes mentioned above, and in some instances required for changes in gene expression in response to water-deficit stress [20]. Extensive literature exists on ABA accumulation upon osmotic stress and recently some of the underlying molecular mechanisms correlated with ABA biosynthesis and its function as

mediator of the cell response to osmotic stress have been elucidated [21, 292]. Most of the information came from expression studies of genes regulated by ABA [reviewed in 293], thus allowing the identification of cis- and trans-acting factors involved in the transcriptional regulation of genes by ABA. However, not all water deficit-induced genes are regulated by ABA. It is now hypothesized that at least four independent signal pathways function in the activation of stress-inducible genes under dehydration conditions: two are ABA dependent and two are ABA independent [222, 294]. Less conclusive information is available on ABA cellular receptor/s and intermediate components of ABA signalling. New information for the understanding of the ABA-signalling cascade was added by the identification of the tobacco *Nt-SYR1* gene, encoding a syntaxin that is associated with the plasma membrane. This gene has been shown to be involved in potassium and chloride ion channel response to ABA in guard cells and in the control of plant transpiration [295]. The screening of *Arabidopsis* genetic mutants allowed for the identification of two serine/threonine phosphatases that are likely to negatively regulate the early stage of ABA signal transduction [296, 297]. These proteins represent potential nodes between different signalling pathways involving ABA [214]. Recent reviews cover the complex topic of ABA signalling and cross-talk among different stress signals that share ABA as common response mediator [21, 214, 224, 248, 293].

Promoter analysis and transcription factors. Substantial progress has been made in the past years in understanding the transcriptional regulation of a number of stress-induced genes, evidencing the important role of ABA as regulator at the transcriptional level of downstream genes involved in protection of cellular structure or in damage repair. The analysis of the promoter region of the stress-induced genes has led to the identification of common regulatory domains to which specific protein factors, possibly activated by phosphorylation or dephosphorylation, bind to drive the transcription of the target genes. Three different cis-acting elements are involved in regulating the expression of ABA-induced genes upon water deficit.

The best characterized cis-element in the context of osmotic stress is the ABA-responsive element (ABRE), which contains the palindromic motif CACGTG with the G-box ACGT core element. The ACGT element has been observed in a multitude of plant genes regulated by diverse environmental and physiological factors [19]. The G box-containing elements are bound by leucine zipper-type transcription factors. These are proteins that contain a basic DNA-binding domain followed by a leucine zipper involved in dimerization. *Arabidopsis* contains at least 58 genes that encode bZIP factors [reviewed in 293]. These proteins may form homo- or heterodimers indicating that they could participate in both positive and negative gene regulation mechanisms.

Two other DNA elements in *Arabidopsis*, MYC-like and MYB-like elements, are involved in regulating the expression of ABA-induced genes in response to severe water-deficit stress. Both of these elements (MYC: ACA-CATGT and MYB: YAAC(G/T)G) were identified in the dehydration-responsive gene *rd22* [298]. A gene that encodes the MYC-related DNA-binding protein RD22 BP1 is induced by water-deficit stress and ABA treatment [298]. An *Arabidopsis* MYB-like protein, ATMYB2, has also been identified.

An inspection of dehydration- and cold-regulated genes in *Arabidopsis* led to the discovery in their promoter of one or multiple copies of the cis-acting dehydration-responsive element (DRE). DRE motifs are involved in drought- and cold-responsive, but ABA-independent, gene expression [299]. Transcription factors (DRE binding: DREB) of the family AP2/EREBP bind this element and activate the transcription of downstream genes. Liu et al. [300] isolated DREB factors interacting with the DRE motif in the promoter region of the *rd29A* gene. Interestingly, DREB1 and DREB2 are differentially induced by low temperature and drought and function as trans-acting factors in two separate signal transduction pathways under low temperature and dehydration conditions, respectively [300]. Studies on dehydration-induced transcription factors in plants are just emerging [182] and for most of the identified transcription factors the target genes are unknown. Only a few of the dehydration-induced genes themselves encode transcription factors. It is presumed that the interaction between these factors and pre-existing factors is what ultimately determines the response leading to gene expression and stress adaptation [224].

9. IMPROVING DROUGHT TOLERANCE THROUGH GENETIC ENGINEERING

Plants employ multiple and coordinated mechanisms to mitigate the negative effects of dehydration. Even with the massive information presently available on the structural and regulatory gene networks involved in plant response to drought, our knowledge of the metabolic changes that contribute to dehydration tolerance is far from complete. This information is essential to successfully apply new strategies to enhance dehydration tolerance in crop plants.

The overexpression of stress-related genes in transgenic plants has been a common approach toward the elucidation of the molecular and physiological bases of water stress tolerance. Moreover, the past decade has witnessed the prospect of using genetic engineering for producing dehydration-tolerant plants and testing their capacity to modulate tolerance altering the levels of both osmolyte and ROS-scavenging enzymes or through the manipulation of genes encoding for transcription factors or signal transduction components (Table 1). Below, we describe some of the most promising results obtained so far as well as the potential and limitations of the gene transfer approach. More comprehensive reviews have been presented by Holmberg and Bulow [301], Bajaj et al. [302] and Chen and Murata [182].

9.1. Examples of plants genetically engineered for drought tolerance

The accumulation of proline has been the target of intense research in plants and microorganisms where the biosynthetic path has been fully elucidated and the crucial genes isolated [272]. Kavi Kishor et al. [303] reported that the overexpression of the gene encoding the mothbean D1-pyrroline-5-carboxylate synthetase (P5CS) in transgenic tobacco led, upon dehydration stress, up to 18-fold accumulation of proline and enhanced root biomass [303]. When the same gene was introduced in

rice under the control of an ABA/stress inducible promoter, the transgenic plants showed an increase in biomass under stress conditions [304]. These results strengthen the notion that proline overaccumulation correlates with the extent of water stress and salinity tolerance.

One of the first metabolic pathways engineered with the aim of developing osmotic stress tolerant plants was the biosynthesis of polyols which include sugar alcohols such as glycerol, sorbitol and mannitol as well as cyclictols (pinitol and D-ononitol) accumulating mainly in halophitic plant species. Transgenic tobacco plants that synthesize and accumulate mannitol have been obtained by introducing a bacterial gene encoding for mannitol-1-phosphate dehydrogenase. Plants overproducing mannitol showed increased drought and salt tolerance [305, 306]. Similarly, a good degree of drought tolerance was obtained in tobacco plants engineered using microbial fructosyl-transferase genes that lead to the accumulation of fructans, high-soluble polyfructose molecules that are produced by many vascular plants and bacteria [307, 308].

Trehalose, a non-reducing disaccharide of glucose, has also been over-produced in tobacco by introducing *TPS1*, a yeast gene encoding trehalose synthase. Transgenic tobacco plants with a threalose concentration of 5 mM in the cytosol showed improved water retention and a drought-tolerant phenotype [309]. When tobacco was transformed with bacterial trehalose-synthesizing enzymes (trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase), the leaves had a better photosynthetic efficiency and a higher dry weight accumulation under drought stress than the controls [310]. Therefore, the transfer into plants of osmolyte-synthesizing genes confirmed their role in stress adaptation, even though the effect of individual genes is often rather small [227]. Since most of the osmolytes did not accumulate in amounts large enough to play a role in osmotic adjustment, the protective mechanism remains presently unclear. Shen et al. [311] demonstrated that mannitol acts in the chloroplast as radical scavenger of ROS that rapidly accumulate in response to different environmental stresses; this action reduces the oxidative cellular damage.

In this context, the increase in the capacity to scavenge ROS by manipulating the level of antioxidant enzymes in transgenic plants resulted in a common strategy to obtain plants tolerant to environmental stresses. Several groups engineered plants with genes induced by oxidative stress (e.g., superoxide dismutase, glutathione-S transferase, catalase and peroxidase) whose expression conferred partial protection from oxidative damages [312, 313]. Positive results were also confirmed in field trials under drought conditions of transgenic alfalfa plants overexpressing an MnSOD from *Nicotiana plumbaginifolia* [314]. Interestingly, ectopic expression of an alfalfa aldose/aldehyde reductase in transgenic tobacco plants provided tolerance to multiple stresses, including drought, with decreased amounts of lipid peroxidation-derived reactive aldehydes [315]. Although more work is needed to understand the actions and the interactions of the different detoxification enzymes during stress, it is conceivable that decreasing oxidative stress offers another avenue for providing protection against drought and other environmental stresses.

Although LEA-related genes are abundantly up-regulated in most plants undergoing osmotic stress, separate ectopic expression of three different members from the resurrection plant *Craterostigma plantagineum* in tobacco did not yield obvious drought-tolerant phenotypes [316]. More recently, Xu et al. [317] and Sivamani et al. [318] produced rice and wheat plants transgenic for the barley LEA gene *hva1*. Transgenic plants accumulated HVA1 proteins in both leaves and roots and showed improved tolerance to drought and salinity. These contrasting results are not surprising considering that drought stress induces an array of different LEA-related proteins in plants and other factors required for the expression of tolerance where LEA proteins are involved.

Since engineering of downstream, stress-induced genes (i.e. end-products of biochemical pathways, structural protective proteins, etc.) has shown small effects on plant stress tolerance, the manipulation of components involved in the signalling cascades was considered as another possible strategy for improving tolerance to multiple stresses [319]. Protein kinases and phosphatases have been shown to play a key role in signalling processes in yeast, animals and also in plants [320-322]. Cloning of genes for kinases and phosphatases has highlighted the central role of these proteins in signal transduction by switching genes on and off through phosphorylation/dephosphorylation of proteins, such as transcription factors. In this context, the identification and characterization of kinases and phosphatases acting in response to specific stimuli is considered a very promising approach. The overexpression of the *At-DBF2* gene encoding a serine-threonine kinase enhanced the level of salt, drought, cold and heat tolerance to a different extent, due to the constitutive expression of several stress-responsive genes [300]. Similarly, a stress-induced rice gene encoding a calcium-dependent protein kinase (OsCDPK7) was expressed ectopically in rice plants resulting in enhanced levels of stress-responsive proteins (LEA-related proteins and glycine-rich proteins) in response to salt and drought but not to cold [319]. Therefore, it was suggested that mechanisms of cold tolerance and salt/drought tolerance differ from each other, sharing OsCDPK7 as a common component.

The discovery of genes for stress-induced transcription factors has opened the possibility of overcoming the pitfalls deriving from the polygenic nature of stress tolerance and the inability to regulate in a coordinated fashion the entire network of genes involved in stress tolerance. In transgenic *Arabidopsis*, the overexpression of genes such as the *DREB1A* [300] or *CFB1* [246] encoding transcription factors able to bind to DNA motifs present in stress-induced genes demonstrated that strong expression of the target downstream genes (LEA genes *rd29A, Kin1, Cor6.6, rd17* and *P5-CS*) was indeed activated in the over-expressing plants. These transgenic plants revealed cold and dehydration tolerance. In many cases, however, the constitutive high level of expression of transcription factors produced strong negative phenotypic effects [300]. This drawback has been bypassed by regulating the expression of genes for transcription factors with inducible promoters [247]. In this way, tolerance genes are activated only when the stress event occurs, minimizing the negative pleiotropic effects associated with a constitutive expression.

9.2. Limitations and future prospects for genetic engineering approaches

The past decade has witnessed the utilization of transgenic approaches to better understand the contribution of the stress-related genes in the plant response to drought and directly obtain drought-tolerant plants. Although the results obtained so far are encouraging, many pitfalls and hurdles need to be overcome before genetic engineering can be used to produce drought-tolerant plants with real commercial value. Though an increase in the level of tolerance to drought has been claimed in many cases under controlled conditions, tolerance of the transgenic plants has only rarely been evaluated in field trials under realistic stress conditions, in which case no obvious advantage has been reported [221, 225, 302]. Additionally, only in few cases have the possible negative pleiotropic effects of genetic manipulation been thoroughly assessed and discussed [300, 324-326]. Sometimes, considerable disagreement among plant molecular biologists and physiologists has been triggered by the misinterpretation of the acquired tolerance [327, 328]. Additional concern on the first wave of stress-tolerant transgenic plants was due to the inadequacy of the constitutive expression of the target genes. This may affect the overall plant metabolism, owing to the strong interactions among crucial metabolic pathways such as those involved in sugar, lipid or protein synthesis. Although at present many patents cover the use of different genes to produce plants tolerant to abiotic stresses, thus far no transgenic variety tolerant to drought and/or other abiotic stresses has been commercially released, as occurred for pathogen-resistant transgenic varieties [329, 330].

A major challenge is the need to introduce and manipulate sets of genes in order to govern the expression of quantitative traits such as tolerance to drought. Current knowledge has broadened the possibilities for genetically engineering plants with multiple genes [331]. As a first attempt, a total of seven genes have been transferred into tobacco plants, including three genes for the biosynthesis of myo-inositol and D-ononitol, one for mannitol accumulation in plastids and trehalose in the cytosol, and two genes for oxidative stress [332]. Recently, the transfer of multiple genes has been demonstrated successfully by Potrykus and co-workers, who introduced three enzymes required for the beta-carotene biosynthetic pathway in rice plants [333]. Alternatively, sequential transformation or sexual crosses between transgenic plants harbouring transgenes of interest are other means of introducing multiple genes, although this is a time-consuming process.

Another compelling priority is to engineer crop plants for stress tolerance without causing detrimental effects to the constitutive plant metabolism. Efforts should be focused on the correct and efficient expression of foreign genes in higher plants [301]. In this direction, the discovery of plant promoters with cell-, tissue- and/or stage-specific, inducible patterns of expression will certainly be useful. Moreover, a genetic strategy should be devised to overcome poor expression of the foreign gene at the protein level and/or low enzymatic activity due to unorthodox post-translation modifications, prosthetic group acquisition and inhibitory cellular environment. Often, the foreign gene expressed in a plant encodes only one member of the set of enzymes governing a metabolic pathway (e.g., osmoprotectants), in

which case any limitation due to precursor availability and/or feedback negative control should be avoided. This last constraint has been elegantly overcome for proline accumulation by mutagenizing the pyrroline-5-carboxylate synthetase gene, so as to disrupt the feedback inhibition on the gamma-glutamyl kinase activity [334] or by antisense expression of proline dehydrogenase, a degradative enzyme which limits proline accumulation [335]. Finally, failure in producing drought-tolerant transgenic varieties could also be ascribed to unpredictable metabolic changes that might be triggered when a foreign gene is expressed, which might lead to degradation of the pool of the desired product or formation of undesired compounds. This possibility has to be evaluated carefully, also in view of the nutritional safety of the new transgenic products.

10. CONCLUDING REMARKS

It seems unlikely that a single strategy will lead to the development of drought tolerant crops adapted to a wide range of environments because of the complexity of adaptive traits and the wide differences in crop biology. Historically, progress has been made in the absence of knowledge of the mechanisms of drought tolerance. However, it will be increasingly necessary to better understand and define the true limits of productivity in dry-land agriculture in order to achieve a more effective manipulation of the limiting factors.

Although in the past two decades our capacity to understand how plants respond to and cope with drought at the molecular level has progressed greatly, little evidence is available as to the direct beneficial role of this knowledge for the release of cultivars with higher and more stable yields under conditions of limited water supply. Analogously, the vast number of crop plants genetically engineered with drought-related genes has not yet produced an improved cultivar. Despite this, efforts to improve our understanding of the intricate pathways regulating the response to drought and tentatives to manipulate such pathways by genetic engineering to release improved cultivars should continue. One of the reasons why the transgenic approach has not delivered improved cultivars more tolerant to drought is because too often the phenotype of the engineered plant is assessed under controlled conditions whose dynamics and intensity provide, at best, only a vague resemblance of the much more complex and variable field conditions. Future work should carefully consider this important aspect. On the same line, experimental conditions in functional genomics studies based on plants grown under controlled conditions should mimic as closely as possible the conditions present in the target environment. Since controlled environments at best only partially mimic field conditions, there is no real substitute to extensive field testing, particularly when trying to assess the agronomic value of novel genotypes. An interesting example is provided by the experimental conditions adopted to study the effects of water deficit on ethylene production: contradictory results were obtained between experiments using plant parts subjected to rapid drying and experiments focusing on intact plants exposed to slow drying, clearly suggesting that great caution should be exercised when extrapolating results of bench experiments to field conditions [147].

Additionally, in other cases the relationships between physiological traits studied in controlled environments and field performance have not been clearly demonstrated.

A contributing factor to the limited success of the physiological approach in improving drought tolerance is that strategies deployed by xerophytes to cope with droughted environments often have little relevance for crop production. Another factor limiting the success of the physiological approach is the difficulty in analysing and comparing data collected from plants at a similar developmental stage and water status. This problem is particularly evident in field experiment when data and samples are concomitantly collected from plants differing in flowering time and/or drought resistance. Consequently, interaction of variation in phenology with the dynamics of the drought episode/s hinders our capacity to dissect and correctly interpret the mechanisms underlying the response to drought.

In terms of physiological traits to be considered in future work to identify QTLs for drought tolerance, a promising approach is to consider traits characterized by a low "genotype x environment" interactions, such as the elongation rate at the base of the leaf [336, 337]. In maize, elongation rate is genotype-specific and is linearly influenced by environmental variables (e.g., water availability, temperature, etc.) whose measurement can reduce the confounding effects of uncontrolled variation in such environmental factors [336, 337]. Crossing genotypes differing in elongation rates can thus lead to the identification of the relevant QTLs.

One of the dilemmas faced by those striving to release improved, drought-tolerant cultivars is what portion of the available resources should be invested in conventional *vs.* the non-conventional approaches herein described. Although conventional approaches will remain the mainstay, increasing attention should be devoted to (i) the identification of chromosome regions important in determining yield and its stability under drought, (ii) the cloning of the genes responsible for such effects, (iii) the identification of agronomically superior alleles at such loci and (iv) their concerted manipulation either directly via genetic engineering or indirectly via MAS. Functional maps obtained with EST and/or cDNA clones of known function coupled with comparative mapping using model species for which the entire genome sequence may become available will dramatically improve our ability to identify candidate genes. The emerging picture for these genes and putatively associated QTLs is that of functional clusters non-randomly distributed along the chromosomes as theorized by Khavkin and Coe [338] who hypothesized that many plant reactions to abiotic stresses rely on such gene clusters. A similar picture has recently emerged from the work of Li and co-workers investigating yield QTLs in rice [132, 133]. These functional clusters may indicate the presence of functionally different alleles at loci encoding for transcription factors regulating a cascade of downstream events affecting the phenotype. In *Arabidopsis* and rice ca. 7 and 6% of all ORFs (Open Reading Frames), respectively, encode for proteins with significant similarity to known classes of plant transcription factors [7]. As more data become available, it will be possible to verify what percentage of major QTLs for drought tolerance are determined by sequence polymorphism (e.g., single nucleotide polymorphisms: SNPs) at loci encoding for transcription factors or at their promoter regions.

It has been suggested that the remarkable increase in crops yield during the past century should be equally attributed to breeding and better agronomic practices. A higher resistance to drought and other stresses, coupled with an improved ability to maximize yield under low-stress conditions have both contributed to such spectacular increases and to stabilize yield across environments with different water availability [339]. Unless new sources of genetic variation are utilized and more effective selection schemes are devised, it may not be possible to maintain the linear gains in yield of the past century, particularly in view of the increasingly higher unpredictability of weather patterns, depletion of irrigation water and its increasing cost. Clearly, enhancing drought resistance will play an increasingly important role for securing an adequate food supply world-wide and for improving the livelihoods and quality of life of farmers more exposed to the consequences of erratic rainfalls in drought-prone areas in the less developed countries. Given the complexity of this challenge, future progress towards a better understanding of the molecular, physiological and morphological bases of yield under drought and a more effective deployment of such information for breeding purposes will only be possible through a close collaboration among scientists of different disciplines.

Table 1. Plant and microbial genes over-expressed in transgenic plants for improving drought tolerance

Gene	Donor	Product	Host plant	References
	PROTECTIVE PROTEINS			
HVA1	plant	lea protein	rice	[317]
	SCAVENGING ENZYMES			
Gst/Gpx	plant	Glutathione transferase/ Glutathione peroxidase	tobacco	[313]
Mn-Sod	plant	Mn-superoxide dismutase	alfalfa, tobacco	[314] [335]
Fe-Sod	plant	Fe-superoxide dismutase	tobacco	[312]
	COMPATIBLE SOLUTES			
MtlD	*E. coli*	mannitol 1-P dehydrogenase	tobacco, *A. thaliana*	[306]
Bet A	*E. coli*	choline dehydrogenase	tobacco	[234]

Table 1. Continued

Gene	Donor	Product	Host plant	References
PEAMT	plant	phospho thanolamine Nmethyltransf erase	tobacco	[315]
SacB	*B. subtilis*	levansucrase	tobacco	[307]
CodA	*A. globiformis*	choline oxidase	*A. thaliana*	[311]
Imt 1	ice plant	myo-inositol-O-methyl-transferase	tobacco	[338]
Tps1	yeast	trehalose 6-phosphate synthase	tobacco	[326] [309]
otsA, otsB	*E. coli*	trehalose 6-phosphate synthase/trehalose 6-P phosphatases	tobacco, potato	[324]
ProDH	plant	proline dehydrogenase	*A. thaliana*	[335]
P5CS	plant	pyrroline 5-carboxylate synthetase	tobacco	[303]
	REGULATORS AND SIGNALING MOLECULES			
At-DBF2	plant	serine/threonine kinase	tobacco	[323]
OSCDPK7	plant	calcium-dependent protein kinase	rice	[319]
AVP1	plant	H(+)-pyrophosphatase	*A. thaliana*	[235]
DREB1	plant	transcription factor	*A. thaliana*	[300] [247]
BIP	plant	chaperone-binding protein	tobacco	[275]
ABF3	plant	transcription factor	*A. thaliana*	[301]

Table 1. Continued

Gene	Donor	Product	Host plant	References
ME	plant	NADP-malic enzyme (ME)	tobacco	[315]
PvNCED1	plant	9-cis epoxycaroteno id dioxygenase (ABA biosynthesis)	tobacco	[317]

ACKNOWLEDGEMENTS

The Scottish Crop Research Institute is funded by grant in aid from the Scottish Executive Environment and Rural Affairs Department. Contribution No. 12 of Institute of Plant Genetics (CNR-IGV), Research Division of Portici, Italy. Contribution of the Interdepartmental Centre for Biotechnology, University of Bologna, Italy.

REFERENCES

1. McNamara, R.S. (1974). *Ann. Rep. Int. Bank Recon. Dev.*, Washington, USA, 15.
2. Edmeades, G.O., Bolanõs, J., Elings, A., Ribaut, J.M., Bänziger, M., and Westgate, M.E. (2000). In: Westgate, M.E., and Boote, K.J., eds., Physiology and Modeling Kernel Set in Maize CSSA Special Publication No. 29, Madison, WI, CSSA, 43.
3. Bartsch, K.P., Miegroet, H., van Boettinger, J., and Dobrowolski, J.P. (2002). *J. Soil Water Cons.*, 57, 29.
4. Greiner, R. (1997). *Land Degrad. Dev.*, 8, 282.
5. Etterson, J.R., and Shaw, R.G. (2001). *Science*, 294, 151.
6. Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yanaguchi-Shinozaki, K., and Shinozaki, K. (2002). *Plant J.*, 29, 417.
7. Goff, S.A., et al. (2002). *Science*, 296, 92.
8. Passioura, J.B. (1977). *J. Austr. Inst. Agric. Sci.*, 43, 117.
9. Austin, R.B. (1999). *Crop Sci.*, 39, 1604.
10. Mechin, V., Argillier, O., Hebert, Y., Guingo, E., Moreau, L., Charcosset, A. and Barriere, Y. (2001). *Crop Sci.*, 41, 690.
11. Yadav, R.S., Hash, C.T., Bidinger, F.R., Cavan, G.P., and Howarth, C.J. (2002). *Theor. Appl. Genet.*, 104, 67.
12. Calderini, D.F., Dreccer, M.F., and Slafer, G.A. (1997). *Field Crops Res.*, 52, 271.
13. Atlin, G.N., and Lafitte, H.R. (2002). In: Saxena, N.P., and O'Toole, J.C., eds., Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice. Proc. of an Int. Workshop on Field Screening for Drought Tolerance in Rice, Dec. 11-14, 2000, ICRISAT, Patancheru, India, 71.
14. Hsiao, T.C. (1973). *Annu. Rev. Plant Physiol.*, 24, 519.
15. Blum, A. (1988). Plant Breeding for Stress Environments. CRC Press, Boca Raton, USA.
16. Ludlow, M.M., and Muchow, R.C. (1990). *Adv. Agron.*, 43, 107.
17. Quarrie, S.A. (1993). In: Close, T.J., and Bray, E.A., eds., Plant Responses to Cellular Dehydration During Environmental Stress. Proc. of the 16th Annual Riverside Symposium in Plant Physiology, Riverside, CA, 224.

18. Passioura, J.B. (1996). *Plant Growth Regul.*, 20, 79.
19. Ingram, J., and Bartels, D. (1996). *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 47, 377.
20. Bray, E.A. (1997). *Trends in Plant Sci.*, 2, 48.
21. Bray, E.A. (2002). *Ann. Bot.*, 89, 803.
22. Bohnert, H.J., and Bressan, R.A. (2001). In: Nössberger, J., ed., Crop Science: Progress and Prospects, CAB Int., Wallingsford, UK, 81.
23. Richards, R.A. (1996). *Plant Growth Regul.*, 20, 157.
24. Mitra, J. (2001). *Curr. Sci.*, 80, 758.
25. Bolaños, J., and Edmeades, G.O. (1993). *Field Crops Res.*, 31, 233.
26. Ceccarelli, S. (1984). *Gen. Agr.*, 38, 43.
27. Fussell, L.K., Bidinger, F.R., and Bieler, P., 1991. *Field Crops Res.*, 27, 183.
28. Blum, A. (1993). In: Cherry, P., ed., Biochemical and Cellular Mechanisms of Stress Tolerance in Plants. Springer Verlag, Berlin, Germany, 315.
29. Dhanda, S.S., Behl, R.K., and El-Bassam, N. (1995). *Landbau Volk*, 45, 159.
30. Blum, A. (2002). In: Saxena, N.P., and O'Toole, J.C., eds., Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice. Proc. of an Int. Workshop on Field Screening for Drought Tolerance in Rice, Dec. 11-14, 2000, ICRISAT, Patancheru, India, 17.
31. van Oosterom, E.J., and Acevedo, E. (1992). *Euphytica*, 62, 29.
32. Price, A.H., Cairns, J.E., Horton, P., Jones, H.G., and Griffiths, H. (2002). *J. Exp. Bot.*, 53, 989.
33. Reynolds M.P., Rajaram, S., and Sayre K.D. (1999). *Crop Sci.,* 39, 1611.
34. Richards, R.A. (2000). *J. Exp. Bot.*, 51, 447.
35. Borrell, A., Hammer, G., and van Oosterom, E. (2001). *Ann. Appl. Biol.*, 138, 91.
36. Blum, A., Golan, G., Mayer, J., and Sinmena, B. (1997). *Field Crops Res.*, 52, 43.
37. Thomas, H., and Howarth, C.J. (2000). *J. Exp. Bot.*, 51, 329.
38. Akhtar, M.S., Goldschmidt, E.E., John, I., Rodoni, S., Matile, P. and Grierson, D. (1999). *J. Exp. Bot.*, 50, 1115.
39. Moser, D., and Matile, P. (1997). J. *Plant Physiol.*, 150, 759.
40. Thomas, H., Schellenburg, M., Vicentini, F., and Matile, P. (1996). *Bot. Acta*, 109, 3.
41. Crasta, O.R., Xu, W.W., Rosenow, D.T., Mullet, J., and Nguyen, H.T. (1999). *Mol. Gen. Genet.*, 262, 579.
42. Xu, W.W., Subudhi, P.K., Crasta, O.R., Rosenow, D,T., Mullet, J.E., and Nguyen, H.T. (2000). *Genome*, 43, 461.
43. Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2000). *Theor. Appl. Genet.*, 101, 733.
44. Bolaños, J., and Edmeades, G.O. (1996). *Field Crops Res.*, 48, 65.
45. O'Toole, J.C., and Bland, W.L. (1987). *Adv. Agron.*, 41, 91.
46. Fukai, S., and Cooper, M. (1995). *Field Crops Res.*, 40, 67.
47. Mohamed, M.F., Keutgen, N., Tawfik, A.A., and Noga, G. (2002). *J. Plant Physiol.*, 159, 31.
48. Ito, O., O'Toole, J., and Hardy, B., eds. (1999). Genetic Improvement of Rice for Water-limited Environments. Proc. of the Workshop on Genetic Improvement of Rice for Water-Limited Environments, Dec. 1-3, 1998, Los Baños, Philippines, Int. Rice Research Institute.
49. Saxena, N.P., and O'Toole, J.C., eds. (2002). Field screening for drought tolerance in crop plants with emphasis on rice. Proc. of an Int. Workshop on Field Screening for Drought Tolerance in Rice, Dec. 11-14, 2000, ICRISAT, Patancheru, India, 208 pp.
50. Premachandra, G.S., Saneoka, H., Fujita, K., and Ogata, S. (1992). *J. Exp. Bot.*, 43, 1569.
51. Morgan, J.M, and Condon, A.G. (1986). *Aust. J. Plant Physiol.* 13, 523.
52. Boyer, J.S. (1996). *Adv. Agron.*, 56, 187.
53. Volaire, F., Conejero, G., and Lelievre, F. (2001). *Aust. J. Plant Phys.*, 28, 743.
54. Wright, G.C., Smith, R.C.G., and McWilliam, J.R. (1983). *Aust. J. Agric. Res.*, 34, 615.
55. Noctor, G., Veljovic-Jovanovic, S., and Foyer, C.H. (2000). *Phil. Trans. R. Soc. Lond. B*, 355, 1465.
56. Foyer C.H., and Noctor, G. (2000). *New Phytol.*, 146, 359.
57. Slafer, G.A., and Araus, J.L. (1998). In: Slinkard, A.E., ed., Proc. of the 9th Int. Wheat Genetics Symposium, Saskatoon, Saskatchewan, Vol. 1, 201.
58. Slafer, G.A., Araus, J.L., and Richards, R.A. (1999). In: Satorre, E.H., and Slafer, G.A., eds., Wheat: Ecology and Physiology of Yield Determination. New York, Food Product Press, 379.
59. Araus, J.L., Slafer, G.A., and Romagosa, I. (1999). *Aust. J. Plant Physiol.*, 26, 345.
60. Araus, J.L., Slafer, G.A., Reynolds, M.P., and Royo, C. (2002). *Ann. Bot.*, 89, 925.

61. Royo, R., Villegas, D., García del Moral, L.F., El Hani, S., Aparicio, N., Rharrabti,, Y., and Araus, J.L. (2001). *Austr. J. Agric. Res.*, 53, 1.
62. Griffiths, H., and Parry, M. (2002). *Ann. Bot.*, 89, 801.
63. Wade, L.J., McLaren, C.G., Quintana, L., Harnpichitvitaya, D., Rajatasereekul, S., Sarawgi, A.K., Kumar, A., Ahmed, H.U., Sarwoto, Singh, A.K., Rodriguez, R., Siopongco, J., and Sarkarung, S. (1999). *Field Crops Res.*, 64, 35.
64. Wade, L.J. (2002). *Field Crops Res.*, 76, 89.
65. Hargrove, T.R. (1978). *Field Crops Res.*, 1, 197.
66. Price, A.H., Young, E.M., and Tomos, A.D. (1997). *New Phytol.*, 137, 83.
67. Price, A.H., Steele, K.A., Moore, B.J., Barraclough, P.B., and Clark, L.J. (2000). *Theor. Appl. Genet.*, 100, 49.
68. Courtois, B., McLaren, G., Sinha, P.K., Prasad, K., Yadav, R., and Shen, L. (2000). *Mol. Breed.*, 6, 55.
69. Tuinstra, M.R., Grote, E.M., Goldsbrough, P.B., and Ejeta, G. (1997). *Mol. Breed.*, 3, 439.
70. Tuinstra, M.R., Ejeta, G., and Goldsbrough, P. (1998). *Crop Sci.*, 38, 835.
71. Price, A.H., Townend, J., Jones, M.P., Audebert, A., and Courtois, B. (2002). *Plant Mol. Biol.*, 48, 683.
72. Bruce, W.B., Edmeades, G.O., and Barker, T.C. (2002). *J. Exp. Bot.*, 53, 13.
73. Tollenaar, M., and Wu, J. (1999). *Crop Sci.*, 39, 1597.
74. Ribaut, J.M., Banziger, M., Betran, J., Jiang, C., Edmeades, G.O., Dreher, K., and Hoisington, D. (2002). In: Kang, M.S., ed., Quantitative Genetics, genomics, and Plant Breeding, CABI Publishing, Wallingford, UK, 85.
75. Bolaños, J., Edmeades, G.O., and Martinez, L. (1993). *Field Crops Res.*, 31, 269.
76. Bolaños, J., and Edmeades, G.O. (1997). In: Edmeades, G.O., Bänziger, M., Mickelson, H.R., and Peña-Valdivia, C.B., eds., Developing drought- and low N-tolerant maize. Proc. of a Symposium, March 25-29, 1996, CIMMYT, El Batán, Mexico, 355.
77. Swanston, J.S., Ellis, R.P., Perez-Vendrell, A., Voltas, J., and Molina-Cano, J.L. (1997). *Cer. Chem.*, 74, 456.
78. Ellis, R.P., Forster, B.P., Gordon, D.C., Handley, L.L., Keith, R.P., Lawrence, P., Meyer. R., Powell, W., Robinson, D., Scrimgeour, C.M., Young, G.R., and Thomas W.T.B. (2002). *J. Exp. Bot.*, 53, 1.
79. Ellis, R.P., Forster, B.P., Robinson, D., Handley, L.L., Gordon, D.C., Russell, J.R., and Powell, W. (2000). *J. Exp. Bot.*, 51, 9.
80. Kirby, E.J.M., and Ellis, R.P. (1980). *J. Agric Sci. Camb.*, 95, 101.
81. Kirby, E.J.M., and Appleyard, M. (1987). Cereal Development Guide 2nd Edition. Coventry, UK, Arable Unit, National Agricultural Centre.
82. Kirby, E.J.M., and Riggs, T.J. (1978). *J. Agric. Sci. Camb.*, 91, 207.
83. Kirby, E.J.M. (1993). *Asp. Appl. Biol.*, 34, 69.
84. Tuberosa, R., Sanguineti, M.C., and Conti, S. (1986). *Plant Breed.*, 97, 345.
85. Laurie, D.A., Pratchett, N., Bezant, J.H., and Snape, J.W. (1995). *Genome*, 38, 575.
86. Bezant, J., Laurie, D., Pratchett, N., Chojecki, J., and Kearsey, M. (1996). *Heredity*, 77, 64.
87. Laurie, D.A. (1997). *Plant Mol. Biol.*, 35, 167.
88. Noli, E., Sanguineti, M.C., Tuberosa, R., Giuliani, S., Maccaferri, M., Salvi, S., Landi, P., and Conti, S. (2000). In: Logue, S., ed., Proc. of 8th Int. Barley Genetics Symposium, Oct. 22-27, Adelaide, South Australia, Vol. III, 87.
89. Ellis, R.P., and Kirby, E.J.M. (1980). *J. Agric. Sci. Camb.*, 95, 111.
90. Ellis, R.P., and Russell, G. (1984). *J. Agric. Sci. Camb.*, 102, 85
91. Russell, G., and Ellis, R.P. (1988). *Ann. Appl. Biol.*, 113, 357.
92. Teulat, B., Monneveux, P., Wery, J., Borries, C., Souyris, I., Charrier, A., and This, D. (1997). *New Phytol.*, 137, 99.
93. Ellis, R.P., and Marshall, B. (1998). *J. Exp. Bot.*, 49, 1021.
94. Teulat, B., Merah, O., Souyris, I., and This, D. (2001). *Theor. Appl. Genet.*, 103, 774.
95. Robinson, D., Handley, L.L., Scrimgeour, C.M., Gordon, D.C., Forster, B.P., and Ellis, R.P. (2000). *J. Exp. Bot.*, 51, 41.
96. Farquhar, G.D., O'Leary, M.H., and Berry, J.A. (1982). *Aust. J. Plant Physiol.*, 9, 121.
97. Ehdaie, B., and Waines, J.G. (1994). *Theor. Appl. Genet.*, 88, 1023.
98. Acevedo, E. (1993). In: Ehleringer, J.R., Hall, A.E. and Farquhar, G.D., eds., Stable isotopes and

plant carbon-water relations. London Academic Press, 399.
99. Richards, R.A. (1988). In: Srivastrava, J., Porceddu, E., Acevedo, E., and Barma, S., eds., Drought tolerance in winter cereals. Wiley, Chicester, England, 133.
100. Lebreton, C., Lazic-Jancic, V., Steed, A., Pekic, S., and Quarrie, S.A. (1995). *J. Exp. Bot.*, 46, 853.
101. Sanguineti, M.C., Tuberosa, R., Landi, P., Salvi, S., Maccaferri, M., Casarini, E., and Conti, S. (1999). *J. Exp. Bot.*, 50, 1289.
102. Ribaut, J.M, Hu, X., Hoisington, D., and Gonzales-de-Leon, D. (1997). *Plant Mol. Biol. Rep.*, 15, 156.
103. Tuberosa, R., Sanguineti, M.C., and Landi, P. (1994). *Crop Sci.*, 34, 1557.
104. Landi, P., Conti, S., Gherardi, F., Sanguineti, M.C., and Tuberosa, R. (1995). *Maydica*, 40, 179.
105. Quarrie, S.A. (1996). *Plant Growth Regul.*, 20, 167.
106. Setter, T.L. (1997). In: Edmeades, G.O., Bänziger, M., Mickelson, H.R., Peña-Valdivia, C.B., eds., Developing Drought- and Low N-tolerant maize. Proc. of a Symposium, March 25-29, 1996, CIMMYT, El Batán, Mexico, 142.
107. Mugo, S.N., Bänziger, M., and Edmeades, G.O. (2000). In: Ribaut, J.M., and Poland, D., eds., Molecular Approaches for the Genetic Improvement of Cereals for Stable Production in Water-Limited Environments. A Strategic Planning Workshop held at CIMMYT, El Batán, Mexico, June 21-25, 1999, 73.
108. Landi, P., Sanguineti, M.C., Conti, S., and Tuberosa, R. (2001). *Crop Sci.*, 41, 335.
109. Westgate, M.E., and Boyer, J.S. (1985). *Planta*, 164, 540.
110. Jones, R.J., and Setter, T.L. (2000). In: Westgate, M.E., and Boote, K.J., eds., Physiology and Modelling Kernel Set in Maize, Crop Science Society of America, Madison, Wisconsin, 25.
111. Saini, H.S., and Westgate, M.E. (2000). *Adv. Agron.*, 68, 59.
112. Agrama, H.A.S. and Moussa, M.E. (1996). *Euphytica*, 91, 89.
113. Gale, M.D., and Devos, K.M. (1998). *Science,* 282, 656.
114. Tanksley, S.D., and Nelson, J.C. (1996). *Theor. Appl. Genet.*, 92, 191.
115. Quarrie, S.A., Steed, A., Semikhdoski, A., Lebreton, C., Calestani, C., Clarkson, D.A., Tuberosa, R., Sanguineti, M.C., Melchiorre, R., Prioul, J.L. (1995). In: Leigh, R.A., and Metchled Blake-Kalff, eds., Proc. of 2nd STRESSNET Conference, Sept. 21-23, Salsomaggiore, Italy, 175.
116. Quarrie, S., Abugalieva, S., Abugalieva, A., Sanguineti, M.C., Turuspekov, Y., and Tuberosa, R. (2003). Abstract of the "Plant & Animal Genome XI" Conference, Jan. 11-15, San Diego, CA, P377, 169.
117. McCouch, S.R., Kochert, G., Yu, Z.H., Wang, Z.Y., Khush, G.S., Coffman, W.R., Tanksley, S.D. (1988). *Theor. Appl. Genet.*, 76, 815.
118. McCouch, S.R., and Doerge, R.W. (1995). *Trends Genet.*, 11, 482.
119. Mackill, D.J., Nguyen H.T., and Zhang, J.X. (1999). *Field Crops Res.*, 64, 177.
120. Champoux, M.C., Wang, G., Sarkarung, S., Mackill, D.J., O'Toole, J.C., Huang, N., and McCouch, S.R. (1995). *Theor. Appl. Genet.*, 90, 969.
121. Lilley, J.M., Ludlow, M.M., McCouch, S.R., and O'Toole, J.C. (1996). *J. Exp. Bot.*, 47, 1427.
122. Price, A.H., Steele, K.A., Gorham, J., Bridges, J.M., Moore, B.J., Evans, J.L., Richardson, P., and Jones, R.G.W. (2002). *Field Crops Res.*, 76, 11.
123. Price, A.H., Steele, K.A., Moore, B.J., and Jones, R.G.W. (2002). *Field Crops Res.*, 76, 25.
124. Yadav, R., Courtois, B., Huang, N., and McLaren, G. (1997). *Theor. Appl. Genet.*, 94, 619.
125. Kamoshita, A., Wade, L.J., Ali, M.L., Pathan, M.S., Zhang, J., Sarkarung, S., and Nguyen, H.T. (2002). *Theor. Appl. Genet.*, 104, 880.
126. Lafitte, H.R., Courtois, B., and Atlin, G.N. (2002). In: Saxena, N.P., and O'Toole, J.C., eds., Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice. Proc. of an Int. Workshop on Field Screening for Drought Tolerance in Rice, Dec. 11-14, 2000, ICRISAT, Patancheru, India, 25.
127. Courtois, B., Sinha, P.K., Prasad, K., and Carandang, S. (1997). *Intern. Rice Res. Notes*, 22, 8.
128. Ali, M.L., Pathan, M.S., Zhang, J., Bai, G., Sarkarung, S., and Nguyen, H.T. (2000). *Theor. Appl. Genet.*, 101, 756.
129. Zhang, W.P., Shen, X.Y., Wu, P., Hu, B., Liao, C.Y. (2001). *Theor. Appl. Genet.*, 103, 118.
130. Tripathy, J.N., Zhang, J., Robin, S., Nguyen, T.T., and Nguyen, H.T. (2000). *Theor. Appl. Genet.*, 100, 1197.
131. Salvi, S., Maccaferri, M., Giuliani, S., Sanguineti, M.C., and Tuberosa, R. (2003). Abstracts of the "Plant & Animal Genome XI" Conference, Jan. 11-15, San Diego, CA, W303, 66.

132. Li, Z.K., Pinson, S.R.M., Paterson, A.H., Park, W.D., and Stansel, J.W. (1997). *Genetics*, 145, 453.
133. Li, Z.K., Shen, L.S., Courtois, B., and Lafitte, R. (1999). In: Ito, O., O'Toole, J., and Hardy, B. Genetic Improvement of Rice for Water-limited Environments, Proc. of the Workshop on Genetic Improvement of Rice for Water-limited Environments, Dec. 1-3, 1998, Los Baños, Philippines, IRRI, 157.
134. Li, Z.K. (2001). In: Kush, G.S., Brar, D.S., and Hardy, B., eds., Rice Genetics IV, Proc. of the Fourth Int. Rice Genetics Symposium, Oct. 22-27, 2000, Los Baños, Philippines, Int. Rice Research Institute, 153.
135. Menz, M.A., Klein, R.R., Mullet, J.E., Obert, J.A., Unruh, N.C., and Klein, P.E. (2002). *Plant Mol. Biol.*, 48, 483.
136. Kebede, H., Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T., 2001. *Theor. Appl. Genet.*, 103, 266.
137. Subudhi, P.K., Magpantay G.B., Rosenow D.T., and Nguyen H.T. (1999). In: Ito O., O'Toole J., and Hardy B., eds., Genetic Improvement of Rice for Water-limited Environments. Proc. of the Workshop on Genetic Improvement of Rice for Water-Limited Environment, Dec. 1-3, 1998, Los Baños, Philippines, Int. Rice Research Institute, 183.
138. Sanchez, A.C., Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2002). *Plant Mol. Biol.*, 48, 713.
139. Davis, G.L., McMullen, M.D., Polacco, M., Grant, D., Musket, T., Baysdorfer, C., Staebell, M., Xu, G., Koster, L., Houchins, K., Melia-Hancock, S., and Coe, E.H. (1998). *Maize Gen. Coop. News.*, 72, 118.
140. Tuberosa, R., Salvi, S., Sanguineti, M.C., Landi, P., Maccaferri, M., and Conti, S. (2002). *Ann. Bot.*, 89, 941.
141. Robertson, D.S. (1985). *J. Theor. Biol.*, 117, 1.
142. Zeevaart, J.A.D., and Creelman, R.A. (1988). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39, 439.
143. Quarrie, S.A. (1991). In: Davies, W.J., and Jones, H.G., eds., Abscisic acid: Physiology and Biochemistry. Bios Scientific Publishers, Oxford, UK, 227.
144. Sharp, R.E. (1996). *Hort. Sci.*, 31, 36.
145. Close, TJ. (1997). *Physiol. Plant.*, 100, 291.
146. Spollen, W.G., LeNoble, M.E., Samuels, T.D., Bernstein, N., and Sharp, R.E. (2000). *Plant Physiol.*, 122, 967.
147. Sharp, R.E. (2002). *Plant Cell. Environ.*, 25, 211.
148. Hose, E., Steudle, E., and Hartung, W. (2000). *Planta*, 211, 874.
149. Tuberosa, R., Parentoni, S., Kim, T.S., Sanguineti, M.C., and Phillips, R.L. (1998). *Maize Gen. Coop. News.*, 72, 72.
150. Tuberosa, R., Sanguineti, M.C., Landi P., Salvi, S., Casarini, E., and Conti, S. (1998). *Theor. Appl. Genet.*, 97, 744.
151. Salvi, S., Tuberosa, R., Sanguineti, M.C., Landi, P., and Conti, S. (1997). *Maize Gen. Coop. News.*, 71, 15.
152. Sanguineti, M.C., Conti, S., Landi, P., and Tuberosa, R. (1996). *Maydica*, 41, 193.
153. Landi, P., Salvi, S., Sanguineti, M.C., Stefanelli, S., and Tuberosa, R. (2002). *Maize Gen. Coop. News.*, 76, 7.
154. Giuliani, S., Landi, P., Salvi, S., Bellotti.M, Sanguineti, M.C., Stefanelli, S., and Tuberosa, R. (2003). Abstracts of the "Plant & Animal Genome XI" Conference, Jan. 11-15, San Diego, CA, P449, 187.
155. Jensen, A.B., Busk, P.K., Figueras, M., Albà, M.M., Peracchia, G., Messeguer, R., Goday, A., and Pagès, M. (1996). *Plant Growth Regul.*, 20, 105.
156. Quarrie, S.A., Lazic Jancic, V., Kovacevic, D., Steed, A., and Pekic, S. (1999). *J. Exp. Bot.*, 50, 1299.
157. McDonald, A.J.S., and Davies, W.J. (1996). *Adv. Bot. Res.*, 22, 229.
158. Tuberosa, R., Sanguineti, M.C., Landi, P., Giuliani, M.M., Salvi, S., and Conti, S. (2002). *Plant Mol. Biol.*, 48, 697.
159. Landi, P., Sanguineti, M.C, Darrah, L.L., Giuliani, M.M., Salvi, S., and Tuberosa, R. (2002). *Maydica*, 47, 233.
160. Tuberosa, R., Sanguineti, M.C., Ribaut, J.M., Landi, P., Giuliani, M., Salvi, S., and Conti, S. (2000). Abstracts of the "Plant & Animal Genome VIII" Conference, Jan. 9-12, San Diego, CA, 5.
161. Ribaut, J.M., Hoisington, D.A., Deutsch, J.A., Jiang, C., and Gonzales-de-Leon, D. (1996). *Theor. Appl. Genet.*, 92, 905.

