ABIOTIC STRESS TOLERANCE IN PLANTS

Abiotic Stress Tolerance in Plants

Toward the Improvement of Global Environment and Food

Edited by

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Preface

Stresses in plants caused by salt, drought, temperature, oxygen, and toxic compounds are the principal reason for reduction in crop yield. For example, high salinity in soils accounts for large decline in the yield of a wide variety of crops world over; ~1000 million ha of land is affected by soil salinity. Increased sunlight leads to the generation of reactive oxygen species, which damage the plant cells. The threat of global environment change makes it increasingly demanding to generate crop plants that could withstand such harsh conditions.

Much progress has been made in the identification and characterization of the mechanisms that allow plants to tolerate abiotic stresses. The understanding of metabolic fluxes and the main constraints responsible for the production of compatible solutes and the identification of many transporters, collectively open the possibility of genetic engineering in crop plants with the concomitant improved stress tolerance. Abiotic Stress Tolerance in Plants is a new book with focus on how plants adapt to abiotic stress and how genetic engineering could improve the global environment and food supply. Especially, the application of biotechnology in Asia and Africa would be important. Environmental stress impact is not only on current crop species, but is also the paramount barrier to the introduction of crop plants into areas not currently being used for agriculture. Stresses are likely to enhance the severity of problems to be faced by plants in the near future.

The present book brings together contributions from many laboratories around the world in order to discuss and compare the current knowledge about the role of stress genes in plant stress tolerance. In addition, strategies to introduce these genes into economically important crops and its effects on plant productivity are discussed.

We express our thanks to all the contributors. Our sincere thanks are especially due to Prof. Tetsuko Takabe for her kind help in going through the contents and its arrangement. Finally, it is a profound pleasure to thank Springer for taking up the publication of this book.

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Ashwani K Rai
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SECTION I

SIGNAL TRANSDUCTION

1. STRESS SIGNAL TRANSDUCTION:

components, pathways and network integration

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Abstract. Drought, high soil salinity, and low temperature are common adverse environmental conditions that limit crop productivity worldwide. Plants respond to these abiotic stresses partly by activating the expression of stress-responsive genes. The products of some of these genes can increase plant tolerance to the stresses. Understanding how stress-responsive genes are activated by abiotic stress will help us to breed or engineer stress tolerant crop plants. Genetic and other studies are revealing components that are involved in the signal transduction forabiotic stresses. The pathways that lead to the activation of stress-responsive genes and the network that integrates these pathways are being discovered in model plant systems. This chapter discusses some recent progresses in the elucidation of abiotic stress signaling mechanisms.

1. INTRODUCTION

Adverse environmental conditions such as drought, high soil salinity, and temperature extremes are found in many agricultural areas. These abiotic stresses can result in severe yield loss to agricultural crops. Plants exhibit various responses to these stresses at the molecular, cellular, and whole plant levels [1-4]. These responses may contribute to increased tolerance to the stresses [5-8]. To breed or genetically engineer plant stress tolerance, it is imperative to identify the genes that control these traits and to understand how these genes and their products are regulated.

With the availability of complete information on a couple of plant genomes and of various genomics and proteomics tools, knowledge on plant abiotic stress responses has been advanced at a great pace in the last few years. In particular, documentation of genes that are regulated by stresses is comprehensive for the model plant Arabidopsis and should be complete for rice soon as well. Nonetheless, abiotic stress signaling mechanisms have been proven to be very complex, and there is still much more to learn. To date, only a handful of genes that play critical roles in plant adaptation to stresses have been identified with confidence. Few pathways that mediate stress responses are revealed in complete. In this chapter, we will give an

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overview of the current knowledge on signal transduction mechanisms responsible for signal perception, amplification, transmission, and final activation of stress responses. We will discuss examples of genetic studies or other studies where there is some supporting genetic evidence. This overview will mainly focus on advances made during the past two years since our last review on abiotic stress signal transduction was published [9].

2. ABIOTIC STRESS-REGULATED GENES

One major response of plants upon encountering abiotic stresses is the activation of stress-responsive genes. Genome-wide transcript profiling with Arabidopsis has identified many genes that are regulated by cold, salt, and drought stress (reviewed in [10]). Similar studies were also conducted with crop plants such as rice, barley, maize, and soybean [11-15]. It was suggested that as many as 30% of the genes in the Arabidopsis genome may be affected by abiotic stress at the transcript level [16]. Some of these genes can be activated by multiple stresses and also by the stress hormone abscisic acid (ABA). Generally, more genes are up-regulated than down-regulated [10,17]. This is also true for gene expression in response to ABA. Using the Agilent long-oligo chips, our microarray assay with Arabidopsis seedlings treated with ABA found that over 2000 genes were up regulated by more than 2-fold, whereas about 500 genes were down regulated (unpublished).

The products of some of these stress-inducible genes may play roles in stress signaling and stress tolerance [5,7,18,19]. These include, for example, enzymes that function in the biosynthesis of compatible solutes (osmolytes) or either directly in detoxification of reactive oxidants or in the biosynthesis of antioxidant compounds, ion transporters, ABA biosynthetic enzymes, etc. The products of some other genes may also have protective roles against stress damages yet their modes of action are unclear. These are mainly the late-embryogenesis-abundant protein (LEA)-like proteins [2]. However, some stress-regulated genes may not play a primary role in stress response. Their induction may be merely a consequence of the stress and stress injuries [20]. In some cases, genes physically associated with certain key stress-induced genes in a chromatin region may be regulated by stress, although these genes may not be related otherwise. One example is the *UFC* (upstream of FLC) gene [21]. *FLC* is a flowering repressor whose transcript level is down regulated by long-term cold treatment (e.g., vernalization). Interestingly, *UFC* is similarly regulated by vernalization yet it does not relate to *FLC* either in sequence or in function. They are merely neighboring genes on the same chromosomal region. This suggests that chromosome location may have a strong influence on the induction of certain genes.

3. AN OVERVIEW ON STRESS SIGNAL TRANSDUCTION

Signal transduction is required for many cellular activities and their coordination. Some signal transduction processes are simple but most others are complex, involving multiple components and occurring in a time and space-dependent

manner. Generally, signal transduction starts with the perception of a stimulus by a specific cellular molecule(s). These sensors or receptors may differ in their molecular identities, modes of signal perception and output, as well as subcellular localizations.

In plant cells, it is also common for receptor activation to result in the generation of second messengers, so called because they represent intracellular signals being translated from the primary external signal. These intracellular messengers are interpreted further by other signaling component(s) and result in the activation of downstream pathways that may have multiple outputs. These pathways usually involve reversible protein phosphorylation. Protein phosphorylation could lead to, among others, the activation of transcription factors that induce the expression of stress-responsive genes.

Signal transduction often requires additional components that recruit and assemble signaling complexes, target signaling molecules, and regulate their lifespan. In many cases, these components themselves are also regulated by the signaling pathways that may have been initiated from the same stress signals. Here we refer to these components collectively as signaling partners.

Figure 1 depicts a genetic signaling pathway that can serve as a framework for anchoring many of the individual signaling components that are increasingly being reported in the literature.

4. SIGNAL TRANSDUCTION COMPONENTS

4.1. Receptors

4.1.1. Complexity of abiotic stress as signals

Receptors are the molecules that first perceive stress stimulus and then relay the signal to downstream molecules to initiate the signal transduction pathway. However, it is not an easy task to find these receptors. Many of the abiotic stress signals are complex in their nature. They may not simply be physical or chemical signals but rather, a mixture of several favours. For example, low temperature may induce both osmotic stress and mechanistic stress. Drought, conditioned by decreased water potential in the soil, may involve osmotic stress, ionic stress, a mechanistic signal, and heat stress in some cases. Very likely, each of these stress attributes may have different weights with regard to the plant status or the severity of the particular stress in question. Thus, a simple water shortage in the soil may in fact impart very complex and different information to the plants.

One can also expect that there may be multiple cellular sensors to perceive a stress signal or one attribute of the signal. The complex nature of abiotic stress as signals and the redundancy of their perception machinery pose great constraints for the identification of cellular machinery that perceives abiotic stress.

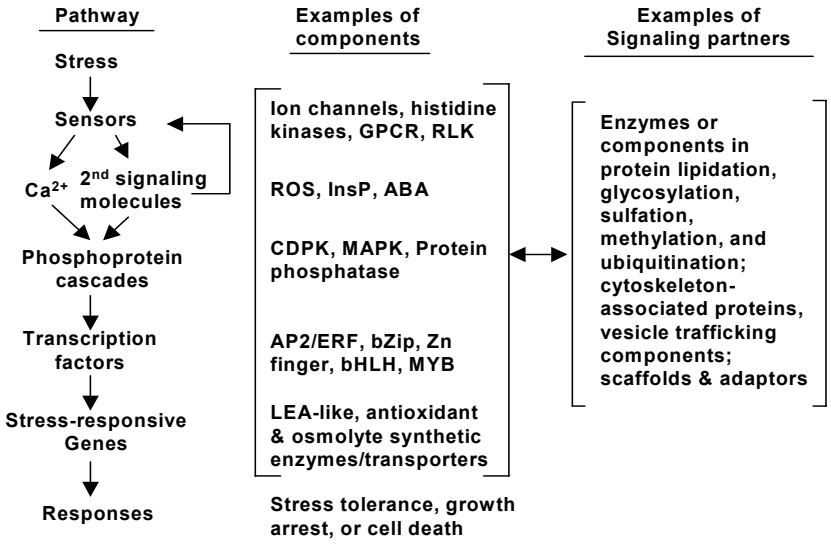

Figure 1. A conceptual signal transduction pathway for drought, cold, and salt stress in plants. Examples of signaling components in each of the steps are shown. It should be noted that none of the indicated receptor components has been confirmed as a stress sensor. Secondary signaling molecules can cause receptor-mediated Ca^{2+} release (indicated with a feedback arrow). Examples of signaling partners that modulate the components in the main pathway are also shown. These partners can be regulated by the main pathway. Signaling can also bypass Ca^{2+} or secondary signaling molecules in early signaling steps. GPCR, G-protein coupled receptor; RLK, receptor-like kinase; InsP, inositol polyphosphates. Please refer to the text for other abbreviations.

4.1.2. Putative sensors in stress signal perception

Studies in other systems have identified several kinds of receptors that function in stress signal perception. These include receptor-like kinases, two-component receptors, receptor tyrosine kinases, G-protein coupled receptors, iontropic channel-related receptors, histidine kinases, and nuclear hormone receptors. By sequence homology, most of these receptor families can be found in sequenced plant genomes. However, a few such as receptor tyrosine kinases and nuclear hormone receptors cannot be identified by sequence homology search. Many people thus believe these signaling components nonexistent in higher plants.

Because of their stress inducibility or, in a few cases, phenotypes conferred by their regulated expression, receptor-like kinases, two-component receptors, histidine kinases, iontropic receptors, and G-protein associated receptors have each been implicated as potential receptors for abiotic stresses or the stress hormone ABA (reviewed in [9,22]). Despite the importance of identifying stress sensors, research effort to find these receptors has been limited. To date, there has been no convincing evidence to support any of the above-mentioned putative receptors as stress sensors.

Thus, it may be helpful to briefly introduce abiotic stress sensors identified in other systems.

In cyanobactieria, histidine kinases were identified as cold-sensors in the activation of selected marker genes [23]. Knockout of these kinases resulted in substantially reduced expression of these genes. In neurons, a TRP Ca^{2+}/cation channel was suggested to be a cold sensor [24]. In fact, similar TRP channels can act as heat sensors as well [25]. However, no TRP channel protein can be found in the sequenced plant genomes using sequence similarity searches. In plant cells, cold-induced Ca^{2+} influx has been documented as an early response to cold [26]. Manipulations of Ca^{2+} influx can affect the expression of cold-regulated genes (e.g., [27]). Nonetheless, the calcium channels responsible for this Ca^{2+} influx have not been identified.

As mentioned above, an abiotic stress may initiate multiple signaling pathways in plants. It may be difficult to directly identify stress sensors through genetic analysis, since knocking out one receptor may not significantly affect the stress signaling outputs. Because ABA is involved in abiotic stress signaling, revealing how ABA is perceived certainly will help reveal how stress signals are sensed. Unfortunately, how ABA is perceived is not known either (reviewed in [22]). Current efforts to uncover ABA perception mechanisms mainly focused on putative receptor-linked components or those putative receptor molecules that are regulated by stress or ABA. For example, Arabidopsis heterotrimeric G-protein α subunit GPA1 was suggested to be involved in ABA response in guard cells since *gpa1* mutants were insensitive to ABA inhibition of stomata opening and ABA regulation of inward K^+ channels, yet it does not function in ABA-induced stomata closure. The *gpa1* mutant seedlings lost water more quickly than the wild type [28]. GPA1 interacts with the G-protein couple receptor-like protein GCR1, yet *gcr1* mutant was more sensitive to ABA than the wild type [29]. The reason for the opposite phenotypes between *gpa1* and *gcr1* mutants and the modes of action for both proteins are unclear. In addition to the heterotrimeric G proteins, there are different classes of small G proteins [30]. One of the ROP family Rho GTPases, ROP10, was proved to be a negative regulator of ABA responses in Arabidopsis [31]. Since another ROP related to ROP10 was shown to be associated with CLV receptor kinase [32] and ROP10 was localized to plasma membrane, it was hypothesized that ROP10 may be associated with an ABA perception complex on the plasma membrane [31].

Abiotic stresses also generate second signaling molecules (see below). Receptors for these signals should exist in plants, yet none has been identified. In contrast, receptors for inositol trisphosphate, cADPR and sphingosine 1-phosphate are well characterized in animal systems.

4.2. Second intracellular signaling molecules

Several intracellular signaling molecules are involved in stress signal transduction. These include reactive oxygen species, lipid phosphates-derived signals, and cyclic

nucleotides-related signals. In addition, some plant hormones also have the characteristics of secondary signal molecules.

4.2.1. Reactive oxygen species

In addition to the reactive species generated during normal photoreactions and cellular biochemical oxidations, plants also produce reactive oxygen species (ROS) during environmental stresses and in response to pathogen attacks (reviewed in [33]). Although these reactive molecules may have damaging effects on cellular membranes and macromolecules, they play important signaling roles in early stages of stress response. These reactive molecules can activate cellular defense mechanisms to mitigate stress damage. Among others, nitric oxide (NO) and hydrogen peroxide (H_2O_2) are suggested to play roles in ABA signaling and may function in abiotic stress response as well.