162. Ribaut, J.M., Jiang, C., Gonzalez-de-Leon, D., Edmeades, G.O., and Hoisington, D.A. (1997). *Theor. Appl. Genet*, 94, 887.
163. Pelleschi, S., Guy, S., Kim, J.Y., Pointe, C., Mahe, A., Barthes, L., Leonardi, A., and Prioul, J.L. (1999). *Plant Mol. Biol.*, 39, 373.
164. Hanft, J.M., and Jones, R.J. (1986). *Plant Physiol.*, 81, 503.
165. Zinselmeier, C., Westgate, M.E., Schussler, J.R., and Jones, R.J. (1995). *Plant Physiol.*, 107, 385.
166. Boyle, M.G., Boyer, J.S., and Morgan P.W. (1991). *Crop Sci.*, 31, 1241.
167. Conti, S., Frascaroli, E., Gherardi, F., Landi, P., Sanguineti, M.C., Stefanelli, S., and Tuberosa, R. (1994). In: Bianchi A., ed., Proc. of the XVI Conference of the Eucarpia Maize and Sorghum Section, June 6-9, 1993, Bergamo, Italy, 212.
168. Frascaroli, E., and Tuberosa, R. (1993). *Plant Breed.*, 110, 250.
169. Teulat, B., This, D., Khairallah, M., Borries, C., Ragot, C., Sourdille, P., Leroy, P., Monneveux, P., and Charrier, A. (1998). *Theor. Appl. Genet.*, 96, 688.
170. Teulat, B., Borries, C., and This, D. (2001). *Theor. Appl. Genet.*, 103, 161.
171. Tanksley, S.D., Grandillo, S., Fulton, T.M., Zamir, D., Eshed, T., Petiard, V., Lopez, J., and Beck-Bunn, T. (1996). *Theor. Appl. Genet.*, 92, 213.
172. Bernacchi, D., Beck-Bunn, T., Eshed, Y., Lopez, J., Petiard, V., Uhlig, J., Zamir, D., and Tanksley, S.D. (1998). *Theor. Appl. Genet.*, 97, 381.
173. Xiao, J., Li, J., Grandillo, S., Ahn, S.N., Yuan, L., Tanksley, S.D., and McCouch, S.R. (1998). *Genetics*, 150, 899.
174. Forster, B. P., Russell, J. R., Ellis, R. P., Handley, L. L., Robinson, D., Hackett, C. A., Nevo, E., Waugh, R., Gordon, D. C., Keith, R., and Powell, W. (1997). *New Phytologist*, 137, 141.
175. Forster, B.P., Ellis, R.P., Thomas, W.T.B., Newton, A.C., Tuberosa, R., This, D., El Enein, R.A., Bahri, M.H., and Ben Salem, M. (2000). *J. Exp. Bot.*, 51, 19.
176. Talamè, V. (2003). Ph.D. thesis, University of Bologna.
177. Moncada, P., Martinez, C.P., Borrero, J., Chatel M., Gauch, H. Jr., Guimaraes, E., Tohme, J., and McCouch, S.R. (2001). *Theor. Appl. Genet.*, 102, 41.
178. Tuberosa, R., Gill, B.S., and Quarrie, S. (2002). *Plant Mol. Biol.*, 48, 445.
179. Somerville, C., and Somerville, S. (1999). *Science*, 285, 380.
180. Deyholos, M., and Galbraith, D.W. (2001). *Cytometry*, 43, 229.
181. Kawasaki, S., Deyholos, M., Borchert, C., Brazille, S., Kawai, K., Galbraith, D.W., and Bohnert, H.J. (2001). *Plant Cell*, 12, 889.
182. Chen, T.H., and Murata, N. (2002). *Curr. Opin. Plant Biol.*, 5, 250.
183. Ozturk, Z.N., Talamé, V., Deyholos, Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N., Tuberosa, R., and Bohnert, H.J. (2002). *Plant Mol. Biol.*, 48, 551.
184. Zinselmeier, C., Sun, Y., Helentjaris, T., Beatty, M., Yang, S., Smith, H., and Habben, J. (2002). *Field Crop Res.*, 75, 111.
185. Liang, P., and Pardee, A.B. (1992). *Science*, 257, 967.
186. Bachem, C.W.B., van der Hoeven, R.S., de Bruijin, S.M., Vreugdenhil, D., Zabeau, M., and Visser, R.G.F. (1996). *Plant J.*, 9, 745.
187. Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995). *Science*, 270, 484.
188. Matsumura, H., Nirasawa, S., and Terauchi, R. (1999). *Plant J.*, 20, 719.
189. Bruce, W., Folkerts, O., Garnaat, C., Crasta, O., Roth, B., and Bowen, B. (2000). *Plant Cell*, 12, 65.
190. Brenner, S., Williams, S.R., Vermaas, E.H., Storck, T., Moon, K., McCollum, C., Mao, J., Luo, S., Kirchner, J.J., Eletr, S., Robert, B., DuBridge, R.B., Burcham, T., and Albrecht, G. (2000). *Proc. Natl. Acad. Sci. USA*, 97, 1665.
191. Bruce, W., Desbons, P., Crast, O., and Folkerts, O. (2001). *J. Exp. Bot.*, 52, 459.
192. Zinselmeier, C., Jeong, B.-R., and Boyer, J.S. (1999). *Plant Physiol.*, 121, 25.
193. Talamè, V., Neslihan, Ö., Bohnert, H.J., and Tuberosa, R. (2003). Abstracts of the "Plant & Animal Genome XI" Conference, Jan. 11-15, 2003, San Diego, CA, W51, 15.
194. Yates, J.R.I. (1998). *J. Mass Spectr.*, 33, 1.
195. Salekdeh, Gh H., Siopongco, J., Wade, L.J., Ghareyazie, B., and Bennett, J. (2002). *Field Crops Res.*, 76, 199.
196. Damerval, C., Maurice, A., Josse, J.M., and de Vienne, D. (1994). *Genetics*, 137, 289.
197. Touzet, P., Winkler, R.G., and Helentjaris, T. (1995). *Theor. Appl. Genet.*, 91, 200.
198. de Vienne, D., Leonardi, A., Damerval, C., and Zivy, M. (1999). *J. Exp. Bot.*, 50, 303.

199. Thiellement, H., Bahrman, N., Damerval, C., Plomion, C., Rossignol, M., Santoni, V., de Vienne, D., and Zivy, M. (1999). *Electrophoresis*, 20, 2013.
200. Consoli, L., Lefévre, C.L., Zivy, M., de Vienne, D., and Damerval, C. (2002). *Plant Mol. Biol.*, 48, 575.
201. Zivy, M., and de Vienne, D. (2000). *Plant Mol. Biol.*, 44, 575.
202. Ribaut, J.M., and Hoisington, D. (1998). *Trends in Plant Sci.*, 3, 236.
203. Young, ND. (1999). *Mol. Breed.*, 5, 505.
204. Salvi, S., Tuberosa, R., and Phillips, R.L. (2001). *Mol. Breed.*, 8, 169.
205. Hash, C.T., Schaffert, R.E., and Peacock, J.M. (2002). *Plant and Soil*, 245, 135.
206. Ribaut, J.M., and Betran, J. (1999). *Mol. Breed.*, 5, 531.
207. Phillips, R.L., Kim, T.S., Kaeppler, S.M., Parentoni, S.N., Shaver, D.L., Stucker, R.I., and Openshaw, S.J. (1992). Proc. of the 47th Annual Corn and Sorghum Research Conference American Seed Trade Association, Washington, D.C., 135.
208. Graham, G.I., Wolff, D.W., and Stuber, C.W. (1997). *Crop Sci.*, 37, 1601.
209. Legare, M.E., Bartlett, F.S., and Frankel, W.N. (2000). *Genome Res.*, 10, 42.
210. Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000). *Plant Cell*, 12, 2473.
211. Takahashi, Y., Shomura, A., Sasaki, T., and Yano, M. (2001). *Proc. Natl. Acad. Sci. USA*, 98, 7922.
212. Vladutu, C., McLaughlin, J., and Phillips, R.L. (1999). *Genetics*, 153, 993.
213. Salvi, S., Tuberosa, R., Chiapparino, E., Maccaferri, M., Veillet, S., van Beuningen, L., Isaac, P., Edwards, K., and Phillips, R.L. (2002). *Plant Mol. Biol.*, 48, 601.
214. Knight, H., and Knight, M.R. (2001). *Trends in Plant Sci.*, 6, 262.
215. Cushman, J.C., and Bohnert, H.J. (2000). *Curr. Opin. in Plant Biol.*, 3, 117.
216. Bohnert, H.J., Ayoubi, P., Borchert, C., Bressan, R.A., Burnap, R.L., Cushman, J.C., Cushman, M.A., Deyholos, M., Fischer, R., Galbraith, D.W., Hasegawa, P.M., Jenks, M., Kawasaki, S., Koiwa, H., Kore-eda, S., Lee, B.-H., Michalowski, C.B., Misawa, E., Nomura, M., Ozturk, N., Postier, B., Prade, R., Song, C.-P., Tanaka, Y., Wang, H., and Zhu, J.-K. (2001). *Plant Physiol. and Biochem.*, 39, 295.
217. Seki, M, Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001). *Plant Cell*, 13, 61.
218. Bray, E.A. (2002). *Plant Cell Environ.*, 25, 153.
219. Leone, A., Costa, A., Tucci, M., and Grillo, S. (1994). *Plant Physiol.*, 106, 703.
220. Grillo, S., and Leone, A. (1996). Physical Stresses in Plants: Genes and their Products for Tolerance. Springer-Verlag, Heidelberg, Germany.
221. Leone, A., Costa, A., Consiglio, F., Massarelli, I., Dragonetti, E., De Palma, M., and Grillo, S. (1999). *Potato Res.*, 42, 333.
222. Shinozaki, K. and Yamaguchi-Shinozaki, K. 1997. *Plant Physiol.*, 115, 327.
223. Zhu, J.K. (2001). *Trends Plant Sci.*, 6, 66.
224. Zhu, J.K. (2002). *Annu. Rev. Plant Biol.*, 53, 247.
225. Ramanjulu, A., and Bartels, D. (2002). *Plant Cell Environ.*, 25, 141.
226. Nuccio, M.L., Rhodes, D., McNeil, S.D., and Hanson, A.D. (1999). *Curr. Opin. Plant Biol.*, 2, 128.
227. Rai, V.K. (2002). *Biologia Plantarum*, 45, 481.
228. Hong, Z., Lakkineni, K., Zhang, Z., and Verma, D.P.S. (2000). *Plant Physiol.*, 122, 112.
229. Niu, X., Bressan, R.A., Hasegawa, P.M., and Pardo, J.M. (1995). *Plant Physiol.*, 109, 735.
230. Hasegawa, P.M., Bressan, R.A., Zhu, J.K., and Bohnert, H.J. (2000). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51, 463.
231. Maurel, C., and Chrispeels, M.J. (2001). *Plant Physiol.*, 125, 135.
232. Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S., and Shinozaki, K. (1992). *Plant Cell Physiol.,* 33, 217.
233. Fray, R.G., Wallace, A., Grierson, D., and Lycett, G.W. (1994). *Plant Mol. Biol.*, 24, 539.
234. Dreyer, I., Horeu, C., Lemaillet, G., Zimmermann, S., Bush, D., Rodriguez-Navarro, A., Schachtman, D., Spalding, E., Sentenac, H., and Gaber, R. (1999). *J. Exp. Bot.*, 50, 1073.
235. Hirsch, R.E., Lewis, B.D., Spalding, E.P., and Sussman, M.R. (1998). *Science*, 280, 918.
236. Dennison, K.L., Robertson, W.R., Lewis, B.D., Hirsch, R.E., Sussman, M.R., and Spalding, E.P. (2001). *Plant Physiol.*, 127, 1012.
237. Shi, H., Ishitani, M., Kim, C., and Zhu, J.K. (2000). *Proc. Natl. Acad. Sci. USA*, 97, 6896.

238. Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B.H., Matsumoto, T.K., Koiwa, H., Zhu, J.K., Bressan, R.A., and Hasegawa, P.M. (2001). *Proc. Natl. Acad. Sci. USA*, 98, 14150.
239. Dure, L. III, Crouch, M., Harada, J., Ho, T.-H., Quatrano, R., Thomas, T., and Sung, Z.R. (1989). *Plant Mol. Biol.*, 12, 475.
240. Cuming, A. (1999). In: Shewry, P.R., and Casey, R., eds., Seed Proteins, Kluwer Academic Publishers, Dordrecht, The Netherlands, 753.
241. Skriver, K., and Mundy, J. (1990). *Plant Cell*, 2, 503.
242. Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (1997). *Crit. Rev. Plant Sci.*, 16, 253.
243. Close, T.J., and Lammers, P.J. (1993). *Plant Physiol.*, 101, 773.
244. Garay-Arroyo, A., Colmenero-Flores, J.M., Garciarrubio, A., and Covarrubias, A.A. (2000). *J. Biol. Chem.*, 275, 5668.
245. Imai, R., Chang, L., Ohta, A., Bray, E., and Takagi, M. (1996). *Gene*, 170, 243.
246. Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998). *Science,* 280, 104.
247. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). *Natl. Biotechnol.*, 17, 287.
248. Xiong, L., and Zhu, J.K. (2002). *Plant Cell Environ.*, 25, 131.
249. Alamillo, J.-M., and Bartels, D. (2001). *Plant Sci.*, 160, 1161.
249. Godde, D. (1999). In: Lerner, H.R., ed., Plant Responses to Environmental Stresses, Marcel Dekker, New York, 449.
250. Schneider, K., Wells, B., Schmelzer, E., Salamini, F., and Bartels, D. (1993). *Planta*, 189, 120.
252. Pruvot, G., Cuine, S., Peltier, G., and Rey, P. (1996). *Planta*, 198, 471.
253. Rey, P., Pruvot, G., Becuwe, N., Eymery, F., Rumeau, D., and Peltier, G.A. (1998). *Plant J.*, 13, 97.
254. Fernando, M.R., Nanri, H., Yoshitake, S., Nagata-Kano, K., and Minakami, S. (1992). *Eur. J. Biochem.*, 209, 917.
255. Levitt, J. (1972). Response of Plants to Environmental Stresses. Academic Press, New York, USA.
256. Zhu, J.K., Damsz, B., Kononowicz, A.A., Bressan, R.A., and Hasegawa, P.M. (1994). *Plant Cell*, 6, 393.
257. Costa, A., Massarelli, I., Dragonetti, E., Leone, A., and Grillo, S. (1999). Abstracts of the 14th Triennial Conference of the European Association for Potato Research, May 2-7, Sorrento, Italy, 44.
258. Guerrero, F.D., Jones, J.T., and Mullet, J.E. (1990). *Plant Mol. Biol.*, 15, 11.
259. Nishio, N., and Satoh, H. (1997). *Plant Physiol.*, 115, 841.
260. Almoguera, C., and Jordano, J. (1992). *Plant Mol. Biol.*, 19, 781.
260. Vierling, E., 1991. Annu. Rev. *Plant Physiol. Plant Mol. Biol.*, 42, 579.
261. Jacob, U., Gaestel, M., Engel, K., and Buchner, J. (1993). *J. Biol. Chem.*, 268, 1517.
263. Zhu, J.K., Shi, S., Bressan, R.A, and Hasegawa, P.M. (1993). *Plant Cell*, 5, 341.
264. Costa, A., Di Giacomo, M., Leone, A., and Grillo, S. (2003). *Plant Mol. Biol.*, submitted.
265. Smirnoff, N. (1993). *New Phytol.*, 125, 27.
266. Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., and Kobayashi, H. (1999). *Plant Cell*, 11, 1195.
267. Guan, L.M., Zhao, J., and Scandalios, J.G. (2000). *Plant J.*, 22, 87.
268. Albinsky, D., Masson, J.E., Bogucki, A., Afsar, K., Vass, I., Nagy, F. and Paszkowski, J. (1999). *Plant J.*, 17, 73.
268. Bowler, C., van Montagu, M., and Inze, D. (1992). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 43, 83.
269. Ruis, H., and Schüller, C. (1995). *Bioassays*, 17, 959.
271. Macario, A.J.L., and Conway de Macario, E. (2000). In: Fink, G., ed., The encyclopedia of stress, Academic Press, San Diego, CA, pp. 350.
272. Bohnert, H.J., Nelson, E.D., and Jensen, R.G. (1995). *Plant Cell*, 7, 1099.
272. Delauney, A.J., and Verma, D.P.S. (1993). *Plant J.*, 4, 215.
273. Hare, P.D., Cress, W.A., and van Staden, J. (1999). *J. Exp. Bot.*, 50, 413.
275. Wurgler-Murphy, S.M., and Saito, S. (1997). *Trends in Biochem. Sci.*, 22, 172.
276. Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida N., and Shinozaki, K. (1994). *Mol. Gen. Genet.*, 224, 331.
277. Maeda, T., Wurgler-Murphy, S.M., and Saito, H. (1994). *Nature*, 369, 242.
278. Hong, S.W., Jon, J.H., Kwak, J.M., and Nam, H.J. (1997). *Plant Physiol.*, 113, 1203.
279. Conley, T.R., Sharp, R.E., and Walker, J.C. (1997). *Plant Physiol.*, 113, 219.

280. Mizoguchi, T., Irie, K., Hiratyama, T., Hayashida, N., Yagamuchi-Shinozaki, K., Matsumato, K. and Shinozaki, K. (1996). *Proc. Natl. Acad. Sci. USA*, 93, 765.
281. Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F., and Dobowolska, G. (2000). *Plant Cell*, 12, 165.
282. Mizoguchi, T,, Ichimura, K., Yoshida, R., and Shinozaki, K. (2000). *Results Probl. Cell Differ.*, 27, 29.
283. Knight, H., Trewavas, A.J., and Knight, M. (1997). *Plant J.*, 12, 1067.
284. Sanders, D., Brownlee, C., and Harper, J.F. (1999). *Plant Cell*, 11, 691.
285. Sheen, J. (1996). *Science*, 274, 1900.
286. Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., and Shinozaki, K. (1999). *Plant Cell*, 11, 1743.
287. Kudla, J., Xu, Q., Harter, K., Gruissem, W., and Luan, S. (1999). *Proc. Natl Acad. Sci. USA*, 96, 4718.
288. Tong, G., Shepherd, D., and Jahr, C.E. (1995). *Science*, 267, 1510.
289. Nakamura, T., Liu, Y., Hirata, D., Namba, H., Harada, S., Hirokawa, T., and Miyakawa, T. (1993). *EMBO J.*, 12, 4063.
290. Pardo, J.M., Reddy, M.P., Yang, S., Maggio, A., Huh, G.H., Matsumoto, T., Coca, M.A., Paino-D'Urzo, M., Koiwa, H., Yun, D.J., Watad, A.A., Bressan, R.A., and Hasegawa, M. (1998). *Proc. Natl. Acad. Sci. USA*, 95, 9681.
291. Liu, J., and Zhu, J.-K. (1998). *Science*, 280, 1943.
292. Xiong, L., and Zhu, J.K. (2001). *Physiol Plant.*, 112, 152.
293. Rock, C.D. (2000). *New Phytol.*, 148, 357.
294. Shinozaki, K., and Yamaguci-Shinozaki, K. (1996). *Curr. Opin. in Biotech.*, 7, 161.
295. Leyman, B., Geelen, D., Quintero, F.J., and Blatt, M.R. (1999). *Science*, 28, 537.
296. Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosikawa, D., and Shinozaki, K. (1997). *Plant Cell*, 9, 1859.
296. Sheen, J. (1998). *Proc. Natl. Acad. Sci. USA*, 95, 975.
297. Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A.R., Vartanian, N., and Giraudat, J. (1999). *Plant Cell*, 11, 1897.
299. Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). *Plant Cell*, 6, 251.
300. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Setsuko, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). *Plant Cell*, 10, 1391.
301. Holmberg, N., and Bülow, L. (1998). *Trends in Plant Sci.*, 3, 61.
302. Bajaj, S., Targolli, J., Liu, L.F., Ho, T.H.D., and Wu, R. (1999). *Mol. Breed.*, 5, 493.
303. Kishor, K.P.B., Hong, Z., Miao, G.H., Hu, C.A.A., and Verma, D.P.S. (1995). *Plant Physiol.*, 108, 1387.
304. Zhu, B.C., Su, J., Chan, M.C., Verma, D.P.S., Fan, Y.-L., and Wu, R. (1998). *Plant Sci.*, 139, 41.
305. Tarczynski, M.C., Jensenn, R., and Bohnert, H.J. (1993). *Science*, 259, 508.
306. Thomas, J.C., Sepahi, M., Arendall, B., and Bohnert, H.J. (1995). *Plant Cell Environ.*, 18, 801.
307. Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jenken, M.J.W., Weisbeek, P.J., and Smeekens, S.C.M. (1995). *Plant Physiol.*, 107, 125.
308. Pilon-Smits, E.A.H., Terry, N., Sears Tobin, K.H., and Van Dun, K. (1999). *Plant Physiol. Biochem.*, 37, 313.
309. Holmstrom, K.O., Mantyla, E., Welin, B., Mandal, A., and Palva, E.T. (1996). *Nature,* 379, 683.
310. Pilon-Smits, E.A.H., Terry, N., Sears, T, Kim, H., Zayed, A., Hwang, S.B., van Dun, K., Voogd, E., Verwoerd, T.C., Krutawagen, R.W.H.H., and Goddijn, O.J.M. (1998). J. *Plant Physiol.*, 152, 525.
311. Shen, B., Jensen, R.G., and Bohnert, H.J. (1997). *Plant Physiol.*, 113, 1177.
312. Van Camp, W., Capian, K., Van Montagu, M., Inzé, D., and Slooten, L. (1996). *Plant Physiol.* 112, 1703.
313. Roxas, V.P., Smith, R.K, Allen, E.R., and Allen, R.D. (1997). *Nature Biotech.*, 15, 988.
314. McKersie, B.D., Bowley, S.R., and Jones, K.S. (1996). *Plant Physiol.*, 119, 839.
315. Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Audits, D., and Horvath, G.V. (2000). *Plant J.*, 24, 437.
316. Ituriaga, G., Schneider, K., Salamini, F., and Bartels, D. (1992). *Plant Mol. Biol.*, 20, 555.
317. Xu, D., Duan, X., Wang, B., Hong T.H. Ho, and Wu, R. (1996). *Plant Physiol.*, 110, 249.
318. Sivamani, E., Bahieldin, A. Wraith, J.M., Al-Niemi, T., Dyer, W.E., Ho, T.-H.D., and Qu, R.D. (2000). *Plant Sci.*, 155, 1.

319. Saijo Y., Hata S., Kyozuka, J., Shimamoto, K., and Izui, K. (2000). *Plant J.*, 23, 319.
320. Smith, R.D., and Walker, J.C. (1996). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47, 101.
321. Mizoguchi, T., Ichimura, K., and Shinozaki, K. (1997). *Trends Biotech.*, 15, 15.
322. Luan, S. (1998). *Trends Plant Sci.*, 3, 271.
323. Lee, J.H., Van Montagu, M., and Verbruggen, N. (1999). *Proc. Natl. Acad. Sci. USA*, 96, 5873.
324. Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., De Graaf, P.T., Van Dun, K., Poels, J., Ponstein, A.S., Damm, B. and Pen, J. (1997). *Plant Physiol.*, 113, 181.
325. Karakas, B., Ozias-Akins, P., Stushnoff, C., Suefferheld, M., and Rieger, M. (1997). *Plant Cell Environ.*, 20, 609.
326. Romero, C., Belles, J.M., Vaya, J.L., Serrano, R., and Culianez-Macià, F.A. (1997). *Planta*, 201, 293.
327. Blum, A., Munns, R., Passioura, J.B., and Turner, N.C. (1996). *Plant Physiol.*, 110, 1051.
328. Sharp, R.E., Boyer, J.S., Nguyen, H.T., and Hsiao, T.C. (1996). *Plant Physiol.*, 110, 1051.
329. Beachy, R.N. (1997). *Curr. Opin. in Biotech.*, 8, 215.
330. Van der Bienzen, E.A., and Jones, J.D.G. (1998). *Trends Biochem. Sci.*, 23, 454.
331. Francois, I.E.J.A., Broekaert, W.F., and Cammue, B.P.A. (2002). *Plant Sci.*, 163, 281.
332. Bohnert, H.J., and Shen, B. (1999). *Scientia Horticolturae*, 78, 237.
333. Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., and Potrykus, I. (2000). *Science*, 287, 303.
334. Zhang, C.S., Lu.Q. and Verma, D.P. (1995). *J. Biol. Chem.*, 270, 20491.
335. Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). *FEBS Letters,* 461, 205.
336. Muller, B., Reymond, M., and Tardieu, F. (2001). *J. Exp. Bot.*, 52, 1259.
337. Reymond, M., Muller, B., Leonardi, A., Charcosset, A., and Tardieu, F. (2003). *Plant Physiol.*, 131, 664.
338. Khavkin, E., and Coe, E. (1997). *Theor. Appl. Genet.*, 95, 343.
339. Duvick, D.N. (1997). In: Edmeades, G.O., Bänziger, M., Mickelson, H.R., and Peña-Valdivia, C.B., eds., Developing Drought- and Low N-tolerant Maize. Proc. of a Symposium, March 25-29, 1996, CIMMYT, El Batán, Mexico, 332.

CHAPTER 5

ANOXIA: THE ROLE OF CARBOHYDRATES IN CEREAL GERMINATION

E. LORETI[a], A. ALPI[b] AND P. PERATA[c]

[a] *Institute of Biology and Agricultural Biotechnology, CNR, via del Borghetto 80, 56124 Pisa, Italy*
[b] *Department of Crop Plant Biology, University of Pisa, Via Mariscoglio 34, Pisa, Italy*
[c] *Department of Agricultural Sciences, University of Modena and Reggio Emilia, Via Kennedy 17, 42100 Reggio Emilia, Italy*

Abstract. Cereal grains are unable to germinate under anaerobic conditions. An exception to this rule is rice, which is able to germinate even under complete anoxia. The ability of rice to germinate in the absence of oxygen is likely the results of several elements, but in the recent years the importance of carbohydrates have been highlighted. In this chapter we outline recent achievements in the study of carbohydrates physiology in cereal grains kept under anoxia.

1. INTRODUCTION

Higher plants are aerobic organisms requiring oxygen for their life. Plants may experience a low oxygen availability (hypoxia) or total absence of oxygen (anoxia) due to flooding of the soil or anatomical structure of some tissues whose histological properties severely limits the permeability to oxygen (tubers, vascular cambium of trees, meristematic tissues etc.) [1]. A few plant species show tolerance to relatively prolonged anaerobic conditions [see 1, 2, 3 for recent reviews]. Among cereals, only rice can germinate under anoxia, showing coleoptile elongation [4]. A similar behavior is observed in some species of the rice field weed *Echinochloa* [5]. The physiology of *Echinochloa* has been reviewed elsewhere, and we will therefore not include reference to this interesting plant species [5]. The great majority of higher plants seeds fails to germinate under anaerobic conditions. Only rice, *Echinocloa* species, *Erithina caffra* and the water plants *Trapa natans* L., *Nuphar luteum* L. and *Scirpus mucronatus* L. can germinate under conditions of total oxygen deprivation such as under nitrogen atmosphere.

The biochemical basis of the ability to germinate under anoxia are not known but the ability to maintain an active fermentative metabolism by fuelling the glycolytic pathway with readily fermentable carbohydrates is likely of importance. All cereals with the peculiar exception of rice show limited anoxia tolerance.

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 123-131.

However maize, barley, and wheat are those most frequently used as experimental material. Most of the data reviewed here refer indeed to these species.

Tolerance to anaerobiosis is difficult to be defined. Firstly, the degree of absence of oxygen is not often described in several research papers. Indeed, plants that may display an intolerant behaviour to anoxia may tolerate to some degree hypoxic conditions. Furthermore, anaerobic conditions can be obtained by flushing glass jars with nitrogen gas, by flooding plants under water or buffer solutions, by partly flooding the plant (anaerobic root system) etc. This variety of approaches to obtain anaerobiosis makes not easy the comparison of data obtained from different laboratories. While the physiology of anaerobiosis tolerance in water plants is often studied by flooding the plant tissue, seed germination of cereals is conveniently studied by placing cereal grains under complete anoxia. Under these conditions most of the aerobic metabolism is switched off, and tolerance to anoxia can be evaluated. Since cereal grains kept under anoxia (e.g. in nitrogen gas) do not have access to air, any adaptive response is to be attributed to tolerance traits unrelated to the plant anatomy, and thus due to biochemical adaptation.

2. RESPIRATION UNDER ANOXIA

There is no doubt concerning the metabolic pathway that is more affected by anoxia: respiration. In the absence of oxygen aerobic respiration cannot proceed, and ATP production will drop from the 32 ATP moles produced for each mole of glucose [6] to only 2 ATP moles under anaerobiosis. This value is valid assuming the conversion of glucose into ethanol through the glycolytic and fermentative pathway, but it should be remembered that only in few cases glucose is an available substrate for the anaerobic metabolism. In most cases, starch and sucrose are the real substrates for plant metabolism, either under aerobic and anaerobic conditions.

2.1. Carbohydrates and the fermentative pathway

Glucose is metabolized under anoxia through glycolysis and fermentation leading to ethanol as the main end product [7]. Glucose is however not a stored carbohydrate, but results from starch degradation. Starch is indeed stored in large amounts in the cereal's endosperm. The site of starch degradation does not necessarily coincide with the site of carbohydrate utilization through fermentation in the cereal's embryo. Indeed, only a very limited amount of glucose resulting from starch breakdown will be fermented in cells nearby the site of starch storage, while most of glucose units arising from starch degradation must be translocated (likely as sucrose) to the other plant organs where starch is not stored but where energy production through fermentation is needed for survival. Sucrose synthesis, transport, and degradation is therefore needed to provide hexose units for fermentation.

2.2. The importance of α-amylases in starch degradation under anoxia

A set of enzymes is needed to carry on starch breakdown: α-amylase, β-amylase, debranching enzyme, and α-glucosidase [8, 9]. Most of the information we have concerning the importance of a metabolic pathway in cereal grains under anoxia arises from a comparison of the enzymatic set present in the anoxia-tolerant rice with that of anoxia-intolerant cereals (wheat, barley) kept under either aerobic or anaerobic conditions. β-Amylase is synthesized *de novo* in the anoxic rice grains [10], while in anoxia-intolerant cereals (wheat and barley) the enzyme is present in the dry seed, stored as a starch-bound form [11, 12, 13]. Under anoxia β-amylase remains in the bound form, and cannot play any role in starch degradation [10, 14]. Even assuming that β-amylase could be released under anoxia, this would have a minor impact on starch degradation, since β-amylase cannot degrade native starch granules [9]. Furthermore, cereal mutants devoid of β-amylase show a normal germination under aerobic conditions [15, 16], indicating that this enzyme plays a minor role even in the aerobic starch degradation. Debranching enzyme and α-glucosidase are both present in the rice dry seed as latent, inactive forms, which are activated during germination under either aerobic or anaerobic conditions [10]. On the other hand, in the anoxia-intolerant cereals these enzymes are not detectable during grain imbibition under anaerobic conditions [10]. Although both α-glucosidase and α-amylase are able to degrade native starch granules, the latter enzyme is considered to play a major role in this process, and is therefore the key enzyme for starch degradation [8, 9]. A detailed review on the effects of anoxia on the induction of α-amylase has been published [17].

The major conclusions reached at that time were as follows: (i) α-amylase is produced in rice seeds under anoxia [18], while it is not induced in the anoxia-intolerant cereals (wheat, barley) [10]; (ii) the successful induction of α-amylase in rice is very likely responsible for the subsequent successful degradation of starch taking place in the endosperm, as the other starch degrading enzymes are unlikely to be able to initiate the process of starch degradation; (iii) in the absence of α-amylase induction (anoxia-intolerant cereals: wheat, barley) starch is not degraded, and the grains suffer soon from sugar starvation [19]; (iv) anoxic rice embryoless half-grains respond to exogenous gibberellic acid (GA_3), while wheat and barley are insensitive to the hormone under anoxia [20]; (v) the induction of α-amylase in rice seeds under anoxia is GA-dependent [20], and this implies that gibberellins are either produced or already present in the grains.

2.3. Recent developments about the role of α-amylase under anoxia

As stated above, the successful induction of α-amylase in rice is possibly responsible for the successful degradation of starch taking place in the endosperm, since other starch degrading enzymes are unlikely to be able to initiate the process of starch degradation. Indeed, in the absence of α-amylase, starch is not degraded, and anoxia-intolerant cereals such as wheat and barley suffer soon from sugar starvation, and eventually die [19]. The importance of α-amylase in anoxia tolerance is likely

not restricted to cereals. Indeed, in a recent report Arpagaus and Braendle [21] demonstrated that α-amylase plays an important role for carbohydrate metabolism also in the anoxia stress tolerant rhizomes of *Acorus calamus* L.. Comparison of α-amylase activities in *Acorus calamus* rhizomes with those detected in the non-tolerant tubers of *Solanum tuberosum* L., revealed the ability of the tolerant plant to maintain a high level of α-amylase activity also under anoxia together with a higher-than-aerobic amount of soluble carbohydrates. Potato tubers suffer instead from sugar starvation, a likely consequence of the low α-amylase activity found in the tubers of these species [21].

The mechanisms allowing the successful production of α-amylase under anoxia in rice seeds is largely unknown. Anoxic rice embryoless half-grains respond to exogenous gibberellic acid (GA_3), but with great delay when compared to the rapid induction triggered by gibberellins under aerobic conditions (Figure 1A). Intriguingly, the appearance of the α-amylase protein is instead unaffected by anoxia (Figure 1B) as demonstrated by immunoblot analysis [22].

In rice, anoxia delays the expression of gibberellin-modulated α-amylase, while wheat and barley are insensitive to the hormone under anoxia [20]. It is worth remembering that rice α-amylases are encoded by at least 10 genes [23], not all necessarily regulated by gibberellins [24]. In germinating rice grains *Amy1A*, *Amy 3B/C*, *Amy3D*, and *Amy3E* are expressed, while *Amy1B*, *Amy1C*, *Amy2A*, and *Amy3A* mRNA level is below the detection limit of northern blot [25]. *Amy1A* shows the higher expression level in aerobic rice grains, while under anoxia other α-amylase genes are expressed at comparable levels, namely *Amy 3B/C*, *Amy3D*, and *Amy3E* [25]. *Amy1A* induction by gibberellins and repression by abscisic acid is well described [26, 27]. Little is known about the mechanisms regulating other α-amylase genes in rice. *Amy3D* and *Amy3E* are sugar-repressed in rice embryos [28], and sugar repression of *Amy3D* transcription in anoxic rice aleurones has also been observed [29].

This complex pattern of expression of α-amylase genes suggests that the model proposed is over-simplified (gibberellins can induce α-amylase in rice, but not in anoxia-intolerant cereals; 30).

Several evidences have been presented supporting this model [see 30], but some additional considerations are certainly needed. Indeed, rice can respond to exogenous GA_3 under anoxia while anoxia-intolerant cereals fail to behave similarly, but it should also be remarked that under anoxia gibberellins cannot be synthesized *de novo*. Membrane-bound monooxygenases needed to oxidize ent-kaurene to GA_{12} require NADPH and oxygen [31].

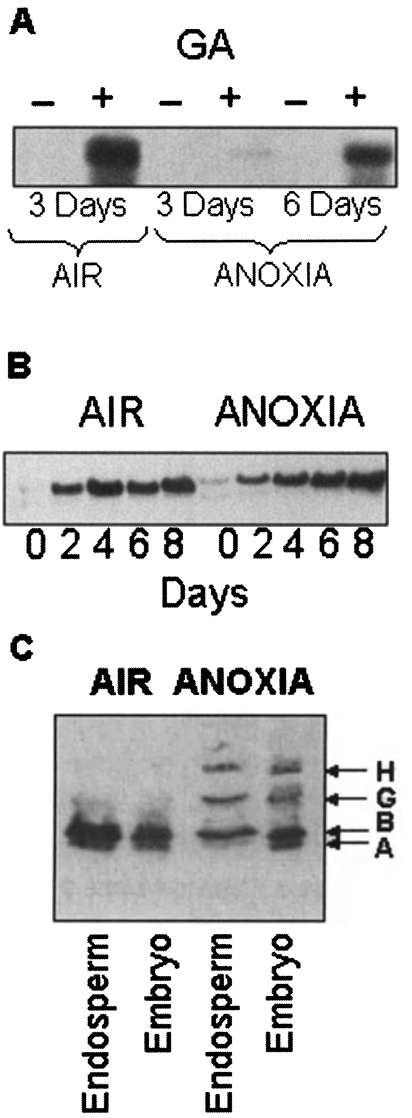

Figure 1. Effects of anoxia on α-amylase mRNA induction by gibberellins, α-amylase protein accumulation and isoforms pattern. A. Effects of air and anoxia on the induction of α-amylase mRNA in embryoless half-grains. Half-grains incubated in the presence of GA for three and six days, followed by northern blot with a rice α-amylase probe. See Perata et al. [20] for details about the experiment. B. Effects of anoxia on α-amylase protein accumulation. Rice grains germinating under aerobic or anaerobic conditions were extracted for proteins, subjected to SDS-PAGE and immunoblot, probed with an antibody against α-amylase. See Guglielminetti et al. [22] for details about the experiment. C. Pattern of aerobic and anaerobic α-amylase isoforms. Rice grains germinating under aerobic or anaerobic

conditions for 8 days were extracted for proteins, subjected to IEF and immunoblot, probed with an antibody against α-amylase. The different isoforms are identified by the letters A, B, G and H. See Guglielminetti et al. [17] for details about the experiment.

The existence of stored gibberellins (or precursors) in the dry grain of rice could be hypothesized, but this would imply that release from the stored form is anoxia-dependent, since aerobic gibberellin-dependent α-amylase gene expression requires exogenous GA_3 if gibberellins biosynthesis is blocked by using specific inhibitors [32]. Experimental evidences obtained in our laboratories are suggestive of anoxic germination of rice as a gibberellin-independent process (unpublished observations). This proposal is supported by the successful germination of a gibberellin-deficient rice mutant (Tan-ginbozu) under anoxia, despite an extremely low level of *Amy1A* transcript detected in this mutant; furthermore, the vigorous expression of *Amy3D* under anoxia (and to a minor extent *Amy3B/C*) compensates for the absence of the gibberellin-modulated *Amy1A*-encoded enzyme.

2.4. Sucrose synthesis and degradation under anoxia

Sucrose is synthesized under anoxia in rice, while this disaccharide is not synthesized in barley grains kept under anoxia [33]. These results are easily explained by the effects of anoxia in barley, which depresses the activity of sucrose phosphate synthase, the key enzyme for sucrose synthesis. In anoxic rice grains glucose resulting from starch degradation accumulates and sucrose can be synthesized [22]. The level of sucrose phosphate synthase is not affected by anoxia in rice [33]. Sucrose synthesis takes place in the scutellum of rice embryos, after glucose resulting from starch degradation is taken up by the epithelium cells. Sucrose synthesis is an energy-consuming process suggesting the importance of sucrose translocation to the growing rice coleoptile (where it will be degraded to hexoses and utilized through the fermentative pathway). Sucrose can be degraded in the citosol through the activity of two distinct pathways, one involving invertase and another with sucrose synthase as key enzyme [34]. One of the most heavily labeled proteins detected after two-dimensional electrophoresis of *in vivo* ^{35}S-methionine labeled protein extracts from anoxic maize seedlings is a 87 kD polypeptide, the product of the *Shrunken* gene (*Sh1*), encoding the SS1 subunit of the tetrameric enzyme sucrose synthase [35]. This indicates that sucrose synthase is synthesized under anoxia in maize [35], but a report by McElfresh & Chourey [36] shows that sucrose synthase is induced only at the transcriptional level in maize, without significant increase in the sucrose synthase protein amount. Therefore, mRNA encoding sucrose synthase is translated under anaerobic conditions [35], but not with a rate resulting in an increased sucrose synthase activity under anoxia [36]. In rice seedlings incubated under anoxia sucrose synthase is induced at both the transcriptional and the translational level [37, 38, 39], suggesting that differences between species showing a different tolerance to anaerobiosis can be observed in the efficiency of translation of mRNA coding for anaerobic polypeptides.

The enzymatic set needed for the operation of the sucrose synthase pathway is fully present in anoxic rice seedlings. Anoxia does not reduce the activity of sucrose synthase, UDP-glucose-pyrophosphorylase, phosphoglucomutase, and fructokinase activities but, on the contrary, enhanced the activity of sucrose synthase, and fructokinase [22]. These enzymatic activities are instead negatively affected in anoxia-intolerant cereal grains [19].

2.5. Sugar availability affects anoxia tolerance in cereal grains

Wheat grains incubated under anoxia show a decreased soluble sugar content. Indeed, the level of glucose, fructose, and sucrose falls to an almost undetectable level within a few days of incubation under anoxia [19]. Sugar depletion correlates with loss of viability of the grains (e.g. barley and wheat grains fail to resume germination if transferred from anoxia to aerobic conditions). Survival is correlated to an adequate supply of fermentable carbohydrates, since energy production under anoxia is restricted to glycolysis/fermentation. Several experimental evidences support this view. Feeding glucose to wheat grains enhances significantly their ability to withstand prolonged anaerobiosis, and even promotes root elongation [18]. Vartapetian *et al.* [40] proposed that rice coleoptiles are tolerant to anoxia as a consequence of their ability to transport organic compounds from the seed to the anaerobic coleoptile. Some authors reported data indicating that carbohydrates supplied exogenously enhance anoxia tolerance of plant tissues, [18, 41, 42, 43] while others proposed an opposite view [44, 45].

Additional evidences suggesting an important role for sugar supply in conferring anoxia tolerance have been recently reviewed [46, and references therein]. These evidences and others can be summarized as follows: (i) rice coleoptiles survival depends upon exogenous sugar supply; (ii) coleoptiles separated from sugar supply (endosperm) suffer of mitochondrial damage, while in cereal roots fed with exogenous sugars show enhanced tolerance to anoxia [42, 47]; (iii) maize root tips fed with glucose show increased adenylate energy charge [48, 49]; (iv) wheat grains fed with glucose or sucrose show increased anoxia tolerance [18]; (v) Ricard *et al.* [39] confirmed the critical role of sucrose synthase in anoxia tolerance in maize seedlings: a double mutant of maize lacking both the sucrose synthase genes shows decreased tolerance to anoxia, suggesting that sucrose synthase is needed for tolerance to anaerobiosis, and that sucrose is a substrate of primary importance for the anaerobic metabolism.(vi) Germain *et al.* [50] demonstrated that sucrose but not glucose or fructose allows anoxic tolerance in tomato roots, a consequence of a marked inhibition of hexokinases in the anoxic tomato roots resulting in the inability of this tissue to utilize hexoses. Sucrose was utilized instead, thanks to a sucrose synthase pathway allowing to by-pass the hexokinase-dependent hexose-phosphorylating step [50].

2.6. Conclusion

Rice grains represent a widely used model to study anoxia tolerance at the level of germination. It should be emphasized that the germination of rice grains under anoxia is far from normal, as described by Alpi and Beevers [37]. Only the anaerobic coleoptile elongates, longer than the aerobic one, but no root and leaves are produced under anoxia; these organs are indeed promptly produced upon transfer of the anaerobic seedlings to aerobic conditions. The ability to withstand prolonged anoxia (up to several days or even weeks) is likely the result of a modified metabolic rate, allowing to save as much energy as possible, together with an efficient pathway of starch degradation coupled sucrose synthesis. Sucrose is transported to the growing coleoptile where degradation occurs through a sucrose synthase pathway granting the recovery of most of the energy stored in this disaccharide. Energy production through fermentation can proceed allowing the maintenance of the subcellular integrity and a prompt recovery when aerobic conditions are restored.

REFERENCES

1. Perata, P., and Alpi, A. (1993). *Plant Sci.*, 93, 1.
2. Armstrong, W., Brändle, R., and Jackson, M.B. (1994). *Acta Bot. Neerl.*, 43, 307.
3. Crawford, R.M.M., and Braendle, R. (1996). *J. Exp. Bot.*, 47, 145
4. Alpi, A., and Beevers, H. (1983). *Plant Physiol.*, 71, 30.
5. Kennedy, R.A., Rumpho, M.E., and Fox, T.C. (1992). *Plant Physiol.*, 100, 1.
6. Taiz, L., and Zeiger, E. (1991). Plant Physiology. The Benjamin/Cummings Publishing Company, Redwood City.
7. Mayne, R.G., and Kende, H. (1986). *Plant Sci.*, 45, 31.
8. Dunn, G. (1974). *Phytochemistry*, 13, 1341.
9. Sun, Z., and Henson, C.A. (1991). *Arch. Biochem. Biophys.*, 284, 298.
10. Guglielminetti, L., Yamaguchi, J., Perata, P., and Alpi, A. (1995). *Plant Physiol.*, 109, 1069.
11. Sopanen, T., and Lauriere, C. (1989). *Plant Physiol.*, 89, 244.
12. Hara-Nishimura, I., Nishimura, M., and Daussant, J. (1986). *Protoplasma*, 134, 149.
13. Lauriere, C., Lauriere, M., and Daussant, J. (1986). *Physiol. Plant.*, 67, 383.
14. Loreti, E., Guglielminetti, L., Yamaguchi, J., Gonzali, S., Alpi, A., and Perata, P. (1998). *J. Plant Physiol.*, 152, 44.
15. Daussant, J., Zbaszyniak, B., Sadowski, J., and Wiatroszak, I. (1981). *Planta*, 151. 176.
16. Kreis, M., Williamson, M.S., Buxton, B., Pywell, B., Hejgaard, J., & Svendsen, I. (1987). *Eur. J. Biochem.*, 169, 517.
17. Perata, P., Guglielminetti, L., and Alpi, A. (1997). *Ann. Bot.* (suppl.), 79, 49.
18. Perata, P., Pozueta-Romero, J., Akazawa, T., and Yamaguchi, J. (1992). *Planta*, 188, 611.
19. Perata, P., Guglielminetti, L., and Alpi, A. (1996). *J. Exp. Bot.*, 47, 999.
20. Perata, P., Geshi, N., Yamaguchi, J., and Akazawa, T. (1993). *Planta*, 191, 402.
21. Arpagaus, S., and Braendle, R. (2000). *J. Exp. Bot.* 51, 1475.
22. Guglielminetti, L., Perata, P., and Alpi, A. (1995). *Plant Physiol.*, 108, 735.
23. Huang, N., Stebbins, G. L., and Rodriguez, R. L. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 7526.
24. Mitsui, T., and Itoh, K. (1997). *Trends Plant Sci.*, 2, 255.
25. Hwang, Y. S., Thomas, B. R., and Rodriguez, R. L. (1999). *Plant Mol. Biol.*, 40, 911.
26. Itoh, K. Yamaguchi, J., Huang, N., Rodriguez, R. L., Akazawa, T., and Shimamoto, K. (1995). *Plant Physiol.*, 107, 25.
27. Loreti, E., Vernieri, P., Alpi, A., and Perata, P. (2002). *Plant Biology* (in press).
28. Yu, S. M., Lee, Y. C., Fang, S. C., Hwa, S. F., and Liu, L. F. (1996). *Plant Mol. Biol.*, 30, 1277.
29. Loreti, E., Yamaguchi, J., Alpi, A., and Perata, P. (2002). *Ann. Bot* (in press).
30. Perata, P., Loreti E., Guglielminetti, L., and Alpi A. (1998). *Acta Bot. Neerl.*, 47, 269.
31. Hedden, P., and Kamiya, Y. (1997). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 48, 431.
32. Mitsunaga, S., and Yamaguchi, J. (1993). *Plant Cell Physiol.*, 34, 243.
33. Guglielminetti, L., Loreti, E., Perata, P., and Alpi, A. (1999). *J. Plant Res.*, 112, 353.

34. Rees, T. (1992). In: C.J. Pollock, J.F. Farrar, A.J. Gordon (eds.). Carbon partitioning within and between organisms. Bios Scientific Publishers, Oxford, pp. 115-131.
35. Springer, B., Werr, W., Starlinger, P., Bennet, D.C., Zokolica, M., and Freeling, M. (1986). *Mol. Gen. Genet.*, 205, 461.
36. McElfresh, K.C., and Chourey, P.S. (1988). *Plant Physiol.*, 87, 542.
37. Ricard, B., Rivoal, J., Spiteri, A., and Pradet, A. (1991). *Plant Physiol.*, 95, 669.
38. Guglielminetti L., Wu, Y., Boschi, E., Yamaguchi, J., Favati, A., Vergara, M., Perata, P., and Alpi, A. (1997). *J. Plant Physiol.*, 150, 251.
39. Ricard, B., VanToai, T., Chourey, P., and Saglio, P. (1998). *Plant Physiol.*, 116, 1323.
40. Vartapetian, B.B., Andreeva, I.N., and Kozlova, G.I. (1976). *Protoplasma*, 88, 215.
41. Andreev, V., Generozova, I.P., and Vartapetian, B.B. (1991). *Plant Physiol. Biochem.*, 29, 171.
42. Webb, T., and Armstrong, W. (1983). *J. Exp. Bot.*, 34, 579.
43. Waters, I., Morrel, S., Greenway, H., and Colmer, T.D. (1991). *J. Exp. Bot.*, 42, 1437.
44. Malki, E., Waisel, Y., and Pesis, E. (1989). *Physiol. Plant.*, **77**, 519.
45. Roberts, J.K.M., Andrade, F.H., and Anderson, I.C. (1985). *Plant Physiol.*, 77, 492.
46. Vartapetian, B.B., and Jackson, M.B. (1997). *Ann. Bot.*, 79, 3.
47. Vartapetian, B.B., Andreeva, I.N., Kozlova, G.I., and Agapova, L.P. (1977). *Protoplasma*, 91, 243.
48. Saglio, P., and Pradet, A. (1980). *Plant Physiol.*, 66, 516.
49. Saglio, P., Raymond, P., and Pradet, A, (1980). *Plant Physiol.*, 66, 1053.
50. Germain, V., Ricard, B., Raymond, P., and Saglio, P.H. (1997). *Plant Physiol.*, 114, 167.

CHAPTER 6

RESPONSE TO HEAVY METALS IN PLANTS: A MOLECULAR APPROACH

L. SANITÀ DI TOPPI[a], P. GREMIGNI[b], B. PAWLIK-SKOWROŃSKA[c], M.N.V. PRASAD[d] AND C.S. COBBETT[e]

[a] *Department of Evolutionary and Functional Biology, Section of Plant Biology, University of Parma, viale delle Scienze 11/A, 43100, Parma, Italy.*
[b] *CSIRO Plant Industry, Centre for Mediterranean Agricultural Research, Wembley, Australia*
[c] *Centre for Ecological Research, Polish Academy of Sciences, Experimental Station at Lublin, Poland*
[d] *Department of Plant Sciences, School of Life Sciences, University of Hyderabad, India*
[e] *Department of Genetics, University of Melbourne, Australia*

Abstract. Heavy metal pollution causes a number of toxic symptoms both in higher plants and algae, e.g. growth retardation, inhibition of photosynthesis, induction and inhibition of enzymes, generation of oxidative stress. All plants cope with heavy metal stress by exploiting broad range of different response mechanisms acting in additive and/or synergistic way. Most common and important mechanisms comprise synthesis of metal-complexing peptides (glutathione, phytochelatins and related peptides), increased antioxidative enzymatic activity, synthesis of stress proteins (HSP), intracellular metal binding to nonprotein metal chelators like organic acids and phytate, release of extracellular, metal -binding exudates (composed of organic acids, amino acids, peptides, sugars and polysaccharides). Phytochelatins are regarded as potential biomarkers of heavy metal stress in plants.