ABA regulation of stomata closure appears to require the generation of H_2O_2. H_2O_2 production is a prerequisite for ABA-induced stomatal closure [34-36]. NAPH oxidase may represent the major source for H_2O_2 production. Mutations in genes that encode catalytic subunits of NADPH oxidase impair ABA-induced ROS production and the activation of guard cell Ca^{2+} channels and stomata closure [37].

In plants, nitric oxide can be generated by enzymatic reactions as well as non-enzymatic reactions [38]. Both nitrate reductases and NO synthases (NOS) can contribute to NO generation. It appears that these two kinds of enzymes do not function redundantly since mutations in either enzyme could confer specific phenotypes. For example, loss-of-function mutations in Arabidopsis NOS, AtNOS1, impair ABA-induced NO production and stomata closure [39].

ROS may affect stress signal transduction in the activation of stress-responsive genes [40,41], in particular those that encode enzymes in the biosynthesis of antioxidants or enzymes that directly detoxify reactive oxidative radicals. Then, how does ROS affect stress signal transduction? It was demonstrated that ROS is involved in the regeneration of Ca^{2+} signals through the activation of Ca^{2+} channels (reviewed in [42]). These secondary Ca^{2+} signals could initiate additional signal transduction via Ca^{2+} mediated pathways [9]. Another route is that reactive oxygen species themselves can directly modify signaling molecules through redox regulation. Molecules with cysteine residues as key active sites could be the targets of redox regulation. These molecules could be potential sensors for ROS [20]. Since tyrosine phosphatases in animals are the potential targets of ROS, and these phosphatases could regulate MAPK cascades, it is believed that MAPK pathways are probably the major pathways mediating ROS signal transduction (see Section 4.5.1). An AGC family protein kinase OXI1 appears to be involved in ROS activation of MPK3 and MPK6 since in *oxi1* mutant, activation of both MAPK was compromised [43].

Cellular redox environment may also modulate cell signaling by regulating the activity of other signaling components. One example is the regulation of the transcription activator NPR1 by redox status. A reduced milieu in the cytosol facilitates the inactive NPR1 oligomer to change into an active monomer form. The active NPR1 may target the TGA zinc finger transcription factors and activates PR

gene expression [44]. In Arabidopsis genome, there are several NPR1-like genes, but it is not clear whether any of these genes would regulate stress responses and interact with ABF-like zinc finger proteins.

Lipids-derived messengers

Membrane lipids may be directly involved in stress response by modulating membrane fluidity or its other physiochemical properties [45]. Yet a more important function of these lipid components is their role in generating intracellular signaling molecules. Lipids and their biogenesis and degradation enzymes play many roles that directly or indirectly regulate or affect plant stress signaling and stress tolerance. For a general discussion of the roles of lipids in cell signaling, readers are referred to a recent review [46]. Some recent advances in lipid signaling of abiotic stress are briefly outlined below.

It is known that phospholipids, the backbone of cellular membranes, can serve as precursors for the generation of second messengers in response to abiotic stresses. While the relevant lipid cleaving enzymes are the phospholipases A2, C, and D, the most studied is the phosphoinositide-specific phospholipase C (PI-PLC). Upon activation, PI-PLC hydrolyzes phosphotidylinositol 4,5-bisphosphate (PIP_2) to produce two important molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). DAG and $InsP_3$ are second messengers that could activate protein kinase C (PKC) and trigger Ca^{2+} release, respectively.

In plants, the role of exogenous $InsP_3$ in releasing Ca^{2+} from cellular stores has been widely reported [47,48]. Inhibition of PI-PLC activity impairs ABA-induced stomata closure [49] and inhibits osmotic stress-induction of the stress-responsive genes *RD29A* and *COR47* [50]. On the contrary, inhibiting the breakdown of $InsP_3$ may increase the expression of stress-inducible genes. This was demonstrated with transgenic plants overexpressing Arabidopsis inositol polyphosphate 5-phosphatases [51,52] and with loss-of-function or conditional mutation in an enzyme of inositol polyphosphate 1-phosphatase FIERY1 [53,54].

Accumulating evidence suggests that phosphatidic acid (PA) is also involved in the transduction of stress signals. PA is generated by the Phospholipase D (PLD) hydrolysis of phospholipids. In guard cell protoplasts, PLD activity mediates ABA–induced stomatal closure [55]. PA produced by PLDα1 may interact with and inhibit the activity of ABI1, which is a negative regulator of ABA signaling (see below). Consequently, PLDα1 knockout mutants become less sensitive to ABA [56]. Interestingly, PLDα1 interacts with G-protein α GPA1 [57]. Therefore, PLDα1 and GPA1 may in fact function together in controlling aspects of ABA signaling. Knockout plants in another PLD isoform PLDζ are more susceptible to freezing damage whereas its overexpression enhances freezing tolerance [58].

Several other secondary signaling molecules including $InsP_6$, sphingosine1-phosphate (S1P), and cADPR were also suggested to regulate ABA responses in guard cells (reviewed in [48]). However, their role in stress signal transduction is unclear.

Phytohormones

Upon encountering abiotic stress, plants may alter their growth and development programs. Cell expansion and division may be halted and thus growth generally slower than under normal growth conditions. Longer-term abiotic stress may also affect plant phase transitions. For example, drought stress can promote flowering. These developmental changes imply that abiotic stress may alter the homeostasis of growth regulators. Although plant hormones are not considered as second messengers, the stress hormone ABA acts like one in many aspects: ABA biosynthesis is activated by abiotic stress [59]; ABA mediates many downstream pathways [22]; and ABA can be subjected to long distance transport and play physiological roles at sites distant from where it is synthesized [60]. In addition to ABA, other plant hormones, in particular ethylene and auxin, are involved in ABA and perhaps abiotic stress responses as well.

The role of ABA in plant stress responses has long been recognized [12,18]. In guard cells ABA regulates ion channels and promotes stomata closure to minimize transpiration water loss [48]. ABA activates the expression of many stress-responsive genes independently or synergistically with stresses. ABA can inhibit the biosynthesis of ethylene and may also potentially reduce the sensitivity of plants to ethylene [61]. The expression of some aquoporin genes or the activity of these water channel proteins may also be regulated by ABA. With the involvement of ABA in these processes, a general consequence is that plants will adapt to the stress with reduced water potential (so that they could lose less and uptake more water) and consequently, a reduced growth rate.

In addition to many physiological and biochemical changes that are mediated by ABA under abiotic stress, ABA may also regulate plant development programs and developmental changes such as root patterning. Our current knowledge in this aspect is limited.

4.3. Ca^{2+} as an intermediate signal molecule

The above-mentioned secondary signaling molecules may activate transient increases in cytosolic Ca^{2+} [26]. The sources of cytosolic Ca^{2+} inevitably are either internal or external. Both sources have much higher concentrations of Ca^{2+} relative to the cytosol. Therefore, the gating of Ca^{2+} channels is the major means to control Ca^{2+} transient increase in the cytoplasm. The complex or pumping of Ca^{2+} into vacuoles (or extracellular space) would be the major route for resetting the signals. Currently, a lot is known about both processes in animal cells but related information in plant cells is limited. Most plant Ca^{2+} channels may have diverged significantly in primary sequences from those of animal Ca^{2+} channels. Nonetheless, a putative two-pore Ca^{2+} channel in Arabidopsis was found to share sequence similarity to a voltage-gated Ca^{2+} channel in rats [62]. A recent study reported the identification of an 'extracellular Ca^{2+} senor' [63], yet the biochemical functionality and its mode of action for this protein is unclear.

Internal Ca^{2+} could also contribute to stress-induced Ca^{2+} transients in the cytosol. In animal cells, several Ca^{2+} channels on ER membranes or other

endomembranes are responsible for transient Ca^{2+} increases in the cytosol. These include the $InsP_3$ receptors and the cADPR ryanodine receptors. Although $InsP_3$ and cADPR also exist and function in plant cells, their plant receptors have not been identified. Since many cell signaling events use Ca^{2+} as an intermediate signaling molecule, it is surprising that no Ca^{2+} channel has been identified in genetic screens that aim to elucidate these signal transduction mechanisms. Given that many putative ion channel genes exist in the Arabidopsis genome, future reverse genetic studies may help to identify potential Ca^{2+} channels.

Cytosolic or organelle Ca^{2+} concentrations are tightly controlled by various Ca^{2+} pumps and transporters. These Ca^{2+} transporters restore cytosolic Ca^{2+} homeostatasis after various stimulus disturbances. One Ca^{2+} transporter is the tonoplast Ca^{2+}/H^+ exchanger (CAX). These exchangers transport Ca^{2+} from the cytosol into vacuoles, a major storage of Ca^{2+} within plant cells. Overexpression of CAX1 resulted in increased freezing sensitivity [64] whereas its knockout mutants exhibited increased efficiency of cold acclimation in that the transcript levels for *CBF/DREB1* transcription factor genes (see Section 3.7) and their downstream stress responsive genes were higher in the mutant than in the wild type. These *cax1* knockout plants are thus more tolerant to freezing stress [65].

4.4. Ca^{2+}-binding proteins

In contrast to the lack of information on Ca^{2+} channels in plants, many Ca^{2+}-binding proteins have been identified in plants [66,67]. These Ca^{2+}-binding proteins possess Ca^{2+}-binding motifs that are homologous to those in animal Ca^{2+} binding proteins. One major Ca^{2+} binding motif is the so-called EF hand motif, which is conserved across organisms. Major plant Ca^{2+} binding proteins include calmodulins, SCaBP (SOS3-like Ca-biniding proteins)/Calcineurin B-like (CBL) proteins, and Ca-dependent protein kinase (CDPK).

Arabidopsis SOS3 (Salt-Overly-Sensitive 3) was identified because of the salt hypersensitive phenotypes of the *sos3* mutant. SOS3 shares sequence similarity with the calcineurin B subunit (CNB) and animal Ca^{2+} sensor, although SOS3 does not function as CNB in the activation of calcineurin. Rather, it acts as a Ca^{2+}-binding protein to interact with and activate the AMPK/SNF-like serine/threonine protein kinase SOS2, whose mutation also confers salt sensitivity. The activated SOS2 phosphorylates and regulates ion transporters such as the plasma membrane-localized Na^+/H^+ antiporter SOS1. This eventually leads to the restoration of ion homeostasis in the cytoplasm under salt stress conditions [68].

In the Arabidopisis genome, there are 9 SOS3 homologs (SCaBP/CBL) and 22 SOS2 homologs (SOS2-like protein kinases -PKS/CBL-interacting protein kinases-CIPK). Individual SCaBP/CBL interacts with PKS/CIPK with different specificities (Gong et al., 2004; Luan et al., 2002). It appears that the various interaction pairs between these two groups of proteins may mediate responses to different abiotic stresses [69,70]. An example is the SCaBP5 and PKS3 interaction that may interpret Ca^{2+} signatures resulting from ABA or drought stress signals.

Mutations in SCaBP5 or PKS3 confer similar ABA hypersensitive phenotypes in the mutants. In addition, it was found that PKS3 interacts with the ABI2, a type 2C protein phosphatase (see below). ABI2 may negatively regulate the signals perceived by the SCaBP5-PKS3, thus potentially preventing over activation of the downstream signaling pathways. The interaction between PKS3 and ABI2 in this case did not result in detected dephosphorylation or phosphorylation of either partner. It is possible that some other component associated with this complex is the target of ABI2 [71]. More recently, Ohta et al. [72] confirmed this interaction and mapped the protein domain of SOS2 that interacts with ABI2. They found that this interaction is sensitive to the abi2 dominant mutation because the mutated form no longer interacted with PKS3, suggesting that the interaction between PKS kinases and ABI phosphatases may be physiologically significant.

Calmodulins have been implicated in several cellular processes through interaction with CaM-binding proteins [67]. The expression of several plant CaM genes is regulated by various environmental stresses such as mechanical stress/touch, cold, salt, or drought stress. Presumably, these CaMs may participate in the transduction of these external stimuli. One of the CaMs, CaM3, was suggested as a negative regulator of *COR* gene expression, since overexpression of this CaM led to reduced transcript levels of stress-responsive genes *RD29A* and *KIN1* [73]. Consistent with this notion, experimental evidence indicates that a CaM binding protein, AtCaMBP25, may act as a negative regulator of osmotic stress tolerance. Transgenic Arabidopsis plants overexpressing AtCaMBP25 are more sensitive to osmotic stress whereas the antisense plants are more tolerant to salt stress [74].

The involvement of CDPK in stress signal transduction has also been implicated. In addition to stress-inducibility for some of the CDPKs, constitutively active CDPK was demonstrated to regulate stress-responsive reporter gene expression under ABA or stress treatments in protoplasts (Sheen, 1996). Overexpression of a CDPK in rice conferred increased tolerance to cold and salt stress [76]. However, there has been no report showing that loss-of-function CDPK may affect stress signal transduction or stress responses.

Other Ca^{2+}-binding proteins or Ca^{2+}-dependent proteins include annexins [77], calnexin and calreticulin. Calnexin and calreticulin may serve as endoplasmic reticulum (ER) chaperones and ER Ca^{2+} reservoirs. The role of these proteins in stress signaling is unclear. However, plants may have similar ER stress responses as do other eukaryotes [78].

4.5. Phosphoproteins at the core of stress signal transduction

In many signal transduction pathways, protein reversible phosphorylation is the major form of signal relay. The enzymes that catalyze these reversible phosphorylation processes are protein kinases and protein phosphatases. In the Arabidopsis genome, there are over 1,085 protein kinases (cited in [79]) and 112 protein phosphatases [80]. Protein kinases and phosphatases can be divided into several categories based on substrate specificity or on the structure or functional

characteristics. In this section, we present several recent examples on genetic studies of the role of phosphoproteins in stress signaling.

4.5.1. MAPK

Aside from its mysterious position in stress signaling, the mitogen activated protein kinase (MAPK) cascades are known to be involved in plant abiotic stress responses. The cascade is characterized by the sequential phosphorylation of a kinase by its upstream kinase in the order of MAPKKK-MAPKK-MAPK. Early studies found that the transcript levels of certain MAPK genes were enhanced by cold and salt stress. Some late studies followed the kinase activities for these proteins and found the activation of MAPK by stress treatments [81]. In several cases, regulated expression of MAPK components was shown to affect stress sensitivity. For example, expression of an active form of a tobacco MPKKK, NPK1, increases freezing tolerance of transgenic tobacco or maize plants [82,83].