L. Sanità di Toppi and B. Pawlik-Skowrońska (eds.), Abiotic Stresses in Plants, 133-156.

1. INTRODUCTION

Heavy metals (HMs) - a group of metals with a density higher than 5 g cm^{-3} - are generally toxic to plants, although a subset of them, at appropriate low concentrations, are essential micronutrients. All plants cope with HM stress (and homeostasis) by exploiting a broad range of different response mechanisms, which may act in an additive and/or synergistic way. The main defence mechanisms involved in HM detoxification were previously discussed in the so-called "fan-shaped" model (Figure 1) [1].

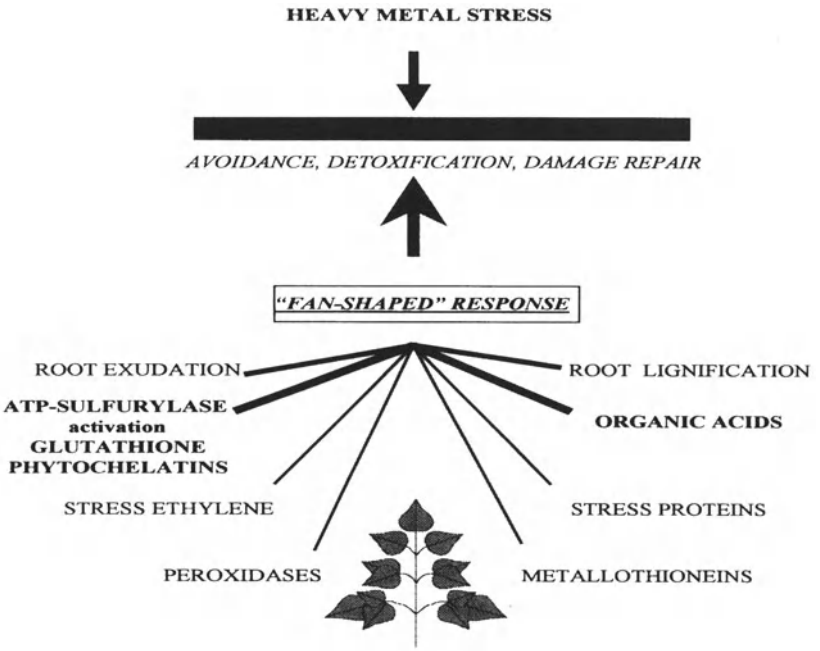

Figure 1. "Fan-shaped" response to heavy metal stress in higher plants. In this multicomponent model, plants are proposed to cope with heavy metal stress by modulating the "expression" of each ray of the fan (after Sanità di Toppi and Gabbrielli, 1999 and; Sanità di Toppi et al., 2002; with permission from Elsevier Science and Kluwer Academic Publishers)

In this chapter, which should be considered complementary and updating of another review-chapter previously published on this topic [2], we have paid particular attention to: 1) phytochelatins; 2) non-protein metal chelators, in particular organic acids and phytate; 3) HM response in algae.

2. PHYTOCHELATINS

2.1. Generalities

Some HMs and metalloids, and a few multi-atomic anions (see below) rapidly induce in the plant cell the cytosolic synthesis of metal-binding thiol peptides, namely phytochelatins (PCs), which may substantially contribute to HM detoxification in algae, fungi, lichens, mosses, ferns, gymnosperms and angiosperms [1-9]. PCs were first isolated and characterized in higher plants by Grill et al. [10], although their presence in Hg-treated tobacco leaves had been brilliantly hypothesized since 1973 [11, 12].

PCs form a family of structures with increasing repetitions of the γ-GluCys dipeptide followed by a terminal Gly: $(\gamma\text{-GluCys})_n$-Gly, where *n* is generally in the range 2 to 5. Sometimes, desGlyPCs, that is PCs lacking the terminal Gly residue, can also be present [13]. In addition, a number of structural variants, for example, $(\gamma\text{-GluCys})_n$-β-Ala (homo-PCs), $(\gamma\text{-GluCys})_n$-Ser, $(\gamma\text{-GluCys})_n$-Glu, etc. (collectively termed as *iso*PCs), have been identified in some plant species. In *iso*PCs and desGlyPCs, *n* values ranging from 2 to 7, more often from 2 to 4, are the most common.

PCs are structurally related to glutathione (GSH; γ-GluCysGly) and various studies have confirmed that GSH (or, in some cases, related compounds, such as γ-GluCys-β-Ala [homo-glutathione]) is the substrate for PC biosynthesis. Genetic studies, for example, in *S. pombe* [14, 15] and *Arabidopsis* [16] have confirmed that GSH-deficient mutants are also PC-deficient and hypersensitive to Cd.

PCs can produce complexes with several HMs, thus preventing their free circulation inside the cytosol, by means of the metal chelating sulfhydryl groups of the Cys residues present in their structure [10] The molecular masses, for instance, of Cd-PC complexes range from about 1,800 to 4,000 Da, but may be as high as 8,000 Da, depending on the ionic strength of the solvent employed for gel-filtration chromatography [9,17, 18] Some Cd-PC complexes can also include acid-labile sulphur (S^{2-}), which gives them increased stability and higher Cd sequestration capacity.

The most effective metal and metalloids in the induction of PC biosynthesis are Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+}. Moderate PC biosynthesis is detected also in response to Au^{+}, Bi^{3+}, Ga^{3+}, In^{3-}, Sb^{3+}, SeO_3^{2-}, SeO_4^{2-} and Sn^{2+}. On the contrary, other metals such as Al^{2+}, Co^{2+}, Cr^{3+}, CrO_4^{2-}, Fe^{2+}, MoO_4^{2-}, Mn^{2+}, Ni^{2+}, Te^{4+} and W^{6+} do not appear to be effective stimulators of PC synthesis. Cd ions are probably the most powerful inducers of PC synthesis [7, 19, 20].

2.2. Biosynthesis

Two features of PCs, namely the presence of a non-translational γ-carboxamide bond as in GSH, and the presence in *iso*-PC-(β-Ala) of an unusual C-terminal amino acid for which no tRNA is available, soon suggested a non-ribosomal origin of these peptides. Other important hints pointing to the non-translational formation of PCs through the extension of the GSH tripeptide were: i) the structural similarity between PCs and GSH, particularly the isopeptide bond; ii) the existence of an inverse relationship between HM-induced (especially Cd) PC accumulation and GSH depletion [17, 21, 22]; iii) the blockage of PC synthesis by buthionine sulfoximine (BSO), a specific inhibitor of γ-EC synthetase [22, 23, 24]. All this strong circumstantial evidence on the origin of PCs were substantiated and extended by the isolation of a homogenously purified enzyme preparation from *Silene vulgaris*, capable of catalyzing the *in vitro* synthesis of PC_{2-4} in the presence of GSH and micromolar Cd^{2+} ion concentrations [25]. The *in vitro* reaction catalyzed by this enzyme involved the transpeptidation of the γ-EC moiety of GSH onto another GSH molecule forming PC_2, or, at later stages, onto a growing PC_n unit to yield a PC_{n+1} oligomer. The first transpeptidation product (formed within a few minutes after Cd addition) was PC_2, followed at 10-20 minute intervals by the appearance of higher order polymers (PC_3, PC_4).

The enzyme responsible for PC synthesis, which can also utilize preformed PCs as its sole substrate, is a γ-glutamylcysteine dipeptidyl transpeptidase (E.C.2.3.2.15), and is commonly referred to as PC synthase [25].

2.3. Identification of PC synthase mutants and genes

Even though PCs were first identified in *S. pombe* [26], and numerous screens for Cd-sensitive mutants of *S. pombe* have identified a range of mutants affected in PC biosynthesis or function [27, 28], none of these mutants identified the PC synthase gene. The first PC synthase mutants were identified in *Arabidopsis*. Cd-sensitive *cad1* mutants are PC-deficient but have wildtype levels of GSH. They also lack PC synthase activity, indicating a defect in the PC synthase gene [29].

The identification of the *cad1* mutants led to the isolation of the *CAD1* (*AtPCS1*) gene using a map-based cloning strategy [30]. Simultaneously, PC synthase genes were isolated from two other groups using the expression of *Arabidopsis* and wheat cDNA libraries in *S. cerevisiae* to identify genes [*AtPCS1* [31] and *TaPCS1* [32], respectively], which conferred increased Cd-resistance. A similar sequence was identified in the genome of *S. pombe* and targeted deletion mutants of that gene are, like *Arabidopsis cad1* mutants, Cd-sensitive and PC-deficient, confirming the analogous function of the two genes in the different organisms [30, 32]. Heterologous expression in yeast or *E. coli* was used to demonstrate the biochemical activity of these *Arabidopsis*, wheat and *S. pombe* gene products. Each was sufficient for GSH-dependent PC biosynthesis *in vitro*,

confirming their identity as PC synthase genes [30, 31, 32]. Full-length or partial cDNA clones encoding PC synthases have also been isolated from other plant species including *Brassica juncea* and rice. In addition, a cDNA encoding a homo-PC synthase (GmhPCS1), which uses homo-glutathione (hGSH) $(\gamma\text{-GluCys})_n\text{-}\beta\text{-Ala}$ as a substrate, has recently been isolated from soybean [33]. A second PC synthase gene, *AtPCS2*, with significant identity to *CAD1/AtPCS1* has also been identified in *Arabidopsis* [34]. Because PCs have not been detected in the severe *cad1-3* mutant after prolonged exposure to Cd, it likely there was only a single active PC synthase in the wildtype [30]. Thus, the presence of a second gene in the genome came as a surprise. A recent study has demonstrated that this gene is transcribed and encodes a functional PC synthase enzyme [34]. The physiological function of this gene, however, remains to be determined. The isolation of a *PCS2* mutant may be required to define its function. It appears that in most tissues *AtPCS2* is expressed at a relatively low level compared to *AtPCS1*. Nonetheless, since AtPCS2 has been preserved as a functional PC synthase through evolution, it must presumably confer a selective advantage. This may indicate that PCS2 is the "predominant" enzyme in some sub-cellular compartment, or in a confined range of cells or tissues, or under a particular set of environmental conditions. It remains to be seen how many plant species express more than one PC synthase.

2.4. Some animals express a PC synthase

PCs have never been identified in an animal species. Thus, another surprise came when database searches identified similar genes in nematodes (*Caenorhabditis* species) and in slime mould, *Dictyostelium discoideum*. Also, partial sequences with homology to the plant and yeast PC synthase genes have been identified in the aquatic midge, *Chironomus*, and earthworm species (unpublished data). Both the *C. elegans* and *D. discoideum* genes encode PC synthase activities. Heterologous expression studies and *in vitro* activity assays have demonstrated conclusively that *CePCS1* is a *bona fide* PC synthase [35, 36]. Similarly, expression of the *D. discoidium* PC synthase in *S. cerevisiae* also confers PC biosynthesis *in vivo* and increased Cd-resistance (C. Cobbett, unpublished data). Significantly, by using the double-stranded RNA interference technique to suppress *CePCS1* expression *in vivo*, Vatamaniuk et al. [36] have shown that CePCS1 plays an essential role in heavy metal detoxification in *C. elegans*. Animals injected with CePCS1 double-stranded RNA are highly sensitive to Cd. Interestingly, while PCs clearly play a wider role in heavy metal detoxification in biology than previously expected, it appears that the genomes of *S. cerevisiae*, *Drosophila melanogaster*, mouse or human, some organisms do not contain a PC synthase gene. More comprehensive information regarding the presence of PC synthase genes in the phylogenies of animals may provide some clue to the selective pressures involved in their maintenance.

2.5. PC synthase enzymes and their regulation

To date, PC synthase genes or cDNAs have been isolated from a range of different species. The molecular weights predicted for the deduced polypeptides range from 42 to 70 kDa. The plant PC synthase sequences can be aligned across their entire length even in comparisons between sequences from a monocot (TaPCS) and a dicot (AtPCS) [32]. In contrast, an alignment of plant, yeast and animal PC synthase sequences shows that the N-terminal regions are similar (40-50% identical), while the C-terminal sequences show little apparent conservation [30]. The C-terminal regions contain multiple Cys residues, often as adjacent pairs or near pairs. However, there is no apparent conservation of the positions of these Cys residues relative to each other. Both the *S. pombe* and *D. discoideum* sequences contain N-terminal extensions which, in the *D. discoideum* sequence, also contains a number of Cys residues.

As reported before, when a PC synthase activity was first identified (from cultured cells of *Silene vulgaris*), it was characterised as a γGluCys dipeptidyl transpeptidase (EC 2.3.2.15) [25]. It catalysed the transpeptidation of the γGluCys moiety of GSH onto a second GSH molecule to form PC_2, or onto an existing PC_n molecule to form a PC_{n+1} derivative. *In vitro* studies using purified epitope-tagged derivatives of cloned PC synthases have confirmed these earlier observations [31, 37]. The recently isolated soybean hPC synthase (GmhPCS1) is able to use both GSH and hGSH as a substrate [33]. In the presence of hGSH only a low level of hPC synthase activity was detected (in contrast to AtPCS1, which was unable to use hGSH only as a substrate). In the presence of GSH only, both enzymes had a high level of PC synthase activity. For GmhPCS1, when GSH was added in the presence of hGSH, the rate of hPC synthase activity increased 100-fold. Further experiments using hGSH and S-methyl-GSH demonstrated that hGSH acts as the acceptor molecule for γGlu-Cys(SCH_3) moieties transferred from the S-methyl-GSH donor molecules (and presumably γGlu-Cys moieties transferred from GSH). Interestingly, AtPCS1 can also effectively synthesise hPCs under the same conditions *in vitro*. This indicates that it is the presence of the substrate GSH or its various isoforms, such as hGSH, *in vivo,* rather than different specificities of the enzymes themselves, that determines the nature of the PCs synthesised.

PC synthesis can be induced by a range of metal ions in *S. pombe* and in both intact plants and plant cell cultures (see above). Kinetic studies using plant cell cultures demonstrated that PC induction is rapid and is independent on *de novo* protein synthesis. Thus, PC synthase appears to be expressed independently of heavy metal exposure and the activity has been detected in extracts of plant cell cultures or tissues grown in the presence of only trace levels of essential heavy metals. These *in vivo* studies suggested PC synthase is primarily regulated by activation of the enzyme in the presence of heavy metals. *In vitro* assays using the partially purified enzyme from *S. vulgaris* demonstrated it was active only in the presence of a range of metal ions, including Cd, Ag, Bi, Pb, Zn, Cu, Hg, and Au cations [20, 25]. Interestingly, among the effective activators are also the anions,

AsO_2^- and AsO_4^{-3}. Broadly, the range of activating metal ions has been confirmed by *in vitro* studies of PC or hPC synthase enzymes expressed and purified from *E. coli* or *S. cerevisiae* [31, 32, 33, 37]. There is some debate about the mechanism through which PC synthase is activated by such a wide range of metal ions. Early models for the activation of PC synthase assumed a direct interaction between metal ions and the enzyme but raised the question of how the enzyme might be activated by such a wide range of metals. With the cloning of PC synthase genes, the expression and purification of different enzymes has led to more comprehensive investigations of the mechanisms of enzyme activation and catalysis. Nonetheless, two recent studies have led to different interpretations.

The data of Vatamaniuk et al. [37] suggested that, in contrast to earlier models of activation, metal binding to the enzyme *per se* is not responsible for catalytic activation. AtPCS1 binds Cd ions at high affinity (Kd = 0.54 ± 0.20 μM) and high capacity (stoichiometric ratio = 7.09 ± 0.94). However, it has much lower affinity for other metal ions, such as Cu, which are equally effective activators [31, 37]. Modelling studies showed that in the presence of physiological concentrations of GSH and micromolar concentrations of Cd, essentially all of the Cd would be in the form of a GSH thiolate, thus suggesting that free Cd would be unlikely to be the activator. They also showed that S-alkylglutathiones can participate in PC biosynthesis in the absence of heavy metals indicating that metal ions were not an absolute requirement for activation and suggesting a model whereby blocked glutathione molecules are both the substrate and the activator for PC biosynthesis.

In contrast, the study by Oven et al. [33] found that S-methyl-GSH activated AtPCS1 to a very limited extent and that this activation was completely inhibited in the presence of Cd ions. This difference was ascribed to the use of different buffers in the reactions. Under their chosen reaction conditions, Oven et al. [33] demonstrated the presence of thiols as an essential requirement for PC synthase activation. Interestingly, these authors found that different combinations of thiols and metal ions influenced activity to greatly different extents indicating that the characteristics of the metal-thiolate complexes are likely to be important in *in vivo* activation of the enzyme.

The conserved N-terminal domain of PC synthase is presumed to be the catalytic domain. This conclusion is supported by the characterisation of a nonsense mutant of *CAD1* (*AtPCS1*) which, while expected to lack most of the C-terminal domain, retains a residual level of activity *in vivo* [29, 30]. This domain probably acts to enhance activity possibly by binding metal glutathione complexes via thiolate bonds with the Cys residues and thereby increasing the kinetics of catalysis. Further *in vitro* studies are required to determine the role of the C-terminal region.

Most studies to date indicate that PC synthase activity is regulated at the level of enzyme activation and suggest that the induction of PC synthase gene expression is unlikely to play a significant role in regulating PC biosynthesis. Indeed, the expression of *AtPCS1/CAD1* showed that levels of mRNA were not influenced by exposure of plants to Cd and other metals, thus suggesting an absence of regulation at the level of transcription [30, 37]. In contrast, analysis of *TaPCS1* expression in roots indicated increased levels of mRNA on exposure to Cd [32] indicating that, in

some species, activity may be regulated at a number of levels. Little is known about the tissue specificity of PC synthase expression and/or PC biosynthesis. In a study on tomato, activity was detected in the roots and stems of tomato plants but not in leaves or fruits [38].

3. NONPROTEIN METAL CHELATORS

3.1. Organic acids

In plants, organic acids may be implicated in detoxification, transport and compartmentalization of heavy metals. Organic acids are present in all living organisms; they are low-molecular weight compounds containing carbon, hydrogen and oxygen, and are characterised by one or more carboxylic groups. The number and the dissociation properties of the carboxylic groups determine the negative charges carried by the molecules, thus either the number of metal cations that can be bound in solution or the number of anions that can be displaced from the soil matrix [39]. The most stable ligand-metal complexes have the highest number of carboxyl groups available for binding metal cations. Metal complexes with citrate^{3-} (tricarboxylate) are more stable than those with malate^{2-}, oxalate^{2-} or malonate^{2-} (dicarboxylate) and acetate^{1-} (monocarboxylate) [40].

In several plant species, organic acids participate in (i) metal exclusion mechanisms, as metal chelators excreted by the root apex outside the plant and (ii) metal hyperaccumulation mechanisms, as metal chelators inside the plant, with various degrees of metal retention within root and shoot [41, 42]. The total concentration of organic acids in the root is generally about 10-20 mM, but may vary depending on the degree of cation-anion imbalance, because organic acids often provide the negative charges which balance excess cations [39].

Within the plant cell, organic acids are mainly synthesised in mitochondria through the tricarboxylic acid cycle, but the site of preferential storage is the vacuole. Usually root vacuoles contain 2-10 fold higher concentrations of malate and citrate than cytosol (5 mM) [39], and organometallic chelates can be found in the cell wall, cytoplasm and vacuoles [9].

3.2. Metal exclusion (organic acids in root exudates)

The composition of root exudates varies greatly depending on environment, plant species and age [39, 43]. Plants growing on strongly acid soils (pH<5) are exposed to Al toxicity, which inhibits root growth thus limiting crop productivity [40, 44, 45]. The fundamental mechanisms of Al toxicity and resistance in plants are not known as yet, but root exudation of organic acids appears to be the main mechanism for Al detoxification in Al-resistant plants [39, 45, 46]. Soil Al^{3+} ions induce the release of organic acids (malate, citrate, oxalate) into the apoplast of the root apex

and the external solution, where they chelate Al^{3+} to prevent damage to cell wall and/or plasma membrane [39, 44, 45].

Other metal cations were reported to induce root exudation of citric acid in *Arabidopsis thaliana* (Cu^{2+}) [47] acetic and succinic acids in durum wheat [48], and oxalic acid in rice (Pb^{2+}) [49]. In some species (wheat, maize, buckwheat) the efflux rate of organic acids depends on transport rather than on internal concentrations of organic acids [40], and may be stimulated by high concentrations of the external dissolved inorganic C incorporated into PEP carboxylase activity [50].

Two patterns for Al-induced secretion of organic acids by plants were described [51]. In pattern I (wheat, buckwheat) the release of organic acids by plant roots exposed to toxic Al^{3+} concentrations is immediate and probably due to the activation of anion channels for organic acids. In pattern II (rye, *Cassia tora*) there is a clear lag phase between Al exposure and organic acid exudation, probably due to induction of genes related to the metabolism of organic acids, anion channels on plasma membrane and/or tonoplast, or organic acid transport from mitochondria [51]. In *Arabidopsis thaliana* the release of organic acids by the root appears to be mediated by an outward rectifying anion channel localized on the plasma membrane of root cells, with its gating possibly regulated by Al^{3+} [52]. Toxic concentrations of Al^{3+} may stimulate the release of organic acids in the rhizosphere either directly or altering organic acid export from mitochondria and/or reduced organic acid transport into the vacuole, leading to increased concentration of organic acids in the cytosol and subsequent excretion from root cells [52]. Al-dependent anion channels have been identified in wheat and maize and seem to be the likely pathway for citrate efflux from the root [40, 46, 53]. Conclusive evidence that organic acid excretion from the root is the main mechanism of Al tolerance in higher plants was given by tobacco (*Nicotiana tabacum*) plants genetically engineered for this trait [54].

Plant species differ greatly in their sensitivity to Al^{3+} stress (Table 1) and a large intraspecific variation in Al^{3+} tolerance is known to occur in some important crops (wheat, maize, soybean) [40].

In the tribe Triticeae, research on Al-resistance mechanisms has focused on wheat (*Triticum aestivum*), rye (*Secale cereale*) and triticale, a synthetic hybrid of wheat and rye. Wheat roots excrete malic acid when exposed to toxic Al concentrations [55-59]. Although rye is the most Al-tolerant species, its Al-resistance mechanism is not known [60]. However, Al^{3+} specifically induced citric acid exudation from the root tip of rye [60]. In triticale, Al-resistance is thought to be inherited from rye and rely on similar mechanisms [60]. Janxi, a variety of buckwheat (*Fagopyrum esculentum* Moench.) which is highly resistant to Al toxicity, excretes oxalic acid from the root tip [61, 62].

Maize (*Zea mays* L.) responds to Al^{3+} toxicity through root excretion of either citrate and malate [46, 63], or oxalate [45]. However, there is some indication that also phenolics may be involved in Al-detoxification [45].

In several varieties of soybean (*Glycine max*) toxic levels of Al specifically induced citric acid exudation from primary root. Furthermore, citric acid concentrations were positively related to the degree of Al-resistance [64]. In contrast, Al-exclusion mechanisms do not seem to operate in lateral roots of

soybean, since Al^{3+} accumulates in the meristem [65]. Citric acid exudation from root tip is the mechanism for Al-resistance also in a variety of snapbean (*Phaseolus vulgaris* L.) [66].

Table 1. Enhanced organic anion exudation from the root of selected species in response to environmental stimuli (modified from [40])

Species	*Major organic acid released*	*Metal*	*Efflux 1 nmol/gFW/h*	*Efflux 2 (units shown)*	*Comments*
Wheat	Malate	+Al	4000[c]	2.0 nmol/apex/h	Root apices
Maize	Citrate	+Al	55[b]	0.25 nmol/apex/h	Root apices
Tabacco	Citrate	+Al	240[d]	0.18 nmol/apex/h	Root apices
Cassia tora	Citrate	+Al	280[a]		Wr
Criticale	Citrate, malate	+Al	7, 9[a]		Wr
Rye	Citrate, malate	+Al	70, 35[a]		Wr
Buckwheat	Oxalate	+Al	70[a]		Wr
Soybean	Citrate	+Al	55		Wr
Taro	Oxalate	+Al	46		Wr
Sunflower	Citrate	+Al		150, 25 nmol/plant/h	
Oats	Citrate	+Al	6[a]		Wr
Radish	Citrate	+Al	23[a]		Wr
Brassica napus	Citrate, malate	+Al	4, 4[a]		Wr
	Citrate	+Al	38[a]		Wr
Paraserianthes falcataria	Citrate	+Al		1.4 nmol/plant/h	Wr
Arabidopsis thaliana	Citrate	+Cu	122[a]		Wr

Assumption used in calculating efflux to common units: (a) DW = 7% FW; (b) root apex = 6 mm × 1 mm diameter; (c) root apex = 2 mm × 0.5 mm diameter; (d) root apex = 6 mm × 0.4 mm diameter. Wr = whole root.

Investigations on Al-resistance mechanisms of *Cassia tora* showed that citrate exudation protects the root apex against Al stress [67]. Similarly, five of ten species of wild herbs (*Deschampsia flexuosa*, *Galium saxatile*, *Rumex acetosella*, *Veronica officinalis* and *Viscaria vulgaris*) were shown to rely on exudation of citrate, malate or oxalate in the rhizosphere for Al detoxification [68].

Al-sensitive mutants of *Arabidopsis thaliana* were analyzed in an attempt to identify genes encoding targets of Al toxicity and characterizing genes for Al resistance [52]. When *Arabidopsis thaliana* was exposed to Al stress, the main organic acids exuded by the root were citric acid in the wild type, and both citric and malic acids in these mutants [52].

3.3. Metal hyperaccumulation (organic acids as metal chelators inside the plant)

Metal hyper-accumulating plants partition the absorbed metals between root and shoot to different degrees, depending on species, metal type and concentration [9]. Toxic metals accumulated in the shoot can easily be removed by leaf fall. In contrast, metals absorbed by perennial organs such as roots are converted into soluble and insoluble fractions. The insoluble fraction is retained by the root (mostly in the cortex), while the soluble fraction is translocated to the leaves and can be subsequently removed at leaf fall [69-71]. Within the cell, organic acids may act as intracellular chelators in the cytosol, the vacuoles, and the cells of the vascular system of either root or shoot [9]. Plants have evolved mechanisms to avoid metal toxicity, either tolerating high concentrations in the cell cytosol or sequestering metals in vacuoles. Generally vacuoles of metal-tolerant and sensitive plants exposed to toxic metals contain high metal concentrations (Ni, Zn, Cu, Pb, Cd) [69]. Organic acids could operate both as metal detoxifying agents and transporters as in the Zn-malate shuttle hypothesis [72, 73], proposed to explain Zn transport from cytosol to vacuoles. According to this model, malic acid would bind Zn in the cytosol, and the resulting Zn-malate complex would be transported across the tonoplast into the vacuole, where Zn would be stored after being complexed by another strong chelator such as citric or oxalic acid, or anthocyanidines when available [72, 73]. However, the pivotal role of malate in Zn intracellular transport is not supported by more recent studies [74, 75].

Perhaps the most unusual Ni hyper-accumulator is the serpentine-endemic tree *Sebertia acuminata* from New Caledonia. This tree produces a dense blue-green sap extraordinarily rich in Ni (257,000 μg/g in the dried material, 112,000 μg/g in the fresh latex) [71, 76, 77]. In the latex, at least 40% Ni is complexed by citrate [77], as previously found also in the leaf sap [78], but the inorganic anion nitrate (NO_3^-) may also participate in Ni complexation [77]. In *S. acuminata*, Ni concentrations are very high also in other plant parts, such as leaves, trunk bark, twig bark, fruits and wood (11,700, 24,500, 11,200, 3000, 1700 μg/g DW Ni) [71]. As hypothesised earlier [79], the Ni absorbed by the root may be complexed by special root membrane "selectors" and then passed onto "transport ligands" such as citrate and/or malate. Alternatively, the Ni-selector complex may form a triple complex with the transport

ligand (Morrison, 1980, as cited by [80]) and the Ni-transport complex released into the xylem, with the selector going back to the root membrane. However, the precise mechanism of Ni detoxification and transport inside the plant of *S. acuminata* needs to be fully elucidated [77].

Most Ni-hyperaccumulating species store Ni as Ni-organic acid complexes in vacuoles of leaf epidermis and other periferic tissues [71, 77, 78, 81-86], either including trichomes [84] or not [86] and using Ni as an inorganic defence system against predators and pathogens [77, 87, 88]. Ni can be complexed by ligands containing carboxyl (COOH) and sulphydril (SH) groups [89], and in the majority of Ni-hyperaccumulators is stored in leaf vacuoles as Ni-citrate or malate complexes, with some involvement of malonate [71]. A Ni-citrate complex was found in three *Homalium* and two *Hybanthus* species, generally accompanied by the Ni aquaion $Ni(H_2O)_6^{2+}$ and hydrated Ca^{2+} and Mg^{2+} ions, except in *Homalium guillainii* [78]. Ni-citrate and traces of malic and malonic acids were also found in leaf extracts of *Alyssum* and *Pearsonia metallifera* [78], and of three subspecies of *Alyssum serpyllifolium* [82]. Similarly, purified extracts of three Philippine Ni-hyperaccumulators contained Ni associated to comparable amounts of either malic acid in *Dichapetalum gelonioides* subsp. *tuberculatum* and *Walsura monophylla*, or malic and citric acids in *Phyllanthus palawanensis* [90]. Several studies on the Ni-hyperaccumulating species *Alyssum bertolonii*, a serpentine endemic in Italy (Tuscany) which can accumulate over 10,000 μg/g Ni in the leaves, unequivocally showed that malic and malonic acids are responsible for Ni^{2+} chelation in leaf sap [81, 84, 91], root sap and xylem too [84]. The finding that malic and malonic acid concentrations in the leaves of *A. bertolonii* decreased dramatically when it was grown in Ni-poor soil further supported this hypothesis [91]. However, the 36-fold increase of the concentration of free amino acid histidine in xylem sap when *A. lesbiacum* was exposed to toxic Ni concentrations and the positive linear correlation between Ni and the free histidine [92] gave compelling evidence that amino acids are involved in Ni complexation in xylem sap. It can be hypothesised that Ni-amino acid complexes may be preferred for Ni transport while Ni-organic acid complexes may be the chemical form of Ni storage [71, 92, 93]. Amino acids were found to be involved in Ni complexation in xylem exudates also in the Ni-hyperaccumulator *Dichapetalum gelonioides* subsp. *tuberculatum* [94]. Ni-hyperaccumulator species are known in the genus *Thlaspi* (*T. goesingense*; *T. montanum* cv. *californicum*, *montanum* and *siskiyouense*; *T. caerulescens*) [95]. Although *T. goesingense* is characterised by rhizosphere exudates rich in citric acid and histidine, neither this strategy nor a fast root-shoot transport of Ni^{2+} appears to be related to Ni tolerance and accumulation in this species [96]. A recent study [97] on Ni speciation in leaf tissues of *T. goesingense* through x-ray absorption spectroscopy showed that 87% of Ni not bound to cell walls was bound and compartimentalized as Ni-citrate in leaf vacuoles. However, a small portion (about 25%) of intracellular Ni resulted bound to histidine in the cytosol, probably for Ni intracellular transport and storage in leaf vacuoles [97]. The phytochemistry of Ni accumulation in other *Thlaspi* species has not been investigated yet.

A few studies focusing on some cobalt (Co) and/or copper (Cu) hyperaccumulating species growing on mineralised outcrops in Zaïre suggested that oxalic acid may chelate Co in the leaves of the Co-hyperaccumulator *Haumaniastrum robertii* (Morrison, 1980 and Morrison et al., 1981 as cited by [71]). Although a similar mechanism for both Ni and Co accumulation in *Alyssum trodii* has been postulated, the phytochemical basis of Co accumulation still needs to be elucidated in this species [95].

Thlaspi caerulescens is the most well known Zn hyper-accumulator. This species and other species of the same genus which accumulate Zn in roots and shoots, organic acids (citrate and oxalate) seem to play a key role in Zn^{2+} long-distance transport and vacuolar storage [95, 98]. It has been found that in leaf epidermal cells, Zn is converted into a soluble compound that is preferentially stored in vacuoles [75]. However, evidence of the involvement of organic acids in Zn compartmentalization, which is the main process responsible for Zn tolerance [99] is still lacking.

Cadmium (Cd) tolerance in plants relies on Cd binding to high molecular weight compounds such as PCs, however both long-distance transport [100, 101, 102] and vacuolar compartmentalization [103] of Cd seem to require organic acids (citrate, oxalate, malate) and probably amino acids [1].

In the Al-resistant buckwheat, not only oxalic acid is secreted by the roots to prevent Al^{2+} from entering the root, but it also has a role in the internal detoxification of Al^{2+} in root and shoot tissues. Both mechanisms contribute to Al-resistance in buckwheat [104].

In conclusion, organic acids in plant tolerance to heavy metals are responsible for metal detoxification when metals enter the root, metal compartmentalization and storage in the cell vacuoles and long-distance transport of metals, although the latter is sustained also by other low molecular weight compounds such as amino acids [105, 106].

3.4. Phytate

Phytate is a salt of phytic acid [*myo*-inositol-1,2,3,4,5,6-hexakis (dihydrogen phosphate)] which belongs to the inositol phosphate family. These compounds have different biological and chemical activities depending on number and relative positions of phosphate groups [107]. Phytate has a strong but not selective cation-chelating ability: the six phosphate groups of each molecule may release 12 hydrogens in water and expose six negatively charged sites on phytic acid that may bind cations of plant macronutrients (P^{2+}, Mg^{2+}, K^{+}, Ca^{2+}) [108] and micronutrients (Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}), thus functioning as a mineral storage compound, and may detoxify cations of heavy metals (Cd^{2+}, Cr^{3+}) [107, 109-112]. Phytate complexes with mineral cations form globoids that constitute 1% or more of the embryo and (or) seed dry weight in wheat, barley and maize, and appear to be important for seedling establishment, when enzymatic degradation of phytate by phytase (phytic acid phosphatase) provides the plant with phosphate, mineral cations

and *myo*-inositol [109,111]. However, phytate can also be found in other plant organs. Roots of several crop plants (soybean, lucerne, lupin, tomato, rapeseed, cabbage, radish, wheat, maize) and of a Zn-tolerant species (*Deschampsia caespitosa*) had high levels of phytate when exposed to high concentrations of Zn. Restricted transport of Zn from root to shoots is probably a strategy to protect the plant from metal toxicity [113,115]. In the root, globular inclusions of Zn-phytate were localized mainly in the endodermis of dicotyledonous species and in the pericycle, stele and inner cortex of monocotyledonous species, after prolonged exposure to toxic Zn concentrations [115]. In contrast, no globular inclusions of Zn-phytate were observed in sunflower, field pea and Italian ryegrass [115], or in the metallophyte *Thlaspi caerulescens* [116] exposed to high Zn concentrations.

Cd stimulated the formation of Cd-phytate globoids in the fronds of some lines of duck weed (*Lemna minor*) [117] and in the root of bracken (*Pteridium aquilinum* L.) infected by mycorrhizae [118], but not in soybean, lucerne and maize [115]. In the *Agrostis capillaris* metal tolerant variety Parys Mountain, Pb was accumulated in the root as Pb-P deposits with a pyromorphite [$Pb_5(PO_4)_3Cl$]-type structure and not as Pb-phytate [$C_6H_{18}O_{24}P_6^{12-}$] complexes [119].

In conclusion, phytate is essential for P storage, but it appears to play only a minor role in metal detoxification in plants.

4. STRESS RESPONSE TO HEAVY METALS IN ALGAE

4.1. Generalities

Algae, a group of diverse organisms of plant-type photosynthesis, are primary producers and essential constituents of aquatic ecosystems, often playing a role as dominant vectors for heavy metals. Algal cells and thalli are important biosorbents influencing the environmental fate of metals, their chemical speciation and bioavailability [120-122]. All metals, even those that are essential nutrients at low concentrations (Zn, Cu, Mn, Co, etc.) may exert toxic impact on algae at high concentrations.

To indicate effects of stressors (including HM) on aquatic organisms such as algae, special acronyms such as LOEC (lowest observed effect concentration), NOEC (no observed effect concentration), MATC (maximum acceptable toxicant concentration), EC (effective concentration), LC (lethal concentration) are used by toxicologists [123]. These parameters are estimates of the stressor concentrations to have the effect on a percentage of the population, or biological response being measured for test that have been conducted for a specified duration. For instance, Schäfer et al. [124] estimated that 0.079 mg Cu/l was 72 h EC_{50}, which inhibited growth of the green alga *Chlamydomonas reinhardtii* by 50 % for 72 h exposure.

A variety of environmental factors are known to modify significantly the uptake and toxic effects of heavy metals on algae [125-127]. They include pH of water [128, 132], light intensity [133, 135], the presence of inorganic nutrients such as

phosphorus, calcium, magnesium [132], inorganic and organic chelators and complexing compounds present in surrounding water [136, 137], exchange reactions between suspended matter and water [138]. Generally, strongly complexed and adsorbed metals like Cd, Pb, Cu, Zn are not available for uptake, and free-cations are most toxic for algal cells. However, some recent reports show that Cd, Zn and Ag may also be available for algae in the form of particular complexes like chlorides or citrates, not only in the form of free-cations [139-141]. Some metals and metalloids like Pb, Hg, Sn, As can occur as lipophilic organometallic compounds, which can be also accumulated by algae and in some cases can exert even stronger toxic effects than inorganic forms of these elements [142, 145]. There are suggestions that they can rapidly pass through algal cell membranes via passive diffusion [146]. Accumulation of organotin compounds was reported for a few freshwater green algae like *Ankistrodesmus falcatus* [147], *Scenedesmus obliquus*, *Chlorella vulgaris* [148] and some blue-green algae [149]. Accumulation of organocomplexes of Pb, Cu, Cr and Tl in neutral lipids and plastoglobuli of the green alga *Cladophora* has also been reported recently [143]. The primary impact of heavy metals on the algal cells is at the biochemical and physiological levels. However, HM-stress affects algae also at organism, population and community levels of biological organization, leading to the elimination of sensitive species, and the dominance of a few resistant/tolerant species (for review see [150]).

4.2. Effects of metals on algal metabolism and growth

At the sub-cellular level heavy metals can interact with macromolecules or disturb cell membrane integrity, leading to inhibition of essential metabolic or physiological process and ultra-structural changes. Uptake of heavy metals in algae can result in inhibition of several enzymes [151]. For example, it was found that Cd, Zn and Hg inhibited NADP-oxidoreductase in *Euglena*, thereby significantly lowering the cell supply of NADPH [152]. High concentrations of Cd, Cu and Zn induced reduction of nitrate assimilation in the green alga *Chlamydomonas reinhardtii*, by inhibition of glutamine synthetase activity [153]. Cu was found to inhibit plasma-membrane H^+-ATPase activity in *Nitella flexis* [154], while Ni and Mn increased activity of the chlorophyll degradation enzyme chlorophyllase (Chlase) in two other green algal species [155]. Cd caused inhibition of carbonic anhydrase in the blue-green alga *Synechocystis aquatilis* [156]. Generally, binding of the metal to the sulphydryl group is proposed as mechanism of metal action on enzymes: strong affinity to SH groups is exhibited particularly by metals as Cd, Pb, Hg [151]. However, as reported for Rubisco in Cd-exposed brown alga *Laminaria saccharina*, Cd does not interact directly with this enzyme, but inhibits *de novo* protein synthesis and general enzyme biosynthesis [157].

Furthermore, heavy metals can disrupt cell transport processes in algal cells by formation of pores and changes in plasma membrane [154]. High concentrations of unessential metals can cause imbalance of essential metals in algae; however, there are competitive interactions also among metals, independently of their physiological

role. For example, high levels of Cu and Zn inhibited cellular Mn uptake, and high Cu inhibited Zn uptake rates in the green alga *Chlamydomonas* spp., by blocking Mn and Zn binding to high-affinity uptake systems [158]. The uptake of toxic amounts of metals such as Cd, Cu, Cr, Hg, Sn, Pb, Zn, etc. may result, among other things, in inhibition of photosynthesis by strong effects on the pigment content, inorganic carbon assimilation and oxygen evolution, both in prokaryotic and eukaryotic algae [132, 144, 156, 159, 160]. Photosystem II was inactivated by elevated Cu and Zn concentrations in *Chlorella pyrenoidosa* [161]. Chlorophyll biosynthesis inhibition and chlorophyll damage by Cd, Hg, Pb, Cu and Zn were reported in many green algae, euglenins and diatoms [162, 164]. In some species of *Euglena* and *Chlorella* cell respiration was also affected by Cd, Hg, Pb and Zn [165-167]. In *Scenedesmus quadricauda*, apart from chlorophylls, total DNA, RNA and protein content were lowered too [168]. Adenylate metabolism was disturbed by Cd in the blue-green alga *Synechocystis aquatilis* [169].

In HM-stressed algal cells several significant ultra-structural and morphological alterations can be observed. For example, Cr(VI) induced formation of large uninucleated cells in *Scenedesmus acutus*, but also formation of multicellular plurinucleated aggregates [170]. Cell enlargement was also observed in Cd-exposed green alga *Stichococcus bacillaris* [171] and Cu-exposed diatom *Ditylum brightwelli*. Heavy metals may affect total cell volume, number and relative volume of polyphosphate bodies, lipids, vacuoles, plasmalemma and cell wall organization in algae belonging to various taxonomic groups [172-175]. Metals caused a disruption of thylakoidal membranes in chloroplasts of the green algae *Chlorella fusca* and disorganization in structure of nucleus and loss of flagella and movement ability in *Haemotococcus lacustris* in the flagellate stage [176, 177]. Deformed algal cells, unable to divide, and broken cells have been often observed [160, 171]. HM-exposed algae revealed inhibition of cell division rates and significant growth inhibition [132, 171, 178-180]. Another toxic metal Al., caused destruction of cell wall and cell vacuolization in green algae *Monoraphidium* and *Stichococcus* [181], as well as inhibition of cytoplasmic streaming in *Vaucheria longicaulis* (Xanthophyta) [182].

HMs may also affect algae life cycles. In the Cr-exposed green alga *S. acutus* cell proliferation and coenobia formations were inhibited [170]. Inhibitory effect of Zn on zoospore release and settling was reported in the periphytic green alga *Stigeoclonium tenue* [183, 184]. In the Cu- and As-exposed brown alga *Macrocystis pyrifera* and *Laminaria saccharina*, germination of zoospores and gametophyte development were inhibited [185, 186].

4.3. Oxidative stress

Exposure to heavy metals also causes alterations at the cellular level of reactive oxygen species (ROS) implicated in many cellular injuries, leading to growth inhibition or cell death [178]. The micronutrient Cu can participate directly in reactions generating ROS because of its redox properties. Cu-induced oxidative

stress, expressed as an increase of activities of antioxidative enzymes such as ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD), was observed in the green microalga *Scenedesmus bijugatus* [187]. Another symptom of Cu-stress was glutathione (GSH) oxidation, expressed as decreased ratio of GSH/GSSG, reported for different algal taxons [160, 188, 189]. A similar effect was evidenced in Hg-stressed green algae *Chlorogonium elongatum* [190] and *Tetraselmis tetrahele* [191]. In the Ni-stressed *Scenedesmus acutus* f. *alternans*, there were significant differences in the expression of oxidative stress between Ni-tolerant and Ni-sensitive algal strains: only in the sensitive strain, Ni induced a decrease of GSH/GSSG ratio [192]. The enzymes of glutathione cycle and metabolisms like γ-glutamylcysteine synthetase (γ-GCS), glutathione synthetase (GS), glutathione disulfide reductase (GSSG-R), GSH-transferase (GST) and GSH-peroxidase (GSH-PX) are involved in glutathione-mediated alleviation of metal stress in algae. In *Scenedesmus bijugatus* Cu stress increased the activities of γ-GCS, GST and GSH-PX and decreased the activity of GSSG-reductase [193]. Toxic effects of nonessential elements like Pb, Cd, and Hg are related to their ability to promote oxidative stress at subcellular levels. Hg and Cd can disrupt electron transport in both respiration and photosynthesis, thus generating ROS in *Euglena gracilis* [194]. In the marine alga *Dunaliella tertiolecta*, dose-dependent stimulation of intracellular reactive oxygen species (ROS) production was also observed with Zn treatment [195], while the Cd-stressed cells of *Chlamydomonas reinhardtii* accumulated free proline acting as an antioxidant [196]. In a number of green algae, diatoms and dinoflagellates, the exposure to Cu, Cd, Hg and Pb caused increase of SOD activity [160, 197]. Fe-SOD and Mn-SOD, but not Cu/Zn-SOD isoforms were induced by pollutant metal treatment. Toxic metals are able to disturb the oxidative balance in chloroplasts of algae. Beside the increased activity of SOD, in the isolated chloroplasts of the unicellular alga *Gonyaulax polyedra*, chronically exposed to Hg, Cd, Cu and Pb, high activity of APX, high GSH content and decreased peridin (dinoflagellate carotenoid) level were observed [198]. In acute HM-stressed cells, increased levels of β-carotene and slightly increased activities of SOD and ascorbate peroxidase were detected. The changes in SOD activity were dependent on metal concentration and time of exposure. In addition, increased oxidative damage to proteins and lipids occurred mainly in the cells under acute stress. Pb was the most damaging toxicant, causing protein oxidation and lipid peroxidation. The increase of APX activity to prevent cell damage in the presence of Cu, Co, Pb and Ni was also reported in the green alga *Selenastrum capricornutum* [199]. For these reasons, heavy metals in a number of different ways may interrupt the normal metabolic processes and integrity of algal cells.

4.4. Cell exudates

Algae are known to produce extracellular organic substances under optimum conditions and increase this exudate production under stress. The production of exudates, which posses metal complexation capacity, is assumed as a potential self-

protective mechanism against heavy metal toxicity in algae [180, 200]. The composition of these compounds usually varies among species; however, generally, they are extracellularly released organic acids, amino acids, peptides, sugars, oligo- and polysaccharides [201]. For instance, Cd-exposed *Selenastrum capricornutum, Scenedesmus quadricauda* and *Chlorella kessleri* released many more proteins and carbohydrates than non-exposed cells [200]. Also Cu-exposed algae, belonging to different taxonomic groups, produced increased amounts of extracellular polysaccharides with parallel increase of Cu concentrations [180]. Furthermore, Corradi et al. [202] showed that strains of *Scenedesmus acutus*, having different sensitivity to Cr(VI), release exudates with different capacity of reducing Cr toxicity. Interestingly, the apparent detoxification capacity was higher in the exudates from the Cr-tolerant strain than those from the normal one. These detoxifying effects seem to be due to a specific Cr/algae/exudates interaction, which occurs when algae are subjected to Cr short-time stress [203].

4.5. PCs, metallothioneins and stress proteins in algae

When they accumulate excessively in the cytosol, both essential and unessential heavy metals (Cu, Zn, Cd, Pb, Ag, Hg, etc.) can induce in both eukaryotic macro- and microalgae [134, 138, 188, 204-207] synthesis of PCs of the general structure $(\gamma Glu\text{-}Cys)_nGly$, n= 2-9 [205, 208], and in some cases their des-Gly derivatives [132]. PC production was so far reported in many classes of algae, with exception of dinoflagellates [180]. Interspecies differences concerning both total levels of these peptides and particular oligomer concentrations have been reported too [134, 205, 209, 210]. Variability of PC production among species may rely on constitutive differences such as the size of cellular GSH pool, rate of metal uptake and mechanisms of metal sequestration in cells [195, 211]. In the green alga *Chlamydomonas reinhardtii* exposed to Cd, PCs were found in both cytosol and chloroplasts [212]. Hu and Wang [213] demonstrated that in *C. reinhardtii* two types of Cd-PC complexes are present: a high molecular weight (HMW) complex and a lower molecular weight complex (LMW). The accumulation of the LMW complex has been suggested as an early sign of Cd stress. PC production in algal cells was an early sign of stress also caused by Pb [132, 207].

In the case of the essential element Zn, no PC production was observed at low concentrations in the diatom *Phaeodactylum tricornutum* [211]. However, at higher Zn concentrations, which are toxic to most freshwater but not to marine algae, PCs were accumulated both in the freshwater alga *Stigeoclonium tenue* [138] and the marine alga *Dunaliella tertiolecta* [214]. When adapted to high Zn concentrations, *S. tenue* produced mainly (besides PCs) novel, PC-related peptides (with additional cystine residue in a molecule), which were analyzed by mass spectrometry [215]. Recently, PC synthesis was reported in *D. tertiolecta* also in response to non-toxic Ni concentrations [214]. Interestingly, in the Hg-exposed unicellular green alga *Tetraselmis tetrathele*, no PCs were synthesized; however, a nonthiol tripeptide Arg-Arg-Glu was found to be abundant [191].

In a number of HM-exposed higher plants, identification of metallothionein-like genes has been reported, but only a few metallothioneins have been characterized up to now in the plant kingdom [2]. Genes for metallotioneins are induced in Cd-stressed prokaryotic blue-green algae [216]. However, in the case of eukaryotic algae, a MT gene was identified for the first time in Cu-exposed marine brown alga *Fucus vesiculosus*. It encoded a protein product which bound Cd and Cu [217].

Little is known so far on stress proteins (also referred to as heat shock proteins, HSP) and ubiquitin induced in algae in response to heavy metals. Under adverse conditions, stress proteins are thought to counter proteotoxic effects by preventing the denaturation of proteins. For example, proteins synthesized in Cu-exposed dinoflagellatae *Prorocentrum micans* and having a MW of 25,000 Da were regarded as stress proteins [218]. In the green microalga *Raphidocelis subcapitata* a dose-dependent induction of HSP 70 synthesis, following exposure to Zn and Se, was reported [219]. HSP 70 expression was also increased with copper exposure of the green marine macroalga *Enteromorpha intestinalis,* however it was relatively insensitive biomarker of copper exposure compared to growth measurements [220].

REFERENCES

1. Sanità di Toppi, L., and Gabbrielli, R. (1999). *Environ. Exp. Bot.* 41,105.
2. Sanità di Toppi, L., Prasad, M.N.V., and Ottonello, S. (2002). Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants. Kluwer Academic Publishers, Dordrecht.
3. Cobbett, C.S., and Goldsbrough, P. (2002). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 53, 159.
4. Hall, J.L. (2002). *J. Exp. Bot.* 53, 1.
5. Pawlik-Skowrońska, B., Sanità di Toppi, L., Favali, M.A., Fossati, F., Pirszel, J., and Skowroński, T. (2002). *New Phytol.* 156,95.
6. Clemens, S. (2001). *Planta* 212, 475.
7. Cobbet, C.S. (2000a). *Plant Physiol.* 123, 825.
8. Cobbet, C.S. (2000b). *Curr. Opin. Plant Biol.* 3, 211.
9. Rauser, W.E. (1999). *Cell Biochem. Biophys.* 31, 19.
10. Grill, E., Winnacker, E.-L., and Zenk, M.H. (1985). *Science* 230, 674.
11. Anelli, G., Pelosi, P., and Galoppini, C. (1973). *Agr. Biol. Chem.* 37, 1579.
12. Pelosi, P., Galoppini, C. (1973). *Atti. Soc. Tosc. Sci. Nat. Mem.* 80, 215.
13. Bernhard, W.R. and Kägi, J.H.R. (1987). *Experientia* 52 (suppl.), 309.
14. Mutoh, N., and Hayashi, Y. (1988). *Biochem. Biophys. Res. Comm.* 151, 32.
15. Glaeser, H.A., Coblenz, R., Kruczek, I., Ruttke, A., Ebert-Jung, A. And Wolf, K. (1991). *Curr.Genet.* 19, 207.
16. Cobbett, C.S., May, M.J., Howden, R.,and Rolls, B. (1998). *Plant J.* 16, 73.
17. Grill, E., Winnacker, E.-L., and Zenk, M.H. (1987). *Proc. Natl. Acad. Sci. USA* 84, 439.
18. Murasugi, A., Wada, C., and Hayashi, Y. (1981). *J. Biochem.* 90, 1561.
19. Kotrba, P., Macek, T., and Ruml, T. (1999). *Collect. Czech Che. Commun.* 64, 1057.
20. Zenk, M.H. (1996). *Gene* 179, 21.
21. Delhaize, E., Jackson, P.J., Lujan, L.D., and Robinson N.J. (1989). *Plant Physiol.* 89, 700.
22. Scheller, H.V., Huang, B., Hatch, E., and Goldsbrough, P.B. (1987). *Plant Physiol.* 85, 1031.
23. Reese, R.N., and Wagner, G.J. (1987). *Plant Physiol.* 84, 574.
24. Steffens, J.C., Hunt, D.F., and Williams, B.G. (1986). *J. Biol. Chem.* 261, 13879.
25. Grill, E., Loeffler, S., Winnacker, E-L., and Zenk, M.H. (1989). *Proc. Natl. Acad. Sci. USA* 86, 6838.
26. Kondo, N., Isobe, M., Imai, K., and. Goto, T. (1983). *Tetrahed. Lett.* 24, 925

27. Ortiz, D.F., Kreppel, L, Speiser, D.M., Scheel, G., McDonald, G., and Ow, D.W. (1992).. *EMBO J.* 11,:3491.
28. Speiser, D.M., Ortiz, D.F., Kreppel, L., and Ow, D.W. (1992). *Mol. Cell. Biol.* 12, 5301.
29. Howden, R., Goldsbrough, P.B., Andersen, C.R., Cobbett, C.S., 1995. *Plant Physiol.* 107, 1059.
30. Ha, S.-B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., and Cobbett, C.S. (1999). *Plant Cell.* 11, 1153.
31. Vatamaniuk, O.K., Mari, S., Lu, Y.-P., and Rea, P.A. (1999). *Proc. Natl. Acad. Sci. USA.* 96, 7110.
32. Clemens, S., Kim, E.J., Neumann, D., and Schroeder, J.I. (1999). *EMBO J.* 18, 3325.
33. Oven, M, Page, J.E., Zenk, M.H., and Kutchan, T.M. (2002). *J. Biol. Chem.* 277, 4747.
34. Cazalé, A.C., and Clemens, S. (2001). *FEBS Lett.* 507, 215.
35. Clemens, S., Schroeder, J.I., and Degenkolb, T. (2001). *Eur. J. Biochem.* 268, 3640.
36. Vatamaniuk, O,K., Bucher, E.A., Ward, J.T., and Rea, P.A. (2001). *J. Biol. Chem.* 276, 20817.
37. Vatamaniuk, O.K., Mari, S., Lu, Y.P., and Rea, P.A . (2000). *J. Biol. Chem.* 275, 31451.
38. Chen, J., Zhou, J., and Goldsbrough, P.B. (1997). *Physiol. Plant.* 101, 165.
39. Jones, D.L. (1998). *Plant Soil* 205, 25.
40. Ryan, P.R., Delhaize, E., and Jones, D.L. (2001). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 527.
41. Kochian, L.V. (1995). *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 46, 237.
42. Herrera-Estrella, L.R., Guevara-García, A.A., and López-Bucio J. (2001). *Enc. Life Sci.*, 4 p. Nature Publishing Group/ www.els.net. 3.
43. Curl, E.A., and Trueglove, B. (1986). The Rhizosphere, Springer-Verlag, Berlin.
44. López-Bucio, J., Nieto-Jacobo, M.F., Ramirez-Rodriguez, V., and Herrera-Estrella, L. (2000). *Plant Sci.*160, 1.
45. Kidd, P.S., Llugany, M., Poschenrieder, C., Gunsé, B., and Barceló, J. (2001). *J. Exp. Bot.* 52, 1339.
46. Kollmeyer, M., Dietrich, P., Bauer, C.S., Horst, W.J., and Hedrich, R (2001). *Plant Physiol.* 126, 397.
47. Murphy, A.S., Eisinger, W.R., Shaff, J.E., Kochian, L.V., and Taiz, L. (1999). *Plant Physiol.* 121, 1375.
48. Cieśliński, G., Van Rees, K.C.J., Szmigielska, A.M., Krishnamurti, G.S.R., and Huang, P.M. (1998). *Plant Soil* 203, 109.
49. Yang, Y.-Y., Jung, J.-Y, Song, W.-Y., Suh H.-S., and Lee, Y. (2000). *Plant Physiol.* 124, 1019.
50. Cramer, M.D., and Titus, C.H.A. (2001). New Phytol.,152, 29.
51. Ma, J. F., Taketa, S., Yang Z.M. (2000). *Plant Physiol.*, 122, 687.
52. Larsen, P.B., Degenhardt, J., Tai, C-Y, Stenzler, L.M, Howell, S.H., and Kochian, L.V. (1998). *Plant Physiol.* 117, 9.
53. Piñeiros, M.A., and Kochian, L.V. (2001). *Plant Physiol.*, 125, 292.
54. de la Fuente, J.M., Ramírez-Rodríguez, V., Cabrera-Ponce, J.L., and Estrella, L.H. (1997). *Science* 276, 1566.
55. Delhaize, E., Craig, S., Beaton, C.D., Bennet, R.J., Jagadish, V., and Randall P.J. (1993a). *Plant Physiol.* 103, 685.
56. Delhaize, E., Ryan P.R., and Randall P.J. (1993b). *Plant Physiol.* 103, 695.
57. Basu, U., Godbold, D., and Taylor, G.J. (1994). *J. Plant Physiol.* 144, 747.
58. Ryan, P.R., Delhaize, E., Randall, P J. (1995a). *Planta* 196, 103.
59. Ryan, P.R., Delhaize, E., Randall, P J. (1995b). *Aust. J. Plant Physiol.* 22, 531.
60. Li, X.F., Ma, J.F., and Matsumoto, H. (2000). *Plant Physiol.* 123, 1537.
61. Ma, J.F., Zheng, S.J., Diradate, S., and Matsumoto, H. (1997). *Nature* 390, 569.
62. Zheng, S.J., Ma, J.F., and Matsumoto, H. (1998). *Plant Physiol.* 117, 745.
63. Pellet, D.M., Grunes, D.L., and Kochian, L.V. (1995). *Planta* 196, 788.
64. Yang, Z.M., Sivaguru, M., Horst, W. J., and Matsumoto, H. (2000). *Physiol. Plant.* 110, 72.
65. Silva, I.R., Smyth, T.J., Raper, C. D., Carter, T.E., and Rufty, T.W. (2001). *Physiol. Plant.* 112, 200.
66. Miyasaka, S.C., Buta, J.G., Howell, R.K., and Foy, C.D. (1991). *Plant Physiol.* 96, 737.
67. Ma, J.F., Zheng, S.J., and Matsumoto, H. (1997). *Plant Cell Physiol.* 38, 1019.
68. Schöttelndreier, M., Norddahl, M.M., Ström, L., and Falkengren-Grerup, U. (2001). *Ann. Bot.* 87, 769.
69. Ernst, W.H.O., Verkleij, J.A.C., and Schat, H. (1992). *Acta Bot. Neerl.* 41, 229.
70. Shier, W.T. (1994). *J. Toxicol.-Toxin Rev.* 13, 205.

71. Brooks, R.R. (1998). Plants That Hyperaccumulate Heavy Metals. R.R. Brooks (Ed.), CAB International, NY, USA.
72. Ernst, W.H.O. (1975). *Int. Conf. Heavy Metals in Environment,* Toronto, 121-136.
73. Mathys,W. (1977). *Physiol. Plant.* 40, 130.
74. Shen, Z.G., Zhao, F.J., and McGrath, S.P. (1997). *Plant, Cell Environ.* 20, 898.
75. Küpper, H., Zhao, F.J., and McGrath, S. (1999). *Plant Physiol.* 119, 305.
76. Jaffré, T., Brooks, R.R., Lee, J., and Reeves, R.D. (1976). *Science*, 193, 579.
77. Sagner, S., Kneer, R., Wanner, G., Cosson, J.P., Deus-Neumann, B., and Zenk, M.H. (1998). *Phytochemistry* 47, 339.
78. Lee, J., Reeves, R.D., Brooks, R.R., and Jaffré, T. (1977) *Phytochemistry* 16, 1503.
79. Still, E.R., and Williams, R.J.P. (1980). *J. Inorg. Biochem.* 13,35.
80. Raskin, I. (1996). *Proc. Natl. Acad. Sci. USA.* 93, 3164.
81. Pelosi, P., Fiorentini, R, and Galoppini, C. (1976). *Agr. Biol. Chem.* 40, 1641.
82. Brooks, R.R., Shaw, S., and Asensi Marfil, A. (1981). *Physiol. Plant.* 51, 167.
83. Mesjasz-Przybyłowicz, J., Balkwill, K., Przybyłowicz, W.J., and Annegarn, H.J. (1994). *Nucl. Instr. Meth. Phys. Res.* B89, 208.
84. Gabbrielli, R., Gremigni, P, Bonzi Morassi, L., Pandolfini, T, and Medeghini Bonatti, P. (1997). Écologie des Milieux sur Roches Uultramafiques at sur Sols Métallifères. T. Jaffré, R.D. Reeves, and T. Becquer (Eds.), ORSTOM, Noumea, NC.
85. Gabbrielli, R, and Gremigni, P. (1988). *Giorn. Bot. Ital.* 122 (suppl.), 168.
86. Psaras, G.K, Constantinidis, T., Cotsopoulos, B., and Manetas, Y. (2000). *Ann. Bot.* 86, 73.
87. Boyd, R.S., and Martens, S.N. (1992). The Vegetation of Ultramafic (Serpentine) Soils. A.J.M. Baker, J. Proctor, and R.D. Reeves (Eds.), Intercept, Andover.
88. Boyd, R.S. (1998). Plants That Accumulate Heavy Metals. R.R. Brooks (Ed.), CAB International, NY, USA.
89. Nieboer, E., and Richardson, D.H.S. (1980). *Environ. Pollut. Series B.* 1, 3.
90. Homer, F.A. (1991). PhD Thesis, University of Oxford, UK.
91. Pancaro, L., Pelosi, P., Vergnano Gambi, O., and Galoppini, C. (1978). *Giorn. Bot. Ital.* 112, 141.
92. Krämer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M., and Smith, J.A.C. (1996). *Nature* 379, 635.
93. Persans, M.W., Yan, X., Patnoc, J-M.M.L., Krämer, U., and Salt, D.E. (1999). *Plant Physiol.* 121, 1117.
94. Homer, F.A., Reeves, R.D., and Brooks, R.R. (1997). *Curr. Topics Phytochem.* 14, 31.
95. Sanità di Toppi, L., Favali, M.A., Gabbrielli, R., and Gremigni, P. (2001). Metals in the Environment – Analysis by Biodiversity, M.N.V. Prasad (Ed.), Marcel Dekker, NY.
96. Krämer, U., Smith, D., Wenzel, W.W., Raskin, I., and Salt, D.E. (1997). *Plant Physiol.* 115,1641.
97. Krämer, U., Pickering, I.J., Prince, R.C., and Salt, D.E. (2000). *Plant Physiol.* 122, 1343.
98. Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., and Pickering, I.J. (1999). *Environ. Sci. Technol.* 33, 713.
99. Brune, A., Urbach, W., and Dietz, K.-D. (1994). *Plant, Cell Environ.* 17, 153.
100. van de Geijn, S.C., and Petit, C.M. (1978). *Planta* 138, 145.
101. Senden, M.H.M.N., Van Paassen, F.J.M., Van der Meer, A.J.G.M., and Wolterbeek, H.T. (1992). *Plant, Cell Environ.* 15, 71..
102. Senden, M.H.M.N., Van der Meer, A.J.G.M., Verburg, T.G., and Wolterbeek, H. T. (1994). *J. Exp. Bot.* 45, 597.
103. Krotz, R.M., Evangelou, B.P., and Wagner, G.J. (1989). *Plant Physiol.* 91, 780.
104. Ma, J.F., Syuntaro, H., and Matsumoto H. (1998). *Plant Physiol.* 117, 753.
105. White, M.C., Decker, A.M., Chaney, R. L. (1981). *Plant Physiol.* 67, 292.
106. Cataldo, D.A., McFadden, C., Garland, T.R., and Wildung, R.E. (1988). *Plant Physiol.* 86, 734.
107. Bebot-Brigaud, A., Dange, C., Fauconnier N., and Gérard, C. (1999). *J. Inorg. Biochem.* 75, 71.
108. Marschner, H. (1995). Mineral Nutrition of Higher Plants, (2nd edition), Academic Press, London, UK.
109. Lott, J.N.A., and West, M.M. (2001). *Can. J. Bot.* 79, 1292.
110. Lott, J.N.A., Greenwood, J.S., and Batten, G.D. (1995). Seed Development and Germination. J. Kigel and G. Galili (Eds.), Marcel Dekker, Inc., New York, Basel, Hong Kong.
111. Hatzack, F., Hübel, F., Zhang, W., Hansen, P.E., and Rasmussen, S.K. (2001*). Biochem. J.* 354, 473.

112. Evans, W.J., and Martin, C.J. (1991). *J. Inorg. Chem.* 41, 245.
113. Vansteveninck, R.F.M., Babare, A., Fernando, D.R., and Vansteveninck, M.E. (1993a). *Plant Soil* 156, 525.
114. Vansteveninck, R.F.M., Babare, A., Fernando, D.R., and Vansteveninck, M.E. (1993b). *Plant Soil* 157, 457.
115. Vansveveninck, R.F.M., Babare, A., Fernando, D.R., and Vansveveninck, M.E. (1994). *Plant Soil* 167, 157.
116. Vázquez, M.D., Poschenrieder, C., Barceló, J., Baker, A.J.M., Hatton P., and Cope G.H. (1994). *Bot. Acta.* 107, 243.
117. Vansteveninck, R.F.M., Vansteveninck, M.E., and Fernando, D.R. (1992). *Plant Soil* 146, 271.
118. Turnau, K., Kottke, I., and Oberwinkler, F. (1993). *New Phytol.* 123, 313.
119. Cotter-Howells, J.D., Champness, P.E., and Charnock, J.M. (1999). *Miner. Mag.* 63, 777.
120. Baccini, P., Ruchti, J., Wanner, O., and Grieder, E. (1979). *Swiss J. Hydrol.* 41, 202.
121. Gächter, R., and Mares, A. (1979). *Swiss J. Hydrol.* 41, 22.
122. Revis, N.J.P., Merks, A.G.A., Valenta, P., and Rützel, H. (1989). *Chem. Spec. Bioavail.* 1, 31.
123. Rand, G.M., and Petrocelli, S.R. (1985). Fundamentals of Aquatic Toxicology: Method and Applications, G.M. Rand and S.R. Petrocelli (Eds.), Hemisphere Publishing, New York.
124. Schäfer, H., Hettler, H., Fritsche, U., Pitzen, G., Röderer, G., and Wenzel. (1994). *Ecotox. Environ.* 27, 64.
125. Skowroński T. (1988). *Post. Mikrob.* 27, 77.
126. Genter, R.B. (1996). Algal Ecology – Freshwater Benthic Ecosystems, R.J. Stevenson, M.L. Bothwell, R.L.Lowe (Eds.), Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Tokyo, Toronto.
127. Pawlik- Skowrońska, B., and Skowroński, T. (1996). *Wiad. Bot.* 40, 170.
128. Campbell, P.G.C., and Stokes, P.M. (1985). *Can. J. Fish. Aquat. Sci.* 42, 2034.
129. Skowroński, T. (1986). *Appl. Microb. Biotech.* 24, 423.
130. Skowroński, T., Szubińska, S., Pawlik, B., Jakubowski, M., Bilewicz, R., and Cukrowska, E. (1991). *Environ. Pollut.* 74, 89.
131. Franklin, N.M., Stauber, J.L., Markich, S.J., and Lim, R.P. (2000). *Aquat. Toxicol.* 48, 275.
132. Pawlik - Skowrońska, B. (2002). *Environ. Pollut.* 119, 119.
133. Skowroński, T. (1984). *Chemosphere.* 13, 1385.
134. Skowroński, T., De Knecht, J.A., Simons, J., and Verkleji, J.A.C. (1998). *Eur. J. Phycol.* 33, 87
135. Gorbi, G., Corradi, M.G., Invidia, M., and Bassi, M. (2001). *Ecotox. Environ. Saf.* 48, 36
136. Morrison, G.M.P., and Florence, T.M. (1989). *Electroanalysis* 1, 107.
137. Skowroński, T., Szubińska S., Jakubowski M., and Pawlik B. (1992). *Environ. Pollut.* 76,163.
138. Pawlik- Skowrońska, B. (2001). *Aquat. Toxicol.* 52, 241.
139. Reinfelder, J.R., and Chang, S.I. (1999). *Environ. Sci. Technol.* 33, 1860.
140. Errecalde, O., and Campbell, P.G.C. (1998). *J. Phycol.* 36, 473.
141. Fortin, C., and Campbell, P.G.C. (2001). *Environ. Sci. Technol.* 35, 2214.
142. Gadd, G.M. (1990). *Experientia* 46, 834.
143. Wong, S.L., Nakamoto, L., and Wainwright, J.F. (1997). *Arch. Environ. Contam. Toxicol.* 32, 358.
144. Pawlik-Skowrońska, B., Kaczorowska, R., and Skowroński, T. (1997). *Environ. Pollut.* 97, 65.
145. Pawlik-Skowrońska, B., Kaczorowska, R., and Skowroński, T. (1998). *Ocean. Stud.* 1, 79.
146. Mason, R.P., Reinfelder, J.R., and Morel, F.M.M. (1996). *Environ. Sci. Technol.* 30, 1835.
147. Maguire, R.J., Wong, P.T.S., and Rhamey, J.S. (1984). *Can. J. Fish.Aquat. Sci.* 41, 537.
148. Huang, G., Bai, Z., Dai, S., and Xie, Q. (1993). *Appl. Organomet. Chem.* 7, 373.
149. Avery, S.V., Codd, G.A., and Gadd, G.M. (1993). *Appl. Microbiol. Biotechnol.* 39, 812.
150. Pawlik – Skowrońska, B., and Skowroński, T. (2001). Metals in the Environment – Analysis by Biodiversity, M.N.V. Prasad (Ed.), Marcel Dekker, Inc., New York.
151. Van Assche, F., and Clijsters, H. (1990). *Plant Cell Environ.* 13, 195.
152. De Filippis, L.F., Hampp, R., and Ziegler, H. (1981). *Arch. Microbiol.* 128, 407.
153. Devriese, M., Tsakaloudi, V., Garbayo, I., León, R., Vílhez, C., and Vigara, J. (2001). *Plant Physiol. Biochem.* 39, 443.
154. Demidchik, V., Sokolik, A., and Yurin, V. (2001). *Planta* 212, 583.
155. Abdel-Basset, R., Issa, A.A., and Adam, M.S. (1995). *Photosynthetica* 31, 421.
156. Pawlik, B., Skowroński, T., Ramazanov, Z., Gardeström, P., and Samuelsson, G. (1993). *Environ. Exp. Bot.* 33, 331.

157. Kremer, B., and Markham, J.W. (1982). *Z. Pflanzenphysiol.* 108, 125.
158. Sunda, W.G., and Huntsman, S.A. (1998). *Limnol. Oceanogr.* 43, 1055.
159. Takamura, N., Kasai, F., and Watanabe, M.M. (1989). *J. Appl. Phycol.* 1, 39.
160. Rijstenbil, J.W., Derksen, J.W.M., Gerringa, L.J.A., Poortvliet, T.C.W., Sandee, A., van den Berg, M., van Drie, J., and Wijnholds, J.A. (1994). *Mar. Biol.* 119, 583.
161. Vavilin, D.V., Ducruet, J.M., Matorin, D.N., Venrdiktov, P.S., and Rubin, A.B. (1998). *J. Photochem. Photobiol.* 42, 233.
162. De Filippis, L.F., Hampp, R., and Ziegler, H. (1981a). *Z. Pflazenphysiol.* 101, 37.
163. Skowroński, T., Jakubowski, M., and Pawlik, B. (1985). *Acta Microbiol. Polon.* 34, 309.
164. Küpper, H., Setlik, I., Spiller, M., Küpper, F. C., and Prasil, O. (2002). *J. Phycol.* 38,429.
165. De Filippis, L.F., Hampp, , R., and Ziegler, H. (1981b). *Arch. Microbiol.* 128, 407.
166. De Filippis, L.F., and Pallaghy, C.K. (1976). *Z. Pflazenphysiol.* 78, 314.
167. Poskuta, J.W., Parys, E., and Romanowska, E. (1996). *Acta Physiol. Plant.* 18, 165.
168. Angadi, S.B., and Mathad, P. (1998). *J. Environ. Biol.* 19, 119.
169. Pawlik, B., and Skowroński, T. (1994). *Environ. Exp. Bot.* 34, 225.
170. Corradi, M.G., and Gorbi, G. (1993). *Ecol. Environ. Saf.* 25, 72.
171. Skowroński, T., Jakubowski, M., and Pawlik, B. (1985). *Acta Microbiol. Polon.* 34, 309.
172. Visviki, I., and Rachlin, J.W. (1994). *Arch. Environ. Contam. Toxicol.* 26, 154.
173. Nassiri, Y., Ginsburger-Vogel, T., Mansot, J.L., and Wéry, J. (1996). *Biol. Cell* 86, 151.
174. Rijstenbil, J.W., and Gerringa, L.J.A. (2002). *Aquat. Toxicol.* 56, 115.
175. Talarico, L. (2002). *Environ. Pollut.* 120, 813.
176. Wong, S.L., Nakamoto, L., and Wainwright, J.F. (1994). *J. Appl. Phycol.* 6, 405.
177. Xylander, M., Fischer, W., and Braune, W. (1998). *Bot. Acta*, 111, 467.
178. Okamoto, O.K., Asano, C.S., Aidar, E., and Colepicolo, P. (1996). *J. Phycol.*, 32, 74.
179. Filho, A.G.M., Karez, C.S., Andrade, L.R., Yoneshigue-Valentin, Y., and Pfeiffer, W.C. (1997). *Ecotox. Environ. Saf.* 37, 223.
180. Pistocchi, R., Mormile, A.M., Guerrini, F., Isani, G., and Boni, L. (2000). *J. Appl. Phycol.* 12, 469.
181. Tönqvist, L. (1989). *Environ. Exper. Bot.* 29, 457.
182. Alessa, L., and Oliveira, L. (2001). *Environ. Exper. Bot.* 45, 205.
183. Harding, J.P.C., and Whitton, B.A. (1977). *Br. Phycol. J.* 12, 17.
184. Pawlik-Skowrońska, B. (2003). *Aquat. Bot.* 75, 189.
185. Chung, I.K., and Brinkhuis, B.H. (1986). *Mar. Pollut. Bull.* 17, 213.
186. Garman, G.D., Pillai, M.C., and Cherr, G.N. (1994). *Aquat. Toxicol.* 28, 127.
187. Nagalakshmi, N., and Prasad, M.N.V. (1998). *Bull. Environ. Contam. Toxicol.* 61, 623.
188. Stauber, J.L., and Florence, T.M. (1986). *Aquat. Toxicol.* 8, 223.
189. Rijstenbil, J.W., Haritonidis, S., Malea, P., Seferlis, M., and Wijnholds, J.A. (1998). *Hydrobiol.* 385, 171.
190. Agrawal, S.B., Agrawal, M., Lee, E.H., Kramer, G.F., and Pillai, P. (1992). *Environ. Exper. Bot.* 32, 145.
191. Satoh, M., Karaki, E., Kakehashi, M., Okazaki, E., Gotoh, T., and Oyama, Y. (1999). *J. Phycol.* 35, 989.
192. Randhawa, V. K., Zhou, F.Z., Jin, X. L., Nalewajko, C., and Kushner, D.J. (2001). *Can. J. Microb.* 47, 987
193. Nagalakshmi, N., and Prasad, M.N.V. (2001). *Plant Sci.* 160, 291.
194. Tschiersch, H., and Ohmann, E. (1993). *Planta* 191, 316.
195. Tsuji, N., Hirayanagi, N., Iwabe, O., Namba, T., Tagawa, M., Miyamoto, S., Miyasaka, H., Takagi, M., Hirata, K., and Miyamoto, K. (2003). Phytochemistry 62, 453.
196. Siripornadulsil, S., Traina, S., Verma, D.P.S., and Sayre R.T. (2002). *Plant Cell.* 14, 2837.
197. Okamoto, O.K., and Colepicolo, P. (1998). *Comp. Biochem. Physiol.* 119C, 67.
198. Okamoto, O.K., Pinto, E., Latorre, L.R., Bechera, E.J., and Colepicolo, P. (2001). *Arch. Environ. Contam. Toxicol.* 40, 18.
199. Sauser, K.R., Liu, J.K., and Wong, Tit-Yee. (1997). *BioMetals* 10, 163.
200. Maršálek, B., and Rojíčková, R. (1996). *Z. Naturforsch.* 51c, 646.
201. Lombardi, A.T., and Vieira, A.A.H. (2000). *Phycol.* 39, 118.
202. Corradi, M.G., Gorbi G., Morsi-Abd-El-Monem, H., Torelli, A., and Bassi M. (1998). *Chemosphere* 37, 3019.
203. Gorbi, G., Corradi, M.G., Invidia, M., Rivara, L., and Bassi, M. (2002). *Wat. Res.* 36, 1917.

204. Hu, S., and Wu, M. (1998). *Mol. Mar. Biol. Biotechnol.* 7, 97.
205. Gekeler, W., Grill, E., Winnacker, E.L., and Zenk, M.H. (1988), Arch. Microbiol., 150, 197.
206. Ahner, B., and Morel, F. (1999). Progress in Phycological Research, F.E. Round, D.J. Chapman (Eds.), Biopress Ltd, 13, 1.
207. Pawlik – Skowrońska, B. (2000). *Aquat. Toxicol.* 50, 221.
208. Torres, E., Cid, A., Fodalgo, P., Herrero, C., and Abalde, J. (1997). *Aquat. Toxicol.* 39, 231.
209. Wikfors, G.H., Neeman, A., and Jackson, PJ. (1991). *Mar. Ecol. Prog. Ser.* 79, 163.
210. Ahner, B.A., Kong, S., and Morel, F.M.M. (1995). *Limnol. Oceanogr.* 40, 649.
211. Morelli, E., and Scarano, G. (2001). *Mar. Environ. Res.* 52, 383.
212. Nagel, K., Adelmeier, U., and Voigt, J. (1996). *J. Plant. Physiol.* 149, 86.
213. Hu, Z.A., and Wang, H.X. (2001). *Acta Bot. Sin.* 43, 111.
214. Hirata, K., Tsujimoto, Y., Namba, T., Ohta, T., Hirayanagi, N., Miyasaka, H., Zenk, M.H., and Miyamoto, K. (2001). *J. Biosci. Bioeng.* 92, 24.
215. Pawlik-Skowronska, B. (2003) *Aquatic Toxicol.* 62, 155.
216. Robinson, N.J., Tommey, A.M., Kuske, C., and Jackson, P.J. (1993). *Biochem. J.* 295, 1.
217. Morris, C.A., Nicolaus, B., Sampson, V., Harwood, J.L., and Kille, P. (1999). *Biochem.J.* 338, 553.
218. Lage, O.M., Parente, A.M., Vasconcelos, M.T.S.D., Gomes, C.A.R., and Salema, R. (1996). *J. Phycol.* 32, 416.
219. Bierkens, J., Maes, J., and Plaetse, F.V. (1998). *Environ. Pollut.* 101, 91.
220. Lewis, S., Donkin, M.E., and Depledge, M.H. (2001). *Aquat. Toxicol.* 51, 277.

CHAPTER 7

PLANT RESPONSE TO ELEVATED CARBON DIOXIDE

Z. TUBA[a], A. RASCHI[b], G.M. LANINI[b], Z. NAGY[a], L. HELYES[a], D. VODNIK[c] AND L. SANITÀ DI TOPPI[d]

[a]*Department of Botany and Plant Physiology, Faculty of Agricultural and Environmental Sciences, Szent Istvan University, 2103 Gödöllõ, Hungary*
[b]*CNR-IBIMET, Piazzale delle Cascine 18, 50100 Florence, Italy*
[c]*Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1001, Ljubljana, Slovenia*
[d]*Department of Evolutionary and Functional Biology, Section of Plant Biology, University of Parma, viale delle Scienze 11/A, 43100, Parma, Italy*

Abstract. In consequence of the current increase in atmospheric CO_2 concentration, a large research effort has been devoted to clarifying the response of plants and ecosystems to this peculiar aspect of global change. The results have often been contradictory, also in consequence of the different experimental techniques. Through recent years, attention has moved from single physiological processes, often investigated in artificial environments with the risk of experimental artefacts, to the analysis of whole ecosystems, examined, as far as possible, in their natural conditions.The chapter reviews the technical development in fumigation facilities, and the main experimental results, focusing in particular on forests and grasslands, including lichens and mosses. The response of glasshouse grown horticultural plants is also outlined. Finally, attention is devoted to the effects of elevated CO_2 on soil and hypogeous growth, mineral nutrition and soil microbial populations; on these topics, many questions are still open, and research appears to be promising.

L. Sanità di Toppi and B. Pawlik-Skowronska (eds.), Abiotic Stresses in Plants, 157-204.

1. INTRODUCTION

The atmospheric CO_2 concentration has increased from about 280 ppm to 360 ppm in the last two centuries [1]. The longest continuous record on atmospheric CO_2 concentration, taken throughout the last 44 years at the Mauna Loa Observatory, shows a yearly increase, with higher concentrations in winter and lower concentrations in summer months. An increasing concern exists about the possible impact on climate by this phenomenon: high atmospheric CO_2 concentrations, together with the increased concentrations of other radiatively active gases, are expected to cause a global warming, by trapping the long wave radiation emitted by the Earth. It is worth noticing that a warning on global warming is found in Arrhenius works [2], dating back to the end of the 19^{th} century; so, if the general concern is recent, the debate is not, and it is still far from its conclusion. Beside the climatic effect, a direct effect of elevated CO_2 is expected; it is obvious that, in a CO_2 richer atmosphere, photosynthesis will be affected; yet, the interaction with other environmental factors (in particular, water and nutrient availability, soil and air temperature, light), the different fate of the extra carbon assimilated (different growth, respiration, addition to soil), the difficulty in discriminating long term adaptive effects and short term acclimation, often affecting the experiments, and finally the typically species-specific response evidenced in many experimental studies, make any predictive summarization extremely difficult [3]. In fact, plants often experience, during their lifespan, different CO_2 concentrations. Atmospheric concentration itself can be much lower than average in grasslands and crops exposed to intense radiation, in particular in the absence of wind, as a result of intense photosynthesis, and it can increase in the early morning, due to soil and plant respiration; this phenomenon is particularly evident at the soil level (A. Raschi, unpublished). Variations up to 300 ppm between day and night in sunny summer days were detected in apple orchards, with a maximum (up to 632 ppm) measured between 2.00 a.m. and 4.00 a.m., and a minimum (down to 328 ppm) measured in the afternoon between 2.00 p.m. and 4.00 p.m. [4]. Variations in aerenchima CO_2 concentrations throughout the day have also been reported, and these can influence the photosynthetic patterns [5, 6]. Other differences are linked to some anatomical and physiological aspects, and are less evident: atmospheric CO_2 enters the leaf through a series of resistances, which may vary in the different stages of development, in relation to plant anatomy and physiology, and to canopy structure. It has been noticed [3] that different CO_2 concentrations may be "seen" by Rubisco in different species, and at different ages of the leaf. In particular, mesophyll or internal leaf conductance can be very low in some species [7], causing low CO_2 concentrations inside the chloroplasts.

In long-term elevated atmospheric CO_2 concentration cannot be considered unambiguously beneficial for natural vegetation and field grown agronomic plants

but rather a profound change in a source the vegetation and outdoor agronomic plants have to acclimate to; and, indirectly, in interaction with other environmental factors it can appear as a stress effect. Further, under current atmospheric CO_2 concentration vegetation and field grown agronomic plants continuously have to face environmental constrains (limited resources, extreme temperatures, herbivores, pollution, fire, etc.), which occur as naturally induced stress effects [8, 9]. On the other hand, there are many indications that plants may generally cope better with various environmental stresses at a higher CO_2 concentration. Thus elevated CO_2 can be considered as an indirect constrain and as a stress alleviator.

Many experimental evidences support the view, that plants growing under natural conditions will not receive direct beneficial effects by elevated CO_2. In the shorter term the effect of CO_2 is mediated by the photosynthesis. However, in the longer term the effect of elevated CO_2 becomes more and more indirect and is mediated by source-sink interactions within plants, resources (nutrients, water), temperature, microbials, herbivores an land use/management practice [10].

As the growth of plants is modified, their competition for natural resources is modified as well. Not only root growth, according to many authors, is increased under elevated CO_2 (see below), but the different above ground response of different species and genotypes [11, 12], may also lead, in the long term, to different responses in competition for light and to different microclimatic conditions. Even in agricultural plant production systems some but far not all of the environmental constraints can be avoided or their limiting effects can be reduced. Horticultural greenhouses are the only plant production system where one can exclude or minimize the effects of all limiting constrains using radiation, temperature, nutrient, water, weed, pest control, etc. Therefore greenhouse plant production represents a system which can provide useful information on plants growing under elevated CO_2 and unlimited environmental conditions. Indeed, it is true that historically the first information about plants under high CO_2 concentrations was obtained from greenhouse cultivation. Although these data are not really relevant to assess the response of ecosystems to global change , they are important in the studying and understanding of the longer term direct effects of elevated CO_2 under less or (un)stressed conditions.

To fully understand the plant and ecosystem responses to elevated CO2, long term experiments conducted in realistic conditions are needed; yet we have to consider that, in any fumigation experiment, a system is submitted to a sudden increase in atmospheric CO_2 concentration; the adaptation of natural ecosystems took place during centuries, while atmospheric CO_2 concentration was progressively increasing. This difference may produce biased experimental results, yet its effects are far from being fully understood. Also the quick changes in atmospheric CO_2 concentration experienced by plants in fumigation experiments can cause species-specific responses, that make extrapolations difficult [13].

2. DIFFERENT FUMIGATION TECHNIQUES

2.1. Growth cabinets and enriched glasshouses

Glasshouse fumigation, aiming to enhance the productivity of horticultural plants, has been in use since the XIX century, and is now a well established practice. Although the inadequacy of controlled environment experiments in assessing the effect of a global CO_2 increase was soon recognised, for quite a long time, experiments on plant responses to elevated CO_2 were still conducted in growth cabinets and enriched glasshouses. This limited, in particular, the reliability of the results obtained on trees; those on crops and herbaceous species have also been critically reconsidered. It must be noticed that many of the earlier experiments were run on plants growing in artificial media, or on sterile substrates, while the interactions between plants and rhizosphere symbionts are known to have a large influence on the overall plant response to elevated CO_2. At the same time, most of the physiological studies, in particular on photosynthesis and respiration, were, and still are performed in individual-leaf chambers, within the laboratory, often at low light levels. Although it is well known that any chamber enclosing plant samples can potentially modify their response to environmental parameters [14], current experimental technology however does not provide less invasive tools, and some experimental artefacts have to be accepted, integrating the short time scale physiological experiments with plant growth tests to be performed in more realistic environments.

2.2. Open top chambers

The use of open top chambers (OTCs), to be placed in the field to enclose portions of ecosystems was the first step towards less artifactual experiments. OTCs [15, 16] were mainly developed for air pollution researches; a chamber is usually a cylindrical or polygonal metallic frame, fixed to the soil, supporting transparent plastic coverings, that encloses a volume of air; a frustum at the chamber's top may reduce the entering of the external air. Air is blown into the chamber by a fan, feeding a perforated pipe that encircles the base of the chamber; CO_2 is added to the air flow, using different tools to improve the mixing of pure CO_2 in the air flow. OTCs have been successfully used to fumigate herbaceous plants and small trees in experiments lasting even for years; although in OTCs the natural microclimatic conditions can be tracked with acceptable precision, yet they have helped only in part to overcome the problems mentioned above: in fact, the internal microclimatic conditions are usually influenced by the presence of plastic walls, that, although transparent, reduce the light intensity, and by the forced ventilation, inducing unnatural air turbulence; inside OTCs air temperature is often higher than in adjoining open spaces, and quite often temperature gradients between the center and

the walls are evident. Homogeneous rainfall distribution is often forbidden by the frustum, while runoff is blocked by the chamber's walls.

The control of an environmental variable is often in contrast to regulating another [15]: for instance, high air flow rates may reduce temperature, but expose the plants to the risk of desiccation. As a matter of fact, chambered plants exposed to ambient CO_2 often behave differently from those living in the open, and in many experiments an "unchambered control" was used to account for these differences; in fact, the chambered plants are exposed to other stimuli, apart of the fumigation treatment. Moreover, the area enclosed by the chambers is usually small, so that these tools are inadequate for ecosystem studies.

2.3. Branch bags

In this technique, a part of the crown of an adult tree is enclosed in a chamber or a bag of transparent plastic material, in which air flows similarly to OTCs. The technique is not new, having been used in the past to submit whole branches to different environmental conditions, or to measure their gas exchanges, using the chamber as a couvette; it has been used extensively in the last decade to fumigate forest trees [17, 18,]; as OTCs, branch bags are robust and simple, they allow inexpensive replicates and allow to study adult trees in their natural environment; the control of the micrometeo parameters displays the same limitations seen for OTCs. Moreover, when just a part of the crown is submitted to elevated CO2, the source-sink relationships are severely altered: nutrients can be allocated differently to the crown parts, in accordance to their conditions, in order to optimise their use. In this respect, the branch bag technique assumes a limited feedback between the branch and the trunk: this hypothesis, the so called branch autonomy hypothesis, is far from being fully verified. It has also been observed [19], that the whole plant feedbacks cannot fully occur if only a small portion of the plant is fumigated.

2.4. Free air fumigation

The need for bold and innovative large scale experiments, to be performed in more realistic conditions became evident very soon [20]; yet, the possibility of long lasting, free air fumigation experiments was for long time limited by the need for large amounts of gas through long time spans, and important investments in expensive equipments. The first FACE experiments were performed on crops with a relatively short life cycle. Only later, the increasing general awareness of the need for more detailed information on responses at the ecosystem level enabled the scientific communities to raise funds for huge, expensive fumigation systems to be applied on trees. Still it is worth noticing that these were used mainly on plantations, and that the response of natural forests is still largely unknown. As with OTCs, free air CO_2 enrichment (FACE) used methods introduced previously to expose plants to other gaseous pollutants [see 21 for a complete review]. The most usual FACE setup

consists of a circular array of vertical pipes, enclosing the experimental area, and emitting CO_2 enriched air toward it through emitter ports. The pipes are connected by on/off valves to a circular toroidal plenum, into which air is injected by a blower. Pure CO_2 is fed into the plenum based on a proportional-integral-differential (PID) algorithm which includes terms for wind speed, measured CO_2 concentration at the center of the array and various performance statistics. Emitter pipe valves are opened and closed according to wind direction, to emit gas from the upwind pipes [22]. FACE systems minimize other disturbances to the plant growth environment, apart from the elevation of CO_2 concentrations; the dimension of the fumigated area allows the performance of multidisciplinary studies. One of the major concerns with FACE technology is the spatial distribution of atmospheric CO_2 concentration within the rings: while short term spatial gradients are unavoidable, the presence of long term spatial gradients, which would result in different mean CO_2 concentrations throughout plant growth, must be avoided [23]. In general, control of CO_2 concentration is easier over a wide range of wind velocities, while it is more difficult in the absence of wind.

FACE systems ranging from 18 to 30 m in diameter have been built by the Brookhaven National Laboratory (BNL) [24, 25]; the system dimensions have been reduced (Mid-FACE: [23]) to find a compromise between the quality of temporal and spatial control, and the reduction of costs. MiniFACE systems represent a further simplification of the FACE concept, and were built for the fumigation of crops [26], and, in particular, of grasslands [27]. Each miniFACE ring consists of a horizontal and circular plenum resting on the soil; air is injected in it by a blower, and is vented through small holes from the plenum itself, or from vertical pipes, according to the crop's height. CO_2 injection is regulated by a series of flow controllers (one per ring), operated by a control algorithm using both CO_2 concentrations measured in the centre of the ring and wind speed data [24]. Ring diameters ranging from 1 to 2 m have been used so far; however, similar systems have been applied also to bigger fumigation rings and to polygonal fumigation arrays used in vineyards.

Recently, different groups in Japan [28] and Italy [29] have modified the design of their FACE systems to release pure CO_2 instead of CO_2-air mixtures, thus reducing the infrastructure costs and limiting further their impact on the system to be studied. The first results obtained are positive, although the control still suffers of some flaws under stable atmosphere.

2.5. CO_2 springs

In geothermal areas, natural CO_2 vents (also called CO_2 springs, or mofettes) are not uncommon; in some cases, they have exposed portions of terrestrial ecosystems to elevated CO_2 for centuries or even millennia. This offers the chance to study long term plant adaptations as well as interspecies competition and changes in ecosystem biogeochemistry. It cannot be denied that CO_2 springs are not perfect experimental tools, in consequence of fluctuations in CO_2 concentration, different concentration at

different heights, and lack of knowledge of past CO_2 concentrations and patterns of distribution: sometimes, in fact, the emission point varies its position because of natural events such as flooding or earthquakes, and the emission intensity can be modified as well. Moreover, in most of the gas vents the presence of sulphur pollutants can strongly affect plant response to elevated CO_2. CO_2 springs are unsuitable for establishing experimental plots to run experiments on crops, due to the variability of soil conditions. Although experiments on potted crop species [30] or transplanted plants [31] have been run in some CO_2 springs, the best way to use these facilities is to work on the existing vegetation, studying the long term adaptations and comparing the adaptive trends with those evidenced in shorter term experiments. From this point of view, CO_2 springs have provided information on many species, and can provide more. Most of the work, so far, has been conducted on a limited number of species in the Mediterranean area [32, 33, 34, 35] (Figures 1 and 2); more work has been carried out in Iceland [36], Slovenia (see below) and New Zealand [37]. There is no doubt that further research would find a number of other springs, suitable for research. In addition, a deeper investigation on sulphur polluted springs could provide interesting insights on long term adaptations to sulphur pollutants.

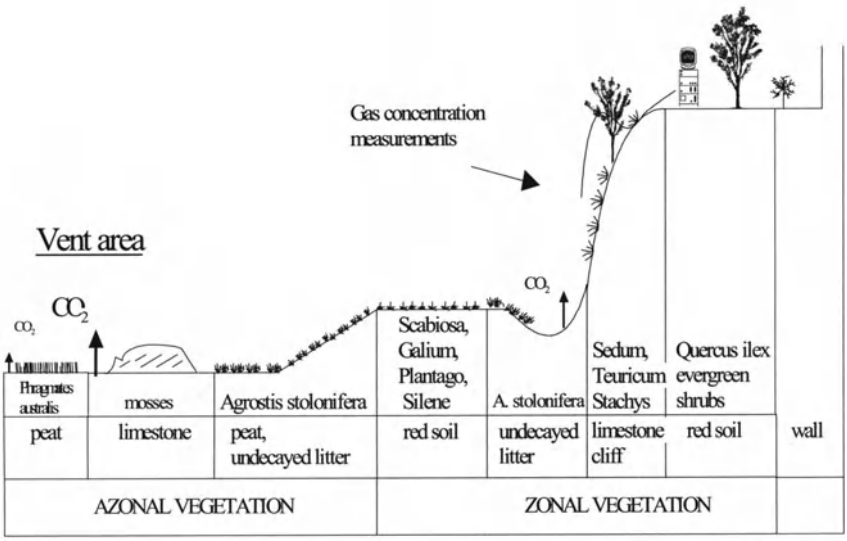

Figure 1. Cross-sectional diagram illustrating the relationship between topography and vegetation at the Bossoleto site (modified from [30]).

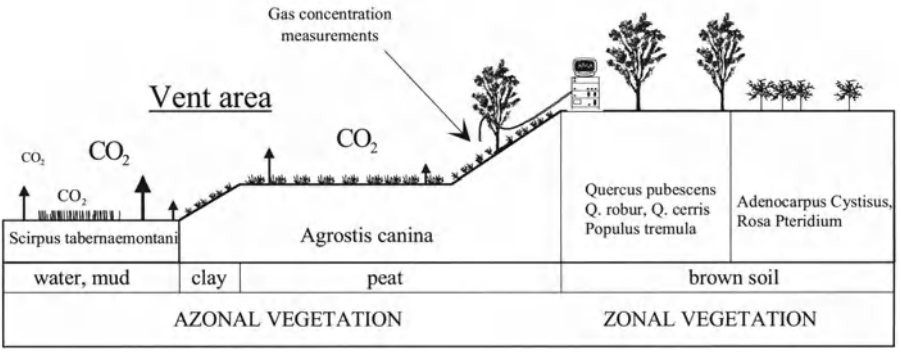

Figure 2. Cross-sectional diagram illustrating the relationship between topography and vegetation at the Solfatara site (modified from [30]).

Other interesting work has been done on the possible role of geothermal CO_2 emissions in the global carbon budget [38]; the study of this aspect is still in its initial stages.

In the following sections we give the overview of the effects of elevated atmospheric CO_2 on grasslands and their desiccation-tolerant cryptogamic lichen and moss components, as well as on forests. Grasslands were selected because after forests they are an important constituent of the Earth's vegetation and they are highly diverse with a very broad range of environmental conditions in all climate types, e.g. from the chronically infertile tropical desert type to the atlantic highly fertile, well watered ones without extreme temperature. Finally a brief overview of the CO_2 enriched horticultural plant responses is given.

3. GRASSLAND ECOSYSTEMS UNDER ELEVATED CO_2 CONCENTRATION

On a global scale grasslands cover about 24% of the Earth's vegetated area [39]. They occur over a very broad range of climatic and soil conditions and vary from natural grasslands to intensively managed sown pastures. Despite their importance, the potential effects of elevated air CO_2 on grasslands have received much less attention than effects on other ecosystems such as forests [40, 41].

Rising atmospheric CO_2 concentration is one of the major concerns when considering the possible effects of global climate change on grasslands. Central to many studies are questions of the future productivity of natural or semi-natural grassland ecosystems and the potential carbon storage capacity of grassland ecosystems [42]. It is estimated that on a global scale, the atmospheric carbon is recycled by the vegetation every 4-6 years [43]. Therefore an altered balance

between CO_2 uptake by photosynthesis and CO_2 emission by respiration in the biosphere could modify the upward trend of the increase in atmospheric concentration [1, 44]. Grasslands account for about 12 % of the total carbon storage in the terrestrial biosphere [43] and might therefore contribute significantly to the changes in carbon cycling [44].

Until the early 1990s the predictions of the effects of future elevated air CO_2 concentrations on grasslands were first of all based on studies carried out with single species. However, scaling from these results, usually obtained on plants growing in artificial substrates and controlled environments, to natural conditions in which competition or symbiosis may play a major role [45], can be often misleading. An attempt to predict the consequences of global change on grassland must therefore take into account both the direct effects of elevated CO_2 and the indirect effects mediated by climatic change and limited sources, as well as their interaction, bearing in mind the complexity of the natural ecosystems in which plants are growing [10].

New experimental data on grasslands and pastures considerably reduced the uncertainties about the effects of elevated CO_2 on production and to a lesser extent on botanical composition and forage quality of intensive grasslands in cool, wet climatic zones, but information on other grazed ecosystems is still limited [46]. Efforts should be concentrated in grasslands of humid/semi-arid margins, wet/dry seasonal and subtropical climates, which are the most sensitive and most subject to global change. Progress is especially needed in the study of the interactive effects of increased air CO_2 and climate change on species dynamics and soil nutrients in grazed pastures.