Recently, a MAPK cascade in Arabidopsis was suggested to be involved in cold and osmotic stress signal transduction. This cascade consists of the MAPKKK MEKK1, the MAPKK MKK2, and two MAPKs, MPK4 and MPK6 [84]. Salt and cold stresses activate MKK2, MPK4 and MPK6, whereas in *mkk2* mutant plants, MPK4 and PMK6 were no longer activated by cold. The *mkk2* mutant plants were also sensitive to freezing and salt stress. Transcript profiling revealed that 152 genes were affected by over-expression of MKK2. These include genes for several transcription factors (such as RAV1, STZ, ZAT10, ERF6, WRKY, and CBF2), disease resistance proteins, cell wall related proteins, enzymes involved in some secondary metabolisms and an ACC (1-aminocyclopropane-1-carboxylate) synthase. Interestingly, several auxin-responsive genes were down regulated. Although this MAPK cascade apparently is involved in stress responses and the *CBF2* transcript level was higher in the overexpressing plants, none of the CRT/DREB class of stress-responsive genes was significantly affected by this cascade. This is consistent with our previous prediction that MAPK pathways appear to be independent of the pathways that up regulate the expression of the *CBF/DREB* class of stress responsive genes [9]. In addition, since genes involved in other hormone biosynthesis (ethylene) and responses (auxin) were altered, it will be important to distinguish the direct targets of this MAPK cascade from those that are regulated by altered hormonal and oxidative stress responses. It is known that the 'cross-talk' between various MAPK cascades is intensive [81] and that various feedback regulations are also common within some pathways. For instance, MAPK6 had previously been shown to affect auxin signaling and stress tolerance [82], disease resistance [43,85], and ethylene biosynthesis [86]. Similarly, MPK3 was also suggested to affect ABA inhibition of seed germination [87] and pathogenesis signaling [43,85]. Identifying the targets of MAPK cascades may prove to be challenging.

4.5.2. Other protein kinases

Certain protein kinases are induced by various abiotic stresses either at the transcript level or at the activity level, implying that they may be involved in transduction of

these stress signals. There have been several reports presenting evidence that suppression or overexpression of some of these kinases resulted in altered stress responses in transgenic plants. However, genetic studies regarding the in vivo functionality for these kinases have been lacking. An exception is the SOS2 group AMPK/SNF like kinase (see Section 3.4), which was grouped into the SNF1-related kinase subfamily 3 (SnRK3) [79]. Some other members in the SnRK family were also shown to affect stress signaling and stress responses [79].

The protein kinase OST1 functions in the ABA signaling pathway upstream ABA-induced ROS production [88]. OST1 is related to the ABA-activated protein kinase AAPK in *Vicia faba* [89] and also related to SNF1 protein kinase [90] and was grouped into the SnRK2 subfamily [79]. The *ost1* mutants showed reduced response to ABA in stomata closure yet did not change in ABA responsiveness during seed germination. Ost1 activity is activated by ABA but its gene expression is not. Proteins similar to Ost1 also exist in several other plants and were reported to have similar roles in regulating osmotic stress and ABA signal transduction [91,92]. Further genetics and biochemistry studies are expected to reveal the roles of these kinases in stress signaling by defining their targets and modes of action in stress signaling.

4.6. Protein phosphatases

Protein phosphatases dephosphoryate phosphoproteins and thereby attenuate the function of protein kinases. Protein phosphatases can be classified by their substrate specificity as serine/threonine phosphatases, tyrosine phospahtases, and dual specificity phosphatases. Among them, serine/threonine phosphatases are the largest group of phosphatases in plants. According to their sequence (structure) characteristics and cation requirements, serine/threonine phosphatases can be classified further into PP1, PP2A, PP2B, and PP2C. Among these phosphatases, some PP2C, PP2A, PTP, dsPPase have been implicated in ABA or stress signal transduction [93,94]. The best-known example is the PP2C involvement in ABA signal transduction.

Early genetic studies using the inhibitory effect of ABA on seed germination identified the ABA-insensitive 1 (ABI1) and ABI2, two homologous 2C type phosphatases (reviewed in [22]). However, due to the dominant nature of both mutations, their roles in ABA signaling were not clear. Mutation analysis and reporter-gene assays in protoplast systems suggested that these ABI may function as negative regulators of ABA signaling [95]. Consistent with this notion, recessive intragenic revertants of *abi1* exhibited hypersensitivity to ABA in seed germination and vegetative growth [96]. Although ABA hypersensitive phenotypes for loss-of-function *abi1* and *abi2* mutants have not been reported, the discovery of other PP2C as negative regulators of ABA signaling [97,98] supports the idea that some PP2C may function as negative regulators of ABA signaling.

Following the isolation of the dominant *abi1-1* and *abi2-1* mutants, many researchers used these mutants in their studies of abiotic stress signaling and plant stress tolerance. It is clear that ABA or stress induction of many ABA and

stress-regulated genes are impaired in *abi1* [99] or *abi2* mutants (reviewed in [22]). Further studies using both mutants have found that *abi1-1* and *abi2-1* have defects in reactive oxygen species generation or their regulation on ion channels. The *abi1*-1 mutants are impaired in ABA-induced ROS production whereas *abi2-1* guard cells are defective in H_2O_2-activated Ca^{2+} channel regulation [35].

To reveal the functionality of ABI1 and ABI2, it is essential to identify their targets. In yeast two-hybrid assays ABI1 interacts with the homeodomain transcription factor AtHB6 [100]. ABI1 and ABI2 also interact in vitro and in vivo with the SOS2 class of protein kinases [71,72] (see above sections). Some signal molecules such H_2O_2 and fatty acids were shown to bind to ABI1 or other PP2C (reviewed in [93]). ABI1 may also be regulated by PA derived from PLDα hydrolysis of phospholipids. PA binding of ABI1 inhibits ABI1 phosphatase activity and therefore will activate ABA signaling in response to ABA [56]. Interestingly, Zhang et al. [56] reported that ABI1 was predominately localized in the cytoplasm but tended to be relocated to plasma membrane in response to ABA treatment. It should be noted that PA is generated upon ABA treatment and is also membrane-localized. Subcellular localization of ABI1 and ABI2 were not reported before. If ABI1 does not localize in the nucleus where AtHB6 is found [100], the interaction between AtHB6 and ABI1 may not occur in vivo.

Previous pharmacological studies suggested that PP2A might be involved in cold stress signaling [101]. Recently, PP2A was shown to play roles in ABA activation of slow anion channels in guard cells because the *rcn1* mutant exhibited ABA-insensitivity to ABA in stomatal closure and was impaired in slow anion channels regulation by ABA [102]. *RCN1* encodes the regulatory subunit of PP2A. Because RCN1 is involved in response to ethylene and auxin [103,104], it is not clear whether the role of RCN1 in ABA signaling is the consequence of the regulation of an ABA signaling component(s) by PP2A or a result of the complex interaction between different plant hormones.

4.7. Transcription factors in stress signaling

Presumably, the targets of some protein kinases will be transcription factors that upon activation will bind to *cis*-elements in the promoters of stress-responsive genes and thus activate their transcription. Transcription factors may themselves be regulated at the transcription level by other upstream transcription factors. These further upstream transcription factors are often in a constitutively active state but are contained by repressors or held physically separate from their target genes (e.g., in cytoplasm or inaccessible to the target regions within the nucleus). Regulation of these transcription factors is therefore an important way to control gene expression. Common means for the release of repression include conformation changes by protein phosphorylation, degradation by ubiquitination, and trafficking between subcellular localizations. Protein kinases could play roles in all these processes, yet examples in plants are still very rare. Nonetheless, a lot has been learned regarding gene activation by transcription factors during abiotic stress signal transduction.

Several classes of transcription factors are involved in the activation of stress-response genes in plants. These include the AP2/ERF (ethylene responsive element binding factor), Zn finger, basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), MYB, and NAC transcription factors. The CBF/DREB transcription factors belong to the AP2/ERF class and have been studied in detail. These transcription factors bind to the C-repeat element (CRT)/dehydration-responsive element (DRE) in the promoters of many stress-responsive genes [3,19]. Nonetheless, CBF/DREB may not be the sole transcription factors in the regulation of CRT/DRE genes. A homeodomain transcription factor, HOS9, regulates cold signal transduction and cold tolerance through a pathway independent of the CBF/DREB transcription factors [105]. Because of their functional redundancy, null mutation in a single CBF/DREB transcription factor may not necessarily give rise to a visible phenotype. On the other hand, several experiments demonstrated that overexpression of CBF/DREB transcription factors could lead to an enhanced expression of stress-responsive genes and increased tolerance to various abiotic stresses [3,19].

Because CBF/DREB transcription factor genes are also induced by stress, upstream transcriptional activators must exist. Recently, it was suggested that CBF2 probably is a negative regulator of other CBF genes, since in *cbf2* knockout mutant, the transcript levels of *CBF1* and *CBF3* were slightly higher than in the wild type, and the *cbf2* mutant seedlings were more resistant to freezing stress [106]. Another putative transcription factor for *CBF/DREB1* genes is ICE1. A dominant mutation in *ICE1* resulted in impaired cold-stress regulation of *CBF* genes, whereas overexpression of *ICE1* increases cold-induced *CBF* and the downstream gene expression. These transgenic plants are also more tolerant to chilling and freezing stress [107].

Several other putative signaling components that regulate the CBF/DREB class of transcription factors were identified in genetic screens for altered stress-inducible *RD29A*::*LUC* (*luciferase)* reporter gene expression. Mutation in the *HOS1* gene resulted in increased stress-responsive gene induction by cold. HOS1 is a novel protein containing a RING finger domain that potentially participates in protein degradation. Since *hos1* mutant seedlings had higher expression of CBF transcription factor genes, it is hypothesized that HOS1 may target positive regulators CBF transcription factors for proteolysis. Other potential regulators include FRY1/HOS2 [54], FRY2/CPL1 [109], and LOS4 [110]. FRY2 encodes a novel RNA polymerase II C-terminal domain (CTD) phosphatase [109,111,112] and may function in the regulation of transcript elongation. FRY1 encodes a bifunctional enzyme with both inositol polyphosphate 1-phosphatase and nucleotidase activities [53]. Both *fry1/hos2* and *fry2* mutants had higher transcript levels of several CBF transcription factor genes and higher level induction of stress-responsive genes [53,54,109], whereas *los4* had a lower transcript level of *CBF* genes [110]. Because of the nature of these proteins, *fry1/hos2*, *fry2/cpl* and *los4* may indirectly regulate the transcription of *CBF* either through upstream signaling pathway regulation or through regulation of the transcription machinery under abiotic stress.

4.8. Chromatin remodeling factors

Because genes are packed in chromatins, remodeling of chromatin structure to allow positive transcriptional regulators access to the genes is thus a critical step toward gene activation. It is conceivable that stress signal transduction involves components in chromatin remodeling. An example in this regard is the activation of the yeast High Osmolarity Glycerol 1(Hog1) pathway. Upon osmotic stress, a MAPK pathway is activated and leads to the phosphorylation of the MAPK Hog1. Activated Hog1 is recruited to specific promoter regions by transcription factors. Hog1, once bound to the promoter complex, then recruits histone deacetylase Rpd3 to deacetylate histone and activate osmoresponsive genes [113]. Currently, little is known about the regulation of chromatin remodeling by most abiotic stresses except for low temperature.

Many plants in the temperate region require an exposure to prolonged low temperature (winter) to promote flowering in the spring, a process referred to as vernalization. In Arabidopsis, vernalization involves the down regulation of the flowering suppressor Flowering Locus C (*FLC*). FLC has a dosage repressing effect on flowering time. Vernalization modifies the *FLC* locus into a repressed state by histone methylation and therefore promotes flowering transition [114]. It should be noted, however, that vernalization and cold acclimation are two different processes [115]. Plants respond to them differently both in terms of gene expression and physiological consequences.

4.9. Posttranscriptional regulation in stress signaling

Gene regulation could occur at the level of transcription, posttranscription, translation, and posttranslation. Current studies of stress gene regulation are mainly focused on the transcription level. Although other processes of gene regulation are also important, it is only until recently that the importance of posttranscriptional regulation of stress responsive genes has become evident. Particularly, genetic studies of ABA and stress signal transduction have demonstrated that aspects of mRNA processing are critical for stress and ABA signal transduction.

In screens for components that affect the activation of the *RD29A::LUC* reporter gene, several mRNA processing factors or RNA-binding proteins were isolated. The *SAD1* (Supersensitive to ABA and Salt 1) encodes a Sm-like U6 small ribonucleoprotein (snRNP) that is required for mRNA splicing and export. The *sad1* mutant plants are hypersensitive to ABA and osmotic stress in gene expression, seed germination, and vegetative growth. The mutant plants are also defective in ABA biosynthesis because drought regulation and self-regulation of ABA biosynthetic genes are impaired in the mutant [59,116]. A second component is the FRY2/CPL1 RNA Pol II CTD phosphatases [109,111,112]. FRY2/CPL1 contains two dsRNA binding domains, suggesting that structured RNA may regulate the FRY2/CPL1 activities [109]. In the same screen, a RNA helicase, LOS4, was found to be required for cold acclimation and cold-regulated gene expression [110] (see Section 3.7). All these studies indicate a potential role of RNA processing in stress and ABA signal transduction.

Using different approaches, several other groups have discovered similar components functioning in ABA signal transduction. ABH1 (CBP80) is an mRNA cap binding protein. The *abh1* mutant was isolated by its hypersensitivity to ABA during seed germination. The *abh1* guard cell ion channels are also hypersensitive to ABA [117]. Because of its enhanced sensitivity to ABA in guard cells, *abh1* plants can withstand water shortage for a longer time than the wild type plants. Mutation in another cap binding protein, CBP20, which is in complex with ABH1, confers phenotypes similar to ABH1 [118]. CBP80 and CBP20 are single copy genes and therefore, their mutations confer pleiotropic phenotypes such as small statue and serrated leaves. The *abh1* mutation also suppresses the late-flowering phenotype conditioned by mutation in *FRIGIDA* [119].

Other RNA-binding proteins that potentially affect ABA signaling include HYL1 and AKIP. The *hyl1* mutant is hypersensitive to ABA during seed germination, and also hypersensitive to cytokinin, auxin, glucose, and salt and osmotic stress[120]. HYL1 is a dsRNA binding protein and appears to affect the levels of several miRNA [121,122]. The ABA-activated protein kinase (AAPK) interacting protein AKIP is similar to heterogeneous nuclear RNA-binding protein A/B in animals [123]. AKIP probably functions in the targeting or trafficking of certain mRNA [123] and may also regulate the stability of mRNA species that encode ABA signaling components.

Another group of molecules that can potentially regulate stress signaling and also plant developmental adaptation to stress is small RNA. Small endogenous RNA such as micro RNA (miRNA) and short interference RNA (siRNA) may regulate the transcript stability of some stress signaling components. The biogenesis of some of these small RNA may also be regulated by stress or ABA [124].

4.10. Regulation of stress signaling components by protein modifiers

The above-mentioned components are directly involved in stress signaling. In many cases, however, their roles in stress signaling may be regulated by other components that are not directly involved in the signal relay. Protein modifiers that are responsible for protein lipidation, glycoslation, methylation, sulfation, and ubiquitination regulate protein targeting, activity and longevity. Some of these processes are known to affect abiotic stress signaling.

Protein lipidation facilitates membrane localization of the modified proteins. Prenylation (including farnesylation and geranylgeranlylation) is particularly required for signal transduction that involves small GTPases. Although the detailed plant pathways that are regulated by prenylation are unclear, it is known that ABA signaling requires that some of its components be modified by prenylation. Mutations in subunits of protein farnesyl transferase or geranylgeranyl transferase made the mutant plants hypersensitive to ABA in seed germination and stomatal regulation [125,126]. Nonetheless, the regulation of the stress-responsive genes *NCED3* and *ABA1* in *era1* mutant does not appear to be hypersensitive to ABA or NaCl [127], suggesting that stress and ABA activation of these genes may not require ERA1.

Myristoylation is another form of protein lipidation. Several proteins in stress responses are known to be modified by myristalation. These include CDPK [128,129] and the Ca^{2+}-bindign protein SOS3. Myristoylation is required for SOS3 function in salt tolerance [130]. Other protein modification processes such glucoslyation, sulfation, and nitrosylation may also affect stress signal transduction, but currently there is little experimental information to confirm this hypothesis. An Arabidopsis mutant defective in an oligosaccharyltransferase that potentially affects protein glycosylation is more sensitive to salt and osmotic stress [78].

Protein ubiquitation is often used by cells to target signaling proteins for degradation, thereby regulating signal transduction. The role of proteolysis in cell signaling was established for several processes such as light signaling, hormone (auxin, ethylene, GA, and ABA) signaling and signaling for pathogenesis [131,132]. Although information regarding the role of protein ubiquitation in stress signaling is limited, its role in ABA signaling is now well documented. Ubiquitination was found to regulate ABA signaling component ABI5 during seed germination and early seedling development. ABI5 is a bZip transcription factor whose mutation confers insensitivity of seed germination to ABA inhibition. Following seed germination, ABI5 is ubiquitinated and the germinated embryos established as seedlings. ABA can stabilize ABI5 and therefore prevent seed germination and seedling establishment [133]. When the 26S proteasome regulatory particle subunit RPN10 was mutated, ABI5 was stabilized and, therefore, the *rpn10* mutant seeds are hypersensitive to ABA inhibition of seed germination [134]. Ubiquitination process involves the ubiquitin activating (E1), conjugating (E2) and ligating enzymes (E3). The SCF (Skp1/Cullin/F-box/Rbx1/2) complexes represent a major type of E3 ubiquitin ligases. F-box proteins in the SCF complexes may define substrate specificity. In Arabidopsis, there are about 700 putative F-box proteins [132]. One F-box protein has been suggested to be involved in ABA signal transduction since the knockout mutant became insensitive to ABA during seed germination [135].

Protein ubiquitination also removes denatured or unfold proteins. These unnatural proteins may become abundant under abiotic stress conditions. Chaperone proteins could restore some of these proteins to their native state [136]. Abiotic stress induces the expression of several heat shock proteins (Hsp). However, the role of these Hsp proteins and other chaperones in stress signaling is not very clear. In animal cells, an important role for some of these chaperones is the assembly of hormone receptor complexes. In plants, all the chaperone proteins such as Hsp90, Hsp70, immunophilins, cyclophilins, TPR (tetratricopepitide repeat) domain adaptor proteins exist, yet no obvious nuclear hormone receptors are found in completely sequenced Arabidopsis and rice genomes. The role of these proteins in stress signaling will await discovery in future studies.

4.11. Role of scaffolds and adaptors and vesicle trafficking in stress signaling

Signal transduction often requires the assembly of protein complexes. This involves recruiting, organizing, and anchoring of individual components. Specific proteins are evolved to play this scaffold and adaptor roles. These proteins usually contain

conserved protein-protein interaction domains. Although the role of scaffold protein in signaling is expected, there has been little information regarding these proteins in plant stress signaling. Other proteins with a physical role in supporting signaling molecules or their trafficking include cytoskeleton and its associated proteins. Cytoskeleton reorganization was suggested to play roles in early steps of cold signal transduction [45,137]

Vesicular trafficking is required for many signal transduction processes. In yeast cells, the barley stress and ABA-induced protein AtHVA22 interacts with vesicle trafficking component, which has high homology with the Arabidopsis RHD3 protein, a protein involved in root hair development [138] and trafficking between ER and Golgi body [139]. Previously, it was shown that drought and ABA affect root hair development in Arabidopsis [140,141]. It is not clear whether this process requires RHD3 or whether RHD3 is defective in drought responses.

Components in other aspects of vesicle trafficking, such as syntaxin-like proteins, are also implicated or demonstrated in ABA and osmotic stress response [142,143]. Rab GTPases that regulate vesicle trafficking [144] may also indirectly affect plant abiotic stress signal transduction and stress tolerance. Indeed, Mazel et al. [145] reported that overexpression of AtRabG3E resulted in increase tolerance to salt stress.

5. SIGNAL TRANSDUCTION PATHWAYS AND NETWORK INTEGRATION

In the previous sections, we presented an overview on individual components involved in stress signal transduction. Needless to say, there are many more components to be discovered, and some of them (such as stress signal receptors) are essential to understanding signal transduction. However, with more and more components being described, an important next task will be to sort out the pathways that integrate these different components. Since individual pathways interact with one another at various levels, the signal pathways actually constitute a signaling network. While integrating these different pathways, one thing often discussed is the interaction (cross-talk) or specificity of various pathways.

Evidently, many genes can be activated by multiple stresses and by the plant hormone ABA (see previous sections). This could result from pathway interactions at any signaling steps (Figure 1). For instance, different stresses many share similar intrinsic attributes (Section 3.11). An obvious example is that drought and salt stress both lead to osmotic stress. In this case, the signaling pathway activated by osmotic stress would likely be shared by both drought stress and salt stress. On the other hand, the ionic stress generated by Na^+ would be specific to salt stress. Additional layers of interactions could occur at the generation of second signal molecules (see Section 3.2). Particularly, various stresses may affect the biosynthesis of and response to various plant hormones such as ABA and ethylene. Abiotic stresses as well as biotic stress also generate reactive species. The signal transduction pathways initiated by a common secondary signaling molecule would be similar in terms of the usage of signaling components and the outputs of the signaling pathways, even if their primary signals are quite different.

Indeed, one reason why so many stress-inducible genes are activated by drought and salt stress (as well as by low temperature) is that all these stresses activate the biosynthesis of ABA [117]. The expression of certain stress-responsive genes appears to require ABA for their activation by stress, since osmotic stress no longer activates the expression of these genes in ABA deficient mutants [146]. Consistent with the observation that short-term cold stress has relatively little effect on ABA biosynthesis, the activation of stress responsive genes by cold was not obviously affected in the ABA biosynthetic mutants [127,146]. As a result, quite a few cold-specific components were identified genetically (e.g., ICE1, HOS1), whereas drought, salt, or ABA specific signaling components are rarely isolated in genetic studies. Transcript profiling also indicates that more than half of the drought-responsive genes are induced by salt stress, whereas only about 10 percent of drought-inducible genes are induced by cold stress [3].

Additional interactions between different stress signal pathways can occur at the level of protein reversible phosphorylation. Many protein kinases and phosphatases can be induced or activated by various abiotic stresses or ABA treatment. Their substrates may also play diverse roles in stress signaling. An example is the MAPK MPK6 (see Section 3.5.1). On the other hand, downstream signaling components such as transcription factors appear to have relatively more signaling specificity. For instance, there are cold specific transcription factors (CBF1 to CBF3), drought/ osmotic stress-specific transcription factors (DREB2), and ABA-specific transcription factors (ABF) [147,148]. Nonetheless, many stress-responsive genes have multiple *cis*-regulatory elements in their upstream regulatory regions. These *cis*-elements may collectively increase the signaling output and also underline the basis for crosstalk among pathways. For instance, The CRT/DRE element was suggested to function also as an ABRE coupling element in the *RD29A* promoter [149]. Therefore, abiotic stress signal transduction pathways are more like an intertwined network. Signal specificity occurs at the local and at the module levels rather than at the global level. Thus, for abiotic stresses, signaling pathway interaction is the rule rather than the exception.

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REFERENCES

1. Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **51,** 463-499.
2. Ramanjulu, S. and Bartels, D. (2002) Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environ*. **25,** 141-151.
3. Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol*. **6,** 410-417.
4. Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **53,** 247-273.

5. Bohnert, H.J. and Cushman, J.C. (2002) Plants and environmental stress adaptation strategies. In: *Plant Biotechnology and Transgenic Plants* (Barz, M. and Oksman-Kaldentey, D., eds). New York: Marcel Dekker, pp. 635-664.
6. Bray, E.A. (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. *J. Exp. Bot*. **55,** 2331-2341.
7. Rontein, D., Basset, G. and Hanson, A.D. (2002) Metabolic engineering of osmoprotectant accumulation in plants. *Metab. Eng*. **4,** 49-56.
8. Sung, D.Y., Kaplan, F., Lee, K.J. and Guy, C.L. (2003) Acquired tolerance to temperature extremes. *Trends Plant Sci*. **8,** 179.-187
9. Xiong, L., Shumaker, K.S. and Zhu, J.K. (2002) Cell signaling during cold, drought and salt stresses. *Plant Cell* **14,** S165-S185.
10. Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr. Opin. Biotechnol*. **14,** 194-199.
11. Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D. and Bohnert, H.J. (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13,** 889-905.
12. Oztur, Z.N., Talame, V., Deyholos, M., Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N< Tuberosa, R. and Bohnert, H.J. (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol. Biol*. **48,** 551-573.
13. Yu, L.X. and Setter, T.L. (2003) Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. *Plant Physiol*. **131,** 568-582.
14. Ueda, A., Kathiresan, A., Inada, M., Narita, Y., Nakamura, T., Shi, W., Takabe, T. and Bennett, J. (2004) Osmotic stress in barley regulates expression of a different set of genes than salt stress does. *J. Exp. Bot*. **55,** 2213-2218.
15. Vodkin, L.O., Khanna, A., Shealy, R., Clough, S.J., Gonzalez, D.O., Philip, R., Zabala, G., Thibaud-Nissen, F., Sidarous, M., Stromvik, M.V., Shoop, E., Schmidt, C., Retzel, E., Erpelding, J., Shoemaker, R.C., Rodriguez-Huete, A.M., Polacco, J.C., Coryell, V.,. Keim, P., Gong, G., Liu, L., Pardinas, J. and Schweitzer, P. (2004) Microarrays for global expression constructed with a low redundancy set of 27,500 sequenced cDNAs representing an array of developmental stages and physiological conditions of the soybean plant. *BMC Genomics* **5,** 73.
16. Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X. and Harper, J.F. (2002) Transcriptome Changes for Arabidopsis in Response to Salt, Osmotic, and Cold Stress. *Plant Physiol*. **130,** 2129-2141.
17. Fowler, S. and Thomashow, M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14,** 1675-90.
18. Bray, E.A. (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the Arabidopsis genome. *Plant Cell Environ*. **25,** 153-161.
19. Thomashow, M.F. (2001) So what's new in the field of plant cold acclimation? Lots! *Plant Physiol*. **125,** 89-93.
20. Xiong, L. and Zhu, J.K. (2002) Molecular and genetic perspective on osmotic stress signal transduction. *Plant Cell Environ*. **25,** 131-139.
21. Finnegan, E.J., Sheldon, C.C., Jardinaud, F., Peacock, W.J. and Dennis, E.S. (2004) A cluster of Arabidopsis genes with a coordinate response to an environmental stimulus. *Curr. Biol*. **14,** 911-916.
22. Finkelstein, R.R., Gampala, S.S. and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell* **14,** S15-S45.
23. Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K. and Murata, N. (2000) The pathway for perception and transcription of low-temperature signals in *Synechocystis*. *EMBO J*. **19,** 1327-1334.
24. Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S. and Patapoutian, A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell* **108,** 705-715.