3.1. Production

Earlier reviews [47, 48] of experiments done under a wide range of conditions have shown that a doubling of atmospheric CO_2 from 330 to 660 ppm may increase the productivity of C_3 non-arborescent (like grassland) species by an average of 33%. However in the view of recent data, overall, grasslands seems to be less affected by higher CO_2 than was originally suggested. The effect of doubled elevated air CO_2 on above-ground average biomass production of grasslands is about +17% [46]. The recent study of Mooney et al. [49] indicates similar figures for the increase (14-16%) of above ground grassland biomass production. However, production responses can vary greatly seasonally and by system, and wide variations can occur in interactions between the effect of high CO_2 and effects of species difference, temperature [50], nitrogen [51, 52], defoliation [52] and water availability [53,54].

Primary production of grasslands has been found to increase in many studies under elevated CO_2 concentrations [55, 51] and much of this increase occurs below ground [55, 56]. The harvestable biomass of the grass swards grown under elevated CO_2 was found to be larger than that of the control swards in several cases [e.g.: 56, 57, 58, 59], but other studies have showed the harvestable (aboveground) biomass to decrease [e.g. 60]. The reason behind some of these observations is the increased allocation to the roots under the high CO_2 treatment.

In temperate xeric grassland in Hungary the total aboveground dry matter production in the high CO_2 stands increased by 15.1 % and in the atlantic perennial ryegrass swards in Ireland the total annual yield stimulations varied between 8 and 28 % year by year [61]. The relatively low increase of the aboveground stand production under elevated CO_2 may have been the consequence of the enhanced C-allocation to the roots. This is a typical high CO_2 allocation response in order to increase both the C-sink capacity and nitrogen uptake in high CO_2 exposed plants and grassland stands [48, 44]. In alpine environments the hypothesis of enhanced growth under elevated CO_2 is doubted [62, 63, 64]. At the same time increases in production of Northern European managed grasslands and agricultural systems are expected [65], although possible changes in the frequency of weather extremes may result in unpleasant surprises, in particular in relation to hardening and dehardening periods [66]. On the other hand, arctic tundra vegetation showed little positive response to elevated CO_2 and associated temperature increase, probably in consequence of lack of nutrients [67] and respiration increase [68]. Considering these responses, it seems that production of grasslands is expected to increase in situations where growth limitation by CO_2 is significant when compared to the other main factors such as nutrient availability or temperature. When one of the latter is seriously limiting plant growth, increased atmospheric CO_2 concentration is not expected to alleviate this limitation.

Although production has been found to increase in many cases, experiments have also indicated increased hazards for plant growth because of the increased frequency of weather extremes such as droughts, extreme temperatures, floods, etc. A greater response to CO_2 is often observed in dry years [54]. In water limited grasslands, elevated CO_2 results in greater water availability for longer during the growing season, therefore the hydrological and water economical consequences of elevated CO_2 in water limited habitats can be greater than the direct effect of high CO_2 on photosynthetic CO_2 assimilation and production [69, 53, 70].

Increased biomass allocation to the roots has also been shown to be caused by the N-limitation in other (tree) species with no CO_2 treatment [71]. Under elevated CO_2, species of Leguminosae have been shown to alleviate the N-limitation on the growth of the grass species [72].

Warmer temperatures increase the CO_2 response in warm-season grasslands such as short grass steppe and tall grass prairie [50]. However, in temperate grasslands, a few (3-4) °C increase in temperature may counterbalance the effect of elevated CO_2 on production [73, 74], or decrease productivity in summer but enhance it in early spring and late autumn [51].

Management practices can also counterbalance the effects of elevated air CO_2 on grasslands; therefore, this interaction should receive greater attention in the future [46]. In semiarid regions in Australia the elevated CO_2 would increase carbon stores by 7-17 %, but only in the absence of fire [75]. Thus, as Campbell et al. [46] stated, radical management changes, excluding grazing and fire, would be needed to obtain substantial carbon sequestration benefits, but this seems to be unlikely in normal practice. It was also shown that the management across the whole of Queensland, Australia, could alter the expected effects of climate change on grazing production at

a regional scale [76]. Defoliation (simulated grazing) substantially decreased the potential biomass gains resulting from the increased water use efficiency in response to elevated CO_2 [77].

The influence of plant biodiversity on the production response to elevated CO_2 and N supply was studied with a series of artificial grassland-like herbaceous community containing grasses, non N-fixing and N-fixing dicots [78]. The results have shown that communities with decreased diversity can acquire less biomass and carbon than plant assemblages with greater diversity. This is in good agreement with the earlier statement of Hunt et al. [10] that plant biomass productivity is severely constrained in plant communities limited by resources (water, nitrogen, phosphorus) and it seems likely that CO_2 effects may occur primarily through interactions with these limiting environmental factors and may take place without major increase in community productivity.

3.2. Below-ground processes and carbon storage

In order to understand the carbon and nitrogen cycling in grasslands under elevated CO_2 we need much more data on below-ground processes from long-term experiments. As the CO_2 concentration in soil is much higher than in the atmosphere, a direct effect may be excluded, while changes in root growth, rhizodeposition and root exudate composition may play a major role. While no effects of elevated CO_2 on bacterial number were found in soils in which C_4 plants occur [79], in soils containing C_3 plants, an increase in microbial biomass and microbial activity has often been reported, although the effects seem to vary throughout the season [80]. It is possible that an increase in the bacterial biomass may immobilise more available soil N, thus causing a shortage of available N for plants [81]. Under high CO_2 the cause of N-limitation on growth has been suggested to be a decreased N-mineralization rate [57] which, in turn, is imposed by the increased soil C:N ratio [72, 82]. Observation of increased growth with decreased elemental content of the dry matter [83] also support the idea of increased cycling of elements in plants under elevated air CO_2 concentration. Under elevated CO_2 the litter decomposition rates are predicted to be slower due to the increased C:N ratios of the leaves and litter [84]. However, recent findings suppose that the C:N ratios in litter are probably lower as it was hypothesized before, because of the reabsorption of nitrogen from the senescence leaf tissues, which in turn could allow a similar litter decomposition rate [49].

There is a close link between the carbon and nitrogen cycles which is supported by the finding that a decline in inorganic nitrogen availability in grass sward contributed to the increased below-ground allocation of the grass [72]. But changes in the soil carbon content are not only the result of the root litter production and decomposition as they are also influenced by root exudation and decomposition rate of the soil organic matter [44, 72]. In grazed or harvested grasslands two factors, the increased (first of all below-ground) litter production due to the increased root mass and the decreased litter quality (increased C:N and lignin:N ratios) contribute the

accumulation of carbon in the soil [85, 86, 44, 60]. It is to be noted, however, that N-content of the litter from a C_4 species is less influenced by elevated CO_2 than that of a C_3 species, with consequences on the N-mineralization rates in the soil [82].

Experiments aiming to assess the effect of elevated CO_2 on specific microbial populations have yielded contrasting results [80], but the differences appear to be linked to the plant species, the enrichment system used and the species of bacteria under investigation; similar contrasting results were reported for soil insect populations that were shown to increase in grasslands (L.Tosi, pers. comm.), both in OTC and in FACE (although with different patterns) but were not affected in soil under a cotton crop [87].

At least in temperate grasslands where the shoots are regularly removed by cut or grazing, carbon sequestration occurs mainly below-ground. Under elevated factors, such as the increased plant productivity, the increased translocation of photosynthates to roots and the decreased decomposability of roots and plant residues, can contribute to the enhanced below-ground carbon sequestration [88, 89, 90]. Despite of the above it is still unclear whether the world's grassland ecosystems could sequester a significant fraction of the increasing atmospheric CO_2. The importance of this question is underlined when the apparent problem of the 'missing sink' for carbon [89, 39] is seen alongside the relatively large proportion that the grassland vegetation contributes to the Earth's terrestrial ecosystems [91]. Increasing temperature and increased precipitation - more exactly, the increased frequency of soil wetting and drying - hasten the decomposition of soil organic matter [92]. Also, the frequency of weather extremes such as drought or flooding are expected to increase with climate change and this may have a serious impact on C-storage, depending upon the sensitivity of the ecosystem concerned. For example, natural grasslands consisting of species highly adapted to the prevailing climatic conditions will tend to respond more moderately in terms of production or changes in C-storage than intensively managed grasslands with introduced species [93].

3.3. Vegetation compositional change

Community structure is expected to change due to elevated CO_2 [94, 56, 10]. Changes in the botanical composition of grasslands, pastures and rangelands are expected to be significant [95], but whether the compositional changes are caused by elevated CO_2 or changes in climate is not well understood today.

Experimental data indicate that species composition change seems to be a key factor of grassland production and its agricultural value [46] and this compositional change can be especially important in drier grasslands [95]. Thus elevated air CO_2 and decreased water availability are predicted to increase grazed woodland thickening, woody shrub invasion and grass suppression, resulting also in decreased nutritive value [96, 95].

Differences between species' acclimation responses can be large and the species-specific responses [10, 97, 59] can cause alterations in species abundance which may lead to altered community structure and, as a consequence, changes of

ecosystem functioning in the long-term [98, 62, 63]. This would result in significant changes when considering the production of the whole community, although considerable changes in ecosystem functioning may occur [64], with enhanced C- [56, 99] and C- and N-turnover [72, 100].

The different physiological acclimation patterns (see Acclimation section) of the various species may have important compositional implications. For example the downward acclimation in *F. rupicola*, which is the dominant species of the temperate loess grasslands, indicates that its dominance in a future high CO_2 climate may be reduced and/or replaced by other, presently less abundant species due to their upward acclimation [101]. However, in the study by Newton et al. [56] the cover of the grasses declined irrespective of photosynthesis type, while that of a leguminous species (*Trifolium ssp.*) increased. This suggests that photosynthesis type is not the crucial factor in future changes of grassland communities, but the role of the apparent N-limitation on growth may become decisive. The apparently contrasting above results may be related to the interactive effects of elevated CO_2 and nitrogen supply mediated through photosynthesis and photosynthates translocation.

The suppressive effect of elevated CO_2 on C_4 species and their less responsiveness to high CO_2 cannot be considered universally observed. Growth responses of C_4 species to CO_2 concentration is similar to that of C_3 species when water shortage limits growth as it usually does in grasslands with C_4 species [102]. Further, in water limited grasslands, a greater availability of soil water due to the water conservation effect of elevated CO_2 may favour the growth of C_4 species later in the growing season [54]. An increase of warmer temperatures and the frequency of warm temperature events are predicted to stimulate the potential for invasion of (subtropical) C_4 species to warm/er temperate grasslands [103, 104, 46]. As a consequence, compositional change results in an increased invasion of C_4 species into temperate grasslands [104] may mean a greater response to temperature in these systems [46].

The outcome of the competition between species in plant communities can contribute significantly to the species composition of the assemblages. According to Campbell and Hunt [105] under elevated CO_2 there are an increased competitive advantage to the CO_2 responsive C_3 species (versus C_4 species), the legume and mycorrhizal species (in N-limited situations), and an increased intensity in the competition for light, water and nutrients as the consequence of the increased biomass production, root/mass allocation and resource capture. However the competitive advantage of C_3 plants in C_3-C_4 grasslands is not universal. Under elevated CO_2, C_4 species have been shown similarly or more responsive to CO_2 as C_3 ones in prairie grasslands [53, 106, 107]. In field microcosm experiments, elevated CO_2 has a significant effect on community composition only if it is accompanied by added nutrients (and/or increased temperature); the nutrient additions lead to much bigger gains in non- mycorrhizal species than in mycorrhizal ones [10].

Mainly in intensive grasslands, there is usually an increase in percentage cover of legumes under CO_2 enrichment. The increase averages about 10 % [46]. The

increase in the proportion of legumes is dependent on the rate of nitrogen fertilisation and the defoliation regime applied [52]. The outcome of the competition between grasses and legumes depends on the nitrogen availability. In FACE plots, the response of *Trifolium repens* to elevated CO_2 was larger that that of *L. perenne* [52]. This resulted in a higher proportion of *T. repens* in the mixed swards at elevated CO_2, although the competition for light among the different species and the management practices may largely counteract the effects of elevated CO_2. Further the predicted competitive advantage of the C_3 and N_2-fixing species is greatly altered by the amount of the available P and/or water.

4. ACCLIMATION RESPONSES

4.1. Photosynthetic acclimation

The effects of elevated air CO_2 on the role of the biosphere in the global carbon cycle depends very much on the altered balance between photosynthesis and respiration of individual plants, communities and ecosystems [44]. This underlines the significance of plant carbon-metabolism (photosynthesis and respiration) responses to elevated atmospheric CO_2.

Higher net photosynthetic rates have been observed in several cases as a result of elevated CO_2 treatment [see 108, for a review]. In fact, in C_3 plants that predominate in temperate climates, CO_2 is captured by the enzyme ribulose bisphosphate carboxylase/oxygenase (rubisco), which also catalyses the oxygenation of ribulose bisphosphate, a reaction that is competitively inhibited by CO_2. Under elevated CO_2, the competition for rubisco is reduced under light saturated conditions, while the photosynthetic quantum efficiency increases under light limiting conditions [109]. At the same time, dark respiration decreases due to the inhibition of the activity of two key enzymes, cytochrome c oxidase and succinate dehydrogenase [110]. However, since the ratio of shoot respiration to photosynthesis is more or less constant [89, 44], the daylight shoot or canopy carbon balance seems to be mainly influenced by the rate of CO_2 assimilation. Concerning the nocturnal shoot respiration under elevated CO_2, an increased rate was reported [111, 112, 44], while the rate of below-ground respiration was largely enhanced in elevated CO_2 and the relative rate of change of below-ground respiration was well correlated with that of gross CO_2 assimilation [44]. This reflected (partly) the control of photo-assimilate supply to the roots over below-ground respiration.

In several cases, species specific acclimation responses by the photosynthetic apparatus have been observed in long-term exposure experiments. Both upward and downward regulation was found in tree species [113] and in herbaceous grassland species [101, 114]. For example, xeric perennial grassland species showed differences in acclimation to elevated CO_2 concentration which varied from marked down-regulation in the grass species to upward regulation in the dicot species [101]. This would be expected to lead to changes in species competition and dominance as

atmospheric CO_2 concentration rises in the future (Vegetation compositional change section).

Downward regulation have been observed at biochemical level [108] with apparent feedback on the photochemical reaction rates [115]. Down regulation, among others, may be coupled to the decreased leaf N-content [116] on a weight basis. However, this change is cancelled out by the decreased specific leaf area, resulting in no change of leaf N-content as a consequence of the high CO_2 treatment on the leaf area basis [60]. The conflicting observations that high CO_2 stimulates net CO_2 uptake and production in some cases [57] but has no significant effect in others [117] may partially be related to species specific response [97, 59]. Elevated CO_2 can exert a positive influence in CO_2 x temperature interactions as has been found in studies on photosynthetic gas exchange and chlorophyll fluorescence [51, 118, 119, 115].

Although CO_2 assimilation in C_4 species is almost saturated at present day air CO_2 concentrations, there are indications that their CO_2 assimilation rate is stimulated in high air CO_2 environment, especially under high irradiation [120, 121, 107]. These studies also indicate that C_4 species benefit from elevated CO_2 levels first through the improved water use efficiency and secondly through the enhanced photosynthetic performance. In the long term CO_2 assimilation in the C_4 species e. g. *Bouteolua gracilis* also displays some acclimation responses, both upward and downward [107].

Gas exchange acclimation responses are influenced by the length of exposure to high CO_2 [108, 114]. Upward acclimation is reported for exposure periods as long as 8 years [122]. Downward acclimation is less probable to occur when sufficient sink strength is available [123]. The significance of the duration of exposure period to high CO_2 is shown by the changing acclimation of the investigated species over an extended period [114]. In *Lolium perenne*, the dominant species of the atlantic ryegrass grassland, the down-regulation is most strongly manifested towards the end of the re-growth periods following cutting [61]. Most of the evidences suggests that accumulation of water soluble carbohydrates or starch in the plants eventually leads to a down-regulation in photosynthetic potential which is also reflected in a decline in Rubisco activity.

Not only species but also canopies of grasslands show acclimation in their photosynthesis. Acclimation of photosynthesis in temperate grass swards showed down-regulation [44]. In general, down-regulation of CO_2 assimilation can be regarded as the most frequent acclimation type both in species and canopies, although complete down-regulation is not reached in all cases.

Studies of photosynthetic acclimation at natural CO_2 springs revealed inconsistent results [124, 125]. According to [124] photosynthetic down-regulation, in terms of photosynthetic capacity, carboxylation efficiency, and Rubisco content, is rarely detected, in spite of acclimation-promotive conditions, which are typical of spring sites [36, 124] (but see f.e. [36]). Yet, clear down regulation has been found in several C3 and C4 plant species at Stavešinci mofette [126,127,128]. *Phleum pratense* growing at different sites of mofette field showed a CO_2-exposure related photosynthetic response. Measurements of plants carefully selected according to the

soil [CO_2] within the rooting horizon revealed a strong correlation between [CO_2] and total plant height. Net photosynthesis, measured at 2000 ppm CO_2 and even more net photosynthesis measured at 350 ppm and 700 ppm CO_2 were higher in the "low-CO_2-grown" plants (0.4% CO_2 in the soil) than in the plants that were exposed to medium (3.3%) or high (26%) soil CO_2 levels during germination and growth. Carboxylation efficiency also drastically differed, being much lower in the high-CO_2-grown plants. Similar photosynthetic response was observed in other C3 (*Dactylis glomerata*, *Alopecurus pratensis*, *Juncus efussus*, *Solidago gigantea*) and C4 plant species (*Setaria pumila,* also in sown maize see [128]) and was generally accompanied with a decrease in leaf nitrogen. Also [36] reports that decrease in photosynthesis found in CO_2-spring grown *Nardus stricta* is related to nitrogen deprivation. Interestingly, analyses of ACi curves in *P. pratense* and *Echinochloa crus-galli* revealed a shift of CO_2 compensation concentration in response to high CO_2 regimes. This indicates that plants adjusted their photosynthetic carbon assimilation and respiration according to CO_2 exposure [126, 127, 128].

4.2. Acclimation of stomata and water relations

It is suggested that long-term exposure to high CO_2 causes a similar acclimation of stomatal regulation and transpiration to that of photosynthesis although transpiration rate in the upward acclimated plants is not increased [101].

One of the significant impacts of elevated CO_2 on plants is to reduce stomatal conductance [129]. It is currently assumed that stomatal aperture decreases with increasing CO_2, with little or no effect on photosynthesis [108], but with a substantial increase in WUE which is expected to range between 60 and 160 % under doubled atmospheric CO_2 concentrations [130]. This occurs consistently in all grassland species and will be of most importance in the summer drought conditions experienced in the temperate-continental and other in seasonally drier climates. It is possible that the greatest impact of elevated CO_2 will be on plant water relations and drought survival rather than on photosynthetic productivity under these summer drought conditions [61, 107]. However, this increase in WUE was found to be smaller in grasses than in herbs at high CO_2 concentration [131]. Higher WUE in the temperate loess grassland dicot species was due to their increased CO_2 assimilation rather than to a decrease in transpiration rate, since the latter were nearly the same in high CO_2 and ambient CO_2 plants. It was remarkable that the increase in WUE occurred at increased or unchanged rates of transpiration and also that the highest increase occurred in species with the highest increase in transpiration rate [61, 132]. At the same time the increased transpiration rate indicated an upward stomatal regulation of the species. Decreased stomatal conductance and increased WUE is documented as well in species with C_4 photosynthesis type [107].

Decrease in stomatal density appears to be a common response, but increases and no change have also been reported [133]. Grasses from CO_2 springs, certainly long acclimated to elevated CO_2, display a lower stomatal conductance with respect to control [35], while stomatal density is unaffected in most of the species. The

effect of stomatal response on the total stand water use may be negligible if LAI is significantly increased by elevated CO_2 [134], although there may be effects on the energy balance of the canopy. On the other hand, in forest trees there may be a substantial reduction in the leaf area per plant [123, 34], enhancing the effect of reduced stomatal conductance on canopy water use.

The protecting effect of elevated CO_2 against water stress under high temperatures [135] is related to the increased water use efficiency [101] and osmotic adjustment [136]. Under elevated CO_2 plants have a greater ability to withstand water stress which is usually related to the partial closure of stomata together with the occurrence of osmotic adjustment mechanisms [137]. In particular, increased concentrations of solutes in leaves growing under elevated air CO_2 are credited with the maintenance of higher relative water content (RWC) and turgor potential (Ψp) [136]. The occurrence of an osmotic adjustment under high CO_2 is reported by several authors in various plant species [138, 137, 139], while [140] found no osmotic adjustment, attributing their finding to the well-watered conditions. However, at present we have data on the occurrence of osmotic adjustment in well watered leaves under elevated CO_2 [136]. Greater retention of high water potential under elevated CO_2 has been reported for several C_3 and C_4 grasses [129, 141]. This behaviour may be related to a correspondent decrease in the stomata conductance, probably due to the partial closure of stomata under increasing atmospheric CO_2 concentrations [142]. The altered leaf and individual plant water relations can modify the stand level water relation responses [123].

4.3. Forage supply and quality

The compositional, production and physiological changes have consequences on the quantity and quality of forage produced in grasslands and this has also implications for food security [46]. In the more productive, intensively managed grasslands an increase in forage supply is predicted under elevated air CO_2; however, decrease in forage supply seems to be likely if regions become drier or temperatures become supra-optimal [46]. Conversely, increase in forage supply is also expected if rainfall and temperature will be favourable for increased production. Concerning forage quality under elevated air CO_2, reduction in leaf nitrogen content and increase in non-structural (starch and soluble sugars) carbohydrates concentration are frequently reported [84, 60, 143, 144, 145, 146]. Changes in cell wall composition of C_3 grasses growing in elevated CO_2 are usually small [147].

4.4. Scaling up from individual species to communities

Processes in grassland vegetation occur over a wide range of spatial and temporal scales; to predict the responses to global change, there is a need to scale upwards [148]. There may be general agreement that increasing global CO_2 concentrations and the changing climate will have direct physiological effects on grasslands, but the

duration of these effects and their impact at the level of the population and ecosystem is still relatively unknown. This is largely because most of the experimental work on grass responses to climate change has involved investigation of single plant responses over time periods of days to weeks. In order to understand how large geographical areas occupied by grasslands will respond to climate change, there is clearly a need to scale up from single plants and plots with dimensions in the order of 1 to 100 m^2, to patches (100-10000 m^2), to landscapes (1-100 km^2) and ultimately to regions (10000 km^2), as well as from relatively short-term to long-term time scales (years to centuries) [94]. The scaling process involves taking information at one scale and using it to derive processes at another scale [149, 150]. However, major problems with this approach are the non-linearity between processes and variables and the heterogeneities in properties that determine the rate of processes. Ultimately, however, the key to scaling is determining what to ignore. The object is not to analyse all of the smaller scale aspects of a process under observation, but to focus instead only on those that have direct importance to the scale under consideration [149]. Bridging the gap between site-level ecological understanding and global scale phenomena will probably be best achieved using a combination of remote sensing studies, the use of geographic information systems, and simulation modelling [151].

In order to reduce the range of predicted responses and to determine which models are most accurate it is necessary to collect appropriate measurements from grassland ecosystems that can confirm or validate the projections of the models. In particular, these include flux measurements of carbon and water exchange over large areas of major grassland types. Measurements over the seasonal cycle and, periodically, over several years to determine fluxes during years with different temperatures and amounts of precipitation are essential. These data can be used to determine whether measured carbon and water fluxes are consistent with values predicted by particular models [152].

Particular emphasis should be placed on carbon and nitrogen pool quantification over time and space. Experimental data suggest a close relationship between the stimulatory effect of elevated CO_2 on growth and long-term changes in soils carbon, nitrogen and water availability [153, 154, 155, 105]. The predictions of the Hurley pasture model are consistent with results of wide range of experiments. The Hurley pasture model predicted increased soil N pools under elevated CO_2, due to the increased rate of mineralisation, N uptake and yield, but only in fertile grasslands, whereas the increase in less fertile system would be very slow [154]. This model predicts that 50-100 years are required to reach equilibrium in less fertile grasslands [156]. Therefore, at present it is still a matter for further research to determine whether soil carbon storage capacity of grasslands as a whole will increase in the long term [157, 100].

5. GRASSLAND LICHENS AND MOSSES UNDER ELEVATED ATMOSPHERIC CO_2 CONCENTRATION

Plant communities in which desiccation-tolerant lichens and bryophytes are an important component of the photosynthesising biomass include arctic and alpine tundras, temperate, mediterranean and sub/tropical grasslands, and non-arborescent communities of arid and semi-arid habitats [158, 159]. For example, even the middle of Europe has areas where the relatively low and unevenly distributed yearly precipitation and the sandy soil with its small water holding capacity result in a semi-arid grassland where small poikilohydric lichens and, first of all, mosses contribute considerably to the total cover (20–80%), forming a poikilohydric mat vegetation. Therefore these plants undoubtedly play an important role in the function of these communities [160]. The lichens and mosses of grasslands (and of many other terrestrial communities) are desiccation tolerant, recovering normal metabolism on remoistening after losing 90-95% of the full-turgor water content of their cells [161, 162].

In order to quantify the carbon cycling of grassland ecosystems enriched with lichens and mosses and to predict their responses to elevated CO_2 concentration, we require information on the responses of lichens and mosses. Studying the effects of rising CO_2 concentration on grasslands without considering the responses of the lichens and mosses creates a large and crucial lacuna in our understanding. Despite this the potential effects of elevated air CO_2 on lichens and mosses have received much less attention than effects on normal vascular plants [94, 108]. Because the water relations and organisation of the photosynthetic tissues are often so different, and because the desiccation/rehydration cycle itself causes profound changes in the photosynthetic activity of these plants [163,164], the effects of increased atmospheric CO_2 concentration on their carbon balance and growth need not necessarily parallel those of non-DT plants [165] .

Soil growing lichens and mosses probably will be exposed to higher air CO_2 concentration as are the upward standing taller plants (see also Soil CO_2 concentration section). As global change also brings elevated temperature, soil and plant respiration are likely to increase, resulting in an even richer CO_2 supply to the soil growing lichens and mosses [166]; a possible counter-effect of elevated CO_2 in depressing soil respiration is not verified [167]. The fact that lichens and bryophytes encounter bright sunlight in metabolically active, hydrated state for only limited periods [168], and that global warming might bring increased cloud cover, makes this CO_2 effect promising for them [169].

Photosynthetic performance of lichens and mosses seems to be influenced by longer term elevated CO_2. The first moss tested in a high-CO_2 experiment was *Polytrichum formosum*, which was exposed to four different CO_2 levels for eleven months in open top chambers [170]. In this case, even photochemical activity was negatively affected by the two highest CO_2 concentrations (700 and 683 ppm). The

higher CO_2 concentrations decreased the chlorophyll content. Obviously, the more common signs of downward regulation could also be detected: lowered Rubisco capacity and elevated levels of soluble sugars and starch. Since moss gametophytes lack stomata, these results suggest that stomata probably play a less important role in the acclimation of photosynthesis in higher plants with stomata as it is supposed [108].

However, the moss *Tortula ruralis* and the green-algal lichen, *Cladonia convoluta* did not display any signs of acclimation in their photosynthesis after four months of exposure to 700 ppm CO_2. In addition to this, elevated CO_2 resulted in a slightly, but significantly higher net photosynthesis rate from the 15th minute of the rehydration period in *T. ruralis*. *C. convoluta* reached the CO_2 compensation point in less than half the time taken at present CO_2 level, and its net carbon assimilation also raised [171]. The difference between the responses of *P. formosum* and the responses of *T. ruralis* and *C. convoluta* may be related to the difference in length of exposure and to the more elaborate morphology of the *Polytrichum*. The lack of acclimation in the latter two species could be a consequence of the intermittent acclimation periods.

Elevated CO_2 proved to be beneficial also at the desiccation end of the dehydration-rehydration cycle in *T. ruralis* and *C. convoluta*. The high CO_2 exposure not only increased the overall carbon gain by about one third but it also prolonged the net photosynthesis without influencing the rate of water loss [163]. Carboxylating enzymes are only inactivated but not degraded on desiccation and bryophytes are able to fix CO_2 at quite low water potentials [172]. Most likely, in desiccation-tolerant plants CO_2 is a limiting factor for carboxylation even at this low hydration level [173].

In green-algal lichen *Parmelia caperata* in the vicinity of a natural CO_2 spring [174] Balaguer and Barnes did not find symptoms of downward acclimation of photosynthesis except for decreased Rubisco content in the algae from the thallus rim. The latter was attributed to the N-starvation of these photobiont cells. In spite of the preserved photosynthetic capacity, there was no sign of increased primary productivity. Instead of non-structural carbohydrates, assimilated carbon was allocated to extracellular phenolic secondary metabolites thus avoiding negative feedback on photosynthesis. It is a speciality of the lichens, that some of them can accumulate phenolics up to 20 % DW [175]. Phenolics protect lichens against parasitic fungi, bacteria and even slugs [175]. They also render the thallus opaque in the desiccated state and thus they offer some photoprotection. On the whole, long-term elevated CO_2 seems to be beneficial for the green-algal lichens. However, the competitive advantage is not exerted by increased biomass but by improved protection.

Most lichens and mosses live in nutrient-poor habitats and this exerts some limitation on their photosynthetic and production responses to elevated atmospheric CO_2. In general, mosses have limited source-sink differentiation, while lichens invest their extra carbon into secondary substances, which has parallels in non-desiccation tolerant higher plants [176]. Lichens and bryophytes are poikilohydric and evergreen. This means that they often have to face sub-optimal environmental

factors and to react immediately to intermittent favourable periods [160]. Lichens and mosses may gain some advantage from elevated CO_2 at both low and excessive water contents [177, 173]. There are also indications that DT plants may generally cope better with heavy metal pollution and other stresses at higher CO_2. [178]. The responses of DT plants to high CO_2 are likely to interact in quite complex ways with other climatic factors and no simple predictions can be made. Broad biogeochemical considerations predict that rising atmospheric CO_2 should result in faster net photosynthetic transfer of carbon from atmosphere to biosphere [43], but this has yet to be equivocally demonstrated and quantified. Much more experimental evidence from long-term experiments would be needed to make a confident forecast of the responses of desiccation-tolerant lichens and mosses to the increased atmospheric CO_2 concentrations that may be expected toward the end of this century.

6. THE RESPONSE OF TREES AND FORESTS TO ELEVATED CO_2

The size and longevity of trees have severely limited the possibility of realistic fumigation experiments performed on whole plants, lasting for a large part of their lifespan, and involving a significant number of samples; therefore, the current views on the response of trees to elevated CO_2 are based mainly on a large number of short term experiments performed throughout the last two decades on seedlings or on tree branches enclosed in bags. The differences in growth rates existing between seedlings and adult trees are well known. In fact, the first grow exponentially, and their response to elevated CO_2 is stronger; the first weeks or months under elevated CO_2 accelerate ontogeny, thus influencing the future growth [19]. Therefore, the extrapolation of relatively short term experiments to assess the long term response of adult trees and forests may be particularly misleading. In addition, many experiments have been biased by unrealistic experimental conditions, with respect in particular to soil and light environment.

It is important to notice that, in the last few years, some FACE experiments fumigating both adult trees and seedlings have been set up. In particular, FACE studies were established on a ten-year-old stand of sweetgum (*Liquidambar styraciflua* L.) in Tennesee [179], on a fifteen years old stand of Loblolly pine (*Pinus taeda*) in North Carolina [180], and on seedling plantations, for instance in Poplar in Italy [181, 12] and Aspen [182]. Several review papers have been published in the last few years about tree response to elevated CO_2 [19, 41]; in this short review, which is by no means exhaustive, we shall focus on the more recent research, pointing out the areas where research is still needed.

6.1. Photosynthesis and growth

As atmospheric CO_2 concentration directly affects photosynthesis, it is quite obvious that many efforts have been devoted to elucidate this topic; yet, univocal conclusions have not been drawn so far. Stimulation of net photosynthesis has been

reported in many studies; yet in the longer term, down regulation was often evidenced [183, 184]. Intriguingly, down regulation was found by some authors [185] in OTC but not in branch bag experiments, suggesting a sink effect. In several experiments, down regulation seems to be related also to stress conditions, for instance to limited access to nutrients in consequence of limited pot dimension [186], or to other stress factors [187], and the response appears to be species specific [188]. Even in field grown trees, under realistic conditions, contrasting results were achieved: for instance, down regulation was not shown in Mediterranean species [189], but was evidenced in *Pinus taeda* [190]. On the other hand, leaf age seems also to play a major role in determining the photosynthetic response to elevated CO_2: in *Pinus radiata*, the current year's needles were shown to display a doubled enhancement in the photosynthetic rates (+63%) with respect to the previous year's needles (+31%) [191].

Moreover, it must be considered that seasonal changes in non structural carbohydrates, VOC emissions and root exudation can play a relevant role in increasing and decreasing the need for photosyntates, thus affecting photosynthesis physiology. The growth patterns (one or more flushes per year) have also been reported to greatly affect the photosynthetic response [41].

Not surprisingly, an increased growth has been evidenced in most studies; contrasting reports exist about the different responsiveness of photosynthesis in conifers and deciduous trees: a higher stimulation in the latter was reported by Ceulemans and Mousseau [41], while more recent bibliographic research on a large number of experiments yielded opposite results [19]: over the average exposure duration of 338 d, conifers increased their biomass by an average of 130 %, while deciduous trees, in a similar fumigation time span, increased it only by 49 %.

Recent research performed in FACE arrays on Sweetgum has evidenced increases by 46% in the net photosynthesis rates, with no decline in photosynthetic enhancement during three years of fumigation [179]. At the same time, stomatal conductance was reduced by 24 % in upper canopy and 14 % in mid canopy leaves, thus increasing water use efficiency by 68 % and 78% respectively. Hymus et al. [180] working on 15-years-old loblolly pine, reported an increase in photosynthesis by 65 % in the warmer months, adding to the normal atmosphere 210 ppm of CO2; a similar increase (63%) was evidenced in loblolly pine seedlings after 4 years of fumigation at 650 ppm CO_2 [191]. Similar effects were evidenced in Ponderosa pine (49 %: [192]) and longleaf pine (50%: [193]).

In branch bag experiments, photosynthesis was enhanced by 50 % in Norway spruce [194] and by 100 % in Sitka spruce [195]. In *Quercus myrtifolia*, a stimulation by 97% has been evidenced [196].

Decreased amounts of Rubisco have been reported in many experiments [197, 198, 199], thus suggesting that:

- plants maintain higher rates of photosynthesis, with lower N investment in Rubisco
- reallocation of N from Rubisco to other processes may substantially improve its use efficiency

- in spite of down regulation, CO_2 increase has a positive effect on growth [186, 108, 19].

As with long term growth response, measurements performed on 35-years-old *Quercus ilex* trees in CO_2 springs evidenced that growth stimulation continued in the long term, being more evident in the first years after coppicing, when competition among resprounts for light and nutrients is more limited [200].

In conclusion, while there is common consensus about photosynthesis stimulation and growth enhancement, differences seem to be related to the different growing conditions: a major role appears to be played by N availability and by temperature, as well as by the presence of water stress. An increase in biomass of ponderosa pines by 42% at low air temperatures and by 62 % at high air temperatures has been reported [201]. In loblolly pine, different stimulation was evidenced in summer and winter months, when it was more limited [180, 202]. Significant differences in the response to elevated CO_2 were also shown at different temperatures in *Quercus myrsinaefolia* [203].

Although the reduction in Rubisco content may allow the plant to reallocate N, it is well know that, if N nutrition is limiting, the effect of elevated CO_2 on tree growth is much reduced; this has been confirmed also by recent research; in peduncultae oak, elevated CO_2 is reported to increase biomass by 140 % and 30 %, respectively under high and low soil N [204]; similar values were evidenced in the stimulation effect on net photosynthesis at two different fertilization levels in aspen [205], and growth differences of 50 % and 25 % were evidenced in the same species [206].

The positive effects of elevated CO_2 were more evident under moderate water stress in red oak [207]. Similar effects were measured in 30 years old *Quercus ilex* trees growing in a CO_2 spring [200].

Although similar effects were reported by many authors, it is sometimes difficult to compare absolute values, due to the different growing conditions, different enrichment levels, and different duration of the experiments, which add to the well known species-specific response not only to elevated CO_2, but to all the above mentioned experimental parameters, and to their interactions.

Anatomical differences were also reported; specific leaf area is often reduced by elevated CO_2, in consequence of an increase in cell size, cell number and the number of cell layers [19]. Wood anatomy is, up to now, less studied; xylem vessel size and number were reported to increase under elevated CO_2 in *Quercus robur*, while in *Prunus avium* an increase in wall thickness was shown [208]. Increases in wood density and in lignin:N ratio were also reported for *Picea abies* [209]. Yet, large differences in lignin concentration response to elevated CO_2 were shown by different authors [210]; the reasons for this are still largely unknown, and, as the potential for variation in this parameter is high, any generalization is difficult.

6.2. Effects on water relations

It is generally assumed that elevated CO_2 reduces stomatal conductance (*gs*), thus reducing transpiration rates and increasing water use efficiency. However, the effect on stomata is more limited in trees, with respect to herbaceous plants. It is intriguing to notice that experiments performed in realistic environments have shown a reduction in stomatal response, with respect to those done on potted plants and in growth cabinets [211, 212, 19], possibly in consequence of experimental artefacts involving root restriction or low light levels. In Mediterranean environments, measurements taken in CO_2 springs did not show any *gs* reduction under extreme water stress, while the differences were more evident in spring and autumn, being more limited in the afternoon, when stomatal conductance was smaller both in enriched and in control plants [213, 214, 215]. On the other hand, the increased leaf area has even been shown to cause, in longleaf pine, an increase in water consumption [193]. The expected reduction in evapotranspiration, with effects on local climate, may therefore be very limited, or counteracted by the increased leaf area. It has been observed that reduction in transpiration may also reduce the water vapour mole fraction in the atmosphere, thus, in turn, stimulating transpiration [216].

Still, in the long term, positive effects of elevated CO_2 have been reported: some species appear to have reduced their stomatal density since preindustrial times [217], and, from the analysis of C isotopes in tree rings, water use efficiency in beech resulted to have increased by about 33 % in the last century [218] .

Xylem conductance adaptations have been examined both in seedlings and in adult plants growing in CO_2 springs. An increase in hydraulic efficiency was found in seedlings of *Quercus robur* when grown at elevated CO_2 concentrations [208], and this response was associated with the observed increase in mean vessel size; however, this relationship was not observed in seedlings of *Prunus avium* and *Prunus pseudocerasus* [208]. According to Heath et al., [219], leaf-area-specific stem hydraulic conductance should closely match the patterns of stomatal conductance found in elevated CO_2, maintaining the balance of water supply and demand; this was confirmed in *Fagus sylvatica* and *Quercus robur* seedlings. An increase in specific hydraulic conductivity was measured in CO_2 spring grown *Quercus pubescens, Quercus ilex* and *Arbutus unedo*, while the opposite tendency was shown by *Fraxinus ornus* and *Populus tremula* [220]. In the same species, differences in xylem vulnerability were small and followed no particular trend. The percent loss of hydraulic conductivity in the different seasons displayed different trends in the different species, with significant differences in *Q. pubescens, P. tremula* and *A. unedo*, and less evident response in *F. ornus* and *Q. ilex*. Only in *Q. pubescens*, this trend was evident through the entire year, while in *F. ornus* and *Q. ilex* the loss of hydraulic conductivity was reduced during the summer under

elevated CO2, and the autumn recovery was more consistent in *P. tremula* and *A. unedo* (see also [221]).

In spring, the production of new functional xylem led to a reduction in xylem embolism, being more evident in *Q. pubescens, F. ornus* and *A. unedo*; intriguingly, these three species had reduced stomatal conductance, when compared with control trees [214, 221, 35]. A less evident trend was shown in *Q. ilex*, in which the effects of elevated CO_2 on stomatal conductance were more limited.

The same trend was shown in Mediterranean shrub species growing in CO_2 springs: *Myrtus communis* and *Erica arborea* were less embolized than the control, while in *Juniperus communis* the differences were more limited [222]. The two former species had also a stronger stomatal response [223].

Finally, it must be noticed that in the long term forest trees can respond to elevated CO_2 by reducing leaf number and dimension, thus down regulating assimilation and reducing water consumption; this phenomenon was seen in CO_2 springs [213, 214, 34]. these differences, which may also alter the resistances to water flow, may deserve further research.

Changes in cell wall elasticity and osmotic adjustment are the main mechanisms sustaining turgor maintenance in plants; it is quite obvious that, as low osmotic potential and high turgor pressure facilitate recovery from stress, changes in these parameters under elevated CO_2 may help trees to cope with the enhanced stress conditions that may be expected in the future. Evidence of osmotic adjustment paralleled by maintenance of higher water content and turgor pressure, were found in severely drought stressed woody species [224, 225], but not in others [226, 227, 228].

On adult CO_2 spring-grown *Q. pubescens* and *Q. ilex* trees, decreased osmotic potential (more negative) was found at the beginning of summer [213].

In *Erica arborea, Myrtus communis* and *Juniperus communis* [216], an increase in turgor pressure was seen, while other parameters showed species-specific responses (for instance, osmotic potential was increased in Juniperus, and decreased in Erica). Changes in photoassimilate concentrations may be offset by higher water contents due to lower transpiration [229], suggesting that the supposed beneficial effects of elevated CO_2 should be considered with caution. The differences could also be related to different strategies in osmotic adjustment and tolerance mechanisms. On the other hand, the three species maintained higher turgor potentials under elevated CO_2 during drought; this may result in enhanced growth, in comparison with control plants.

6.3. Perspectives for research

Among the main uncertainties about forest response to CO_2, we should mention:
- Forests are characterized by very complex light environments, difficult to simulate in chamber experiments; little is still known about shade leaf response to elevated CO_2. In FACE grown sweetgum seedlings, where elevated CO_2 stimulated the canopy closure, CO_2 induced photosynthetic stimulation was greater in the upper

canopy (+26%) and lower in the lower canopy (+3%). This was associated with a reduction in Rubisco that was more evident in the lower canopy (28% vs. 50%) [179]. An improved reallocation of N in shade tolerant trees has been shown in several species [230, 231], as well as improved growth under low light [11]. The effects of elevated CO_2 on growth at low light intensities have been shown to persist and to be species specific [232].

- Great differences exist between seedlings and adult trees. Adults have large respiratory losses and carbon investments in fruit and seed production, and their leaves are often anatomically different from those of seedlings. As their stomatal regulation is strongly conditioned by resistances to water transport, the patterns of the tradeoff between stomatal and xylem conductance might be modified.
- Apart from monospecific plantations, forest trees are usually exposed to competition among species. Although some competition experiments with seedlings have been done, little is yet known about adult tree responses; the study of multi specific systems is still at the beginning [233, 234].
- Although experiments have been done on the most economically important species, it has also been shown that different genotypes react in different ways; a deeper knowledge of these behaviours could have relevant economic implications.
- Very limited attention has been devoted so far to wood quality and structure, and to its mechanical properties; this might have implications on both economy and forestry.
- Finally, it is still matter of debate as to which extent the behaviour of trees, generated in "normal" atmosphere, and exposed to elevated levels of CO_2, is comparable with that of trees growing in a progressively increasing CO_2 concentration. The long-term evolutionary effects are also very difficult to foresee. Seedling from acorns collected in CO_2 springs displayed whole plant biomass values that were bigger both growing in normal and in enriched atmospheres [235].

7. HORTICULTURAL PLANTS' RESPONSES TO ELEVATED AIR CO_2 CONCENTRATIONS

7.1. The historical aspects

Greenhouse crop production under CO_2 enrichment started in the early 1920's. In spite of this, there was rather little interest for CO_2 enrichment in the 1930-40's and it was not practiced to any great extent until the late 1950's [236]. The main reason was probably the use of soil rich in organic matter, emitting large amounts of CO_2. At present, CO_2 enrichment is widely used in horticultural crop production. Initially, directed fired evaporative kerosene burners were used, followed by propane fire burners.

CO_2 can be provided by:

1. Kerosene burning: CO_2 from kerosene burning is recommended on roses, lettuce and spinach. However, serious damage has been observed in some

vegetables (cucumber, pepper and tomato) mainly due to harmful gases such as C_2H_4, CO, or SO_2 [236].

2. Pure liquid CO_2: the most common source for CO_2 enrichment in Norway and in the Netherlands is pure CO_2, because it contains no harmful gases for the plants and is easily used in greenhouses. the use of pure liquid CO_2 is especially recommended on horticultural crops, which are sensitive to ethylene [236, 237].
3. Propane burning: 40 years ago propane burning was the most common method of supplying CO_2. Nowadays, growers in Scandinavia have been sceptical of the use of propane [236].
4. Charcoal burning: charcoal contains about 15% CO_2, but the concentration of ethylene, CO and nitrogen oxides is too high for many species, such as beans, tomato, roses, etc.[238].

7.2. CO_2 concentrations applied in greenhouse practices

About 20 years ago it was common practice to enrich greenhouses to 2000-3000 ppm CO_2 because it was believed that the higher the concentration the better. Later, a CO_2 concentration of 1000-1500 ppm was recommended. In the last few years, it has been shown in a number of experiments that concentrations above 900 ppm very seldom lead to any beneficial effect [239, 240, 241, 242]. In some cases plant injuries (necrosis, chlorosis and curling of the leaves) have been observed at concentration above 1000 ppm [243]. Today, recommended CO_2-levels in horticultural practice, from sunrise until sunset as long as the greenhouse is not ventilated, are: for young plant propagation (600-700 ppm), for cucumber, pepper, tomato (700-800ppm) and for leaf vegetables -lettuce, spinach etc. (1000-1500 ppm) [243, 244].

It is clear that not only the CO_2-concentration, but also other factors involved in greenhouse production influence the effect of CO_2 enrichment; a significant interaction between irradiance and CO_2 concentration was found [245], as they affect dry weight accumulation of *Chrysanthemum*. When the air temperature exceeds 28 °C, the CO_2 enrichment is usually interrupted and the greenhouse ventilated, [246], thereafter the daily CO_2 enrichment period varies according to the weather pattern. In greenhouses, CO_2 enrichment was usually maintained for 4 h in the morning and 3 h in the afternoon, in winter and early spring. In horticultural practice, the growers are often advised to increase the ventilation temperature by 2-4 °C when CO_2 enrichment is used [247]. Rising, the CO_2 concentration reduces the transpiration of plants by 20-40% [248, 130]. Water consumption is thus significantly reduced by CO_2 enrichment at the same time as photosynthesis is increased [243].

7.3. Physiological effects

The physiological responses of horticultural plants to elevated air CO_2 are similar to the responses of other groups of plants.

7.3.1. Photosynthesis

As CO_2 is the substrate of the photosynthetic reaction, the rate of photosynthesis increases with increasing CO_2 concentration. In short-term experiments, photosynthetic rates rise rapidly as the CO_2 concentrations increase above ambient levels [249]. At about 1000 ppm CO_2 the response becomes saturated. The extra photosynthesis with CO_2-enrichement increases with increasing light intensity and optimum temperature [250]. The stomata control the internal CO_2-concentration in the leaf. At very high CO_2-concentration stomata are closing, concentration and aperture depending on crop and other environmental factors. Photosynthesis in potato was found to decrease as the O_2 concentration increased from 2 to 21% [251]. This decrease was nearly compensated for by a 2-fold increase in CO_2 concentration. Mortensen and Moe [245] reported net photosynthesis to increase by 51% when O_2 was decreased from 21% to 2% at 330 ppm CO_2 but the increase was only 9% at 1500 ppm CO_2. In spite of this, sometimes the assimilation rate in high CO_2 plants was lower than those grown at ambient concentration [252, 253]. Protein-nitrogen content is often lower in plants grown at elevated CO_2 concentrations than at present ones [123].

7.3.2. Transpiration and water use efficiency

Stomata gradually closed [250, 254] and transpiration decreased as CO_2 concentration increased. At low light intensity high CO_2-concentration may reduce transpiration. Low transpiration at high light intensity increases the risk of excessively high leaf temperature because of reduced transpirative cooling. In this case the CO_2 level should be decreased [255]. Short-term exposure to high CO_2 caused increased CO_2 assimilation and decreased the rate of transpiration with a resultant increase in water use efficiency [256, 257]. Stomatal aperture and stomatal conductance are also often reduced in long-term high CO_2 treated plants [258] which in turn results in lower intensities of transpiration [259].

7.3.3. Respiration

Respiration is the reversed process of photosynthesis. Maintenance respiration is needed to keep the plant in good condition. At very high CO_2-levels (> 3000ppm) the stomata get poisoned and open completely. Respiration is very temperature sensitive and doubles with 10 °C temperature increase [255, 244]. Experiments on the effects of high CO_2 concentrations on dark respiration show mixed results. It has

been proposed that mitochondrial respiration may increase in plants under high CO_2 in response to sucrose accumulation in leaves [259]. Naturally, the physiological effects are similar to the effects observed on other crops.

7.3.4. Production responses

The CO_2 concentrations inside closed greenhouses are low at daytime when plants are cultivated. In a greenhouse without ventilation and without CO_2 application, the concentration might fall to below 200 ppm [260, 261]. At low CO_2 concentrations, the photosynthetic rates of vegetable crops decrease obviously. At 1000 ppm, they drop to 20-40% compare with those of 300 ppm. Flowers and vegetables plants show very positive effects from CO_2 enrichment by increased dry weight, plant height, number of leaves and lateral branching [243]. For the grower, the question of which period and which concentration of CO_2 should be used the most economically, depends on the source of the extra CO_2 and its costs.