25. Voets, T., Droogmans, G., Wissenbach, U., Janssens, A., Flockerzi, V. and Nilius, B.(2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **430,** 748-754.
26. Knight, H. (2000) Calcium signaling during abiotic stress in plants. *Int Rev Cytol*. **195,** 269-324.
27. Monroy A.F. and Dhindsa, R.S. (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25 degrees C. *Plant Cell* **7,** 321-331.
28. Wang, X.Q., Ullah, H., Jones, A.M. and Assmann, S.M. (2001) G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. *Science* **292,** 2070-2072.
29. Pandey S. and S.M. Assmann, (2004) The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein alpha subunit GPA1 and regulates abscisic acid signaling. *Plant Cell* **16,** 1616-1632.
30. Yang, Z. (2002) Small GTPases: versatile signaling switches in plants. *Plant Cell* **14,** S375-S388.
31. Zheng, Z.L., Nafisi, M., Tam, A., Li, H., Crowell, D.N., Chary, S.N., Schroeder, J.I., Shen, J. and Yang, Z. (2002) Plasma membrane-associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis. *Plant Cell*. **14**, 2787-2797.
32. Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11,** 393-406.
33. Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. **7,** 405-410.
34. Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G.J., Grill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* **406,** 731-734.
35. Murata, Y., Pei, Z.M., Mori, I.C. and Schroeder, J.I. (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in the abi1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell* **13,** 2513-2523.
36. Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W. and Song, C.P. (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol*. **126,** 1438-1448.
37. Kwak J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S. and Jones, J.D. (2003) Schroeder, NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J*. **22,** 2623-2633.
38. Bethke, P.C., Badger, M.R. and Jones, R.L. (2004) Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* **16,** 332-341.
39. Guo, F.Q., Okamoto, M. and Crawford, N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100-103.
40. Vandenabeele, S., Van Der Kelen, K., Dat, J., Gadjev, I., Boonefaes, T., Morsa, S., Rottiers, P., Slooten, L., Van Montagu, M., Zabeau, M., Inze, D. and Van Breusegem, F. (2003) A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. *Proc. Natl. Acad. Sci. USA* **100,** 16113-16118.
41. Pastori, G.M. and Foyer, C.H. (2002) Common components, networks, and pathways of cross-tolerance to stress: the central role of "redox" and abscisic acid-mediated controls. *Plant Physiol.* **129,** 460-468.
42. Mori, I.C. and Schroeder, J.I. (2004) Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiol*. **135,** 702-708.
43. Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature* **427,** 858-861.
44. Mou, Z., Fan, W. and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113,** 935-944.
45. Browse, J. and Xin, Z. (2001) Temperature sensing and cold acclimation. *Curr. Opin. Plant Biol*. **4,** 241-246.
46. Wang, X. (2004) Lipid signaling. *Curr. Opin. Plant Biol*. **7,** 329-336.

47. Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell* **14,** S401-S417.
48. Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. and Waner, D. (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **52,** 627-658.
49. Hunt, L., Mills, L.N., Pical, C., Leckie, C.P., Aitken, F.L., Kopka, J., Mueller-Roeber, B., McAinsh, M.R., Hetherington, A.M. and Gray, J.E. (2003) Phospholipase C is required for the control of stomatal aperture by ABA. *Plant J.* **34,** 47-55.
50. Takahashi, S., Katagiri, T., Hirayama, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. *Plant Cell Physiol*. **42,** 214-222.
51. Sanchez, J.P. and Chua, N.H. (2001) Arabidopsis PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell* **13,** 1143-1154.
52. Burnette, R.N., Gunesekera, B.M. and Gillaspy, G.E. (2003) An Arabidopsis inositol 5-phosphatase gain-of-function alters abscisic acid signaling. *Plant Physiol*. **132,** 1011-1019.
53. Xiong, L., Lee, B., Ishitani, M., Lee, H., Zhang, C. and Zhu, J.K. (2001) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes Dev*. **15,** 1971-1984.
54. Xiong, L., Lee, H., Huang, R. and Zhu, J.K. (2004) A single amino acid substitution in Arabidopsis FIERY1/HOS2 protein confers cold signaling specificity and lithium tolerance. *Plant J*. **40,** 536-545.
55. Jacob, T., Ritchie, S., Assmann, S.M. and Gilroy, S. (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proc. Natl. Acad. Sci. USA* **96,** 12192-12197.
56. Zhang, W., Qin, C., Zhao, J. and Wang, X. (2004) Phospholipase D alpha 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. *Proc. Natl. Acad. Sci. USA* **101,** 9508-9513.
57. Zhao, J. and Wang, X. (2004) Arabidopsis phospholipase Dalpha1 interacts with the heterotrimeric G-protein alpha-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. *J. Biol. Chem*. **279,** 1794-1800.
58. Li, W., Li, M., Zhang, W., Welti, R. and Wang, X. (2004) The plasma membrane-bound phospholipase D delta enhances freezing tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol*. **22,** 427-433.
59. Xiong, L. and Zhu, J.K. (2003) Regulation of abscisic acid biosynthesis. *Plant Physiol.* **133,** 29-36.
60. Sauter, A., Davies, W.J. and Hartung, W. (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *J. Exp. Bot.* **52,** 1991-1997.
61. Sharp, R.E. (2002) Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant Cell Environ*. **25,** 211-222.
62. Furuichi, T., Cunningham, K.W. and Muto, S. (2001) A putative two pore channel AtTPC1 mediates Ca^{2+} flux in Arabidopsis leaf cells. *Plant Cell Physiol*. **42,** 900-905.
63. Han, S., Tang, R., Anderson, L.K., Woerner, T.E. and Pei, Z.M. (2003) A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells. *Nature* **425,** 196-200.
64. Hirschi, K.D. (1999) Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. *Plant Cell* **11,** 2113-2122.
65. Catala, R., Santos, E., Alonso, J.M., Ecker, J.R., Martinez-Zapater, J.M. and Salinas. J. (2003) Mutations in the Ca^{2+}/H^{+} transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in Arabidopsis. *Plant Cell* **15,** 2940-2951.
66. Harper, J.F.,. Breton, G. and Harmon, A. (2004) Decoding Ca^{2+} signals through plant protein kinases. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **55,** 263-288.
67. Yang, T. and Poovaiah, B.W. (2003) Calcium/calmodulin-mediated signal network in plants. *Trends Plant Sci*. **8,** 505-512.
68. Zhu, J.K. (2003) Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol*. **6,** 441-445.
69. Gong, D., Guo, Y., Schumaker, K.S. and Zhu, J.K. (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis. *Plant Physiol*. **134,** 919-926.

70. Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. and Gruissem, W. (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell* **14,** S389-S400.
71. Guo, Y., Xiong, L., Song, C.P., Halfter, U. and Zhu, J.K. (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis. *Dev. Cell* **3,** 233-244.
72. Ohta, M., Guo, Y., Halfter, U. and Zhu, J.K. (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. *Proc. Natl. Acad. Sci. USA* **100,** 11771-11776.
73. Townley, H.E. and Knight, M.R. (2002) Calmodulin as a potential negative regulator of Arabidopsis COR gene expression. *Plant Physiol*. **128,** 1169-1172.
74. Perruc, E., Charpenteau, M., Ramirez, B.C., Jauneau, A., Galaud, J.P., Ranjeva, R. and Ranty, B. (2004) A novel calmodulin-binding protein functions as a negative regulator of osmotic stress tolerance in *Arabidopsis thaliana* seedlings. *Plant J.* **38,** 410-420.
75. Sheen, J. (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science* **274,** 1900-1902.
76. Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.* **23,** 319-327.
77. Lee, S., Lee, E.J., Yang, E.J., Lee, J.E., Park, A.R., Song, W.H. and Park, O.K. (2004) Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. *Plant Cell* **16,** 1378-1391.
78. Koiwa, H., Li, F., McCully, M.G., Mendoza, I., Koizumi, N., Manabe, Y., Nakagawa, Y., Zhu, J., Rus, A., Pardo, J.M., Bressan, R.A. and Hasegawa, P.M. (2003) The STT3a subunit isoform of the Arabidopsis oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. *Plant Cell* **15,** 2273-2284.
79. Hrabak, E.M., Chan, C.W., Gribskov, M., Harper, J.F., Choi, J.H., Halford, N., Kudla, J., Luan, S., Nimmo, H.G., Sussman, M.R., Thomas, M., Walker-Simmons, K., Zhu, J.K. and Harmon, A.C. (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiol*. **132,** 666-680.
80. Kerk, D., Bulgrien, J., Smith, D.W., Barsam, B., Veretnik, S. and Gribskov, M. (2002) The complement of protein phosphatase catalytic subunits encoded in the genome of Arabidopsis. *Plant Physiol*. **129,** 908-925.
81. Jonak, C., Okresz, L., Bogre, L. and Hirt, H. (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Curr. Opin. Plant Biol*. **5,** 415-424.
82. Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* **97,** 2940-2945.
83. Shou, H., Bordallo, P., Fan, J.B., Yeakley, J.M., Bibikova, M.,. Sheen, J. and Wang, K. (2004) Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. *Proc. Natl. Acad. Sci. USA* **101,** 3298-3303.
84. Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L. and Hirt, H. (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. *Mol. Cell* **15,** 141-152.
85. Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415,** 977-983.
86. Kim, C.Y., Liu, Y., Thorne, E.T., Yang, H., Fukushige, H., Gassmann, W., Hildebrand, D., Sharp, R.E. and Zhang, S. (2003) Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. *Plant Cell* **15,** 2707-2718.
87. Lu, C., Han, M.H., Guevara-Garcia, A. and Fedoroff, N.V. (2002) Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. *Proc. Natl. Acad. Sci. USA* **99,** 15812-15817.
88. Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14,** 3089-3099.

89. Li, J., Wang, X.Q., Watson, M.B. and Assmann, S.M. (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* **287,** 300-303.
90. Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., Ecker, J.R. and Shinozaki, K. (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant Cell Physiol.* **43,** 1473-1483.
91. Kobayashi, Y., Yamamoto, S., Minami, H., Kagaya, Y. and Hattori, T. (2004) Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* **16,** 1163-1177.
92. Shen, Q., Chen, C.N., Brands, A., Pan, S.M. and Ho, T.H. (2001) The stress- and abscisic acid-induced barley gene *HVA22*: developmental regulation and homologues in diverse organisms. *Plant Mol. Biol*. **45,** 327-340.
93. Schweighofer, A., Hirt, H. and Meskiene, I. (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci*. **9,** 236-243.
94. Luan, S. (2003) Protein phosphatases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **54,** 63-92.
95. Sheen, J. (1998) Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci. USA* **95,** 975-980.
96. Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N. and Giraudat, J. (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* **11,** 1897-1910.
97. Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M.P., Nicolas, C., Lorenzo, O. and Rodriguez, P.L. (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. *Plant J.* **37,** 354-369.
98. Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G. and Schroeder, J.I. (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* **16,** 596-615.
99. Hoth, S., Morgante, M., Sanchez, J.P., Hanafey, M.K., Tingey, S.V. and Chua N.H. (2002) Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. *J. Cell Sci.* **115,** 4891-4900.
100. Himmelbach, A., Hoffmann, T., Leube, M., Hohener, B. and Grill, E. (2002) Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. *EMBO J.* **21,** 3029-3038.
101. Monroy, A.F., Sangwan, V. and Dhindsa, R.S. (1998) Low temperature signal transduction during cold acclimation: Protein phosphatase 2A as an early target for cold-inactivation. *Plant J.* **13,** 653-660.
102. Kwak, J.M., Moon, J.H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A. and Schroeder J.I. (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. *Plant Cell* **14,** 2849-2861.
103. Rashotte, A.M., DeLong, A. and Muday, G.K. (2001) Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. *Plant Cell* **13,** 1683-1697.
104. Larsen, P.B. and Cancel, J.D. (2003) Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. *Plant J*. **34,** 709-718.
105. Zhu, J., Shi, H., Lee, B.H., Damsz, B., Cheng, S., Stirm, V., Zhu, J.K., Hasegawa, P.M. and Bressan, R.A. (2004) An Arabidopsis homeodomain transcription factor gene, *HOS9*, mediates cold tolerance through a CBF-independent pathway. *Proc. Natl. Acad. Sci. USA* **101,** 9873-9878.
106. Novillo, F., Alonso, J.M., Ecker, J.R. and Salinas, J. (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **101,** 3985-3990.
107. Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M. and Zhu J.K. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev*. **17,** 1043-1054.

108. Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. and Zhu, J.K. (2001) The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo--cytoplasmic partitioning. *Genes Dev*. **15,** 912-924.
109. Xiong, L., Lee, H., Ishitani, M., Tanaka, Y., Stevenson, B., Koiwa, H., Bressan, R.A., Hasegawa, P.M. and Zhu, J.K. (2002) Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **99,** 10899-10904.
110. Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B. and Zhu, J.K (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA* **99,** 11507-11512.
111. Koiwa, H., Barb, A.W., Xiong, L., Li, F., McCully, M.G., Lee, B., Sokolchik, I., Zhu, J., Gong, Z., Reddy, M., Sharkhuu, A., Manabe, Y., Yokoi, S., Zhu, J.K., Bressan, R.A. and Hasegawa, P.M. (2002) C-terminal Domain Phosphatase-like Gene Family Members (AtCPL) Differentially Regulate Arabidopsis thaliana Abiotic Stress Signaling, Growth and Development. *Proc. Natl. Acad. Sci. USA* **99,** 10893-10898.
112. Koiwa, H., Hausmann, S., Bang, W.Y., Ueda, A., Kondo, N., Hiraguri, A., Fukuhara, T., Bahk, J.D., Yun, D.J., Bressan, R.A., Hasegawa, P.M. and Shuman, S. (2004) Arabidopsis C-terminal domain phosphatase-like 1 and 2 are essential Ser-5-specific C-terminal domain phosphatases. *Proc. Natl. Acad. Sci. USA* **101,** 14539-14544.
113. De Nadal, E., Zapater, M., Alepuz, P.M., Sumoy, L., Mas, G. and Posas, F. (2004) The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. *Nature* **427,** 370-374.
114. Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A. and Dean, C. (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* **427,** 164-167.
115. Sung, S. and Amasino, R.M. (2004) Vernalization and epigenetics: how plants remember winter. *Curr Opin Plant Biol*. **7,** 4-10.
116. Xiong, L., Gong, Z., Rock, C., Subramanian, S., Guo, Y., Xu, W., Galbraith, D. and Zhu, J.K. (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in Arabidopsis. *Dev. Cell* **1,** 771-781.
117. Hugouvieux, V., Kwak, J.M. and Schroeder, J.I. (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. *Cell* **106,** 477-487.
118. Papp, I., Mur, L.A., Dalmadi, A., Dulai, S. and Koncz, C. (2004) A mutation in the Cap Binding Protein 20 gene confers drought tolerance to Arabidopsis. *Plant Mol. Biol*. **55,** 679-686
119. Bezerra, I.C., Michaels, S.D., Schomburg, F.M. and Amasino, R.M. (2004) Lesions in the mRNA cap-binding gene ABA HYPERSENSITIVE 1 suppress FRIGIDA-mediated delayed flowering in Arabidopsis. *Plant J.* **40,** 112-119.
120. Lu, C. and Fedoroff, N. (2000) A mutation in the Arabidopsis *HYL1* gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* **12,** 2351-2366.
121. Han, M.H., Goud, S., Song, L. and Fedoroff, N. (2004) The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proc. Natl. Acad. Sci. U S A* **101,** 1093-1098.
122. Vazquez, F., Gasciolli, V., Crete, P. and Vaucheret, H. (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr. Biol*. **14,** 346-351
123. Li, J., Kinoshita, T., Pandey, S., Ng, C.K., Gygi, S.P., Shimazaki, K. and Assmann, S.M. (2002) Modulation of an RNA-binding protein by abscisic-acid-activated protein kinase. *Nature* **418,** 793-797.
124. Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* **16,** 2001-2019.
125. Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. *Science* **273,** 1239-1241.

126. Running, MP., Lavy, M., Sternberg, H., Galichet, A., Gruissem, W., Hake, S., Ori, N. and Yalovsky, S. (2004) Enlarged meristems and delayed growth in plp mutants result from lack of CaaX prenyltransferases. *Proc. Natl. Acad. Sci. USA* **101,** 7815-7820.
127. Xiong, L., Lee, H., Ishitani, M. and Zhu, J.K. (2002) Regulation of osmotic stress-responsive gene expression by the *LOS6/ABA1* locus in Arabidopsis. *J. Biol. Chem.* **277,** 8588-8596.
128. Chehab, E.W., Patharkar, O.R., Hegeman, A.D., Taybi, T. and Cushman, J.C. (2004) Autophosphorylation and subcellular localization dynamics of a salt- and water deficit-induced calcium-dependent protein kinase from ice plant. *Plant Physiol*. **135,** 1430-1446.
129. Martin, M. and Busconi, L. (2000) Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation. *Plant J*. **24,** 429-435.
130. Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Shi, W. and Zhu, J.K. SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. *Plant Cell* **12,** 1667-1678.
131. Hare, P.D., Seo, H.S., Yang J.Y. and Chua, N.H. (2003) Modulation of sensitivity and selectivity in plant signaling by proteasomal destabilization. *Curr. Opin. Plant Biol*. **6,** 453-462.
132. Vierstra, R.D. (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends Plant Sci*. **8,** 135-142.
133. Lopez-Molina, L., Mongrand, S. and Chua, N.H. (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **98,** 4782-4787.
134. Smalle, J., Kurepa, J., Yang, P., Emborg, T.J., Babiychuk, E., Kushnir, S. and Vierstra, R.D. (2003) The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signaling. *Plant Cell* **15,** 965-980.
135. Lai, C.P., Lee, C.L., Chen, P.H., Wu, S.H., Yang, C.C. and Shaw, J.F. (2004) Molecular analyses of the Arabidopsis TUBBY-like protein gene family. *Plant Physiol*. **134,** 1586-1597.
136. Boston, R.S., Viitanen, P.V. and Vierling, E. (1996) Molecular chaperones and protein folding in plants. *Plant Mol. Biol*. **32,** 191-222.
137. Orvar, B.L., Sangwan, V., Omann, F. and Dhindsa, R.S. (2000) Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *Plant J.* **23,** 785-794.
138. Wang, H., Lockwood, S.K., Hoeltzel, M.F. and Schiefelbein, J.W. (1997) The ROOT HAIR DEFECTIVE3 gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in Arabidopsis. *Genes Dev*. **11,** 799-811.
139. Zheng, H., Kunst, L., Hawes, C. and Moore, I. (2004) A GFP-based assay reveals a role for RHD3 in transport between the endoplasmic reticulum and Golgi apparatus. *Plant J.* **37,** 398-414.
140. Schnall, J.A. and Quatrano, R.S. (1992) Abscisic acid elicits the water-stress response in roothairs of Arabidopsis. *Plant Physiol*. **100,** 216-218.
141. Vartanian, N., Marcotte, L. and Giraudat, J. (1994) Drought Rhizogenesis in *Arabidopsis thaliana* (Differential Responses of Hormonal Mutants). *Plant Physiol.* **104,** 761-767.
142. Zhu, J., Gong, Z., Zhang, C., Song, C.P., Damsz, B., Inan, G., Koiwa, H., Zhu, J.K., Hasegawa, P.M. and Bressan, R.A. (2002) OSM1/SYP61: A syntaxin protein in Arabidopsis controls abscisic acid-mediated and non-abscisic acid-mediated responses to abiotic stress. *Plant Cell* **14,** 3009-3028.
143. Leyman, B., Geelen, D., Quintero, F.J. and Blattm, M.R (1999). A tobacco syntaxin with a role in hormonal control of guard cell ion channels. *Science* **283,** 537-540.
144. Vernoud, V., Horton, A.C ,Yang, Z. and Nielsen, E. (2003) . Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant Physiol*. **131,** 1191-1208.
145. Mazel, A., Leshem, Y., Tiwari, B.S. and Levine, A. (2004) Induction of salt and osmotic stress tolerance by overexpression of an intracellular vesicle trafficking protein AtRab7 (AtRabG3e). *Plant Physiol*. **134,** 118-128.
146. Xiong, L., Ishitani, M., Lee, H. and Zhu, J.K. (2001) The Arabidopsis *LOS5/ABA3* locus encodes a molybdenum cofactor sulfurase and modulates cold and osmotic stress-responsive gene expression. *Plant Cell* **13,** 2063-2083
147. Choi, H., Hong, J., Ha, J., Kang, J. and Kim, S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *J Biol. Chem*. **275,** 1723-1730

148. Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. USA* **97,** 11632-11637.
149. Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis *rd29A* gene in response to dehydration and high-salinity stresses. *Plant J.* **34,** 137-148.

2. IDENTIFICATION OF SALT-RESPONSIVE GENES IN MONOCOTYLEDONOUS PLANTS:

from transcriptome to functional analysis

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Abstract. Plants regulate expression of a set of genes in response to environmental stresses. Hence, it is important to decipher the information on comprehensive expression analysis under stress condition. We have studied salt-inducible genes in barley by differential display and the function of some candidates using heterologous expression system. Total 218 and 102 cDNA clones were identified as a salt-inducible gene in barley roots and leaves under salt stress, respectively. Among these, transgenic Arabidopsis overexpressing peroxisomal ascorbate peroxidase or proline transporter showed tolerance toward heat or salt stress. Using barley salt-responsive genes, we have also fabricated barley custom cDNA microarray system to monitor the transcriptomes in barley and rice. Comparative analysis reveals the differences in gene expression pattern between the two plants during the initial phase of salt stress. Especially, divergent responses were observed in expression profiles involving in osmoregulation and ion homeostasis. Furthermore, transcript of methionine synthase is increased in barley, but not in rice. This is identical to abundance of methionine synthase protein that examined by Western blot analysis. Thus, direct comparison in transcriptome is useful to narrow the differences in the two plants, and give information on genetic improving of plant stress tolerance.

1. INTRODUCTION

Plants have developed the adaptation mechanisms to changes in their surroundings during a long evolutional process. Under environmental stress conditions (high salinity, drought, high and low temperature or light, UV, etc.), plants show the ingenious adaptations at physiological level, accompanied with the change of various gene expressions. For example, biosynthesis of glycinebetaine (betaine) or proline, a well-known osmoprotectant, is triggered by salt or drought stress, and expression level of the gene encoding betaine aldehyde dehydrogenase (BADH) or pyrroline-5-carboxylate synthetase (P5CS), a component of betaine or proline synthetic pathway, is also increased [1-3]. Stress-mediated modification in gene expression pattern is achieved by components of a signal transduction pathway, such as the sensor proteins [4] or transcriptional regulator [5]. It was estimated that

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Arabidopsis has 1,533 transcriptional regulators in genome and it accounts for 5.4% of the whole genes [6]. The ratio of transcriptional regulator in Arabidopsis genome is larger than that of *C. elegans* (3.5%), *D. melanogastor* (4.5%) or *S. cerevisiae* (3.5%). Some classes of transcriptional regulator, AP2/EREBP, WRKY, ARF-Aux/IAA and Dof, are specific to plants [7], and plants seem to have the unique transcriptional system. Including these transcriptional regulators, it is important to study global changes of gene expression in response to environmental stimulus for improvement of plant stress tolerance.

Recently, by the advancement of molecular biology, various approaches are developed to identify the candidates that are differentially regulated at transcript level. cDNA subtraction and differential screening are often used to obtain stress-inducible genes. With the refinement in sensitivity of detection, differential display is also the effective tool to get differentially expressed genes [8-10]. Since differential display does not need expensive equipments, it is widely applied to various purposes from microorganisms to plants and animals. On the other hand, cDNA microarray enables to handle more than several thousand cDNA clones at once for genome-wide expression analysis [11]. Serial analysis of gene expression (SAGE) is also developed for the large scale and quantitative expression analysis that is sequencing the arrayed short tags derived from 3'-UTR of cDNA [12]. Although these have both merits and demerits, many reports utilized above mentioned approaches are published for identification of stress-responsive genes.

Crop productivity is irreversibly inhibited by environmental stresses, and especially, salt stress is one of the serious problems in world agriculture. Salt stress causes both water relation and ion homeostasis. For improving salt tolerance in plants, many approaches of genetic engineering were made attempts until now. It was reported that the intensification in ability of synthesis of osmoprotectant or exclusion sodium ion from cytosol are effectively to improve salt tolerance in Arabidopsis or rice [13-17]. One of the effective strategies is to invest the new traits derived from tolerant plants into salt-sensitive plants. In such sense, a model crop plant, rice, is a good target to invest salt tolerance with genetic information of closely related and salt-tolerant plants, such as barley, wheat, or another monocotyledonous plants. To obtain useful candidates for improving salt tolerance in plants, we have examined salt-inducible genes in barley leaves and roots by differential display and the role of some genes was identified. Then, we have dissected expression profiles of barley and rice under salt stress by cDNA microarray that was fabricated using barley salt-responsive genes and compared their transcriptomes with physiological responses.

2. SCREENING SALT-INDUCIBLE GENES IN BARLEY BY DIFFERENTIAL DISPLAY

Differential display is one of the powerful approaches to identify the differentially regulated genes between two samples. Firstly, Pardee and Liang developed differential display RT-PCR technique combined with oligo-dT primer and short arbitrary primers [8]. Some variations of differential display method were reported,

such as the simple version with RAPD primer [18], utilization of fluorescence-labeled primer [19] and restriction fragment-coupled version [20]. Basically, differential display is one of the mRNA fingerprintings and consists of two steps, reverse transcription and PCR with arbitrary primer. Specifically expressed genes are partially amplified by PCR, and the resulting DNA fragments are easily subcloned and sequenced. One of the points is which primers should be used, oligo-dT or random primer for reverse transcription, and what kind of and how many arbitrary primers for PCR (10-12 mer). Logically, emergent frequency of 10 mer sequence of arbitrary primer is less than 1,000 kbp in target cDNA. Size of cDNA amplified by PCR is usually ranged from 100 bp to 2,000 bp, suggesting probability of annealing to cDNA with arbitrary primer is quite low. However, mismatch annealing would actually occur due to the lower annealing temperature (approx. 40^{o}C). This complexity is still argumentative [21]. In comparison with other approach to identify the differentially expressed genes, differential display has the virtue of high sensitivity due to application of RT-PCR. On the other hand, sometimes, false clones might emerge because of inaccurate PCR-amplification. Therefore, expression level of positive candidates obtained by differential display should be confirmed by alternative approaches, such as RT-PCR or Northern blot analysis.

2.1. Salt-inducible genes in roots

Plant root is the tissue that primarily perceives salt signaling from soil. During imposition of salt stress, root cells are exposed to high salinity environment, hence dynamic changes in gene expression might occur in roots under salt stress. Differential display was performed with 480 species of RAPD primers (random 12 mer) and first strand cDNAs transcribed from poly $(A)^{+}$ RNA using random hexamer. Detailed information was described in previous report [10]. Total 218 salt-inducible genes were obtained by differential display using 6 days stressed barley roots (100 mM NaCl for 3 days, and then 200 mM NaCl for 3 days) (Table 1). Of these, 133 cDNA clones have similarity to known protein such as the components of signal transduction (26 genes), membrane protein (17 genes), cytochrome P450 (16 genes), sugar, amino acid, C/N relations (16 genes), stress tolerance (13 genes), RNA functions (11 genes), proteases (4 genes) and others (30 genes). These includes some of typical stress-responsive genes, phosphatidylinositol-4-phosphate-5-kinase (PIP5K), mitogen activated protein kinase (MAPK), heat shock protein (HSP), polyubiquitin, etc. It was reported that some of these genes is useful to improve salt tolerance by transgenic approach, such as inorganic pyrophosphatase [22], proline transporter (HvProT) [23], glutathione reductase [24], trehalose-6-phosphate synthase [25] and translation initiation factor [26]. Many novel genes are identified as a salt-inducible candidate in plants, such as AMSH (Associated Molecule of SH3 domain of STAM), putative SET1 (Su(var)3-9, Enhancer-of-zeste, Trithorax) -domain protein, splicing factor, nonsense-mediated mRNA decay trans-acting factor, apoptosis protein Ma-3, and so on. By Northern blot analysis, expression of approximately 70% of salt-inducible genes obtained from stressed roots is

up-regulated in roots but not in leaves. This indicated different regulation system of gene expression in roots and leaves in response to salt stress. Interestingly, it was shown that transcripts of HvProT and aldehyde oxidase, catalyzes the last step for ABA biosynthesis, are localized in root cap cell [3, 27]. Function of root cap cell is not clear under salt stress although it is expected to play roles as the place of plant hormone biosynthesis and the sensors for gravitropism and hydrotropism, and secretion of various compounds into soil. Further analysis needs to be done to show the function of root cap cell under salt stress.

Table 1. Up-regulated genes in barley roots under long-term salt stress.