With CO_2 enrichment to 600-700ppm a shortening of propagation period can be reached of about 10 % in winter conditions, and the quality of the young plant will improve, because the dry matter content of the young plants will increase and the leaves will be thicker. On the other hand the most striking effect of CO_2 enrichment was precocious flower bud formation, for example in the case of tomato and cucumber [244]. Effects of CO_2 on flowering time are usually minor but not necessarily solely due to change in photosynthate supply [262]. The greatest response to CO_2 enrichment was reported in plants grown under the lowest N condition and in the cultivars with a more concentrated fruit set [263]. Kimball and Mitchell [247] also report a greater response to enrichment at the lower of two fertility levels.

Production time was reduced (15 - 25%) by elevated CO_2 level in the cases of lettuce and kohlrabi [264, 265, 266]. Cucumber yield was found to be greater during the first 45 days of picking when temperatures were allowed to increase to 33 °C prior to ventilation instead of 28 °C [267]. Sweet pepper yield was significantly higher (+33%) when CO_2-level was 1000 ppm [268]. Tomato yield was lower when the start of enrichment was delayed in the morning compared to early termination in the afternoon [239]. Hand and Soffe [269] found 32% to 72% higher tomato yields after six weeks of harvesting when plants were grown in 1200 ppm CO_2. In general the total tomato fruit set increased only slightly with CO_2 enrichment but the fruit weight under CO_2 enrichment was significantly bigger [263]. The application of CO_2 on melon resulted in increased leaf area and increased leaf dry matter. If the CO_2 content of the air increases by about 300 ppm, an increase in crop productivity of about 30% is forecast, with varied manifestations in different species [270].

Application of CO_2 through irrigation water is of recent use in greenhouse. In melon forcing, an increase in melon yield (+20%) was found [271], in consequence mainly of increased fruit size and not of greater number of fruits. On the other hand, CO_2 application through irrigation water did not affect the chemical characteristics (°Brix, total acidity and pH) of the fruits at harvest. CO_2 application doubled leaf K concentration but reduced leaf Fe concentration.

CO_2 enrichment in greenhouses has been shown to increase yields of many species significantly and therefore has become a commercial horticultural practice.

In the open field, turbulent dispersion by wind movement reduces the CO_2 enrichment effect with regard to above ground release. In a field experiment involving irrigation with carbonated water, a significant increase (20%) in wheat yield was observed [272]. Pulsation of CO_2 above and below ground as well as temporary bicarbonate ion increase in soil solution and temporary decrease in soil pH would be expected when considering only the crop water requirement [273]. Nevertheless, reduction in soil pH may increase the activity of plant growth stimulating microorganisms [274]. The effect of irrigation with and without carbonated water was tested [275] in Colorado on mulched and unmulched tomato. Water was applied twice weekly by drip irrigation during the growing season. According to the results, marketable fruit yield was increased (18%) ($P<0.05$) by the carbonated water treatment in a mulched experiment, but in an unmulched experiment, the increase was not evident. On the other hand Zn was found to be significantly higher ($P<0.05$) in the carbonated water (CW) treatment and soil pH was lower in the CW treatment (reduced from 6.4 to 4.5).

High CO_2-level can reduce the minimum temperature at which a plant grows and completes its life- cycle. For example, the tropical vegetable okra was unable to complete its life–cycle in normal CO_2 at temperature below 23/17 °C (day/night), while plants grown in 1000 ppm CO_2 at 20/14 °C matured and produced fruit [276].

7.3.5. Concluding remarks

CO_2 enrichment of greenhouse vegetable crop production affects both yield quantity and quality. Significant yield increases for most vegetables are observed with CO_2 enrichment. Greatest response to CO_2 appears to occur when irradiance levels are high. A CO_2 concentration of 700-900 ppm might generally be recommended. Above this level, yield and growth increases are relatively seldom observed. Nevertheless, in spite of recent increases in scientific knowledge, there is still a need for more research on vegetable crop production in CO_2 enriched greenhouses.

8. EFFECTS OF ELEVATED CO_2 ON SOIL AND IPOGEOUS GROWTH

8.1. Soil CO_2 concentration

The partial pressure of CO_2 in soil may differ vastly from that in normal air. Due to the respiratory activity of plant roots and soil biota, most frequently 2-3 fold higher CO_2 concentration compared to air [CO_2]can be measured. However, much higher values can also be detected. The partial pressure of CO_2 in the soil varies seasonally and diurnally [277, 278] and can be strongly influenced by several factors, f.e. a soil water content [279], temperature [278] and vegetation [280, 281, 278, 282]. The dynamics of CO_2 in soil profiles was studied at two sites with different vegetation [208] and was found to increase with the depth, reaching the highest concentrations

of 0.87 % and 0.65% at the depth of 1.0 and 1.5 m, respectively. Several studies have shown that CO_2 concentrations in the rhizosphere can reach percentage values [280, 283, 284, 285]. CO_2 partial pressure can significantly increase also when the soil is flooded. In the flooded soil supporting the growth of desert succulents, values of 0.2-0.4 % were measured [286].

The soil CO_2 concentration at natural CO_2 springs can substantially exceed the concentrations measured at non-enriched sites. At the Stavešinci geothermal mofette, soil [CO_2] was measured using a portable landfill gas analyser [126, 127, 128]. In the rooting horizon of timothy grass *Phleum partense,* (depth 20 cm) CO_2 concentrations ranged from 0.4 to 26% (w/v). When CO_2 concentrations increased, soil oxygen dropped concomitantly. Directly at, or in the close neighbourhood of gas vents, soil oxygen decreased to 10 or 5% (or eventually even to zero). Similar CO_2 concentrations can be expected in the soil of other CO_2 springs [83, 124], or even in degassing areas, where emissions are too faint to produce an enriched atmosphere [38].

8.2. Root growth

Analysing the response of crop roots to elevated CO_2 Pritchard and Rogers [287] concluded that roots in a high CO_2 environment will be larger and more highly branched (see also [288, 289]).The increased C gain under elevated [CO_2] might increase root length density and promote shallower root systems by stimulating lateral root production over primary root elongation [290, 287, 291].

However, upon prolonged exposure to elevated [CO_2] the effects on root biomass are often scarce or absent [292, 293]. Growth enhacement of roots might be a transient response of the plant similar to that discussed for the stimulation of leaf level photosynthesis and growth enhacement of shoots [294, 295, 287]. Changes in total belowground biomass as evaluated at the end of various experiments do not necessarily represent a continued increase in input to the belowground pool. In natural systems finite resource availability might constrain the long-term flux of C into a plant and soil system [288, 296]. On the contrary, [297] it has been hypothesized that elevated [CO_2] favours investment of biomass in roots only if nutrients cannot be absorbed in proportion to the CO_2 enhanced growth. No effect on the fraction of biomass allocated to the root is found if nutrients are supplied at an optimum level [297, 298, 299]. It is therefore not clear whether stimulation of root growth depends on increasing allocation of carbon to the roots or whether it is an indirect effect of nutrient and water limitation.

Root turnover can significantly contribute to the net acquisition of carbon and is important for global budgets as well as for nutrient cycling. The few studies dealing with the root dynamics under elevated [CO_2] yielded inconsistent results [300, 301, 302, 303, 304]. Accelerated root turnover in two different grasslands, which differed also in root dynamics, has been reported [300, 301]. In the study by Arnone et al. [302] root production and mortality were monitored in a species rich calcareous grassland community. CO_2 had no effect on the production and mortality of root

biomass in the top 18 cm, where most roots occur. However, elevated [CO_2] was associated with an upward shift in the root length density: under elevated [CO_2] a greater proportion of roots were found in the upper 0-6 cm soil layer, and a lower proportion of roots in the lower 12-18 cm, than under ambient [CO_2]. A similar response was observed in other studies [305, Raschi et al., unpub.]. Carbon and nutrient cycling may be thus shifted closer to the soil surface [302]. Seasonal weather conditions have been shown to influence the effect of elevated [CO_2] on root turnover [64, 306]. A modest, if any, increase in root productivity under CO_2-enrichment was reported in a recent study on loblolly pine (*Pinus taeda*) [304]. The factors driving changes in the root distribution and longevity with depth under elevated [CO_2] might be related to increases in soil moisture under elevated [CO_2], interacting with vertical patterns in soil temperatures. Reduced tissue N concentration and reduced root maintenance respiration, both of which are predicted to result from elevated [CO_2], should contribute to slightly longer root life spans [307]. It is likely, as hypothesised [308], that increasing atmospheric [CO_2] does not exert a significant direct effect (resulting from a greater C supply) on root turnover. Indirect effects, mediated by shifts in plant water and nutrient relations are more probable.

Compared to the extensive data on the shoot/root relationship in crop plants as influenced by artificially elevated [CO_2] [309, 310, 287], little is known on the allocation of carbon below ground in natural CO_2 springs plants. No *in situ* study dealing with this problem has been performed, also in consequence of the soil difformity often characterizing CO_2 springs. Reciprocal transplant design was used at a natural CO_2 spring in New Zealand in order to generate combinations of atmospheric and soil conditions that represent both short- and long term elevated CO_2 conditions [37]. The growth of three different plants, *Lotus uliginosus*, *Paspalum dilatatum*, and *Plantago lanceolata*, was stimulated by elevated [CO_2] (estimated mean pCO_2 574 ppm) when they were grown in high CO_2 developed soil, i.e. at conditions of long-term exposure. No increase in total mass under high [CO_2] was found if the plants were growing in the soil developed in near global ambient CO_2 concentrations (spring site with ca. 372 ppm CO_2, the situation similar to short term exposure). For this treatment the highest root mass fraction was measured. These results showed that there is not a simple relationship between transient and equilibrium (long-term) responses to elevated [CO_2].

In a study by Fordham and Barnes [295] different populations of *Agrostis canina* and *Plantago major*, originating from a near-ambient or 'high' long term CO_2 concentration, were grown in controlled conditions at 350 and 700 μmol mol^{-1} CO_2. The study revealed that long-term adaptation to growth at elevated [CO_2] maybe associated with a potential for increased growth and higher carbon allocation to the roots [311, 312, 295]. According to Badiani et al. [124] such an effect could be of evolutionary value as one of the mechanisms preventing feedback inhibition of photosynthetic capacity in natural CO_2 spring species.

8.3. Mineral nutrition

It is to be expected that higher growth rate in response to elevated [CO_2] will increase plant nutrient demand. Averaged over the literature data available, a low soil nutrient supply was found to reduce the proportional growth stimulation of elevated [CO_2] [313, 314].

On the other hand, elevated [CO_2] could elicit compensatory adjustments so that acquisition capacity for minerals increases in concert with carbon uptake. Compensatory adjustments such as increases in (i) root mycorrhizal infection, (ii) root-to-shoot ratio and changes in root morphology and architecture, (iii) root nutrient absorption capacity, and (iv) nutrient-use efficiency, can enable plants to meet an increased nutrient demand under high [CO_2] [314].

Nitrogen and phosphorus are the nutrients most likely to be influenced by rising CO_2 since relatively high quantities of both are needed in the photoreductive C cycle and the photo-oxidative cycle [289]. When nitrogen is low or marginal, the acceleration of growth in elevated [CO_2] may drive the plant into nitrogen deficiency. The nitrogen content of plant tissues is very commonly reduced, which has been observed both in artificial [315, 290, 316, 317, 318] and natural [116, 319, 320] CO_2 enrichment.

The decrease in tissue nitrogen concentration and increased C/N ratios indicate that plant biomass can be increased with elevated CO_2 independent of changes in the nutrient supply. Both higher nitrogen use efficiency (NUE), calculated from total leaf N content and plant dry mass, and higher photosynthetic N use efficiency (PNUE), calculated from leaf CO_2 assimilation rates and leaf N concentrations, are reported for plants grown in the CO_2 enriched atmosphere [321, 322, 298, 323, 324]. Rogers et al. (1999) state that N requirement is reduced under CO_2 enrichment [289]. Changes in photosynthetic nitrogen-use efficiency may be a critical determinant of competition within low nutrient ecosystems and low input agricultural systems [321]. In this context, however, other complexities of nitrogen nutrition must also be considered. Studies that have measured the root N uptake capacity commonly report a differential effect of elevated [CO_2] on NH_4^+ and NO_3^- [325, 314, 318]. This can have different effects on plant species that differ in preferences for each form of nitrogen. In addition, as stated below, nitrogen fixation can significantly contribute to a plant's nutritional response to elevated [CO_2].

For phosphorus, in contrast to nitrogen, higher tissue concentrations are required for an optimal productivity under elevated [CO_2] [289, 326]. At the low P supply, white clover plants grown at twice ambient p(CO_2) lost their stimulation of photosynthesis, accumulated non-structural carbohydrates in the leaves, and their growth rate was not stimulated, in contrast to high P grown plants [326]. This and other studies indicate a C-sink limitation of growth. This limitation could be overcome by mycorrhiza [327, 328, 329], but several studies failed to prove that. [330, 331, 332]. In P-limited sour orange under the additional [CO_2] availability, mycorrhized plants had greater CO_2 assimilation, growth and nutrient uptake than non-mycorrhized plants [333] and growth depression of mycorrhized Citrus seedlings grown at the high phosphorus supply was mitigated by elevated [CO_2]

[334]. An increase in phosphatase activity by plant roots at elevated [CO_2] could also contribute to higher P availability [335], however this effect was not widely confirmed [326].

Altered concentrations of other mineral nutrients in response to elevated atmospheric [CO_2] were also observed [336, 337]. Very often decreased nutrient concentrations are a result of a dilution effect due to accumulation of nunstructural carbohydrates [338]. The decline in nutrient absorption per unit growth may be a result of a shortage of these nutrients in the root environment. Root specific activity can also be down-regulated. According to [287] roots under elevated [CO_2] are less efficient in nutrient uptake due to i) production of less efficient root architectures, ii) limitations imposed by anatomical characteristics, iii) reduced mass flow of water through the soil-plant air continuum, iv) inefficient or unbalanced plant C and N relations or v) reduction in the competitive ability to acquire nutrients.

On the other hand, an increased supply of sugars in the high CO_2 grown plants could positively influence the uptake of mineral nutrients [339, 318]. A limited number of studies that have examined the effects of elevated CO_2 on nutrient uptake mechanisms yielded very variable results [340, 341, 342, 325, 339, 343, 344, 298, 345, 318]. Interspecies differences in uptake kinetics under high [CO_2] which were confirmed in these studies, might have important consequences for plant and ecosystem responses [346]. They might also explain why some species do not exhibit a commonly observed decline in tissue nutrient concentrations at high [CO_2].

It has been shown that the initial reduction in leaf mineral concentration gradually disappeared over longer (56 months) exposure to elevated [CO_2] [347]. This emphasizes the importance of conducting long-term experiments. Again, natural CO_2 springs offer an opportunity to study long term effects and potential long-term adjustment or acclimation of leaf mineral concentration over a period of several generations. In timothy grass grown at different locations, i.e. CO_2 exposures at the Stavešinci mofette, the content of N, S, P, K, and Zn decreased the closer the plants grew to the emitting vents. The decrease was approximately 40% in N and P, and 20% in K and S for the most exposed plants (26% CO_2 in the soil, [126]. At the same time total carbon content was constant, which indicates a dilution effect. Leaf mineral concentrations were also measured in three Mediterranean species (*Erica arborea, Myrtus communis* and *Juniperus communis*) growing at the Lajatico natural CO_2 spring and in a nearby control site [320]. Leaves were sampled every two months for one year. Multivariate principal component analyses based on the leaf elemental concentrations clearly differentiated the two sites and the three species. Lower concentrations at the spring site were not the result of a dilution effect by increased structural or non-structural carbon. In contrast to most experimental studies of CO_2 enrichment, mainly conducted for short periods, several of these elements had greater concentrations in the CO_2 spring site. In general, changes in the element composition under the elevated [CO_2] of the spring site were species- and element-specific and time dependent. Therefore, caution should be exercised in generalizing the effect of CO_2 on mineral nutrition.

8.4. Elevated CO_2 and mycorrhiza

Mycorrhizal fungi may play an important role in the sequestration of carbon in the soil under elevated [CO_2] deposition. Plants allocate an estimated 10-20 % of net photosynthate to mycorrhizal fungi, although this number can range from 5% to 85% among systems [348]. By acting as a sink for plant carbon and by promoting plant phosphorus uptake, mycorrhizal symbioses could alleviate photosynthetic down-regulation [349, 329]. It has been pointed out that [350] the spatiotemporal complexity of mycorrhizae should be taken into account when its role in responses to elevated atmospheric [CO_2] is studied. The ways in which mycorrhizal fungi can potentially influence responses to CO_2 at the various levels include: i) influencing the homeostatic adjustment of individual host plants to elevated [CO_2], ii) altering the variability of responses to CO_2 within a plant population, iii) differentially responding to and providing feedbacks to different plant species within a plant community and to different plant functional assemblages in an ecosystem, iv) providing an increased sink of carbon in the soil, and influencing nutrient cycling patterns [350].

The effects of elevated [CO_2] on mycorrhizal growth have been studied by different approaches and at different scopes [329, 351, 350, 352, 353]. Most mycorrhizal studies under elevated [CO_2] quantify changes in the mycorrhizal colonization percentage root length (tips) or total root length colonized per plant. Although frequently increased, mycorrhizal infection might not necessarilly change under elevated [CO_2]. However, as root biomass tends to rise, total mycorrhizal biomass per plant might do so as well [351]. Still in Citrus elevated [CO_2] counteracted the depressive effect of P on intraradical AM colonization [333].

Because extraradical hyphae account for a large portion of fungal biomass they can constitute a sizable pool of carbon in the soils of many terrestrial ecosystems. In arbuscular mycorrhiza (AM) an even more significant pool of fungus-related carbon in the soil is glycoprotein glomalin. Glomalin is very abundant and apparently only produced in significant amounts by AM fungus hyphae [354]. In a sorghum (*Sorghum bicolor*) field FACE experiment a significant increase in soil hyphal lengths of AM fungi and easily extractable glomalin, in response to CO_2 was found [355]. The soil aggregate water stability was also increased. Although a causal relationship between the hyphal length, glomalin and aggregate stability was not demonstrated, the present data do suggest that AM fungi could mediate changes in the soil structure under elevated [CO_2] [355, 356]. Arbuscular mycorrhizae were also studied along an atmospheric [CO_2] gradient (from ca 350 to 700 $\mu mol\ mol^{-1}$) [357] at a CO_2 spring in New Zealand [358, 359] (Hakanoa Springs). Percent root colonization by arbuscular mycorrhizal fungi, AM fungi soil hyphal length, and soil concentrations of the glomalin increased linearly along a CO_2 gradient. These results are an important confirmation of numerous short-term studies, and present the first test of the resource balance model, applied to AM fungi, after long-term elevated CO_2 exposure.

There are no other studies on mycorrhizal functioning under long-term natural CO_2 enrichment. The mycorrhizal research at natural springs could contribute to the

knowledge of the ecosystem response to global change. In addition, it is tempting to speculate on the functioning of the symbiosis at higher partial pressures of CO_2. Little is known about the sensitivity of mycorrhizal fungi to soil hypoxia. In the study of Beck-Nielsen and Madsen [360] the lower redox potential of the soil was associated with the reduction in density of hyphae in the soil and was consistent with lower AM infection of the plants. At natural CO_2 springs, relatively highly infected plants can be found close to the CO_2 vents (Vodnik, unpublished). A detailed analysis of rhizosphere processes would contribute to the quality of mycorrhizal studies in such conditions.

8.5. Elevated CO_2 effects on N_2 fixation

CO_2 enhanced plant growth may result in an increased N-sink strength. In legumes it is to expect that an increased N demand will be translated into an increased N_2 fixation per plant. On the other hand, symbiotic N_2 fixation is a highly energy-demanding process that constitutes a significant sink for photosynthate, which could significantly contribute to the photosynthetic rates in N_2-fixing plants.

Low rhizosphere (below 100 ppm) CO_2 concentrations result in a significant decline in the nitrogenase activity of legume nodules [361]. There is, on the other hand, no evidence of direct nitrogenase stimulation by increased rhizospheric [CO_2]. For actinorrhizal black alder (*Alnus glutinosa*) little effect of low rhizospheric p(CO_2) (0.5 kPa) was found [362], whereas 3.0 kPa CO_2 reduced nitrogenase activity by 31-35%.

Still, elevated [CO_2] leads to an increase in nitrogen fixing activity within several days and increases a total nitrogenase activity in the long term. It is presumed that the long term effect is caused by an increase in the number and in the individual weight of the nodules and is not a result of increased nitrogenase activity per unit nodule dry weight [72].

As carbon input to the rhizosphere may be significantly increased under elevated [CO_2] [363], a response to elevated [CO_2], similar to that in the symbiotic N_2-fixators, can be expected also in the non-symbiotic N_2 fixing diazotrophs. Despite their importance in N-cycling there are only few studies dealing with the effect of elevated [CO_2] on N_2 fixation by the bacteria/non-legume association [364, 365]. This research suggests increased nitrogen fixation in response to the increased [CO_2].

Several studies indicate a decline in the leaf nitrogen concentration under elevated [CO_2] will be much milder in N_2-fixing species than in no N_2-fixing species [366]. This could lead to consequences in litter decomposition processes [367] and nitrogen cycling.

8.6. Root respiration

Two different responses of plant respiration to elevated [CO_2] can be distinguished [368]. Direct (short-term) effects are observed when [CO_2] is rapidly increased,

resulting in changes in respiratory metabolism, including enzyme activity [369, 370]. Indirect (long-term) effects on respiration are observed after long-term growth at elevated [CO_2] and are mediated through effects on the leaf growth rate, a non-structural carbohydrate concentration, and tissue composition. In comparison to the number of studies dealing with the effect of elevated [CO_2] on the respiration of green tissues [see 371 for a review] the information on root respiration is much more scarce. Soil [CO_2] can significantly influence the respiration activity of roots [277]. The inhibition of root respiration of *Pseudotsuga menziessii* [372] and *Pinus strobus* [277] was found for a concentration range normally found in soil. Respiration rates of white pine fine roots were immediately reversed after returning to alternate high (1200 μL CO_2 L^{-1}) and low (air)-high CO_2 concentrations (high-low-high and vice versa), suggesting a direct effect of elevated [CO_2] on apparent respiration. Relatively high sensitivity was found in some desert succulents, where the inhibition of root respiration was found at concentrations which can be measured in the flooded soil [286, 373]. On the other hand, several plant species can sustain relatively high CO_2 concentrations [374, 375, 376] and [297] it is presumed that most plant species are less sensitive to a high CO_2 concentration in root environments [297, 377, 378, 379]. Decreased [301, 380], increased [381, 382], and non-changed [383, 384] respiration rates are reported by different CO_2-enrichment studies. Frequently no effect from elevated [CO_2] is observed when root respiration is expressed per unit root dry weight [385, 377]. According to Lambers et al. [377] increased root dry weight under elevated [CO_2] decreases the specific rate of root respiration but increases the carbon requirement of root respiration relative to that fixed in photosynthesis. Recent leaf respiration studies also confirm that the influence of elevated [CO_2] on plant respiratory carbon flux is primarily through increased biomass [386]. It has been [387] concluded that direct effects of CO_2 on leaf respiration are small. However, direct effects of CO_2 on respiration could be of particular significance in the environments where CO_2 is variable. In this respect natural CO_2 springs are extremes, known for dramatic short-term fluctuations [388, 124]. Soil CO_2 measurements at the Stavešinci mofette area showed non-homogeneously distributed CO_2 emissions over the whole ranging from 0.3 to 100% [126, 127]. Beside spatial variability, temporal changes in local soil gaseous conditions can be expected, as a consequence of rapid changes in the atmosphere and changing soil characteristics, soil drying for example. In the respiration response of plants growing at naturally elevated [CO_2] combined direct effects including suppression of respiratory enzymes, diversion of electron transport pathway to cyanide-resistant pathway, refixation of respired CO_2 and alterations of intercellular pH [368] could be involved. Preliminary measurements of root respiratory potential (measured as electron transport activity) and root respiration (measured by using oxygen electrodes) indicate, however, relatively low susceptibility of CO_2 spring plants to high CO_2 (low O_2) concentrations [389]. As it is speculated that short-term responses to elevated [CO_2] are related to the CO_2 history of the plant [368], further investigation of root respiration under natural CO_2 enrichment is of particular interest.

ACKNOWLEDGEMENTS

The support to Zoltan Tuba by a DAAD (Bonn) professorial fellowship and NATO senior fellowship at Botanical Institute II of Karlsruhe University is gratefully acknowledged. The authors are indebted to Koppány Tuba and Zsolt Ürmös for their help in preparing of reference list.

REFERENCES

1. Keeling, C.D., Whorf, T.P., Wahlen, M., and van der Plicht, J. (1995). *Nature* 375, 666.
2. Arrhenius, S. (1896). *London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science (Series 5)* 41, 237.
3. Amthor, J.S. (1995). *Global Change Biology* 1, 243.
4. Hamacher, M., Blanke, M.M., and Lenz, F. (1994). *Environmental Experimental Botany* 34, 385.
5. Constable, J.V.H., and Longstreth, D.J. (1994). *Plant Physiology* 106,1065.
6. Constable, J.V.H., Grace, J.B., and Longstreth, D.J. (1992). *American Journal of Botany* 79,415.
7. Loreto, F., Harley, P.C., Di Marco, G., and Sharkey, T.D. (1992). *Plant physiology* 98, 1437.
8. Geider, R. J., Delucia, E. H., Falkowski, P. G., Finzi, A. C., Grime, J. P., Grace, J., Kana, T. M., LaRoche, J., Long, S. P., Osborne, B. A., Platt, T., Prentice, I. C., Raven, J. A., Schlesinger, W. H., Smetacek, V., Stuart, V., Sathyendranath, S., Thomas, R. B., Vogelman, T. C., Williams, P., and Woodward, F. I. (2001). *Global Change Biology* 7, 849.
9. Lichtenthaler, H.K. (1996). *Journal of Plant Physiology* 148, 4.
10. Hunt, R., Grime J. P., Diaz S., Spring G. M., Cornelissen J. H. C., and Colasanti R. L. (1997). *Abstracta Botanica* 21, 275.
11. Bazzaz F. A., and Miao, S. L. (1993). *Ecology* 74,104.
12. Tognetti, R., Longobucco, A., Miglietta, F., Raschi, A., and Fumagalli, I. (1999). *Annals of Forest Science* 56, 493.
13. Cardon, Z.G., Berry, J.A., and Woodrow, I.E. (1995). *Journal of Biogeography* 22, 203.
14. Sestak, Z., Catsky, J., and Jarvis, P.G. (eds.) (1971). Plant Photosynthetic production- Manual of Methods. Dr. W. Junk Pub., The Hague.
15. Leadley, P.W., and Drake, B.G. (1993). *Vegetatio* 104/105, 3.
16. Ashenden, T. W., Baxter, R., and Rafarel, C. R. (1992). *Plant, Cell and Environment* 15, 365.
17. Barton, C. V. M., Lee, H. S. J., and Jarvis, P. G. (1993). *Plant, Cell and Environment* 18,1149.
18. Dufrêne, E., Pontailler, J. Y., and Saugier, B. (1993). *Plant, Cell and Environment* 16, 1131.
19. Saxe, H., Ellsworth, D.S., and Heath, J. (1998). *New Phytologist*, 139, 395.
20. Mooney, H. A., Drake, B.G., Luxmoore, R.J., Oechel, W.C, and Pirelka, L. F. (1991). *Bioscience* 41, 2, 96.
21. McLeod, A.R. (1993). In: Schulze, E.D., and Mooney, H.A. (eds.) Design and Execution of Experiments on CO_2 enrichment. Proceedings of a Workshop held at Weidenberg, Germany, 26-30 October 1992. Ecosystem Research Report 6. C.E.C./E.GuyotSA, Brussels, Belgium, pp.353-366.
22. Hendrey, G.R., Lewin, K.F., and Nagy, J. (1993). *Vegetatio* 104/105, 17.
23. Miglietta, F., Lanini, M., Bindi, M. and Magliulo, V. (1997). *Global Change Biology* 3, 417.
24. Lewin, K.F., Hendrey, G. and Kolber, Z. (1992). *Critical Reviews in Plant Sciences* 11, 135.
25. Blum, H. (1993). In: Schulze, E.D., and Mooney, H.A. (eds.) Design and Execution of Experiments on CO_2 enrichment. Proceedings of a Workshop held at Weidenberg, Germany, 26-30 October 1992. Ecosystem Research Report 6. C.E.C./E.GuyotSA, Brussels, Belgium, pp.367-370.
26. Miglietta, F., Giuntoli, A., and Bindi, M. (1996). *Photosynthesis Research* 47, 281.
27. Miglietta, F., Hoosbeek, M.R., Foot, J., Gigon, F., Hassinen, A., Heijmans, M., Peressotti, A., Saarinen, T., van Bremen, N., and Wallen, B. (2001). *Environmental Monitoring and Assessment* 66, 107.
28. Okada, M., Lieffering, M., Nakamura, M., Yoshimoto, M., Kim, M., and Kobayashi, K. (2001).

New Phytologist 150, 251-260.
29. Miglietta, F., Peressotti, A., Vaccari, F.P., Zaldei, A., De Angelis, P., and Scarascia-Mugnozza, G. (2001). *New Phytologist* 150, 465.
30. Miglietta, F., Raschi, A., Bettarini, I., Resti, R., and Selvi F. (1993). *Plant, Cell and Environment* 16, 873.
31. Tognetti R., Sebastiani L., Vitagliano C., Raschi A., and Minnocci, A. (2001). *Photosynthetica* 39, 403.
32. Miglietta, F., Raschi, A., Resti, R. and Badiani, M., (1993). *Plant, Cell and Environment* 16, 909.
33. Miglietta, F., and Raschi, A. (1993). *Vegetatio* 104/105, 391.
34. Hattenschwiler, S., Miglietta, F., Raschi, A., and Korner, C. (1997). *Acta Oecologica* 18 (3), 361.
35. Bettarini, I., Vaccari, F.P., and Miglietta, F. (1997). *Global Change Biology* 4, 17.
36. Cook A.C., Tissue D.T., Roberts S.W., and Oechel W.C. (1998). *Plant, Cell and Environment* 21 417.
37. Newton, P. C. D., Clark, H., Edwards, G. R. and Ross, D. J. (2001). *Ecology Letters* 4, 344.
38. Morner, N-A, and Etiope, G. (2002). *Global and Planetary Change*, in press.
39. Goudriaan, J. (1995). In: Beran, A.M. (ed.) Carbon sequestration in the biosphere. Springer Verlag. Berlin, Heidelberg. NATO ASI series, 133, pp. 3-18.
40. Eamus, D., and Jarvis, P.G. (1989). *Advances in Ecological Research* 19, 1.
41. Ceulemans, R., and Mousseau, M. (1994). *New Phytologist* 127, 425.
42. Sala, O.E., Laurenroth, W.K., and Burke, I.C. (1996). In: Breymeyer, A.I., Hall, D.O., Melillo, J.M., and Agren, G.I. (eds) Global Change: Effects on coniferous forests and grasslands. John Wiley & Sons, Chichester New York Brisbane Toronto Singapore, pp.101-120.
43. Schlesinger, W. H. (1991). Biogeochemistry: an analysis of global change. New York: Academic Press.
44. Casella, E., and Sousanna, J.F. (1997). *Journal of Experimental Botany* 48, 1309.
45. Simard, S.W., Perry, D.A., Jones, M.D., Myrold, D.D., Durall, D.M., and Molina, R. (1997). *Nature* 388, 579.
46. Campbell, B. D., Stafford Smith, D. M., Ash, A. J., Fuhrer, J., Gifford, R. M., Hiernaux, P., Howden, S. M., Jones, M. B., Ludwig, J. A., Manderscheid, R., Morgan, J. A., Newton, P. C. D., Nösberger, J., Owensby, C. E., Soussana, J. F., Tuba, Z., and ZouZhong, C. (2000). *Agriculture, Ecosystems and Environment* 82, 39.
47. Kimball, B. A., and Idso, S B. (1983). *Agricultural Water Management* 7, 55.
48. Cure, J.D., and Acock., B. (1986). *Agriculture and Forest Meteorology* 38, 127.
49. Mooney, H. A., Canadell, J., Chapin, E. S., Ehleringer, J., Körner, Ch., McMutrie, R., Parton, W. J., Pirelka, L., and Schulze, E. D. (1999). In: Walker, B. H., Steffen, W. L., Canadell, J., and Ingram, J. S. I. (eds.). The terrestrial Biosphere and Global Change: Implications for Natural and Managed Ecosystems. IGBP Book Series No. 4., Cambridge University Press, Cambridge, UK, pp. 141-189.
50. Coughenour, M.B., and Chen, D.X. (1997*). Ecological Applications* 7, 802.
51. Casella, E., J. F., Soussana, Loiseau, P., and Ackerly, D.D. (1996). *Plant and Soil* 182, 83.
52. Hebeisen, T., Löscher, A., Zanetti, S., Fischer, B.U., Hartwig, U.A., Frehner, M., Hendrey, G.R., Blum, H., and Nösberger, J. (1997). *Global Change Biology* 3, 149.
53. Owensby, C.E., Ham, J.M., Knapp, A.K., Bremer, D., and Auen, L..M. (1997). *Global Climate Change* 3, 189.
54. Owensby, C.E., Ham, J.M., Knapp, A.K, and Auen, L.M. (1999). *Global Change Biology* 5, 497.
55. Ross, D.J., Tate, K.R., Newton, P.C.D., and Anderson, J.M. (1995). *Plant and Soil* 176, 37.
56. Newton, P. C. D., Clark, H., Bell, C.C., Glasgow, E.M., Tate, K.R., Ross, D.J., Yeates, G. W., Saggar, S., and Baker, C.J. (1995). *Journal of Biogeography* 22, 235.
57. Chen, D. X., Hunt, H.W., and Morgan, A.J. (1996). *Ecological Modelling* 87,11.
58. Hunt, H. W., Elliott, E.T., Detling, J.K., Morgan, J.A., Chen, D.X., and Acock, B. (1996). *Global Change Biology* 2, 35.
59. Jongen, M., and Jones, M. B. (1997). *Abstracta Botanica* 21, 249.
60. Soussana, J. F., Casella, E., Loiseau, P., and Billes, G. (1996). *Plant and Soil* 182, 101.
61. Tuba, Z., Jones, M.B., Szente, K., Nagy, Z., Garvey, L., and Baxter, R. (1998). *Annals of the New York Academy of Sciences* 851, 241.
62. Leadley, P.W., and Körner, Ch. (1996). In: Körner, Ch., and Bazzaz, F.A. (eds). Carbon dioxide, Populations and Communities. Academic Press, San Diego. p 159-175.
63. Leadley, P.W., and Stöcklin, J. (1996). *Global Change Biology* 2, 389.

64. Schäppi, B., and Körner, C. (1996). *Oecologia* 105, 43.
65. Hannukkala, A. (1997). *Abstracta Botanica* 21, 261.
66. Junttila, O. (1996). *Agriculture and Food Science in Finland* 5:251.
67. Tissue, D.T., and Oechel, W.C. (1987). *Ecology* 8, 401.
68. Hilbert, D.W., Prudhomme, T.I., and Oechel, W.C. (1987). *Oecologia*, 72, 466.
69. Campbell, B.D., Stafford Smith, D. M., and McKeon, G.M. (1997). *Global Change Biology* 3, 177.
70. Field, C.B., Lund, C.P., Chiarello, N.R., and Mortimer, B.E. (1997). *Global Climate Change* 3, 197.
71. Zitzer, S. F., Archer, S. R., and Boutton, T. W. (1996). *Journal of Applied Ecology* 33,1125.
72. Soussana, J. F., Hartwig, U.A., and Arnone,J.A. (1996). *Plant and Soil*, 187, 321.
73. *Jones, M., and Jongen, M.* (1996). *Agriculture and Food Science in Finland* 5, 271.
74. Lilley, J.M., Bolger, T.P., and Gifford, R.M. (1997). Proceedings of the XVIII International Grasslands Congress, pp. 9.21-9.22
75. Howden, S.M., McKeon, G.M., Carter, J.O., Beswick, A. (1999). In: Eldridge, D., and Freudenberger, D. (eds.), Proceedings of VI International Rangelands Congress. pp.41-43.
76. Hall, W.B., McKeon, G.M., Carter, J.O., Day, K.A., Howden, S.M., Scanlan, J.C., Johnston, P.W., and Burrows, W.H. (1998). *Rangeland Journal* 20, 177.
77. Walker, L., Ash., Brown, J. (1999). In: Eldridge, D., and Freudenberger, D. (eds.). Proceedings of VI International Rangelands Congress. pp.262-263.
78. Reich, P. B, Knops, J., Tilman, D., Craine, J., Ellsworth, D., Tjoelker, M., Lee, T., Wedin, D., Naeem, S., Bahauddin, D., Hendrey, G., Jose, S., Wrage, K., Goth, J., and Bengston, W. (2001). *Nature* 410, 809.
79. Whipps, J. M. (1985). *Journal of Experimental Botany* 36, 644.
80. Sadowsky, N. J., and Schortemeyer, M. (1997). *Global Change Biology* 3, 217.
81. Diaz, S., Grime, J.P., Harris, J., and McPherson, E. (1993). *Nature* 364, 616.
82. Ball, B. G. and Drake, A. S. (1997). *Global Change Biology* 3, 29.
83. Raschi, A., Menconi, M., Cosentino, V., Sgherri, C. L. M., and Navari-Izzo, F. (1997). *Abstracta Botanica* 21, 289.
84. Owensby, C. (1993). *Water Air and Soil Pollution* 70, 413.
85. Jongen, M, Jones, M.B., Hebeisen, T., Blum, H., and Hendrey, G. (1995). *Global Change Biology* 1, 361.
86. Gorissen, A., van Ginkel, J.H., Keurentjes, J.J.B., and van Veen, J.A. (1995). *Soil Biology and Biochemistry* 27, 117.
87. Runion, G.B., Curl, E.A., Rogers, H.H., Backman, P.A., Rodriguez-Kabana, R., and Helms, B.E. (1994). *Agricultural and Forest Meteorology*, 70, 117.
88. Field, C.B., Chapin, III F.S., Matson, P.A., and Mooney, H. (1992). *Annual Review of Ecology and Systematics* 23, 201.
89. Gifford, R. M. (1994). *Australian Journal of Plant Physiology*, 21, 1.
90. Luo Y, Field C.B., and Mooney, H.A. (1994). *Plant, Cell and Environment* 17, 1195.
91. Long, S.P., Jones, M.B., and Roberts, M.J. (1992) Primary Productivity of grass ecosystems of the tropics and the sub-tropics. Chapmann & Hall, London, New York, Melbourne, Madras. pp. 267.
92. Stevenson, F.J. (1982) Humus chemistry. John Wiley & Sons, New York, Chicester, Brisbane, Toronto, Singapore. pp 375.
93. Postl, W. F., Bolhár-Nordenkampf, H. R., Preinl N., Kamnalrut, A., and Meister, M. (1997). *Abstracta Botanica 21*, 235.
94. Körner, C., and Bazzaz, F. A. (eds.) (1996) Carbon Dioxide, Populations, and Communities. Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto.
95. Polley, H.W., Morgan, J.A., Stafford Smith, M., and Campbell, B. (2000). In: Crop Ecosystem Responses to Global Climate Change. CAB International, UK.
96. Stafford Smith, M., McKeon, G., Buxton, R., and Breen, J. (1999) The integrated impacts of price, policy and productivity changes on land use in northern Australia. Proceedings of VI International Rangelands Congress. pp. 864-866.
97. Stirling, C.M., Williams T. G., Jones M. L., and Ashenden, T. W. (1997). *Abstracta Botanica* 21, 265.
98. Körner, Ch., Bazzaz, F.A., and Field, C.B. (1996). In: Körner, Ch., and Bazzaz F.A. (eds). Carbon dioxide, Populations and Communities. Academic Press, San Diego. p. 443-456.
99. Luo, Y. Q., Jackson, R.B., Field, C. B., Mooney, H.A., and Bowden, R. D. (1996). *Oecologia* 108, 130.

100. Hungate, B. A., Holland, E.A., Jackson, R.B., Chapin,F.S., Mooney, H.A., and Field, C.B. (1997). *Nature* 388, 576.
101. Tuba, Z., Szente, K., Nagy, Z., Csintalan, Zs., and Koch, J. (1996a). *Journal of. Plant Physiology* 148, 356.
102. Samarakoon, A.B., and Gifford, R.M. (1996). *Journal of. Plant Physiology* 23, 63.
103. Zólyomi, B., and Fekete, G. (1994). *Abstracta Botanica* 18, 29.
104. Szente, K., Nagy, Z., Tuba, Z., and Fekete, G. (1996). *Photosynthetica* 32, 399.
105. Campbell, B.D., and Hunt, D.Y. (2001). In: Tow, P.G., and Lazenby A. (eds.). Competition and Succession in Pastures, CAB International, UK. p 233-259.
106. Fredeen, A.L., Randerson, J.T., Holbrook, N.M., and Field, C.B. (1997). *Journal of the American Water Resources Association* 33, 1033.
107. Morgan, J.A., Lecain, D.R., Mosier, A.R., and Milchunas, D.G. (2001). *Global Change Biology* 7, 451.
108. Drake, B. G., Gonzalez Meler, M. A., Long, S.P., and Ackerson, R.E. (1997). *Annual Review of Plant Physiology and Plant Molecular Biology* 48, 609.
109. Long, S.P. (1991). *Plant, Cell and Environment* 14, 729.
110. Gonzáles-Meler, M.A., Ribas-Carb, M., Siedow, J.N., and Drake, B.G. (1997). *Plant Physiology* 112, 1349.
111. Ryle, G. J. A., and Stanley, J. (1992). *Annals of Botany* 6, 563.
112. Schapendonk, A.H.C.M., and Goudriaan, J. (1995). In: Proceedings of the Tenth International Photosynthesis Congress, Montpellier, Kluwer Academic Publishers, Dordrecht Boston London
113. Atkinson, C. J., Taylor, J. M., Wilkins, D., and Besford, R. T. (1997). *Tree Physiology* 17, 319.
114. Nagy, Z., Szente K, and Tuba, Z. (1997). *Abstracta Botanica* 21, 297.
115. Barták, M., Nijs, I., and Impens, I. (1997). *Abstracta Botanica* 21, 317.
116. Körner, Ch., and Miglietta, F. (1994). *Oecologia* 99, 343.
117. Parton, W. J., Scurlock, J. M. O., Ojima, D. S., Schimel, D. S., Hall, D. O., and Bailey, R. G. (1995). *Global Change Biology* 1, 13.
118. Nijs, I., Impens, I., and Allen, S. G. (1996). *Journal of Experimental Botany* 47, 915.
119. Vu, J. C. V., Allen, L. H., Boote, K. J., Bowes, G., and Baker, J.T. (1997). *Plant Cell and Environment* 20, 68.
120. Sionit, N., and Patterson, D.T. (1984). *Oecologia* 65, 30.
121. Wand, S. J.E., Midgley, G. F., Jones, M. H., and Curtis, P.S. (1999). *Global Change Biology* 5, 723.
122. Jacob, J. , Greitner, C., and Drake, B. G. (1995). *Plant, Cell and Environment* 18, 875.
123. Jarvis, P. G. (1993). In: Borghetti, M., Grace M. J., and Raschi, A (eds.). Water Transport in Plants under Climatic Stress. Cambridge University Press, Cambridge, pp. 1-13.
124. Badiani, M., Raschi, A., Paolacci, A.R., and Miglietta, F. (1999). In: Agrawal S.B. and Agrawal M. (eds). Environmental Pollution and Plant Responses. Lewis Publishers, Boca Raton Ann Arbor London Tokyo. pp. 45-81.
125. Van Gardingen, P.R., Grace, J., Jeffree, C.E., Byari, S.H., Miglietta, F., Raschi, A., and Bettarini, I. (1997). In: Raschi, A., Miglietta, F., Tognetti, R. and van Gardingen, P.R. (eds.). Plant responses to elevated CO_2 – Evidence from Natural Springs. Cambridge University Press, Cambridge, pp. 69-86.
126 Pfanz, H., Vodnik., D., Wittmann, C., Aschan, G., and Raschi, A. (2003). *Progress in Botany*, in press.
127 Vodnik, D., Pfanz, H., Maček, I., Kastelec, D., Lojen, S., and Batič, F. (2002). *Photosynthetica* 40, 575.
128 Vodnik, D., Šircelj, H., Kastelec, D., Maček, I., Pfanz, H., and Batič, F. (2003) The effects of natural CO_2 enrichment on the growth of maize. *Journal of Crop Production*, in press.
129. Rogers, H.H., Sionit, M., Cure, J..D., Smith, J..M., and Bingham, G.E. (1984). *Plant Physiology* 74, 233.
130. Morison, J. I. L. (1985). *Plant Cell Environment* 8, 467.
131. Beerling, D.J., and Woodward, F.I. (1995a) .*Functional Ecology* 9, 394.
132. Szente, K., Nagy, Z., and Tuba, Z. (1998). *Photosynthetica* 35, 637.
133. Beerling, D.J., and Woodward, F.I. (1995b). *Annals of Botany* 75, 507.
134. Dugas, W.A., Heuer, M.L., Hunsaker, D., Kimball, M. A., Lewin, K.F., Nagy, J., and Johnson, M. (1994). *Agricultural and Forest Meteorology* 70, 231.
135. Thornley, J. H. M., Cannell, M. G. R., and Met, O. F. F. (1996). *Plant, Cell and Environment* 19, 1331.