Category	Number	Description
Signal transduction	26	Serine/ threonine protein kinase (6), Receptor protein kinase (6), SET-domain transcriptional regulator (2), SCARECROW (2), Protein phosphatase (2), Transcription factor (2), MAPK, AMSH, Casein kinase, etc.
Membrane protein	17	ABC transporter (7), Inorganic pyrophosphatase (3), Amino acid permease (2), Proline transporter, Sugar transporter, etc.
Cytochrome P450	16	CYP99A1 (5), P450 monooxygenase (5), CYP72A1 (2), CYP71C4, Trans-cinnamic acid hydroxylase, etc.
Metabolism	16	Sucrose synthase (2), Phosphogluconate dehydrogenase, Fd-GOGAT, PEPCase, GAPDH, etc.
Stress tolerance	13	HSP90, HSP70, Trehalose-6-phosphate synthase, Glutathione reductase, Phosphoethanolamine-N methyltransferase, etc.
RNA functions	11	Nonsense-mediated mRNA decay trans-acting factor (2), Splicing factor, Translation initiation factor, Elongation factor, etc.
Protease	4	Subtilisin protease (2), Clp protease, Aspartic proteinase
Others	30	GcpE protein (4), Oxalate oxidase (3), Proline rich protein (2), Kaurene synthase (2), Rf2 nuclear restore protein (2), Apoptosis protein Ma-3, Dynamin, Tubby like protein, Brassinosteroid insensitive 1 gene, Mannose/glucose-binding lectin, etc.
Unknown	85	

2.2. Salt-inducible genes in leaves

Leaf salt-inducible genes are screened under short- or long-term salt stress. At 30 min of 200 mM NaCl stress (short-term), 60 cDNA clones were obtained, including the genes related to signal transduction (4 genes), membrane protein (4 genes), RNA functions (4 genes), defense (4 genes), cell wall biosynthesis (3 genes), photosynthesis (2 genes), protease (1 gene), others (16 genes) and function unknown protein (22 genes) (Table 2). On the other hand, smaller number of salt-inducible genes (42 candidates) was identified by long-term salt stress treatment (100 mM NaCl for 3 days, and then 200 mM NaCl for 3 days) [28, 29]. Calcium dependent protein kinase (CDPK) was up-regulated under both short- and long-term stress conditions, suggesting that CDPK would play important roles for stress signaling during high salinity condition. Actually, it was revealed that overexpression of

Table 2. Up-regulated genes in barley leaves under short- or long-term salt stress.

Category	Number	Description
Short-term salt stress		
Signal transduction	4	Calcium dependent protein kinase, Serine/threonine protein kinase, Response regulator, Elicitor inducible protein kinase
Membrane protein	4	Sugar transporter, Vacuolar H+ translocating inorganic pyrophosphatase (2), PDR5-like ABC transporter
RNA function	4	Nucleic acid binding protein, RNA polymerase C, Ribonuclease III, RNA dependent RNA polymerase
Defence	4	Lipoxygenase, Chitinase, Catalase, LRR resistance protein
Cell wall	3	Nucellain, Cellulose synthase, Extensin
Photosynthesis	2	PSI P700 apoprotein, Phosphoribulokinase
Protease	1	Cystein protease
Others	16	Envelope protein, α-tubulin 2, Phosphoribosylamine-glycine ligase, Thiamine biosynthetic enzyme, Pol polyprotein, S222, Oxido-reductase, Steroid dehydrogenase, Herbicide safener binding protein, Non-functional folate binding protein, NADH dehydrogenase F subunit, Neuroblastome-amplified protein, Antisense basic fibroblast growth factor, RecF, Sperm tail-specific protein, Pherophorin-S
Unknown	22	
Long-term salt stress		
RNA function	6	Nonsense-mediated mRNA decay protein (2), Translation elongation factor eEF-1α(2), eEF2, RNA helicase
Signal transduction	4	Serine/threonine protein kinase, Sok1, Calcium dependent protein kinase, Mesotocin receptor
Membrane protein	4	Chloroplast membrane-associated protein, Cell wall-plasma membrane linker protein, H^+-ATPase, K^+ transporter
Amino acid synthesis	4	Pyrroline-5-carboxylate synthetase, Tryptophan synthase, Methionine synthase, Asparagine synthetase
Protease	2	N-acetylated α-linked acidic dipeptidase, Ubiquitin-specific protease
Defence	1	Ascorbate peroxidase
Others	9	Ribosome-sedimenting protein, Acetolactate synthase, Galectin-3, Histidine rich glycoprotein, Gag-pol protein, GP900, Plastid fusion/translocation factor, Nodulin-like protein, Nuclear antigen
Unknown	12	

CDPK can enhance tolerances toward salt, drought and cold stresses [30]. Up-regulation of peroxisomal ascorbate peroxidase indicated increasing in activity for scavenging of reactive oxygen species (ROS) generated under salt stress. Some components of amino acid biosynthesis, such as proline, tryptophan, methionine and asparagine, are up-regulated under long-term salt stress. Expression of RNA metabolism/maturation genes encoding RNA helicase, ribonuclease III, RNA-dependent RNA polymerase, nonsense-mediated mRNA decay and translation elongation factors are induced in leaves, in addition to translation initiation factor,

RNase L inhibitor and splicing factor in roots. This indicated that activity of mRNA metabolism/ maturation might be increased to adapt to salt stress.

3. FUNCTIONAL ANALYSIS OF SALT-INDUCIBLE CANDIDATES

3.1. Peroxisomal ascorbate peroxidase (pAPX)

Production of ROS is also triggered by secondary effect of salt stress and excessive ROS affects plant productivity. To eliminate ROS, plants have the ROS-scavenging enzymes, such as ascorbate peroxidase (APX), superoxide dismutase, catalase, glutathione reductase, etc. Especially, APX is the ubiquitous ROS-scavenging enzyme, localized in cytosol, stroma and thylakoid membrane in chloroplast, mitochondria and peroxisome. APX catalyzes the reaction, ascorbate and H_2O_2 to monodehydroascorbate and H_2O. Many reports described the importance of APX as a ROS scavenger under oxidative stress. Recently, it was reported that the Arabidopsis mutant lacking cytosolic APX isoform showed growth suppression during normal development, and APX has multiple functions in plants [31]. However, the present fragmentary information can not still established the integrative insight which isoform could play a critical role under each situation.

We have obtained the cDNA encoding peroxisomal APX (*HvAPX1)* in salt-stressed barley by differential display [28]. Plant peroxisomal (glyoxysomal) APX is characterized from Arabidopsis, cotton and spinach, but information of its function under environmental stress is still limited. Expression of *HvAPX1* mRNA is induced by not only salt, but also heat stress [32]. Overexpression of *HvAPX1* in Arabidopsis leads to enhanced tolerance toward heat stress rather than salt stress, suggesting that HvAPX1 would play important roles to scavenge H_2O_2 generated by heat stress on peroxisomal membrane. Why can overexpressing *HvAPX1* enhance heat tolerance in Arabidopsis? Under heat stress, heat responsive gene expression is rapidly modified through the function of heat shock element (HSE). Expression level of Arabidopsis pAPX is not quickly increased under heat stress, due to lacking functional HSE in promoter regions [33]. Hence, HvAPX1 would be effectively acting in Arabidopsis under heat stress, although it is little known about the mechanism of H_2O_2 production in peroxisome under heat stress. Since acquisition of heat tolerance by *HvAPX1* is observed during both vegetative and reproductive stages in Arabidopsis and rice (unpublished data), the common scavenging system might be useful in broad plant species.

3.2. Proline transporter

Proline is one of the well-known osmoprotectants, and it is accumulated in many plant species under various stress conditions [34]. Transgenic approaches revealed that proline accumulation leads to enhanced stress tolerance [16]. However, its overaccumulation causes abnormal development in yeast and plant [35]. Therefore, it is considered that proline concentration should be properly regulated according to

environmental condition. Proline is taken into the cells through transporter proteins, such as proline transporter (high affinity) or amino acid transporter (low affinity). We have characterized salt-inducible proline transporter (*HvProT*) from barley roots [3]. By the assay of yeast mutant lacking proline permease (*put4*), substrate specificity of HvProT was determined and HvProT is a proline specific transporter (Km = 25.1 μM) unlike betaine/proline transporter in Arabidopsis or tomato. *HvProT* mRNA is strongly induced in roots by salt stress, and its induction is earlier than that of P5CS, the limiting step of proline synthesis. Under salt stress, *HvProT* transcript is abundant in root tip region, especially root cap and cortex cells. These results suggested that proline might be transported into root tip cells in barley under salt stress. In tomato pollen, it seems that proline accumulation is accomplished by tomato ProT, but not de novo synthesis of proline by P5CS [36]. Therefore, transportation and tissue-specific accumulation might contribute to stress tolerance.

Unexpectedly, growth suppression was observed by overexpression of *HvProT* gene in Arabidopsis (35S-ProT plant), particularly in aerial tissues [23]. Since growth suppression in 35S-ProT plants is recovered by exogenous addition of proline in the medium, it could be due to deficiency of endogenous proline. On the other hand, 35S-ProT plants were more tolerant than wild type plants toward salt stress, and 35S-ProT plants can grow on MS medium containing 125 mM NaCl. Under non stress condition, proline content is increased in roots, and decreased in shoot of 35S-ProT plants. This indicated that endogenous proline homeostasis is disturbed by overexpressing *HvProT*.

3.3. Methionine synthase

Methionine is one of the essential amino acids in all organisms and synthesized by methionine synthase. Methionine and its derivatives have important roles as substrates in metabolic cycle of one-carbon, donor of methyl group and syntheses of betaine and polyamine, and these functions are crucial for not only stress tolerance, but also normal development. Plant methionine synthase gene is cloned from Arabidopsis, ice plant and maize, but its function is still limited under stress condition. Barley methionine synthase (*HvMS*) is identified by differential display under salt stress [37]. Yeast mutant lacking methionine synthase showed salt-sensitive phenotype, and it was restored to the same extent of wild type strain by overexpressing *HvMS*. This suggested that methionine synthase is functionally conserved in yeast and plant. Expression of *HvMS* is induced by various stress treatment, such as salt, drought, cold, ABA and H_2O_2, although it is controlled by circadian rhythm. Important finding is that amount of HvMS protein is increased by salt stress, but not in rice (as described below). In potato, diurnal changes in transcript amount of methionine synthase do not have influence on protein amount [38]. With posttranscriptional regulation of methionine synthase, genetic engineering of increasing capacity of methionine synthesis is the subject in future.

3.4. Plasma membrane protein 3

The gene encoding plasma membrane protein 3 (AcPMP3) is obtained by differential display from a stress tolerant wild plant, sheep grass (*Aneurolepidium chinense* (Trin.) kitag). Expression of AcPMP3 gene is induced by salt, drought, ABA, cold, H_2O_2 and salicylic acid [39]. Barley also has PMP3-like genes and its expression is strongly induced by salt and osmotic stress treatments [40, 41]. It was reported that PMP3 deletion confers sensitivity to cytotoxic cations, including NaCl and hygromycin B. To examine the function of AcPMP3 genes, complementation test was carried out with yeast mutant (Δ*pmp3*, Δ*nha1*, Δ*pmr2*) lacking Na^+/H^+ antiporter and Na^+-ATPase that shows more sensitive to salt stress than Δpmp3 single mutant. It was proposed that loss of PMP3 protein causes membrane hyperporalization, and activates Na^+ influx system [42]. Under the control of strong promoter, AcPMP3 gene functionally substituted for deletion of pmp3 in yeast. Furthermore, to determine the physiological roles of PMP3 in higher plants, the effect of knockout mutations in *RCI2A*, homologous to PMP3, on Na^+ uptake and salt tolerance was investigated at both the cellular and whole plant levels in Arabidopsis plants [43]. Although the growth of *RCI2A* mutants was comparable with that of wild type under normal conditions, high NaCl treatment caused increased accumulation of Na^+ and more reduction of the growth of roots and shoots of *RCI2A* mutants than that of wild type. AcPMP3 protein is a small hydrophobic peptides and it consists of 54 amino acids. Hence, it does not seem to be a transporter protein. In sheep grass, transcript for AcPMP3 gene was observed in root cap and epidermis cells. These facts suggested that PMP3 plays a role directly or indirectly for avoiding over-accumulation of excess Na^+ ion in such outer cells of roots, and contributes to plant salt tolerance.

4. COMPARATIVE EXPRESSION ANALYSES OF BARLEY AND RICE DURING THE INITIAL PHASE OF SALT STRESS BY CDNA MICROARRAY

cDNA microarray is the advanced tool for comprehensive transcriptome analysis and it enables to monitor expression profile of more than several thousand cDNAs at once. Hence, it is effective to identify genome-wide gene expression, targets for a transcription factor/regulator or crosstalk of signal transduction [44]. Many researches have reported on transcriptome analysis of model plants, such as Arabidopsis and rice, using cDNA microarray [45,46]. In comparison to the established bio-resource in model plants, that of another crops, such as barley, wheat, corn, etc., is just partly available. Therefore, reports on comprehensive expression analysis are still limited using such plants. We have prepared cDNA microarray system using barley salt-responsive genes and investigated barley transcriptome under salt stress. Furthermore, we have attempted to analyze rice transcriptome with barley cDNA microarray. Nucleotide sequences are highly conserved in barley and rice [47], and it would allow heterologous hybridization. Comparative expression analysis demonstrated the differences in early responses to salt stress at molecular level.

The concept of cDNA microarray is based on DNA dot blot analysis. In both dot blot and microarray analyses, probe cDNAs are spotted onto the supporting apparatus, nylon membrane or glass slide. Target cDNAs are transcribed from total RNA or mRNA with ^{32}P, ^{33}P or fluorescent dye (Cy3/Cy5), and then hybridized with probes. Sometimes complexity of hybridization status raises the background and false signals due to cross-hybridization of family gene and non-specific hybridization. On the other hand, heterologous microarray analysis is also useful for closely related two species [48]. In this case, optimized experimental condition should be determined to increase hybridization efficiency because it is usually lower than that of homologous hybridization. In comparison to dot blot analysis, one of the advantages of cDNA microarray is to enable monitoring large-scale gene expression (more than several thousands) at a time. Microarray experiment needs a series of expensive system, such as DNA arrayer and fluorescent image scanner, and it is still disputable to get repetitive results or normalize array data. Nevertheless, the researches using microarray system are increasing in recent years, suggesting that cDNA microarray is the powerful and effective tool for large-scale expression analysis.