136. Menconi, M., Raschi, A., Cosentino, V., Sgherri, C.L.M., and Navari-Izzo, F. (1997). *Abstracta Botanica* 21, 305.
137. Conroy, J.P., Virgona, J.M., Smillie, R.M., and Barlow, E.W. (1988). *Plant Physiology* 86, 1108.
138. Thomas, J.F., and Harvery, C.N. (1983). *Botanical Gazette* 144, 303.
139. Sasek, T.W., and Strain, B.R. (1989). *Weed Science* 37, 23.
140. Reekie, E.G., and Bazzaz, F. A. (1989). *Oecologia* 79, 212.
141. Wray, S.M., and Strain, B.R. (1986). *American Journal of Botany* 73, 1486.
142. Tyree, M.T., and Alexander, J.D (1993). *Vegetatio* 104/105, 47-62.
143. Jones, M., Jongen, M., and Doyle, T. (1996*). Agricoltural and Forest Meteorology* 79, 243.
144. Zanetti, S., Hartwig, U.A., van Kessel, C., Luscher, A., Hebeisen, T., Frehner, M., Fischer, B. U., Hendrey, G.R., Blum, H., and Nösberger, J. (1997). *Oecologia* 112, 17.
145. Schenk,U., Jäger, H.-J., and Weigel, H.-J. (1997). *Grass and Forage Science* 52, 232.
146. LeCain, D.R., and Morgan, J.A., (1998). *Physiology of Plant* 102, 297.
147. Soussana, J.F., and Loiseau, P. (1997). *Abstracta Botanica* 21, 223.
148. Parton, W. J., Coughenour, M. B., Scurlock, J. M. O., Ojima, D. S, Gilmanov, T. G., Scholes, R.J., Schimel, D. S., Kirchner, T. B., Menaut, J.-C., Seastedt, T. R., Garcia Moya, E., Kamnalrut, A., Kinyamario, J. I., and Hall, D. O. (1996). In: Breymeyer, A. I., Hall, D. O., Melillo, M., and Agren, G. I. (eds). Global change: Effects on coniferous forests and grasslands. John Wiley and Sons Ltd, Chicester, U.K. pp. 229-266.
149. Wessman, C.A. (1992). *Annual Review of Ecology and Systematics* 23, 175.
150. Jarvis, P.G. (1995). *Plant, Cell and Environment* 18, 1079.
151. Cernusca, A., Bahn, M., Chemini, C., Graber, W., Siegwolf, R., Tappeiner, U., and Tenhunen, J. (1999). *Ecological Modelling* 113, 167.
152. Wohlfahrt, G., Bahn, M., Tappeiner, U., and Cernusca, A. (2001). *Agricultural and Forest Meteorology* 106, 261.
153. Lutze, L.J. and Gifford, R.M. (1998). *Global Change Biology* 4: 851.
154. Cannell, M.G.R., and Thornley, J.H.M. (1998). *Global Change Biology* 4, 431.
155. Williams, M.A., Rice, C.W., and Owensby, C.E. (2000). *Plant and Soil* 227, 127.
156. Thornley, J H M and Cannell, M G R (1997). *Annals of Botany* 80, 205.
157. Hungate, B. A., Jackson, R.B., Field, C.B., and Chapin, F.S. (1996). *Plant and Soil* 187, 135.
158. Hawksworth, D.L., and Ritchie, J.M. (1993) Biodiversity and Biosystematic Priorities: Microorganisms and Invertebrates. CAB International, Wallingford, UK. Pp. 70.
159. Smith, A.J.E. (1982) Bryophyte Ecology. Chapman & Hall, London.
160. Csintalan, Zs., Takács, Z., Proctor, M. C. F., Nagy, Z. and Tuba, Z. (2000). *Plant Ecology* 151, 51.
161. Kappen, L. (1973). In: Ahmadjia, V., and Hale, M.E. (eds). The Lichens. Academic Press, New York, London. pp. 311-380.
162. Proctor, M.C.F. (1982). In: Smith, A.J.E. (ed). Bryophyte Ecology. Chapman and Hall, London. pp.333-367.
163. Tuba, Z., Proctor, M.C.F., and Csintalan, Zs. (1998b*). Plant Growth Regulation* 24, 211.
164. Tuba, Z., Csintalan, Zs., and Proctor, M.F.C. (1996b). *New Phytologist* 133, 353.
165. Tuba, Z., Proctor, M.C.F., and Takács, Z. (1999). *Zeitschrift für Naturforschung* 54, 88.
166. Peterson, K. M., and Billings, W. D. (1975). *Naturalist* 94, 88.
167. Oberbauer, S. F., Oechel, W. C., Riechers, G. H. (1986). *Plant and Soil* 96, 145.
168. Proctor, M.C.F. (1990). Botanic Journal of Linnean Society 104, 61.
169. Svejnbjörnsson, B., and Oechel, W.C. (1992). In: Bates, J.W., and Farmer, A.M. (eds.). Bryophytes and Lichens in a Changing Environment. Clarendon Press, Oxford, pp. 77-102.
170. Csintalan, Z., Tuba, Z., and Laitat, E. (1995). In: Mathis, P. (ed.). Photosynthesis from Light to Biosphere, Vol. V. Kluwer Academic Publishers, Dordrecht, pp. 925-928.
171. Csintalan, Zs., Takács, Z., Tuba, Z., Proctor, M. C. F., Smirnoff, N., and Grace, J. (1997). *Abstracta Botanica* 21, 309.
172. Dilks, T. J. K., and Proctor, M. C. F. (1979). *New Phytologist*, 97, 82.
173. Tuba, Z., Csintalan, Zs., Szente, K., Nagy, Z., and Grace, J. (1998). *Functional Ecology* 12, 39.
174. Balaguer, L., and Barnes, J. D. (1999) Potential impacts of long-term CO_2 enrichment on green-algal lichens. In: Raschi, A., Vaccari, F.P, Miglietta, F. (eds.). Ecosystem response to CO_2 the maple project results. European commission, Luxembourg. pp. 106-116.
175. Lawrey, J. D. (1995). In: Inderjit, D., Dakshini, K. M. M., and Einhellig, F. A. (eds.). Allelopathy. Organisms, processes and applications. American Chemical Society, Symposium Series 582.,

Washington DC. pp 26-38.
176. Cipollini, M.L., Drake, B.G., and Whigham, D. (1993). *Oecologia* 96, 339.
177. Lange, O. L., and Tenhunen, J. D. (1981). *Oecologia* 51, 426.
178. Takács, Z., Csintalan, Zs., and Tuba, Z. (1999). *Zeitschrift für Naturforschung* 54, 797.
179. Gunderson, C. A., Sholtis, J. D., Wullschleger, S. D., Tissue, D. T., Hanson, P. J., and Norby, R. J. (2002). *Plant, Cell and Environment* 25, 379.
180. Hymus, G. J., Ellsworth, D. S., Baker, N. R., and Long, S. P. (1999*). Plant Physiology* 120, 1183.
181. Taylor, G., Ceulemans, R., Ferris, R., Gardner, S. D. L., and Shao, B. Y. (2001). *Environmental Pollution* 115, 463.
182. Takeuchi, Y., Kubiske, M. E., Isebrands, J. G., Pregitzer, K. S., Hendrey, G., and Karnosky, D. F. (2001). *Plant, Cell and Environment* 24, 1257.
183. Ceulemans, R., Taylor, G., Bosac, G., Wilkins, D., and Besford, R. T. (1997). *Journal of Experimental Botany* 48, 1681.
184. Gunderson, C. A., and Wullschleger S. D. (1994). *Photosynthesis Research* 39, 369.
185. Marek, M. V., and Kalina, J. (1996). *Photosynthetica* 31, 129.
186. Sage, R. F. (1994). *Photosynthesis research* 39, 351.
187. Curtis, P. S. (1996). *Plant, Cell and Environment* 19, 127.
188. McConnaughty, K. D. M., Nicotra, A. B., and Bazzaz, F. A. (1996). *Ecological Applications* 6, 619.
189. Scarascia Mugnozza G. E., De Angelis, P., Sabatti, M., Matteucci, G., Kuzminsky, E., Larbi, H., Valentini, R., Calfapietra, C., Oro, T., Tamantini, M. (1996) *Plant Cell Environment*, 19: 643.
190. Ellsworth, D. S., LaRoche, J., and Hendrey, G.R. (1998) Elevated CO_2 in a prototype free-air CO_2 enrichment facility affects photosynthetic nitrogen use in a maturing pine forest. Brookhaven National Laboratory Report vol. BNL 52545. Brookhaven National Laboratory, Upton, New York, USA.
191. Turnbull, M. H., Tissue, D. T., Griffin, K. L., Rogers, G. N. D., Whitehead, D. (1998). *Plant, Cell and Environment*, 21:1019.
192. Houpis, J. L. J., Anderson, P. D., Pushnik, J. C., and Anschel, D. J. (1999). *Water, Air, and Soil Pollution* 116, 403.
193. Runion, G.B., Mitchell, R. J., Green, T. H., Prior, S. A., Rogers, H. H., and Gjerstad, D. H. (1999). *Journal of Environmental Quality* 28, 880.
194. Roberntz, P., and Stockforns, J. (1998). *Tree physiology* 18, 233.
195. Barton, C. V. M., and Jarvis, P., G. (1999). *New Phytologist* 144, 233.
196. Li, J. H., Dijkstra, P., Hymus, G. J., Wheeler, R. M., Piastuchi, W. C., Hinkle, C. R., and Drake, B. G. (2002). *Global Change Biology* 6, 727.
197. Norby, R.J., Cotrufo, M.F., Ineson, P., O'Neill, E.G., and Canadell, J.G. (2001). *Oecologia* 127, 153.
198. Rey, A., and Jarvis, P. G. (1998). *Tree physiology* 18, 441.
199. Gielen, B., Jach, M.E., and Ceulemans, R. (2000). *Photosynthetica* 38, 13.
200. Hättenschwiler, S., Miglietta, F., Raschi, A., and Körner, C. (1997) *Global Change Biology* 3, 463.
201. Maherali, H., DeLucia, and E. H. (2000). *American Journal of Botany* 87, 243.
202. Tissue, D.T., Thomas, R.B., and Strain, B.R. (1997) Plant, Cell and Environment, 20: 1123.
203. Usami, T., Lee, J., and Oikawa, T. (2001). *Plant, Cell and Environment* 24, 1007.
204. Maillard, P., Guehl, J. M., Muller, J. F., and Gross,P. (2001). *Tree Physiology* 21, 163.
205. Curtis, P. S., Vogel, C. S., Wang, X. Z., Pregitzer, K. S., Zak, D. R., Lussenhop, J., Kubiske, M., and Teeri, J. A. (2000). *Ecological Applications* 10, 3.
206. Mikan, C. J., Zak, D. R., Kubiske, M. E., and Pregister, K. S. (2000). *Oecologia* 124, 432.
207. Anderson, P. D., and Tomlinson, P.T. (1998). *New Phytologist* 140, 477.
208. Atkinson, C. J., Taylor, J. M. (1996). *New Phytologist* 133, 617.
209. Hättenschwiler, S., Schweigruber, F. H., and Körner, C. (1996). *Plant, Cell and Environment* 19, 1369.
210. Couteaux, M.M., Kurz, C., Bottner, P., and Raschi, A. (1999). *Tree Physiology* 19, 301.
211. Ellsworth, D. S., Oren, R., Huang, C., Phillips, N., and Hendrey, G.R. (1995). *Oecologia* 104, 139.
212. Paoletti, E., Miglietta, F., Raschi, A., Manes, F., and Grossoni, P., (1997). In: Miglietta, F., Raschi, A., and van Gardingen, P. (eds.). Responses to Elevated Carbon Dioxide: Evidence from Natural Springs. Cambridge University Press, Cambridge. pp. 267.
213. Tognetti, R., Giovannelli, A., Longobucco A., Miglietta, F., and Raschi, A. (1996). *Annales de Sciences Forestières* 53, 475.
214. Tognetti, R., Longobucco, A., Miglietta, F., and Raschi, A. (1998). *Plant, Cell and Environment* 21,

613.
215. Tognetti, R., Longobucco, A., Miglietta, F., and Raschi, A. (1999). *Tree Physiology* 19, 261.
216. Miglietta, F., and Amthor, J. S. (1996). *Weather*, 51, 322.
217. Woodward, F. I. (1987). *Nature* 327, 617.
218. Duquesnay, A., Breda, N., Stievenard, M., and Dupouey, J. L. (1998). *Plant, Cell and Environment* 21, 565.
219. Heath, J., Kerstiens, G., and Tyree, M. T. (1997*). Journal of Experimental Botany* 48, 1487.
220. Tognetti, R., Longobucco, A., and Raschi, A. (1999). *Tree Physiology* 19, 271.
221. Jones, M. B., Brown, J. C., Raschi, A., and Miglietta, F. (1995). *Global Change Biology* 1, 295.
222. Tognetti, R., Longobucco, A., Raschi, A., and Jones, M.B. (2001). *Australian Journal of Plant Physiology* 28, 1.
223. Tognetti, R., Minnocci, A., Peñuelas J., Raschi, A., and Jones, M.B. (2000). *Journal of Experimental Botany* 51, 1135.
224. Morse, S.R. (1993). *Oecologia* 95, 599.
225. Vivin, P., Guehl, J. M., Clément, A., and Aussenac G. (1996). *Annales des Sciences Forestiéres* 53, 447.
226. Tschaplinski, T.J., Norby, R.J., and Wullschleger S.D. (1993). *Tree physiology* 13, 283.
227. Tschaplinski, T.J., Stewart, D.B., and Norby, R.J. (1995). *New Phytologist* 131, 169.
228. Polley, H.W. (1997) *Journal of Range Management*, 50, 561.
229. Allen, L.H., Valle, R.R., Jones, J.W., and Jones, P.H. (1998). *Agronomy Journal* 90, 375.
230. Kubiske M.E., and Pregitzer K.S. (1996). *Tree Physiology* 16, 351.
231. Kubiske M.E., and Pregitzer K.S. (1997). *Functional Ecology* 11, 24.
232. Hättenschwiler, S. (2001). *Oecologia* 129, 31.
233. Bucher, J.B., Tarjan, D.P., Siegwolf, R.T.W., Saurer, M., Blum, H., and Hendrey, G.R. (1997). *Chemosphere* 36, 777.
234. Körner, C. (1996). In: Walker, B.H, Steffen, W.L. (eds.). Global Change and Terrestrial Ecosystems. Cambridge University Press, Cambridge. pp 20-42.
235. Polle, A., McKee, I., and Blaschke, L. (2001). *Plant, Cell and Environment* 21, 1075.
236. Moe, R. (1984). *Acta Horticulturae* 162, 217.
237. Walls, I. G.(1989) Growing tomatoes. David and Charles Newton Abbot, London. p. 197.
238. Mortensen, L. M. (1982). *Gartenbauwissenschaft* 47, 14.
239. Calvert, A., and Slack, G. (1975). *Journal of Horticultural Science* 51, 401.
240. Mortensen, L. M. (1983). *Meld. Norges Landbrukshoegskole* 62, 1.
241. Mortensen, L.M., and Ulsaker, R. (1985). *Scientia Horticulturae* 27, 133.
242. Alvino, A. (1990). *Acta Horticulturae* 278, 15.
243. Mortensen, L.M. (1987). *Scientia Horticulturae* 33, 1.
244. Fuchs, H.W.M (1991) Plant physiology in greenhouse growing IAC. The Netherlands, Wageningen. p.24.
245. Mortensen, L.M., and Moe, R. (1983) *Scientia Horticulturae* 19, 133.
246. Enoch, H.Z., Carmi, I., Rounick, J.S., and Magaritz, M. (1984). *Plant Physiology* 76, 1083.
247. Kimball, B.A., and Mitchell, S.T. (1979). *Journal of the American Society of Horticulture Science* 4, 515.
248. Jones, P., Allen, L.H., Jones, J.W., and Valle, R. (1985). *Agronomy Journal* 77, 119.
249. Kramer, P. J. (1981). *BioScience* 31, 29.
250. Enoch, H.Z., and Hurd, R.G.(1977). *Journal of Experimental Botany* 28(102), 84.
251. Ku, S.B., Edwards, G.E., Tanner, C.B. (1977). *Plant Physiology* 59, 868.
252. DeLucia, E.H., Sasek, T.W., and Strain, B.R. (1985). *Photosynthesis Research* 7, 175.
253. Sasek, T.W., DeLucia, E.H., and Strain, B.R. (1985). *Plant Physiology* 78, 619.
254. Enoch, H. Z., and Hurd, R. G. (1979). *International Journal of Biometeorology* 23(4), 343.
255. Yamaguchi, M. (1983) World Vegetables AVI. Publishing Company. Westport CT., p. 382.
256. Farquhar, G.H., and Sharkey T.D. (1982). *Annual Review of Plant Physiology* 33, 317.
257. Sage, R.F., and Sharkey, T.D. (1987). *Plant Physiology* 84, 658.
258. Rogers, H.H., Thomas, J.F., and Bingham, G.E. (1983). *Science* 220, 428.
259. Tolbert, N.E., and Zelitch, I. (1983). In Lemon, E.R. (ed.). CO_2 and Plants: The responses of plants to rising levels of atmospheric carbon dioxide. AAAS Selected Symposia, 84, 21-64. Westview Press, Boulder, Colorado.
260. Sebesta, Z., and Reiersen, D. (1981). *Acta Horticulturae* 115, 409.

261. Slack, G., and Hand, D.W. (1985). *Journal of Horticultural Science* 4, 507.
262. Marc, J., and Gifford, R.M. (1983). *Canadian Journalof Botany* 62, 9.
263. Peet, M.M., and Willits, D.H. (1984). *Acta Horticulturae* 162, 121.
264. Daunicht, H.J.(1961). *Gartenbauwissenschaft* 26, 133.
265. Wittwer, S.H., and Robb, W.M. (1964). Economic. Botany 18, 34.
266. Hand, D.W., Slack, G., and Sweeney, D.G. (1981) Lettuce crop responses to controlled levels of CO_2 enrichment. Reports of the Glasshouse Crops Research Institute, 58.
267. Ito, T. (1978). *Acta Horticulturae* 87, 139.
268. Vijverberg, A.J., and van Uffelen, J.A.M. (1977). *Acta Horticulturae* 58, 293.
269. Hand, D.W., and Soffe, R.W. (1971). *Journal of Horticulture Science* 46, 381.
270. Kimball, B.A. (1983). *Agronomy Journal* 75: 779.
271. Pinto, J.M., Botrel, T.A., Machado, E.C., and Feitosa Filho, J.C. (2000). *Acta Horticulturae* 537, 267.
272. Nakayama, F.S., and Bucks, D.A. (1980) Using subsurface trickle system for carbon dioxide enrichment. In Proceedings of XV Natural Agri. Plastics Conference, University of Arizona, Tucson..
273. Moore, F.D. (1990). *Acta Horticulturae* 278, 171.
274. Baker, R. (1988). *Critical Reviews in Biotechnology* 7(2), 97.
275. D'Andria, R., Novero, R., Smith, D.H., Shanahan, J.F., and Moore, F.D. (1990). *Acta Horticulturae* 278, 179.
276. Sionit, N., Strain, B.R., and Beckford, H.A. (1981). *Crop Science* 21, 885.
277. Clinton, B.D., and Vose, J.M. (1999). *Tree Physiology* 19, 475.
278. Hamada, Y., and Tanaka, T. (2001). *Hydrological Processes* 15, 1829.
279. Bouma, T.J., and Bryla, D.R. (2000). *Plant and Soil* 227 (1-2), 215.
280. Brook, G.A., Folkoff, M.E., and Box, E.O. (1983). *Earth Surface Processes and Landforms* 8, 79.
281. Sands, R., Nugroho, P.B., Leung, D.W.M., Sun, O.J., and Clinton, P.W. (2000). *Plant and Soil* 225, 213.
282. Song, L.H., and Liang, F.Y. (2001). *Acta Geologica Sinica-English Edition* 75(3), 288.
283. Kiefer, R.H., and Amey, R.G. (1992). Concentrations and controls of soil carbon dioxide in sandy soil in the North Carolina coastal plain. *Catena* 19, 539.
284. Mattson, K.G. (1994). In: Hatfield. J.L. and Stewart B.A (eds.) Advances in Soil Sciences, Soils and Global Change. Lewis Publishers, Boca Raton, FL. pp. 329-341.
285. Yavitt, J.B., Fahey, T.J., and Simmons, J.A. (1995). *Soil Science Society of America Journal* 59, 796.
286. Nobel, P.S., and Palta, J.A. (1989). *Plant and Soil* 120(2), 263.
287. Pritchard, S.G., and Rogers, H.H. (2000). *New Phytologist* 147, 55.
288. Bernston, G.M., and Bazzaz, F.A. (1996). *Plant and Soil* 187, 119.
289. Rogers, H.H., Runion, G.B., Prior, S.A.., and Torbert, H.A. (1999). In: Luo, Y., and Mooney, H. A. (eds.). Carbon dioxide and Environmental Stress. Academic Press, San Diego. pp. 215- 244.
290. Rogers, H.H., Prior, S.A., Runion, G.B., and Mitchell, R.J. (1996). *Plant and Soil* 187, 229.
291. Tingey, D.T., Phillips, D.L., and Johnson, M.G. (2000). *New Phytologist* 147, 87.
292. Lambers, H., Van den Boogaard, R., Veneklaas, E.J., and Villar, R. (1995). *Global Change Biology*. 1, 397.
293. Stulen, I., and Den Hertog, J. (1993). *Vegetatio* 104/105, 99.
294. Fonseca, F., DenHertog, J., and Stulen, I. (1996). *New Phytologist* 133, 627.
295. Fordham, M., and Barnes, J. (1999). In: Raschi, A., Vaccari, F.P, Miglietta, F. (eds.). Ecosystem response to CO_2 the maple project results. European commission, Luxembourg. pp. 143-157.
296. Canadell, J. (1996). *Plant and Soil* 187, 391.
297. Lambers, H., Atkin, O.K., and Scheurwater, I. (1996). In: Waisel, Y., Eshel, A., Kafkafi, U. (eds). Plant Roots: The Hidden Half. Marcel Decker, Inc., New York, USA. pp 232-362..
298. Stitt, M., and Krapp, A. (1999). *Plant, Cell and Environment* 22, 583.
299. Farrar, J.F., and Jones, D.L. (2000). New *Phytologist* 147(1), 43.
300. Fitter, A.H., Self, G.K., Wolfenden, J., van Vuuren, M.M.I., Brown, T.K., Williamson, L., Graves, J.D., and Robinson, D. (1996). *Plant and Soil* 187, 299.
301. Fitter, A.H., Graves, J.D., Wolfenden, J., Self, G.K., Brown, T.K., Bogie, D., and Mansfield, T.A. (1997). *New Phytologist* 137, 247.
302. Arnone, J.A., Zaller, J.G., Spehn, E.M., Niklaus, P.A., Wells, C.E., and Korner C. (2000). *New*

Phytologist 147, 73.
303. Gavito, M.E., Curtis, P.S., and Jakobsen, I. (2001). *New Phytologist* 149, 283.
304. Pritchard, S.G., Rogers, H.H., Davis, M.A., Van Santen, E., Prior, S.A., and Schlesinger, W.H. (2001). *Global Change Biology* 7, 829.
305. Van Vuuren, M.M.I., Robinson, D., Fitter, A.H., Chasalow, S.D., Williamson, L., and Raven, J.A. (1997). *New Phytologist* 135, 455.
306. Sindøj, E., Hansson, A.C., Andrén, O., Kätterer, T., Marissink, M., Pettersson, R. (2000). *Plant and Soil* 223, 255.
307. Eissenstat, D.M., Wells, C.E., Yanai, R.D., and Whitbeck, J.L. (2000). *New Phytologist* 147, 33.
308. Van Noordwijk, M., Martikainenm, P., Bottner, P., Cuevas, E., Rouland, C., and Dhillion, S.S. (1998). *Global Change Biology* 4, 759.
309. Gregory, P.J., Palta, J.A., Batts, G.R. (1996). *Plant and Soil* 187, 221.
310. Rogers, G.S., Milham, P.J., Thibaud, M.C., and Conroy, J.P (1996). *Australian Journal of Plant Physiology* 23, 119.
311. Fordham, M.C., Barnes, J.D., Bettarini, I., Polle, A., Slee N. Raines, C., Miglietta, F., and Raschi, A. (1997). *Oecologia* 110, 169.
312. Fordham, M.C., Barnes, J.D., Bettarini, I., Griffiths, H.G., Miglietta, F., and Raschi, A. (1997). In: Raschi, A., Miglietta, F., Tognetti, R., and van Gardingenm P.R. (eds.). The impact of elevated CO_2 on the growth of Agrostis canina and Plantago major adapted to contrasting CO_2 concentrations. Cambridge University Press, Cambridge, p.174.
313. Poorter, H., and Perez-Soba, M. (2001). *Oecologia* 129, 1.
314. BassiriRad, H., Gutschick, V.P., and Lussenhop, J. (2001). *Oecologia* 126, 305.
315. Coleman, J.S., McConnaughay, K.D.M., and Bazzaz, F.A. (1993). *Oecologia* 93, 195.
316. Cotrufo, M.F., Ineson, P., Scott, A. (1998). *Global Change Biology* 4, 43.
317. Geiger, M., Haake, V., Ludewig, F., Sonnewald, U., Stitt, M. (1999). *Plant, Cell and Environment* 22, 1177.
318. Matt, P., Geiger, M., Walch-Liu, P., Engels, C., Krapp, A., and Stitt, M. (2001). *Plant, Cell and Environment* 24, 1119.
319. Cotrufo, M.F., Raschi, A., Lanini, M., Ineson, P. (1999). *Functional Ecology* 13, 343.
320. Penuelas, J., Filella, I., and Tognetti, R. (2001). *Global Change Biology* 7, 291.
321. Davey, P.A., Parsons, A.J., Atkinson, L., Wadge, K., and Long, S.P. (1999). *Functional Ecology* 13, 21.
322. Centritto, M., and Jarvis, P.G. (1999). *Tree Physiology* 19, 807.
323. Zerihun, A., Gutschick, V.P., and Bassirirad, H. (2000). *Annals of Botany* 86, 723.
324. Rudmann, S.G., Milham, P.J., and Conroy, J.P. (2001). *Annals of Botany* 88, 571.
325. Bassirirad, H., Thomas, R.B., Reynolds, J.F., Strain, B.R. (1996). *Plant, Cell and Environment* 19, 367.
326. Almeida, J.P.F., Luscher, A., Frehner, M., Oberson, A., and Nosberger, J. (1999). *Plant and Soil* 210, 159.
327. Jongen, M., Fay, P., and Jones, M.B. (1996). *New Phytologist* 132, 413.
328. Rouhier, H., and Read, D.J. (1998). *New Phytologist* 139, 367.
329. Fitter, A.H., Heinemeyer, A., and Staddon, P.L. (2000). *New Phytologist* 147, 179.
330. Sanders, I.R., Streitwolf-Engel, R., van der Heijden, M.G.A., Boller, T., and Wiemken, A. (1998). *Oecologia* 117, 496.
331. Staddon, P.L., Graves, J.D., and Fitter, A.H. (1999). *Functional Ecology* 13, 190.
332. Gavito, M.E., Curtis, P.S., Mikkelsen, T.N., and Jakobsen, I. (2000). *Journal of Experimental Botany* 51, 1931.
333. Syvertsen, J.P., and Graham, J.H. (1999). *Plant and Soil* 208, 209.
334. Jifon, J.L., Graham, J.H., Drouillard, D.L., and Syvertsen, J.P. (2002). *New Phytologist* 153,133.
335. Barrett, D.J., Richardson, A.E., Gifford, R.M., (1998). *Australian Journal of Plant Physiology* 25, 87.
336. Baxter, R., Gantley, M., Ashenden, T.W., and Farrar, J.F. (1994). *Journal of Experimental Botany* 45, 1267.
337. Fangemeier, A., Gruters, U., Hogy, P., Vermehren, B., Jager, H.J. (1997). *Environmental Pollution* 96, 43.
338. Kuehny, J.S., Peet, M.M., Nelson, P.V., and Willits, D.H. (1991). *Journal of Experimental Botany* 42, 711.

339. BassiriRad., H, Griffin, K.L., Reynolds, J.F., Strain, B.R. (1997). *Plant and Soil* 190, 1.
340. Newbery, R.M., Wolfenden, J., Mansfield, T.A., and Harrison, A.F. (1995). *New Phytologist* 130, 565.
341. Jackson, R.B., and Reynolds, H.L. (1996). *Oecologia* 105, 74.
342. Bassirirad, H., Griffin, K.L., Strain, B.R., Reynolds, J.F. (1996). *Tree physiology* 16, 957.
343. Bassirirad, H., Reynolds, J.F., Virginia, R.A., and Brunelle, M.H. (1997). *Australian Journal of Plant Physiology* 24, 353.
344. BassiriRad, H., Prior, S.A., Norby, R.J., and Rogers, H.H. (1999). *Plant and Soil* 217, 195.
345. Rothstein, D.E., Zak, D.R., Pregitzer, K.S., and Curtis, P.S. (2000). *Tree Physiology* 20, 265.
346. Bassirirad, H. (2000). *New Phytologist* 147, 155.
347. Penuelas, J., Idso, S.B., Ribas, A., and Kimball, B.A. (1997). *New Phytologist* 135, 439.
348. Allen, M.F. (1991) The ecology of mycorrhizae. Cambridge University Press, New York. Pp. 184
349. Staddon, P.L., Fitter, A.H., Robinson, D. (1999). *Journal of Experimental Botany* 50, 853.
350. Rillig, M.C., and Allen, M.F. (1999). *Mycorrhiza* 9, 1.
351. Treseder, K. K., and Allen, M. F. (2000). *New Phytologist* 147, 189.
352. Staddon, P.L., and Fitter A.H. (1998). *Trends in Ecology and Evolution* 13, 455.
353. Diaz, S. (1996). *Plant and Soil* 187, 309.
354. Wright, S.F., Franke-Snyder, M., Morton, J.B., and Updahyaya, A. (1996). *Plant and Soil* 181, 193.
355. Rillig, M.C., Wright, S.F., Kimball, B.A., Pinter, P.J., Wall, G.W., Ottman, M.J., and Leavitt, S.W. (2001). *Global Change Biology* 7, 333.
356. Rillig, M.C., Wright, S.F., Allen, M.F., and Field, C.B. (1999). *Nature* 400, 628.
357. Rillig, M.C., Hernandez, G.Y., and Newton, P.C.D. (2000). *Ecology Letters* 3, 475.
358. Newton, P.C.D., Bell, C.C., and Clark, H. (1996). *New Zeland Journal of Agricoltural Research* 39, 33.
359. Ross, D.J., Tate, K.R., Newton, P.C.D., Wilde, R.H., and Clark, H. (2000). *Global Change Biology* 6, 779.
360. Beck-Nielsen, D., and Madsen, T.V. (2001). *Aquatic Botany* 2, 141.
361. Bethenod, O., Prioul, J.L., and Deroche, M.E. (1984). *Physiologie. Végetale* 22, 565.
362. Schwintzer, C.R., and Tjepkema, J.D. (2001). *Canadian Journal of Botany* 79, 1010.
363. Cheng, W. (1999). In: Luo, Y., and Mooney, H.A. (eds.). Carbon dioxide and Environmental Stress. Academic Press, San Diego. pp. 245-264.
364. Whiting, G. J., Gandy, E.L., and Yoch, D.C. (1986). *Applied and Environmental Microbiology*. 52, 108.
365. Dakora, F.D., and Drake, B.G. (2000). *Plant, Cell and Environment* 23, 943.
.366. Hartwig, U.A., Luscher, A., Daepp, M., Blum, H., Soussana, J.F., and Nosberger, J. (2000). *Plant and Soil* 224, 43.
367. Norby, R.J., Long, T.M., Hartz-Rubin, J.S., and O'Neill, E.G. (2002). *Plant and Soil* 224, 15.
368. Amthor, J.S. (1991). *Plant, Cell and Environment* 14, 13-20.
369. Gonzalez-Meler, M.A., Ribas-Carbo, M., Siedow, J.N., and Drake, B.G. (1996). *Plant Physiology* 112(3), 1349.
370. Gonzalez-Meler, M.A., and Siedow, J.N. (1999). *Tree Physiology* 19(4-5), 253.
371. Drake, B.G., Azcon-Bieto, J., Berry, J., Bunce, J., Dijkstra, P., Farrar, J., Gifford, R.M., Gonzalez-Meler, M.A., Koch, G., Lambers, H., Siedow, J., and Wulschleger, S. (1999). *Plant, Cell and Environment* 22, 649.
372. Qi J.E., Marshall, J.D., and Mattson, K.G. (1994). *New Phytologist* 128(3), 435.
373. Palta, J.A., and Nobel, P.S. (1989). *Annals of Botany*, 63(6), 651.
374. Good, B.J., and Patrick, W.H. (1987) Root-water sediment interface processes. Aquatic Plants for Water Treatment and Resource Recovery. Magnolia Publishing, Pineville. pp. 359-371.
375. Stolwijk, J.A.J., and Thimann, K.V. (1957). *Plant Physiology* 32, 513.
376. Williamson RE, and Splinter WE (1968). *Agronomy Journal* 60, 365.
377. Lambers, H., Stulen, I., and van der Werf, A. (1996b). *Plant and Soil* 187, 251.
378. Bouma, T.J., Nielsen, K.L., Eissenstat, D.M., and Lynch, J.P. (1997). *Plant and Soil* 195(2), 221.
379. Bouma, T.J., Nielsen, K.L., Eissenstat, D.M., and Lynch, J.P. (1997). *Plant, Cell and Environment* 20(12), 1495.
380. Nijs, I., Roy, J., Salager, J.L., and Fabreguettes, J. (2000). *Global Change Biology* 6(8), 981.
381. Matamala, R., and Schlesinger, W.H. (2000). *Global Change Biology* 6(8), 967.
382. Janous, D., Pokorny, R., Brossaud, J., and Marek, M.V. (2000). *Biologia Plantarum* 43(1), 41.

383. Poorter; H., Gifford, R.M., Kriedemann, P.E., and Wong, S.C. (1992). *Australian Journal of Plant Physiology* 40, 501.
384. Yoder, C.K., Vivin, P., Defalco, L.A., Seemann, J.R., and Nowak, R.S. (2000). *New Phytologist* 145, 245.
385. Leverenz, J.W., Bruhn, D., and Saxe, H. (1999). *New Phytologist* 144(3), 437.
386. Hamilton, J.G., Thomas, R.B., and DeLucia, E.H. (2001). *Plant Cell and Environment* 24(9), 975.
387. Amthor, J. S. (2000). *Tree Physiology* 20, 139.
388. Raschi, A., Vaccari, F.P., and Miglietta, F. (1999). Ecosystem response to CO2: The MAPLE Project results. European Commission, Directorate-General for Research, Brussels. Pp. 205.
389. Maček, I., Pfanz, H., Batič, F., and Vodnik, D. (2003*). Acta Biologica Slovenica,* in press.

CHAPTER 8

OZONE: A NOVEL PLANT "PATHOGEN"

G. LORENZINI[a] AND C. SAITANIS[b]

[a] *Dipartimento Giovanni Scaramuzzi, Università di Pisa, Via del Borghetto, 80 – 56124 Pisa, Italy*
[b] *Laboratory of Ecology and Environmental Sciences, Agricultural University of Athens, Iera Odos 75 – Botanikos, 11855, Athens, Greece*

Abstract. Tropospheric ozone (O_3) is predominantly produced by photochemical reactions involving precursors generated by man's activities, especially vehicular traffic. There is also evidence for a trend towards increasing levels of tropospheric O_3. Although the levels are not threatening to human life, this gas is known to be highly phytotoxic: exposure can result in both symptomatic and subtle effects (i.e. changes in growth, yield and likely in quality of biomass produced); these may affect crops, forest plants and natural communities. Several biological, physical and chemical factors influence the plant response.
The phytotoxic mechanisms of O_3 are complex. Stomatal uptake is a prerequisite for its toxicity. A series of 'Reactive Oxygen Species' (ROS) are produced soon after exposure in the apoplast, unsaturated fatty acids and proteins being their major targets. Although the formation of ROS is generally considered to be detrimental to cellular function, these species are also formed in normal cell metabolism and their production and destruction is a regulated cellular phenomenon. Defence reactions involve detoxifying enzymes (superoxide dismutase, peroxidases, catalase) and non-catalytic scavengers (like ascorbic acid, α-tocopherol, glutathione). Life-long exposure to sublethal levels of O_3 will become a common condition for our plants. This pollutant will pose a challenging problem to world food, fibre and timber production, and to the conservation of natural plant communities, including their species diversity. Economical and ecological aspects of these interactions deserve special attention.

1. INTRODUCTION

Air is a fascinating mixture of gases and vapours, also containing many kinds of minute particles; and every living terrestrial organism is exposed to that mixture. In the last two centuries, man's activities have altered the composition of the atmosphere both locally and globally. The introduction of air pollutants poses new challenges to biota. The burning of fossil fuels releases sulphur dioxide and nitrogen oxides into the atmosphere; industrial activities produce a large amount of chemical waste; millions of vehicles emit nitrogen oxides and hydrocarbons, which give rise

L. Sanità di Toppi and B. Pawlik Skowrońska (eds.), Abiotic stresses in Plants, 205-229.

to an array of reactions which cause the formation of noxious molecules; the level of carbon oxides is growing, and new molecules (*e.g.* benzene) are dispersed. Interactions between plants and air pollutants are complex, as plants may be [2]:

- *victims* (targets) of the toxic activity of chemical stressors, which act alone or in combination;
- *gates of insertion* of persistent chemicals *in the food chain*, thus involving animals and humans;
- *agents of detoxification and bioremediation*, as they can metabolise and neutralize noxious chemicals;
- *biological indicators* of the presence of toxic agents in the environment, such as O_3, by exhibiting characteristic symptoms (qualitative signals);
- *biological monitors* of environmental health, as they may accumulate toxic agents, such as heavy metals, which can be measured in the tissues and related with the levels of those agents in the atmosphere (quantitative signals);
- *producers of biogenic molecules* (*e.g.* hydrocarbons), which may be involved in atmospheric chemistry and the production of secondary pollutants.

Of all the chemical pollutants of the atmosphere, one of them, O_3, is by far the most prevalent and causes more damage to vegetation than all the others combined [3]. Since 1958 O_3 has been recognized as a phytotoxic agent ('*grape leaf stipple*' was the first evidence, [4]). However, some years before, Californian phytopathologists had described visible foliar injury to several crops species due to air pollution [5], in which we now know that O_3 plays a major role. In many areas the presence of relevant O_3 levels during the warm season is persistent. Recently, this gas has become a subject of great interest because, besides playing a role in damaging plants, materials and manufactured goods, and causing irritation of mucous membranes, it also enhances the so-called 'greenhouse effect' (*global warming phenomenon*) and is responsible for reduced visibility.

2. OZONE IN THE ATMOSPHERE: WHAT, WHERE, WHEN AND WHY

Ozone is a triatomic allotropic form of oxygen. It is characterized by a very high chemical reactivity because it is a strong oxidant agent (*i.e.* it acquires electrons from other atoms) due to its elevated standard redox potential (+2.07 V), and plays an important role in atmospheric chemistry. It is formed when oxygen molecules are dissociated by ultraviolet light (this happens in the high atmosphere, the so-called 'ozonesphere') or by electric discharges (*i.e.* thunderbolts). Downward transport of O_3 enriched air masses from the stratosphere is a common phenomenon. Therefore, O_3 is a natural component of our atmosphere, and its presence has been known since 1840. Background mixing ratios usually range from 10 to 20 ppb (parts per billion, *i.e.* $1 \cdot 10^{-9}$, in volume; for O_3, 1 ppb = 1.96 $\mu g \cdot m^{-3}$ at 20 °C and 101.3 kPa). As the O_3 concentration is much higher in the stratosphere (10,000 ppb) than in the

troposphere (some tens ppb), on a quantitative base, tropospheric O_3 is known to account for about 10% of the vertical O_3 column above the Earth's surface [6, 7].

Apart from these natural mechanisms, O_3 is also produced another way: the chain of reactions in '*photochemical smog*' (or '*photosmog*'). This phenomenon occurs especially in heavily polluted urban and industrial areas in the presence of solar radiation.

A simplified set of some of the key reactions involved in photochemical smog formation is given here:

$$N_2 + O_2 \rightarrow 2\,NO \qquad (\textit{inside engine})$$

$$2\,NO + O_2 \rightarrow 2\,NO_2 \qquad (\textit{in atmosphere})$$

$$NO_2 + h\nu \ (\lambda \leq 420\ nm) \rightarrow NO + {}^1O_2$$

$${}^1O_2 + O_2 \xrightarrow{(M)} O_3$$

$$NO + O_3 \rightarrow NO_2 + O_2$$

The starting point is the emission of nitric dioxide (NO_2), resulting from the reaction between atmospheric nitrogen and oxygen under the high temperatures typically reached in the combustion chambers of endothermal engines. NO_2 is easily dissociated by the ultraviolet component of solar radiation into nitrogen monoxide (nitric oxide, NO) and singlet oxygen ($^1O_2$), which spontaneously reacts with molecular oxygen to give rise to O_3. This reaction requires the presence of a third inert body (usually indicated as 'M') to conserve energy. Under these conditions, the lifetime of O_3 is very short because a 'return' reaction with NO takes place to produce NO_2 and O_2 again. Therefore, there is a steady-state balance between O_3 formation and degradation, and the system represents a very rapid null-cycle in O_3 [8].

A remarkable increase in O_3 levels occurs when there are also non-methanic hydrocarbons (HC) present in the atmosphere, which are also derived mainly from vehicle exhausts, but may also be of biogenic nature (they are regularly emitted by several plants, [9]). In these conditions – which are common in all urban atmospheres – NO, instead of reducing O_3, reacts with HC to produce toxic PANs and many other organic substances:

$$NO + HC + O_2 \rightarrow \quad NO_2 + PAN\ (\textit{and other molecules})$$

Thus, (*i*) a net increase in O_3 budget takes place, and (*ii*) several organic chemical species are formed, many of which are regarded as harmful to human and environmental health. Peroxyacethyl nitrate (PAN, $CH_3C(O)OONO_2$) is the most common of them. It appears that O_3 is not only an important pollutant by itself, but it is also an indicator of photosmog.

Tropospheric O_3 concentrations in the Northern Hemisphere have been steadily increasing throughout the industrial era [8, 10]. In polluted urban areas O_3 is an intrinsic component of the so-called *chemical climatology* and its concentration may easily exceed the level of 100-150 ppb for many hours of the day, during the photochemical season (May to September in the Mediterranean). Although most O_3 is formed in urban areas, O_3 itself, as well as its precursors, may travel from cities towards rural and remote areas for hundreds of kilometres. An apparent paradox has been repeatedly reported: O_3 concentrations in the outskirts of cities are higher than in downtown areas. The average O_3 concentrations in city centres are often only 30-50% of those occurring in the surroundings [11]. This is due to (*a*) the lower concentrations of O_3 destroying pollutants (*i.e.* dust particles, NO, etc.) in the outskirts and (*b*) the fact that photochemical formation of this gas continuously takes place during the transportation of the primary pollutants from the source region – downtown – to the outskirts, leading to a gradual increase of O_3 concentration. Thus, O_3 is a major threat not only to urban plants, but also to field crops, forests and natural vegetation. The areas at the highest risk are those characterized by high degree of motorization and long, dry, hot sunny seasons. Most Mediterranean areas meet these conditions [12], and it is known that the problem is severe in many other countries, including developing ones.

Atmospheric stability, high temperatures and particularly intense solar radiation (which depend on the latitude of the region, on the time of the day and on the season of the year,) are the driving forces leading to O_3 formation [8].

The presence of near ground O_3 is expected to be at higher levels in lower latitude regions, which are characterized by higher solar radiation, a presupposition factor for the photochemical activity. It is well known, for instance, that O_3 levels in Mediterranean countries are much higher in comparison to those occurring in central and northern European countries [13]. However, the results of the ICP-Vegetation program have documented the occurrence of plant damaging O_3 episodes throughout Europe and indicated that a range of crops are potentially at risk from pollution [13]. Recently, Bytnerowicz et al. [14], who investigated the distribution of O_3 in 32 forest sites across the Carpathian Mountains, reported that potential phytotoxic effects of O_3 on trees and understory vegetation are expected in almost the entire region.

It is clear that O_3 is produced only during the daytime, with a typical diurnal cycle, and exhibits strong seasonal variation (Figure 1). In low altitude regions, O_3 usually exhibits a diurnal bell-shaped pattern: it peaks during midday and early afternoon hours and gradually decreases during late afternoon and evening hours. This cycle is mainly governed by *in situ* photochemical O_3 formation. The nearby O_3, however, is gradually destroyed by dry deposition. During the daytime, turbulent mixing in the planetary boundary layer leads to surface O_3 enrichment from the free

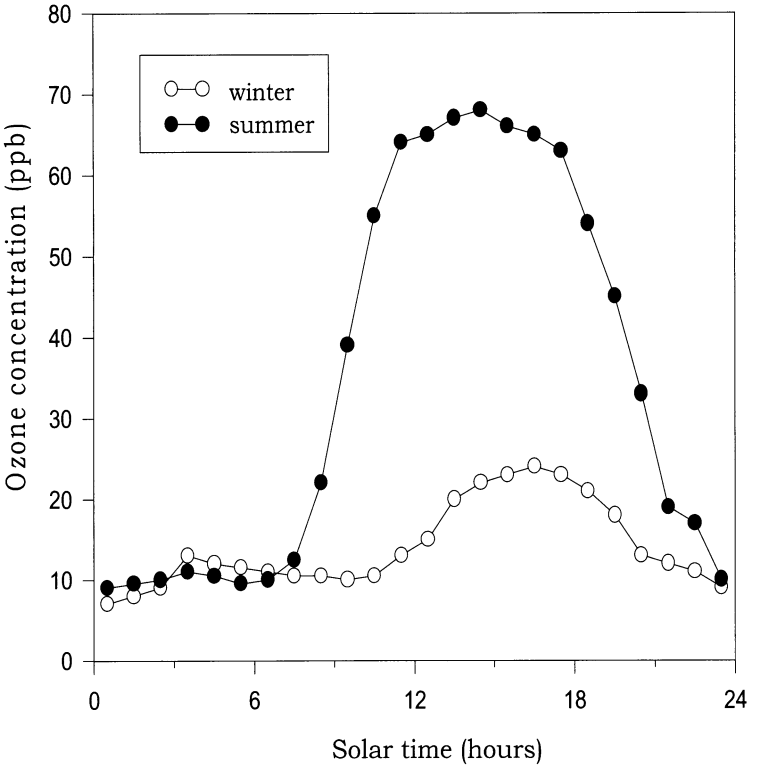

Figure 1. *Typical ozone seasonal and diurnal behaviour (average of hourly measurements) in Pisa (Italy) (after Lorenzini et al., 1994 [97]).*

troposphere, which, to a degree, replenishes the O_3 losses due to dry deposition. During night hours, O_3 cannot be formed due to the lack of light. Moreover, the lower boundary layer becomes thermally stratified and stable, impeding the replenishment of surface O_3 losses due to dry deposition. Thus, surface O_3 concentration decreases, reaching levels of a few tens ppb. The occurrence and stabilization of the boundary layer stratification depend on the local topography. At high altitude (mountainous) regions, where the nocturnal inversion layer occurs only rarely, O_3 concentrations remain relatively constant during both daytime and night-time hours [11].

As to seasonal variation, O_3 levels are higher during the late spring and summer period, mainly due to higher global radiation and to longer daytime, both of which favor photochemical formation [15, 16], but also due to more intensive atmospheric turbulences, occurring mainly during early spring months, which feed troposphere with stratospheric O_3. However, the precise contribution of the stratospheric source to tropospheric O_3 levels remains an open question. Model studies have indicated that, on an overall basis, about half of the tropospheric O_3 load results from downward transport from the stratosphere and the other half from the photochemical formation in the troposphere [15]. In urban areas anthropogenic photosmog is by far predominant. However, it is known that the levels of O_3 and their seasonal and daily patterns depend on geographical characteristics and local topography. All these factors are important and should therefore be considered in studies of the effects of O_3 on plants.

3. OZONE ATTACKS THE PLANT

Ozone is more toxic for vegetation than any other pollutant because of several characteristics: in addition to its redox potential, it has approximately the same coefficient of diffusion as CO_2 and therefore encounters the same resistance when penetrating the leaves; its solubility in water (and in cellular fluid) is ten times higher than that of oxygen [17]. Plants' response to O_3 depends on a sequence of events, which begin with uptake, followed by perturbation, homeostasis and finally injury; repair is possible.

Atmospheric O_3 is deposited onto plant canopies by diffusion; as for all gases, the main route of entry of O_3 into a leaf tissue is via open stomata, which constitute a minimal fraction (2%) of leaf surface [6]. Cuticular absorption is regarded as negligible under natural conditions [18]. Therefore, stomatal density, stomatal conductance and environmental parameters, which affect the stomatal behaviour (especially water availability), are crucial factors controlling the rate of O_3 entry.

Once uptaken, O_3 decomposes quickly in the apoplast into several derivatives, which are regarded as its actual 'toxic principles'. They are collectively indicated as '*Reactive Oxygen Species*' (ROS) (other terms frequently used are '*reactive*' (or '*active*') '*oxygen intermediates*' or '*reactive oxygen metabolites*'). They are all extremely reactive and cytotoxic in all organisms. Thus, oxygen provides a paradox, in that it is essential for aerobic life (it is the terminal receptor of electrons during respiration, which is the main source of energy), yet, in its reduced form, it is one of the most toxic substances with which life on Earth must cope [19]. The interest of biologists in ROS has grown impressibly during the past few decades. At present, there is evidence for their involvement in over 100 human disease states (including arteriosclerosis, cancer, and aging), as well as in many normal biological processes [20].

The main metabolites of oxygen can be classified in two groups. They include [21]:

a) *radicals containing oxygen with unpaired electron(s)*, like the superoxide ($O_2 + e^- \rightarrow O_2^{\cdot -}$), whose protonation produces the hydroperoxyl radical ($HO_2^{\cdot}$); the metal-catalysed 'Haber-Weiss' reaction between $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2) produces the hydroxyl radical ($HO^{\cdot}$), which is the most potent oxidant known (it can react indiscriminately with any biological molecule and its half-life is only fractions of a microsecond); peroxyradicals (RC-O-O$^{\cdot}$) are also generated from the oxidation of lipids;

b) *products containing molecular oxygen*, like $^1O_2$ and H_2O_2.

The superoxide radical and H_2O_2 are less reactive oxidants than $HO^{\cdot}$ but have a longer life span, which allows them to react with molecules in locations far from the site where the ROS were produced [22]. The presence of ROS can be advantageous for cells, as some of them are normally produced in plants (*see the following section*). However, when there is an excess of ROS or when antioxidant defence systems are weakened for some reason, cellular damage may appear.

The primary targets of ROS are double bonds in fatty acids of lipids. They may be subjected to ozonolysis and/or peroxidation, with production of malondialdehyde in both cases, and although ozonolysis and peroxidation are sometimes thought to be synonymous, they are quite distinct processes. The former creates H_2O_2, involves no peroxylradicals in the initial attack and does not bring double bonds into conjugation with each other. Lipid peroxidation involves initial attack by radicals rather than by O_3 itself and does not form H_2O_2 [23].

Proteins are also damaged: two molecules of cystein are linked by S-S bridges (starting from –SH groups) to form cystine; in methionine the S atom is oxidized to sulphoxide; in triptophan the pyrrolic ring is broken. As a result, integrity and functioning of biomembranes are altered. In addition, oxidized proteins increase their hydrophobicity and sensitivity to proteolysis [24].

Enzyme structure and activity is another important aspect of O_3 phytotoxicity. The disturbance in spatial orientation in key aminoacids may be critical if, for example, they form part of the reaction centre of an enzyme [23].

DNA lesions and mutations, often leading to irreparable metabolic dysfunctions and cell death, are induced by hydroxyl radicals [25].

Ozone affects several key biochemical and physiological functions of the plant in a very complex manner, including photosynthesis, water use efficiency, dry matter production, flowering, pollen tube extension, yield, etc. [1]. For example the O_3-induced photosynthesis impairment may be due to [26]:

- stomatal closure (leading to decreased conductivity), as a result of the water loss in the guard and neighbour cells, because of increased membrane permeability caused by O_3. A direct consequence of stomatal closure is the reduction of CO_2 absorption. Decreases in conductivity may also be due to an excess of CO_2 in the hypostomatous chamber, due to reduced mesophyll assimilation;
- the damage of several components of the light-harvesting complex in the chloroplast, as has been repeatedly demonstrated by alterations in the chlorophyll fluorescence parameter, which leads to diminution of energy production in photosystems;

- the decrease in CO_2 assimilation, due to reduced amount and/or activity of active carboxylating (acceptance of CO_2) enzymes. So, the effects on ribulose biphosphate carboxylase (Rubisco) can be considered as either direct oxidation (*i.e.* increased susceptibility to proteolysis), or indirect suppression of mRNA production;
- ultrastructural lesions to chloroplast components;
- the reduced availability of NADPH, if it is diverted to the regeneration of oxidized glutathione.

As a result of these interactions, the plant may develop visible foliar symptoms, whose characteristics (intensity and gross features) depend on plant species (or variety), leaf age, nutrition and environmental factors, as described below.

Nowadays visible symptoms have little biological relevance, as it is widely believed that they only represent a sort of "tip of the iceberg": most of the toxicants act in an inconspicuous way. Foliar visible symptoms may or may not result in crop yield reductions; conversely, yield reductions may occur without visible symptoms [27].

The final result of O_3 insult depends on several factors, from genotypical features to sanitary status (Table 1).

Table 1. Main factors that may affect plant response to ozone (modified after Kley et al. 1999, [3]).

Factors	*Effects*
Biological Factors	
Genetic diversity	Homogeneous plants give uniform responses; species, cultivars, and clones react differently to O_3 in terms of visible injury and/or biomass production
Stage of plant and organ development	Response depends on the stage of plant development and the physiological leaf age; leaves are most sensitive when they have just reached their full size
Health conditions	The presence of diseases (viral, fungal) may modify the response
Physical factors	
Soil moisture and relative humidity	Water stress induces stomatal closure and thus lower O_3 absorption
Temperature	Injury increases in a range from 3 to 30 °C
Air movement	Influences the boundary layer resistance and gas uptake
Light, UV radiation, photoperiod	Contradictory results
Chemical factors	
Ozone levels and pre-exposure	Response depends on the dosage; pre-exposure may modify the response

Table 1. Continued

Factors	*Effects*
Chemical factors	
Carbon dioxide	Injury decreases with elevated CO_2 levels
Other chemical pollutants	Synergism may take place and injury can be more severe
Agronomic practices	
Nutrition	Contradictory results; in bulk, optimal nitrogen minimizes foliar injury
Pesticides and other chemicals	Ethylendiurea (EDU) and other chemicals (*e.g.* some systemic fungicides) counteract visible injury

Biomass reduction is a common response of a plant exposed to O_3: the partitioning of assimilates is frequently changed at the expense of roots, so an altered root/shoot ratio is usually found in exposed plants [28, 29].

The O_3-induced effects may persist for a long time in the plant, so a 'memory effect' is possible in perennial crops, like grapevines [30], and in wooden species, like conifers [3].

Of special interest appears to be the production of 'stress ethylene', which is an early event, associated with O_3 exposure and could be involved in accelerated foliar senescence [31].

4. THE PLANT DEFENCES AGAINST THE ENEMY

The toxicity of a chemical to biota is generally determined by three processes: uptake, biochemistry and cellular defence reactions. The response of plants to O_3 depends on the genotype: species, cultivars and clones react in different ways. In addition, the leaves of a plant show a marked differential response, related to ontogenesis, with very young leaves often showing insensitivity (Table 1).

Plant cells have means to counteract the phytotoxic mechanisms of O_3, as the oxidative stress is also frequent in undisturbed individuals; for instance, they encounter several ROS, which are commonly produced within biological systems - viz. through the mitochondrial respiratory electron transport chains [32]; O_2^- and $^1O_2$ are commonly produced in illuminated chloroplasts by the occasional transfer of an electron from an excited chlorophyll molecule to molecular O_2 [25].

In general, the defensive attribute ('*resistance*') may be of the following types [33]:

- *stress avoidance* ('exclusion'), when the organism is able to avoid the physical interaction (*i.e.* the entry) of the adverse factor. In the case of a toxic gas, like O_3, this should imply an active mechanism, able to allow the normal leaf gas exchange, but selectively impair the uptake of toxic gas

over a certain threshold. This does not represent a common mechanism of plant defence against O_3, although some experimental evidence has been reported [34];

- *stress tolerance*, in terms of strain *exclusion*, when the noxious agent penetrates the host, but is prevented from causing injurious chains of reactions. In the case of chemicals, this implies detoxification. This is believed to represent the major mechanism of plant defence against O_3;
- *stress tolerance*, in terms of *repair* of the biochemical lesions induced by the stressor. So far only little evidence has been collected for O_3.

The quick activation of biochemical defences, which effectively neutralize ROS, is regarded as a crucial mechanism of the response of plants to O_3. During evolution, all aerobes acquired a number of distinct biochemical mechanisms to efficiently and rapidly cope with oxidative stresses, and to remove damaging species from different cellular compartments. The plant antioxidant system, which scavenges naturally occurring ROS, could act as a primary mechanism to alleviate the oxidative burden resulting from O_3 exposure. So, non-enzymatic scavengers of ROS include a number of composts with high reducing potentials, such as ascorbic acid (AA, vitamin C), and α-tocopherol (vitamin E), β-carotenes, polyamines and the tripeptide glutahione in its reduced form (GSH) [35]. Glutathione and ascorbic acid act in combination with enzymes such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) to modify the state of cell's oxidation. The ascorbate-glutathione cycle (Halliwell-Asada pathway) is a cooperative defensive chloroplastic system, involving several protective agents (Figure 2): H_2O_2, generated from ROS metabolism, is converted to H_2O at the expenses of NADPH.

The role of GSH as an antioxidant may actually be multifold: in addition to its function as substrate for the regeneration of AA, it could play a role in membrane stabilization by removing acylperoxides formed during lipid peroxidation [36].

Other well studied antioxidant enzymes are also known to play a crucial role in scavenging ROS. These include multiple forms (containing different metal cofactors, Cu/Zn, Mn or Fe) of superoxide dismutase (SOD), which convert $O_2^{\cdot -}$ into H_2O_2 ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$) at a rate 10^4 times higher than spontaneous dismutation; catalase and peroxidases, which further metabolize H_2O_2 to H_2O. Catalase is primarily localized in the peroxisomes; isoforms of peroxidases and SOD are distributed through the cell and can be found in cytosolic (Cu/Zn SOD), mitochondrial (Mn SOD) and chloroplastic (Cu/Zn and/or Fe SOD) compartments. A detailed description of the antioxidant enzyme systems in higher plants is reported by Scandalios [25].

Contradictory results have so far been gathered on the role of the above mentioned mechanisms in providing resistance to O_3, and many questions, relating to the enzyme scavenging hypothesis for protection against exogenous oxidants, have been left unanswered. Puzzling evidence indicates that increased, decreased and unaltered levels of these enzymes may constitute the response of plants to O_3. The presence of several isoforms of scavengers and integration between different scavenging systems are required to counteract the action of ROS [37].

Overexpression of native forms of SOD in transgenic plants has been achieved in several laboratories, with apparently contradictory results [38]. To understand these mechanisms, it is essential to identify the responsive genes and to investigate their structure, regulation, and expression.

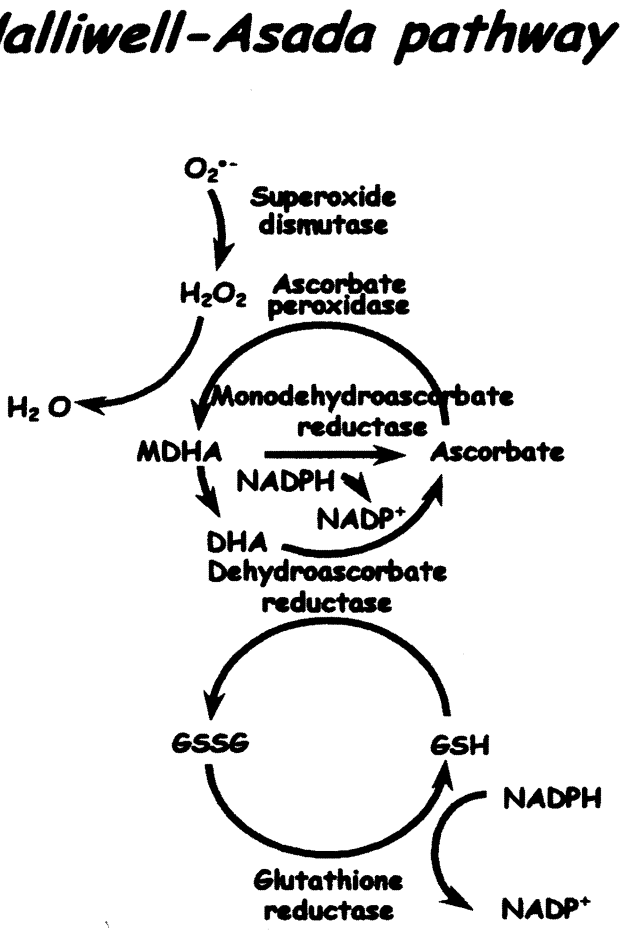

Figure 2. The ascorbate-glutathione (Halliwell-Asada) pathway for neutralization of H_2O_2. Ascorbic acid reacts with H_2O_2 in the presence of ascorbate peroxidase, to form monodehydroascorbate (MDHA). Ascorbate can be regenerated from MDHA directly by the action of monodehydroascorbate reductase, or by spontaneous disproportionation of MDHA into ascorbate and dehydroascorbate (DHA). Ascorbic acid is regenerated from DHA in a reaction catalysed by dehydroascorbate reductase, where reduced glutathione is oxidized into

GSSG. GSH is regenerated in the presence of NADPH by glutathione reductase. (after Sharma & Davis, 1997, [35]).

Repair processes would involve the resynthesis of membrane components which have been injured as a consequence of the action of ROS. To date, the bioenergetics of these operations have not been fully understood [39].

The overall impact of O_3 on plant physiology is complex. A possible relationship between the formation of ethylene and the sensitivity of plants to O_3 was described in 1971 [40]. This phytohormone is released in higher rates by those plants which are more sensitive. One standing hypothesis is based on the possibility that the reactions of ethylene with O_3 initiate the formation of radicals which promote cell injury [41]. Polyamines have been reported as acting as radical scavengers that protect plants from O_3 injury [42]. An additional positive role of theirs could be related to their antagonism against ethylene synthesis.

It is also important to understand how O_3 affects secondary metabolism in plants, with special reference to substances which are known to play an important role in the plant defence against pathogens (pathogenesis-related proteins, phytoalexins, cellular barriers, salicylic acid and other signal substances) [3, 33, 44].

5. GUILTY FINGERPRINTS

Unlike other gases, O_3 is not accumulated in the leaves and thus, after attack, there is no evidence of 'guilt'. Moreover, it does not have a radioactive isotope, so it is difficult to locate the initial point of attack in leaf tissues. In addition, the 'symptomatological divergence' in O_3 phytotoxicity is very wide and differs between broad-leaved plants and conifers and also among species. In some cases, O_3 symptoms are very similar to those induced by other causes: solar radiation may cause bronzing symptoms on bean, similar to those caused by O_3; or O_3 symptoms on tobacco plants are similar to those caused by phosphorus imbalance, and so on [45]. Thus, for the majority of plant species, the recognition of symptoms under field conditions is very difficult and therefore, special care must be exercised when diagnosing symptoms. The most commonly observed macroscopic and microscopic symptoms are described below.

5.1. Macroscopic symptoms

In broad-leaved plants, symptoms of O_3 toxicity firstly appear on the adaxial foliar surface. This is due to the fact that palisade mesophyll cells are more sensitive to O_3 (and so collapse early) than the underlying spongy mesophyll cells. Necrotic spots occur only between the main veins. In severely injured leaves, spots may coalesce and eventually become bifacial.

Ontogenic factors modify the specific leaf sensitivity (Table 1): the most sensitive leaves of a plant are those recently matured. Newly expanding or over-mature leaves are usually insensitive to O_3. It has been found that, under ambient conditions, the leaves of Bel-W3 tobacco become sensitive after they reach 85-90% of their final full expansion size [46] and remain sensitive until the time when they become over-mature. If two leaves overlap, then the shaded leaf area does not exhibit injury ('shade effect'), due to reduced absorption of the toxic gas [47]. Late in the growing season, foliar injury may progress to leaf yellowing or premature senescence and defoliation. In conifer species, chlorotic mottle due to chronic exposure frequently appears in second-year and older needles. The macroscopic appearance of symptoms differs among species. Krupa et al. (1998) [45] have listed the several O_3-induced symptoms for broad-leaved plants as follows: *bleaching,* small pale necrotic spots, usually limited to the adaxial surface, palisade being the main cellular target; *flecking*, small necrotic areas, metallic or brown, fading to tan, grey, or white; *stippling*, tiny punctate spots where a few palisade cells are dead or injured, which may be white, black, red or reddish-purple in colour; *bronzing or pigmentation,* reddish-brown to brown coloration of leaves due to phenolic pigment accumulation after chronic exposure to O_3.

In conifer species, acute O_3 exposure symptoms have been described as *banding*, clear bands of chlorotic tissues developed across the needles; and *tipburn,* necrosis and dying of the tips of expanding young needles. Chronic exposure induces *chlorotic mottling*, small yellowing patches of injured tissues interspersed with green tissue of old needles and premature or early senescence leading to the needles shedding. Finally, the dominant symptom of chronic O_3 exposure syndrome is *early senescence,* almost impossible to be diagnosed under field conditions.

5.2. Microscopic symptoms

Investigations of O_3 induced symptoms, conducted by light, fluorescent, transmission and low temperature electron microscopy, have revealed certain underlying anatomical alterations. For instance, earlier studies have shown O_3 induced ultrastructural changes in birch (*Betula pendula*), including abnormal and spherically shaped chloroplasts, swelling and curling of thylacoid membranes, and increased density of stroma [48]. Recently, Günthardt-Goerg et al. [49] have investigated the visible and microscopic injury in leaves of several deciduous tree species induced by current critical O_3 levels in Switzerland. They observed that, in the O_3-treated plants, the mesophyll cells collapsed earlier than epidermal cells. Also, collapsed single lower epidermal cells or groups of cells caused stippling. When mesophyll cells collapsed in groups, the visible light-green or red spots turned into necrotic spots. They also noticed an increased electron-density of cytoplasm, a darkened and reduced size of chloroplasts, smaller or missing starch grains, a reduction of thylakoid membranes, and increased number, size and electron lucidity of plastoglobuli in palisade cells. Moreover, the tonoplasts formed vesicles; the vascular content became inhomogenous; and some groups of cells appeared

"empty", plasmolysed, their walls invaginated, cell content condensed, disintegrated and finally collapsed. Earlier studies also reported reduced mesophyll starch formation with increasing O_3 injury and the accumulation of starches along small leaf veins [50].

5.3. Secondary effects

In addition to direct primary negative effects, pollutants may induce secondary effects in plants. This is the case of the impairment of defences against other stress factors (*e.g.* parasitism by fungi and insects, frost, drought), a phenomenon often observed in plants exposed to O_3 [51, 52]. Because of the reduced vitality consequent to exposure to the pollutant gas plants can become more susceptible to pathogens. For instance, it has been reported that O_3 can increase the susceptibility of pine seedlings to root-rot pathogens [53]. However, the interactive effect of O_3 and fungi is rather complex and depends on fungi and host plant species, O_3 levels and other interplaying factors. It has been reported, for instance, that powdery mildew (*Sphaerotheca fuliginea*) was severe on bottle gourd (*Lagenaria siceraria*) grown in areas polluted with mild levels of O_3 (<50 ppb) whereas, at 100 ppb O_3 or more, the disease was suppressed [54]. The fungus infection partially protected the plants from injury by O_3 at 200 ppb.

5.4. Plant protectors against ozone

Substances like EDU (ethylen-diurea) and some systemic fungicides like benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate), have been found to lessen the adverse visible impact of O_3 on plants, even if the intimate bases of the interactions are still a matter of debate [55, 56]. Moreover, a number of agrochemicals such as other fungicides and herbicides have been shown to possess somewhat antiozonant properties. It has been reported, for instance, that two modern fungicides (*azoxystrobin* and *epoxiconazole*) may reduce the impact of O_3 on barley by enhancing the plant antioxidant systems (increasing the activities of superoxide dismutase, catalase, ascorbate-peroxidase and glutathione reductase) [57]. However, economic and ecological reasons do not allow a routine application of chemicals to alleviate the effects induced by O_3 on plants. Some of them may play a certain role in diagnosing and evaluating the yield effects of the presence of the pollutant, by comparing treated and untreated plants.

6. SURVEYING WHAT IS SUSPECT

Studies of the O_3 pollution effects on plants may adopt two major approaches. In the first, the ambient concentrations of O_3 are monitored and their possible effects on plants can be estimated, provided that a relationship between exposure and plant

injury is already known. In the second approach, O_3 presence is detected with bioindicator plants.

6.1. Instrumental monitoring

Several physical and chemical methods have been developed for the measurement of O_3 concentrations, and very sophisticated techniques with semiautomatic or fully automatic apparatuses (automatic O_3 analysers, electronic O_3 sensors) are available. Passive samplers are also used. In an attempt to quantify the relationship between exposure to O_3 and plant response, several exposure-response functions have been considered. These functions relate a measure of exposure with a measure of response. The most common measure of exposure is "dose", defined as the product of O_3 concentrations *in* the organism, and the duration of exposure. The most frequently used surrogate measure of internal O_3 concentration is the ambient concentration. As a measure of response the final biomass production or the yield are usually used. Many investigations have shown that the O_3 dose is not sufficient enough to describe the exposure-response relationship. It has been found that the potential of O_3 dose is non-linear; for the same dose, a higher O_3 concentration for a shorter duration (acute exposure) is more effective than a lower O_3 concentration for a longer exposure (chronic exposure).

European and American groups have proposed and tested a large number of alternative exposure indices, related to the biological response to O_3, and relationships to translate yield change into economic effects [58, 59]. The most modern is the 'Accumulated exposure Over the Threshold of 40 ppb', the so-called 'AOT40' index, which is calculated as the sum of the differences between the hourly O_3 concentrations in ppb and 40 ppb for each hour when the concentration exceeds this threshold. It has been found that AOT40s calculated for a given period of time give the best agreement between O_3 levels and effects on crops [60]. Only exposure during the daytime (*i.e.* solar radiation at least 50 Wm^{-2}) is evaluated, due to the necessity for O_3 to enter the plant through open stomata; for the majority of plants, stomata closed during the night. The 'critical level' concept, which refers to the exposure that significantly impairs the quantitative performance of the biological targets, has been set up. Nowadays, an AOT40 of 3 ppm·h for 3-months exposure is regarded as the critical level for crop plants and for natural vegetation; while for forest plants, a value of 10 ppm·h on a 6-month span is assumed. For example, in Pisa (Central Italy, 100,000 inhabitants, about 50,000 circulating vehicles) the weekly AOT40 burden, during a typical summer, is about 1 ppm·h.

The weak point of all exposure indices proposed is that they do not incorporate the interactive effects of concurrent exposure to other stresses like drought, high or low temperature, relative humidity, direct sunlight, UV radiation, other pollutants, etc., which should also be considered in evaluating the exposure-response relationship [61]. Besides, other factors, like genetical traits of plants and agronomic practices are not considered. Moreover, the indices based on O_3 concentration, despite providing useful information about the potential phytotoxicity of ambient O_3,

could not be easily put into agronomic practice, due to their disadvantages: expensive infrastructure, need of electricity and technical support, and advanced knowledge. These disadvantages make the indices undesirable for growers and policy makers who need specific information about the risk of the species cultivated and the agronomic measures necessary to minimize loss of income.

6.2. Ozone phytodetection

Some alternative methods have been developed for local evaluation of O_3 phytotoxicity, based on the use of O_3 detection by bioindicator plants, exhibiting distinct symptoms following O_3 exposure. *(We introduce the word "phytodetection", for the first time here, to describe the use of higher plants (bioindicators or phytodetectors) in detecting O_3 occurrence at potentially phytotoxic levels, instead of the word "biomonitoring" which is extensively used but whose meaning is wider and less specific).* The Bel-W3 tobacco variety is used world wide for ozone phytodetection [47] (Figure 3). The sensitivity threshold of this variety is about 40-45 ppb of exposure for a few hours. The extent of symptoms – visually estimated as the percentage of leaf area showing necrosis – may be correlated with the actual levels of the pollutant.

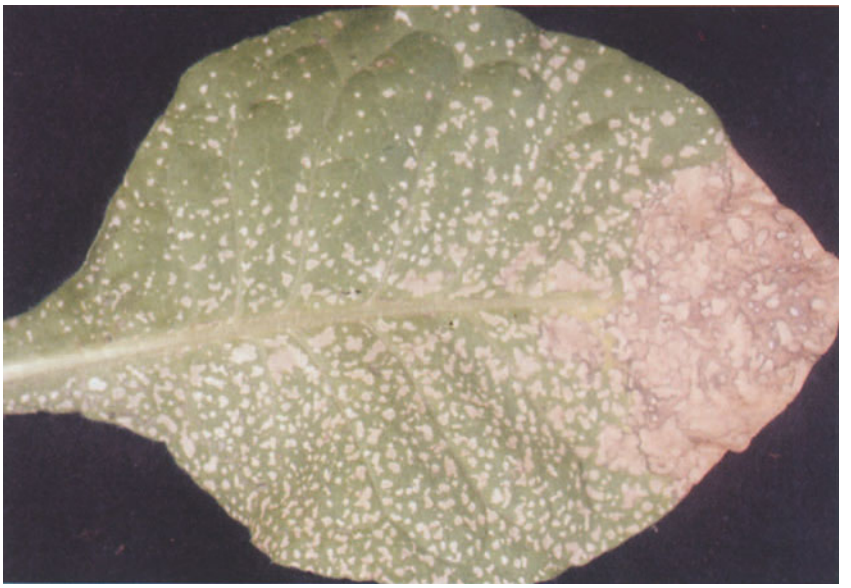

***Figure 3**: A leaf of tobacco variety Bel-W3, a super-sensitive plant worldwide utilised as a bioindicator of ground-level ozone distribution; the picture has been taken after a 7-day exposure of the plant at ambient air in Pisa, Italy.*

A more complete O_3 protocol includes two more tobacco varieties: Bel-B (of intermediate sensitivity) and Bel-C (resistant). Potted and well watered plants of Bel-W3 or of all three varieties have been used in many studies to indicate potentially phytotoxic O_3 levels. Lorenzini [62] suggested the use of miniaturized kits with Bel-W3 seedlings instead of adult plants, as a simple but very sensitive and reliable method in large scale integrated monitoring campaigns [63]. Design details of this method are given by Lorenzini [62]. In summary, seedlings of Bel-W3 and Bel-B are grown in tissue culture plates in an O_3-free environment before being transplanted to the monitoring sites for 7-days' exposure. The percentage of the area of cotyledons and first leaves showing O_3 induced symptoms is assessed visually, by comparison with spot standards. The application of this simple and cost effective method is recommended for O_3 detection in large areas and has been adopted in some European countries.

An alternative technique, suggested by Heagle et al. [64], is the use of two clones of white clover (*Trifolium repens* cv. Regal), one sensitive and one resistant to O_3, in terms of biomass production. The ratio of the above-ground biomass of the sensitive clone to that of the resistant one, produced over 28 days, could be used as an index of O_3 harmful effect. This index has the advantage of quantitatively estimating the yield loss in clover due to O_3, avoiding risks of subjective evaluation of symptom severity and providing a reliable indication of the effects of pollution on crop yield.

7. ITS MEDITERRANEAN TEMPERAMENT

All the above mentioned techniques have been used in the assessment of O_3 threat in agricultural areas in several countries. The most widely used technique, in network schemes for the investigation of O_3 distribution countrywide, is the phytodetection with Bel-W3 potted plants, often coupled with instrumental recording of O_3 concentrations. A characteristic example is the Dutch National Monitoring Network [65], which has shown that ambient O_3 levels are high enough to affect plants and reduce the yield of several field-grown crops in The Netherlands. Tonneijck and Van Dijk [66], using subterranean clover, revealed a persistent phytotoxic O_3 occurrence in four rural areas, over the growing seasons 1994-96. A recent survey of spatial distribution of rural O_3 in the U.K. has indicated that the critical levels for crop and semi-natural vegetation is exceeded in over 71% of the countryside; for forests, the critical level are exceeded in over 8% [11]. Phytotoxic O_3 levels have also been detected in Switzerland [49, 67], India [55], Ukraine [68], Egypt [69] and several other countries. Investigations conducted through Europe, in the framework of the UN-ECE (United Nation Economic Commission for Europe; Convention on Long-Range Transboundary Air Pollution) and the European Open-Top Chamber projects, have shown that O_3 occurs over the entire continent, showing higher phytotoxicity in the southern European (Mediterranean) countries [41, 70]. There is growing evidence that in many rural areas in Spain, Italy and Greece, ambient levels

of O_3 during growing season, often exceed substantially the UN-ECE critical levels for the protection of vegetation [71, 72, 73, 74, 75, 76].

Spanish researchers assert that few areas of Europe are at greater risk from O_3 than Catalunya, Valencia, Murcia and Almera [73]. Using open-top chambers and enclosed ambient air control plots, they investigated the impact of photochemical oxidants (primarily ambient O_3) on the yield and physiological characteristics of a watermelon commercial cultivar, widely grown in a rural site in Eastern Spain. They observed that plants exposed to ambient air developed visible symptoms characteristic of O_3 injury, similar to those recorded in commercial watermelon fields in other parts of Eastern Spain [77, 78, 79].

In Italy, since 1992, the results of a wide co-ordinated research programme, using open-top chambers and bioindicator plants, have suggested that photochemical air pollution was widespread at the regional level in northern and central parts of the countryside, at levels well above the threshold for phytotoxicity, with O_3 being the most important gas [80]; southern areas were not covered by the investigation, but the problem is likely to be even more severe there. Lorenzini et al. [81] using transportable miniaturised kits with Bel-W3 seedlings, monitored phytotoxic O_3 levels over Tuscany. This method was also successfully used to demonstrate a long-distance transport of O_3, across a wide geographical area over a land-sea transect in Italy [71]. Nali et el. [82] conducted a surface O_3 monitoring in Florence, integrated with a phytodetection campaign with Bel-W3 and Bel-B plants, and concluded that the values observed are sufficient to reduce significantly the quantitative and qualitative vigour of vegetation. It has also been estimated that the yield loss attributable to O_3, in some key crop species in the Florence district, varied from 8% for corn and alfalfa to 27% for soybean [83].

In Greece, a Mediterranean country where a high risk of O_3 injury is plausibly expected, the number of studies concerning effects of air pollution on plants is quite limited [25, 84, 85, 86]. Ozone levels have been continually monitored for many years in Athens and recently also in some suburban regions [87]. However, few data on air quality are so far available to confirm the occurrence of O_3 phytotoxic episodes in rural areas. High rural O_3 levels, up to 70 ppb, have been recorded at Messorougion-Achaia [88] and at Finokalia-Crete (130 m altitude) [89]. Velissariou et al. [72] reported that phytotoxic concentrations of O_3 occurred throughout Attica, within a 75 km radius around the city of Athens. Saitanis and Karandinos [75], who conducted a countrywide pilot investigation using Bel-W3 potted plants, reported typical O_3-induced phytotoxic symptoms. They also recorded potentially phytotoxic O_3 levels (high AOT40s) at three rural sites of Corinth (to the west of Athens) and ascertained symptoms on Bel-W3 indicator plants at 11 sites across the agricultural area of Corinth [90] and at 28 sites on Mt Pelion (a forest of particular natural beauty) and in the surrounding area [76]. They also observed symptoms on other cultivated plant species, mainly on grapevines. Fumagalli et al. [74] went over a great deal of "peer-reviewed" and "grey" literature and reported a list of 25 commercial agricultural and horticultural crops in the Mediterranean basin which have been reported to be macroscopically injured by O_3, many of them in Greece (Table 2). Among these, watermelon, pepper, onion, spinach, lettuce, grapevine,

corn, tobacco and wheat are included. All these reports suggest that, in the Mediterranean basin, O_3 is not simply an imminent enemy: it is present and diffuse.

Table 2. List of plant species known to have varieties or clones sensitive to ozone. For those with an asterisk, there are reports of evidence of visible injury in commercial cultivations in Mediterranean countries [74]

Crops	Woody species
Allium cepa (Onion)*	*Ailanthus altissima* (Ailanthus)
Arachis hypogea (Peanut)*	*Amelanchier alnifolia* (Saskatoon Serviceberry)
Avena sativa (Oat)	*Fraxinus americana* (White Ash)
Beta vulgaris (Red beetroot)*	*Fraxinus pennsylvanica* (Green Ash)
Citrullus lanatus (Watermelon)*	*Juglans regia* (Walnut)
Cynara scolymus (Artichoke)	*Larix decidua* (European Larch)
Glycine max (Soybean)*	*Liriodedron tulipifera* (Tulip-tree)
Gossypium hirsutum (Cotton)*	*Rhododendron kaempferi* (Hinodegiri Azalea)
Hordeum vulgaris (Barley)	*Rhododendron kurume* (Snow Azalea)
Lactuca sativa (Lettuce)*	*Rhododendron poukhanensis* (Korean Azalea)
Lycopersicon esculentum (Tomato)*	*Pinus banksiana* (Jack Pine)
Medicago sativa (Alfalfa)	*Pinus jeffreyi* (Jeffrey Pine)
Nicotiana tabacum (Tobacco)*	*Pinus nigra* (Austrian Pine)
Petunia hybrida (Petunia)	*Pinus ponderosa* (Ponderosa Pine)
Phaseolus vulgaris (Bean)*	*Pinus radiata* (Monterey Pine)
Poa annua (Annual blue grass)	*Pinus strobus* (White Pine)
Populus tremuloides (Quaking Aspen)	*Pinus taeda* (Loblolly Pine)
Raphanus sativus (Radish)*	*Pinus virginiana* (Virginia Pine)
Solanum tuberosum (Potato)*	*Platanus occidentalis* (American Sycamore)
Spinacea olearacea (Spinach)*	*Prunus serotina* (Black Cherry)
*Trifolium alexandrinum**	*Quercus alba* (White Oak)
Trifolium repens (White clover)*	*Quercus gambelii* (Gambel Oak)
Trifolium subterraneum (Subterraneum Clover)	*Rhus aromatica* (Fragrant Sumac)
Triticum aestivum (Wheat)*	*Rubus spp.* (Blackberry)
Vitis vinifera (Grapevine)*	*Salix babylonica* (Weeping Willow)
Zea mays (Maize)*	*Sorbus aucupatia* (European Mountain Ash)
Native Species	*Spirea vanhoutii* (Bridal Wreath)
Coriandrum sativum (Coriander)	*Symphoricarpos alba* (Snowberry Alba)
Urtica spp. (Nettle)	*Syringa chinensis* (Chinese Lilac)

8. HOW MUCH DOES THIS GAME COST?

There is no doubt that plant vigour is reduced by long-term exposure to commonly occurring O_3 levels. So far, there is little evidence concerning the impact of O_3 on the quality of plant derivatives (*e.g.* on nutritional value), and only quantitative aspects will be considered here. Unfortunately, our knowledge in the field is very sparse. Dose-response functions are not easily derived in the case of O_3 under typical field conditions, due to the multitude of interacting factors. The scaling up of experimental results from young individuals to adult plants, from single individuals to communities, from one species (and a cultivar) to another is virtually impossible. In addition, the results coming from experiments carried out under laboratory conditions may not be fully representative of what happens under field conditions.

The accurate estimation of crop yield loss is essential to produce useful evaluations of the economic impact of pollutants. A number of experimental approaches have been used to assess the impacts of chronic O_3 exposure and crop response. Among these, the open-top chambers method has been widely used for many years [91]. Long-term studies performed in the U.S.A., in the framework of the National Crop Loss Assessment Network (NCLAN) project, have estimated the crop loss from air pollutants [92]. Murphy et al. [93] estimated that, for the U.S.A., the benefits to the agricultural sector gained from completely eliminating O_3 precursor emissions from vehicles would range between $ 3.5 - 6.1 billion annually.

In a similar attempt, European researchers established a network of Open Top Chambers in which two key species, wheat for crops and beech for forest plants [70], were used. As depicted in Figure 4, significant yield and biomass reductions were found to be associated with realistic exposures to O_3. Unfortunately, relevant data are only available for very few plants and the data base is too small to derive meaningful and reliable effective dose-yield response relationships [94].

Experiments conducted in Northern Italy [95], based on the Open-Top Chambers exposure technique, showed the relevant beneficial effect of filtering ambient air on the productivity of several crops (barley, bean, radish, pumpkin and wheat) when O_3 was the prevalent air pollutant.

This biological information should be coupled with statistical data relevant to the distribution of agricultural species and to economic input related to market prices, to establish the true monetary impact of O_3 pollution on plants [96]. This approach, however, should also take into account some basic economic laws to recognize the actual economic victim of pollution, which will probably be the consumer and not the producer (if the supply of products is reduced because of pollution, yet demand is inelastic, prices rise).

9. WHAT NEXT?

The main air pollutant in most areas is O_3, and its importance will increase in the near future, due the global growing trend in motorization, the major source of its precursors (*i.e.* nitrogen oxides and hydrocarbons). In view of the harmful effects of

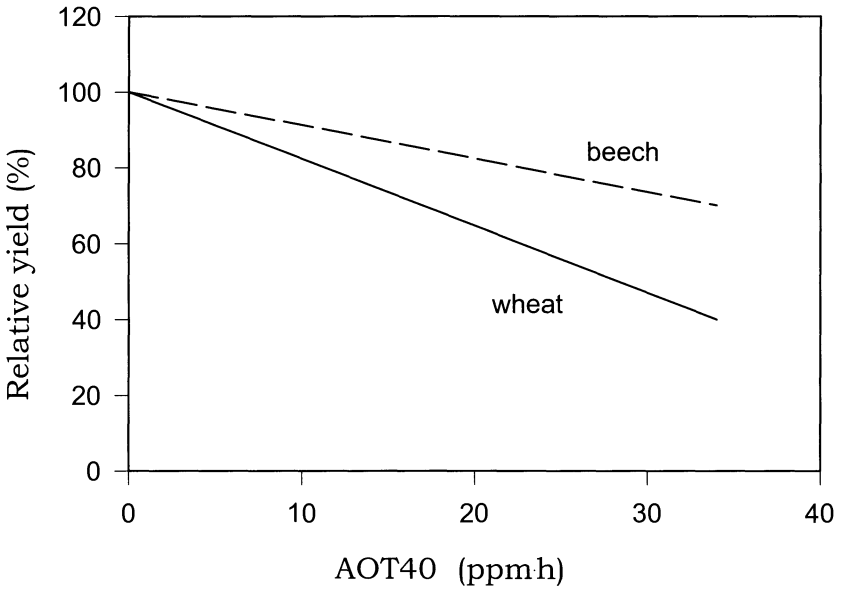

Figure 4. Exposure-response relationships for ozone expressed as AOT40 and relative grain yield in wheat (3-month exposure), and for biomass production in beech (6-month exposure) (redrawn from Kärenlampi and Skärby, 1996 [70]).

air pollution of the troposphere, the European Council adopted a specific Directive on O_3 pollution (Directive 2002/3/EC, 12 February 2002). According to this directive, the threshold target AOT40 values of O_3 concentrations, for the protection of vegetation, (calculated from 1 h values from May to July) has been set at 9,000 ppb·h (about 18,000 μgm^{-3} h) as an average over five years; the long-term objective (to be reached within 2020) has been fixed at 3,000 ppb·h (about 6,000 μgm^{-3} h).

Many investigations have shown that O_3 concentrations regularly exceed these thresholds in urban and suburban areas across Europe and probably also in most rural areas. Moreover, the O_3 surface measurements available for Europe suggest an upward O_3 trend of about 5-20% per decade [43]. So, plants will have to cope with increasing levels of O_3 even in the developing countries.

Furthermore, the effects of this pollutant on the plant defence system allow us to imagine that O_3 may be regarded as a new experimental tool for studying plants' response to oxidative stress. In addition, as topics arising from oxygen activation

and its toxicity in plant, animal and microbiological systems [97] are related, the study of the intimate bases of the O_3-plant interactions may be regarded as a powerful tool in investigations of the biological role of oxygen radicals.

Life-long exposure to sub-lethal levels of O_3 will become a common-condition for our plants. This pollutant will pose a critical threat and a challenging problem to world food, fibre and timber production and conservation of natural plant communities, including their species diversity. In these terms, plant pathology will face new features as in human medicine: here 'classical' pathogens (historical infectious causal agents of diseases) have been flanked and often surpassed in importance by 'new type disorders', linked to agents with a low intrinsic causal potential, but able to interfere with the normal physiology of the host, to reduce its defensive mechanisms against other stress factors, to induce a subtle pathological condition, where a 'cause-effect' relationship is hard to be established. In other terms, O_3 is candidate to become an omnipresent risk factor for plant life, even in the developing countries.

A detailed analysis of the phytopathological and ecological roles of tropospheric O_3 could help to better understand its overall impact in terms of 'costs *vs.* benefits'.

ACKNOWLEDGEMENTS

We thank Professor Michael Karandinos for revising the manuscript and making valuable suggestions.

REFERENCES

1. Krupa, S., McGrath, M.T., Andersen, C.P., Booker, F.L., Burkey, K.O., Chappelka, A.H., Chevone, B.I., Pell, E.J., and Zilinskas, B.A. (2001). *Plant Disease*, 85, 4-12.
2. Lorenzini, G. (2000). 3rd European Conference on Applied Climatology, Pisa (*official CD*).
3. Kley, D., Kleinmann, M., Sandermann, H., and Krupa, S. (1999). *Environmental Pollution*, 100, 19-42.
4. Richards, B.L., Middleton, J., and Hewitt, W. (1958). *Agronomy Journal*, 50, 559-561.
5. Middleton, J.T., Kendrick, J.B., and Schwalm, H.W. (1950). *Plant Disease Reporter,* 34, 245-252.
6. Krupa, S.V., and Manning, W.J. (1988). *Environmental Pollution*, 50, 101-137.
7. Fishman, J. (1990). *Journal of Geophysical Research*, 95, 3599-3601.
8. Crutzen, P.J., Lelieveld, J., and Bruel, C. (1994). Pages 63-93 in: *Environmental Oxidants*, Ed. by J.O. Nriagu and M.S. Simmons, Wiley, New York.
9. Kesselmeier, J., and Staudt, M. (1999). *Journal of Atmospheric Chemistry*, 33, 23-88.
10. Anfossi, D., Sandroni, S., and Viarengo, S. (1991). *Journal of Geophysical Research*, 96, 17349-17352.
11. Coyle, M., Smith, R. I., Stedman, J.R., Weston, K.J., and Fowler, D. (2002). *Atmospheric Environment*, 36, 1013-1024.
12. Lorenzini, G. (1993). *Medit*, 4, 53-59.
13. Benton, J., Fuhrer, J., Gimeno, B.S., Skärby, L., Palmer-Brown, D., Ball, G., Roadknight, C., and Mills, G. (2000). *Agriculture, Ecosystems and Environment*, 78, 19-30.
14. Bytnerowicz, A., Godzik, B., Fraczek, W., Grodzińska, K., Krywult, M., Badea, O., Barančok, P., Blum, O., Černy, M., Godzik, S., Mankovska, B., Manning, W., Moravčik, P., Musselman, R., Oszlanyi, J., Postelnicu, D., Szdźuj, J., Varšavova, M., and Zota, M. (2002). *Environmental Pollution*, 116, 3-25.
15. Guicherit, R., and Roemer, M. (2000). *Chemosphere - Global Change Science*, 2, 167-183.

16. Millán, M.M., Sanz, M.J., Salvador, R., and Mantilla, E. (2002). *Environmental Pollution*, 118, 167-186.
17. Colbeck, I., and MacKenzie, A.R. (1994). *Air Pollution by Photochemical Oxidants*, Elsevier, Amsterdam, 376 pp.
18. Karstiens, G., and Lendzian, K.J. (1989). *New Phytologist*, 112, 13-17.
19. Scandalios, J.G. (1990). *Advances in Genetics*, 28, 1-41.
20. Eberhardt, M.K. (Ed.) (2000). *Reactive Oxygen Metabolites*, CRC Press, Boca Raton, 591 pp.
21. Peñarrubia, L., and Moreno, J. (1999). Pages 769-794 in: *Handbook of Plant Crop Stresses*, Ed. by M. Pessarakli, Dekker, New York.
22. Martìnez-Cayuela, M. (1995). *Biochimie*, 77, 147-161.
23. Wellburn, A. (1988). *Air Pollution and Acid Rain: The Biological Impact*, Longman, Harlow, 274 pp.
24. Peñarrubia, L. and Moreno, J. (1990). *Archivies of Biochemistry & Biophysics*, 281, 319-323.
25. Scandalios, J. (1993). *Plant Physiology*, 101, 7-12.
26. Pell, E.J., Eckardt, N.A., and Glick, R.E. (1994). *Photosynthetic Research*, 39, 453-462.
27. Krupa, S.V., Grünage, L., Jäger, H.-J., Nosal, M., Manning, W.J., Legge, A.H., and Hanewald, K. (1995). *Environmental Pollution*, 87, 119-126.
28. Cooley, D.R., and Manning, W.J. (1987). *Environmental Pollution*, 47, 95-113.
29. Saitanis, C.J. and Karandinos, M.G. (2002). *Journal of Agronomy and Crop Science*, 188, 51-58.
30. Soja, G., Eid, M., Gangl, H., and Redl, H. (1997). *Phyton,* 37, 265-270.
31. Pell, E.J., Schlagnhaufer, C.D., and Arteca, R.N. (1997). *Physiologia Plantarum*, 100, 264-273.
32. Fridovich, I. (1995). *Annual Review Biochemistry*, 64, 97-112.
33. Levitt, J. (1999). *Responses of Plants to Environmental Stresses*, Academic Press, New York, 315 pp.
34. Heath, R.L. (1994). *Photosynthetic Research*, 39, 439-451.
35. Sharma, Y.K., and Davis K.R. (1997). *Free Radical Biology & Medicine*, 23, 480-488.
36. Pell, E., and Reddy, G.N. (1991). Pages 67-75 in: *Active Oxygen/Oxidative Stress and Plant Metabolism,* Ed. by E. Pell and K. Steffen, American Society Plant Physiology.
37. Mehlhorn, H. (1992). *Air Pollution Research Report* (CEC, Brussels), 46, 321-337.
38. Foyer, C.H., Descourvières, P., and Kunert, K.J. (1994). *Plant, Cell & Environment*, 17, 507-523.
39. Alscher, R.G., and Amthor, J.S. (1988). Pages 94-115 in: *Air Pollution and Plant Metabolism*, Ed. by S. Schulte-Hosted, N. M. Darrall, L. W. Blank and A.R. Wellburn, Elsevier, London.
40. Cracker, L.E. (1971). *Environmental Pollution*, 1, 299-304.
41. Mehlhorn, H., and Wellburn, A.R. (1987). *Nature*, 327, 417-418.
42. Bors, W., Langebartels, C., Michel C., and Sandermann, H. (1989). *Phytochemistry*, 28, 1589-1595.
43. Sandermann, H., Ernst, D., Heller, W., and Langebartels, C. (1998). *Trends in Plant Science*, 3, 47-50.
44. Paolacci, A.R., D'Ovidio, R., Marabottini, R., Nali, C., Lorenzini, G., Abenavoli M. R., and Badiani, M. (2001). *Australian Journal of Plant Physiology*, 28, 425-428.
45. Krupa, S.V., Tonneijck, A.E.G., and Manning, W. (1998). Pages 2.1-2.28 in: *Recognition of Air Pollution Injury to Vegetation: A Pictorial Atlas*, Ed. by R.B. Flager, Air & Waste Management Association, Pittsburgh.
46. Karandinos, M.G., and Saitanis, C. (1992). 6th International Conference on "Bioindicators Deteriorisationis Regionis", Ceske Budejovice, Czech Republic, 1991.
47. Heggestad, H.E. (1991). *Environmental Pollution*, 74, 264-291.
48. Pääkkönen, E., Vahala, J., Holopainen, T., Karjalainen, R., and Karenlampi, L. (1996). *Tree Physiology*, 16, 597-605.
49. Günthardt-Goerg, M.S., McQuattie, C. J., Maurer, S. and Frey, B. (2000). *Environmental Pollution*, 109, 489-500.
50. Pääkkönen, E., Gunthardt, G.M.S., and Holopainen, T. (1998). *Annals of Botany*, 82, 49-59.
51. Manning, W.J., and von Tiedemann, A. (1995). *Environmental Pollution*, 88, 219-245.
52. Badiani, M., Lorenzini, G., Paolacci, A.R., and Varvaro, L. (1993). *Fresenius Environmental Bulletin*, 2, 596-601.
53. Bonello, P., Heller, W., and Sandermann, H.J. (1993). *New Phytologyst*, 124, 653-663.
54. Khan, M.R., and Khan, M.W. (1998). *Agriculture, Ecosystems & Environment*, 70, 109-118.
55. Varshney, C.K., and Rout, C. (1998). *Bulletin of Environmental Contamination & Toxicology* , 61, 188-191.

56. Calatayud, A., and Barreno, E. (2001). *Environmental Pollution*, 115, 283-289.
57. Wu, Y., and von Tiedemann, A. (2002). *Environmental Pollution*, 116, 37-47.
58. Kickert, R.N., and Krupa, S.V. (1991). *Environmental Pollution*, 70, 271-383.
59. Heagle, A.S., and Stefanski, L.A. (2000). *Atmospheric Environment*, 34, 735-744.
60. Fuhrer, J., Skärby L., and Ashmore, M.R. (1997). *Environmental Pollution*, 97, 91-106.
61. Zierl, B. (2002). *Environmental Pollution*, 119, 55-68.
62. Lorenzini, G. (1994). *Applied Biochemistry & Biotechnology*, 48, 1-4.
63. Toncelli, M.L., and Lorenzini, G. (1999). *Environmental Monitoring & Assessment*, 55, 445-458.
64. Heagle, A.S., Miller J. E., and Sherril D. E. (1994). *Journal of Environmental Quality*, 23, 613-621.
65. Tonneijck, A.E.G., and Posthumus, A.C. (1987). VDI Berichte Nr. 609, 205-215.
66. Tonneijck, A.E.G., and Van Dijk., C.J. *Environmental & Experimental Botany* (*in press*).
67. VanderHeyden, D., Skelly J., Innes J., Hug C., Zhang J., Landolt W. and Bleuler P. (2001). *Environmental Pollution*, 111, 321-331.
68. Blum, O., Bytnerowicz A., Manning W., and Popovicheva, L. (1997). *Environmental Pollution*, 98, 299-304.
69. Ali, E.A. (1993). *Environmental Pollution*, 81, 251-255.
70. Kärenlampi, L., and Skärby, L. (Eds.) (1996). *Critical Level for Ozone in Europe: Testing and Finalizing the Concepts*, University of Kuopio, Kuopio, 306 pp.
71. Lorenzini, G., Nali C., and Biagioni, M. (1995). *The Science of the Total Environment*. 166, 193-199.
72. Velissariou, D., Davison, A.V., Barnes, J.D., Pfirrmann, T., and MacLean, D.C. (1992). *Atmospheric Environment*, 26, 373-370.
73. Gimeno B.S., Bermejo, V., Reinert, R. A., Zheng, Y., and Barnes J. D. (1999). *New Phytologist*, 144, 245-260.
74. Fumagalli, I., Gimeno, B.S., Velissariou, D., De Temmerman, L., and Mills, G. (2001). *Atmospheric Environment*, 35, 2583-2587.
75. Saitanis, C., and Karandinos, M.G. (2001). *Chemosphere*, 44, 813-821.
76. Saitanis, C., Katsaras, D., Lekkas, A.N. Riga-Karandinos, and G. Arapis. (2002). Pages 1867-1870 in: Proc. Int. Conf. Protection and Restoration of the Environment. IV. (Eds. A.G. Kungolos, A.B. Liakopoulos, G.P. Korfiatis, A.D. Koutsospyros, K.L. Katsifarakis, A.C. Demetracopoulos), Skiathos, Greece.
77. Salleras, J.M. (1987). *Fruticultura Profesional*, 12, 123-126.
78. Reinert, R., Sanchez, B., Salleras, J.M., Bermejo, V., Ochoa, M.J., and Tarruel, A. (1992). *Agriculture, Ecosystems & Environment*, 38, 41-49.
79. Gimeno, B.S., Peñuelas, J., Porcuna, J.L., and Reinert, R.A. (1995). *Water Air & Soil Pollution*, 85, 1521-1526.
80. Schenone, G., and Lorenzini, G. (1992). *Agriculture, Ecosystems & Environment*, 38, 51-59.
81. Lorenzini, G., Nali, C., and Biagioni, M. (1995). *Environmental Monitoring & Assessment*, 34, 59-72.
82. Nali, C. (2001). *Environmental Monitoring. Assessment*, 69, 159-174.
83. Nali, C., Pucciariello, C., and Lorenzini, G. (2002). *Agriculture, Ecosystems & Environment*, 90, 277-289.
84. Chronopoulos, J., Haidouti, C., Chronopoulou-Sereli, A., and Massas, I. (1997). *The Science of the Total Environment*, 196, 91-98.
85. Heliotis, F.D., Karandinos, M.G., and Whiton, J.C. (1988). *Environmental Pollution*, 54, 29-40.
86. Saitanis, C.J., Riga-Karandinos, A.N., and Karandinos, M.G. (2001). *Chemosphere*, 42, 945-953.
87. Kalabokas, P.D., Viras, L.G., Bartzis, J.G., and Repapis C.C., (2000). *Atmospheric Environment*, 34, 5199-5208.
88. Glavas, S. (1999). *Atmospheric Environment*, 33, 3813-3820.
89. Kouvarakis, G., Tsigaridis, K., Kanakidou, M., and Michalopoulos, N. (2000). *Journal of Geophysical Research*, 105, 4399-4407.
90. Saitanis, C.J. (2003). *Chemosphere*, 51, 913-923.
91. Mathy, P. (1988). Pages 505-514 in: *Assessment of Crop Loss from Air Pollutants*, Ed. by W. W. Heck, O. C. Taylor and D. T. Tingey, Elsevier, London.
92. Heck, W.W., Taylor, O.C., Adams, R., Bingham, G., Miller J., Preston E., and Weinstein, L. (1982). *Journal of the Air Pollution Control Association*, 32, 353-361.

93. Murphy, J.J., Delucchi, M.A., McCubbin, D.R., and Kim, H. J. (1999). *Journal of Environmental Management*, 55, 273-289.
94. Grünhage, L., Jäger, H.-J., Haenel, H.-D., Löpmeier, F.-J., and Hanewald, K. (1999). *Environmental Pollution*, 105, 163-173.
95. Adams, R.M., and Crockers, T.D. (1989). *Journal of Environmental Management*, 28, 295-307.
96. Elstner, E.F. (1982). *Annual Review Plant Physiology*, 33, 73-96.
97. Lorenzini, G., Nali, C., and Panicucci, A. (1994). *Atmospheric Environment*, 19, 3155-3164.

Subject index