4.1. Early salt-responsive genes in barley and rice roots

Ranscriptome analyses were examined at 1 h and 24 h after 200 mM for barley or 150 mM NaCl stress for rice, respectively (Fig. 1) [41]. In barley roots, 13 and 43 genes were significantly up-regulated at 1 h and 24 h, respectively. Of these, the genes encoding tryptophan synthase, PMP3, hypothetical protein, non-functional folate binding protein, cytochrome P450, methionine synthase and no homolog, are up-regulated at both 1 h and 24 h. The transcript amount of typical stress-responsive genes, such as proline rich protein (PRP), P5CS, aldehyde dehydrogenase and inorganic pyrophosphatase, are increased at 24 h. Especially, transcript amounts of PMP3, cytochrome P450 and PRP under salt stress are highly increased to 5.3, 19.4 and 17.6 times in comparison to those under control condition. On the other hand, it was observed that expression of only 5 genes was induced in rice roots. PMP3 and inorganic pyrophosphatase, the genes related to maintaining ion homeostasis, are commonly up-regulated in both barley and rice roots, but these inductions were not sustained in rice at 24 h. In yeast, it was shown that loss of PMP3 gene causes salt-sensitive phenotype [42]. This indicates that adaptive mechanisms for regulating ion homeostasis are partly conserved in the two species, but it seems that rice can not sustain cellular ion homeostasis for a long time like barley.

In roots, similar number of the genes was down-regulated in barley (14 genes) and rice (13 genes). Some genes, such as oxalate oxidase and phosphogluconate dehydrogenase, were down-regulated in both plants. Interestingly, expression levels of both plasma membrane water channel 1 and 2 were rapidly decreased in barley. Under drought stress, overexpression of water channel protein in tobacco causes detrimental effects [49], and it would be due to enhanced symplastic water permeability. Therefore, early down-regulation of water channels might contribute to protection from sudden dehydration. Another difference is that, in contrast to

barley, suppression of tryptophan synthase and methionine synthase was observed in rice roots during the initial phase of salt stress.

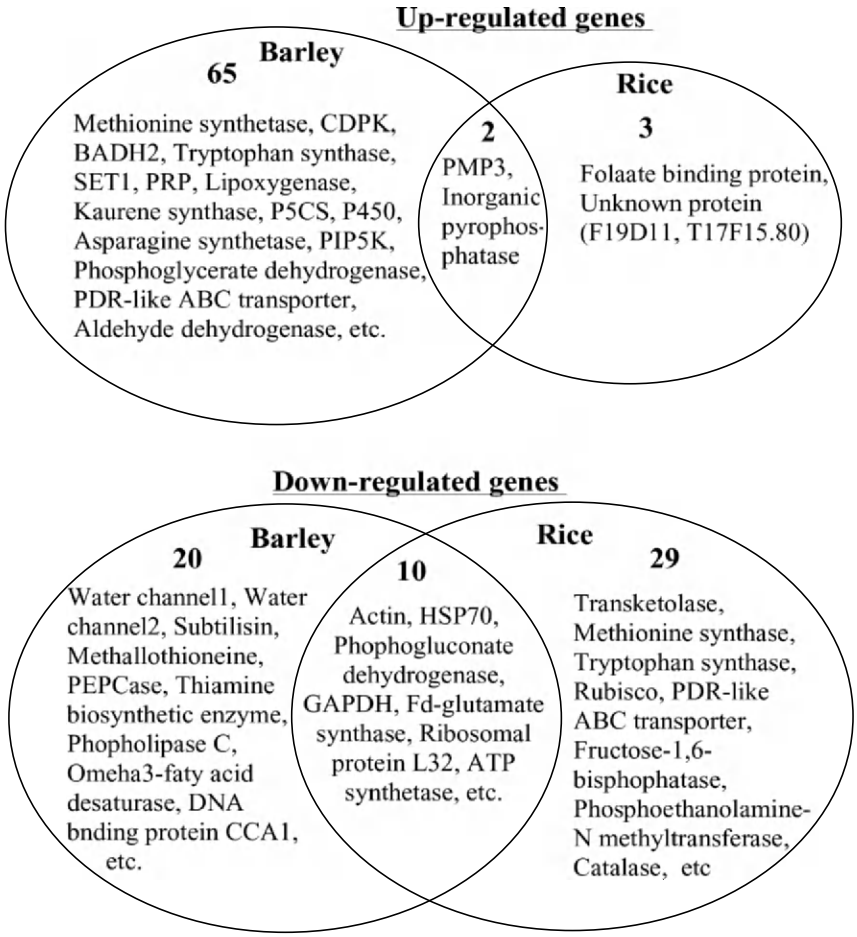

Figure 1. Comparative expression analysis in barley and rice under salt stress. Transcriptome was examined at 1 h and 24 h of 200 mM NaCl stress for barley and 150 mM NaCl stress for rice.

4.2. Early salt-responsive genes in barley and rice leaves

The number of up-regulated genes in salt-stressed leaves is also lesser in rice than in barley. Only PMP3 gene was up-regulated in rice leaves at 1 h, but its induction was not sustained at 24 h. In contrast, 15 and 11 genes were up-regulated at 1 h and 24 h

in barley under salt stress. These genes are including CDPK, PIP5K, PDR5-like ABC transporter, BADH2, asparagine synthetase, P5CS, methionine synthase, PMP3 and lipoxygenase. On the other hand, 21 and 32 genes were down-regulated in barley and rice, respectively. It was found that the transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Fd-glutamate synthase, ATP synthase, DnaK like protein and Ribosomal protein L32 were decreased in both plants. Furthermore, down-regulation of transketolase, fructose-1,6-bisphosphatase, phosphoethanol-amino-N-methyltransferase, S-adenosyl-methionine-sterol-C-methyltransferase and Rubisco, is observed in rice leaves. As seen in the root transcriptome, methionine synthase and tryptophan synthase were down-regulated in only rice leaves, but not in barley. To confirm the difference in transcriptome between barley and rice, western blot analysis of methionine synthase protein was carried out [41]. Obvious increasing of methionine synthase protein was detected in barley, but not in rice. In barley, various stresses and treatments, such as heat, drought and hydrogen peroxide, triggered expression of methionine synthase mRNA. Although contribution of methionine synthase to stress tolerance is not clear in plants, inducible feature of both mRNA and protein levels in barley is markedly under stress conditions, unlike in rice.

4.3. Divergent responses in barley and rice under salt stress

Comparative transcriptome analyses reveal that expression of a different set of the genes is regulated in barley and rice under salt stress. In our research, generally, the number of up-regulated genes is larger in barley and down-regulated genes are larger in rice. One reason is this difference in response to salt stress might be due to the evolutional variation in cis- or trans-acting element of such genes. Also, barley and rice show different physiological responses under salt stress. Two obvious differences in physiological responses were observed during the early phase of salt stress. Leaf water potential was decreased in both plants under salt stress. However, it recovered in barley after 24 h of salt stress, but not in rice. This might be partly explained by expression profiles of the genes involving in osmotic adjustment. Early down-regulation of water channel 1 and 2 genes in barley would contribute to decreasing water permeability across plasma membrane. Transgenic tobacco plants overexpressing plasma membrane-localized water channel showed more sensitive toward drought stress, and this indicated that increment in water permeability is unfavorable trait under water deficit condition. Up-regulation of osmoprotectant syntheses (betaine and proline) is induced in barley. Combined regulation of osmotic adjustment should differentiate the degree of salt tolerance in the two plants. The other finding is tissue-specific pattern of sodium accumulation under salt stress. In barley, sodium content is higher in roots than in leaves. Contrastive pattern is observed in rice. Our comparative microarray analysis does not provide clear frame to explain it although expression manner of inorganic pyrophosphatase and PMP3 genes is different in barley and rice. Some genes involved in photorespiration and glycolysis, are down-regulated in both plants, but the genes encoding amino acid biosynthetic enzyme (proline, methionine, tryptophan, asparagine, serine) are

up-regulated in barley. They seem to reconstitute metabolic balance to adapt to stress condition.

We demonstrated the different response in barley and rice using barley cDNA microarray. This array carries barley salt-responsive genes, and it is useful to examine comparative expression analysis between barley and other close species. Genome project is still running for barley and microarray analysis covering the whole cDNAs will make the clear map of expression profilings. Then, it will contribute to endue unique traits to rice.

Finally we would like to mention brieflyabout glycinebetaine synthesis in monocotyledonous plants. It was reported that glycinebetaine synthesis occurs in chloroplasts in chenopods, since choline monooxygenase(CMO) and BADH are found to be localized in the chloroplasts [50]. However, localization of glycinebetaine synthesis is totally unknown in other dicotyledonous plants and monocotyledonous plants. We have been characterizing BADHs in Gramineae plants, since BADH catalyzes the last step of glycinebetaine synthesis and it must determine localization of glycinebetaine synthesis. We purified the enzyme from salt-stressed barley leaves. We have found that the major BADH does not have any signal peptide targeting to organelle, i.e. amino acid sequence of N-terminal of the major BADH is consistent with that of the deduced amino acid sequence from *BADH2* cDNA [51]. Furthermore, CMO is still mysteriously unknown in monocotyledonous plants.

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REFERENCES

1. Ishitani, M., Nakamura, T., Han, S.Y. and Takabe, T. (1995) Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol. Biol*. **27,** 307-315.
2. Yoshiba, Y., Kiyosue, T., Katagiri, T., Ueda, H., Mizoguchi, T., Yamaguchi-Shinozaki, K., Wada, K., Harada, Y. and Shinozaki, K. (1995) Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. *Plant J*. **7,** 751-760.
3. Ueda, A., Shi, W.M., Sanmiya, K., Shono, M. and Takabe, T. (2001) Functional analysis of salt-inducible proline transporter of barley roots. *Plant Cell Physiol*. **42,** 1282-1289.
4. Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. *Plant Cell* **11,** 1743-1754.
5. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotech*. **17,** 287-291.
6. Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K. and Yu, G. (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science* **290,** 2105-2110.

7. Riechmann, J.L. and Ratcliffe, O.J. (2000) A genomic perspective on plant transcription factors. *Curr. Opin. Plant Biol*. **3,** 423-434.
8. Liang, P. and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257,**. 967-971.
9. Shi, W.M., Ueda, A., Ozaki, K., Inada, M., Takamatsu, A. and Takabe, T. (2002) Analysis of heat stress responsive genes in *Aneurolepidium chinense* leaves by differential display. *Plant Prod. Sci.* **5,** 229-235.
10. Ueda, A., Shi, W., Nakamura, T. and Takabe, T. (2002) Analysis of salt-inducible genes in barley roots by differential display. *J. Plant Res*. **115,** 119-130.
11. Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270,** 467-470.
12. Velculescu, V.E., Zhang, L., Vogelstein, B. and Kinzler, K.W. (1995) Serial analysis of gene expression. *Science* **270,** 484-487.
13. Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993) Stress protection in transgenic tobacco producing a putative osmoprotectant, mannitol. *Science* **259,** 508-510.
14. Kishor, P., Hong, Z., Miao, G.H., Hu, C. and Verma, D. (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol*. **108,** 1387-1394.
15. Takabe, T., Nakamura, T., Nomura, M., Hayashi, Y., Ishitani, M., Muramoto, Y., Tanaka, A. and Takabe, T. (1998) Glycinebetaine and the genetic engineering of salinity tolerance in plants. In *Stress Responses of Photosynthesis Organisms* (Satoh, K. and Murata, N.eds). Amsterdam: Elsevier Science, pp. 115-131.
16. Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science* **285,** 1256-1258.
17. Shi, H., Lee, B.H., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol*. **21,** 81-85.
18. Yoshida, K.T., Naito, S. and Takeda, G. (1994) cDNA cloning of regeneration-specific genes in rice by differential screening of randomly amplified cDNAs using RAPD primers. *Plant Cell Physiol*. **35,** 1003-1009.
19. Ito, T., Kito, K., Adati, N., Mitsui, Y., Hagiwara, H. and Sakaki, Y. (1994) Fluorescent differential display: arbitrarily primed RT-PCR fingerprinting on an automated DNA sequencer. *FEBS Lett*. **351,** 231-236.
20. Fischer, A., Saedler, H. and Theissen, G. (1995) Restriction fragment length polymorphism-coupled domain-directed differential display: a highly efficient technique for expression analysis of multigene families. *Proc. Natl. Acad. Sci. USA* **92,** 5331-5335.
21. Yang, S. and Liang, P. (2004) Global analysis of gene expression by differential display: a mathematical model. *Mol. Biotechnol*. **27,** 197-208.
22. Gaxiola, R.A., Fink, G.R., Hirschi, K.D. (2002) Genetic manipulation of vacuolar proton pumps and transporters. *Plant Physiol.* **129,** 967-973.
23. Ueda, A., Inada, M. and Takabem, T, (2003) Evaluation of abiotic stress tolerance in transgenic Arabidopsis overexpressing barley proline transporter (HvProT) gene. *Plant Cell Physiol.* **44,** S47 (Supplement).
24. Aono, M., Saji, H., Fujiyama, K., Sugita, M., Kondo, K. and Tanaka, K. (1995) Decrease in activity of glutathione reductase enhances paraquat sensitivity in transgenic *Nicotiana tabacum*. *Plant Physiol*. **107,** 645-648.
25. Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V. and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. USA* **99,** 15898-15903.
26. Rausell, A., Kanhonou, R., Yenush, L., Serrano, R. and Ros, R. (2003) The translation initiation factor eIF1A is an important determinant in the tolerance to NaCl stress in yeast and plants. *Plant J.* **34,** 257-267.
27. Yamamotom, Y., Ueda, A. and Takabe, T. (2001) Gene cloning and characterization of salt-inducible aldehyde oxidase in barley. Proceeding of 12th International Congress on Photosynthesis.
28. Muramoto, Y., Nakamura, T., Alvarez-Nakase, A. and Takabe, T. (1998) Isolation of salt-induced cDNA clones in barley leaves using differential display. In *Photosynthesis: Mechanisms and Effects* (Garab G. ed), Kluwer Academic Publishers, Vol. IV, pp. 3043-3046.

29. Nakamura, T., Muramoto, Y., Yokota, S., Ueda, A. and Takabe, T. (2004) Structural and transcriptional characterization of a salt-responsive gene encoding putative ATP-dependent RNA helicase in barley. *Plant Sci*. **167,** 63-70.
30. Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.* **23,** 319-327.
31. Pnueli, L., Liang, H., Rozenberg, M. and Mittler, R. (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient *Arabidopsis* plants. *Plant J*. **34,** 187-203.
32. Shi, W.M., Muramoto, Y., Ueda, A. and Takabe, T. (2001) Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. *Gene* **273,** 23-27.
33. Panchuk, II., Volkov, R.A. and Schoffl, F. (2002) Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in Arabidopsis. *Plant Physiol*. **129,** 838-853.
34. Delauney, A.J. and Verma, D.P. (1993) Proline biosynthesis and osmoregulation in plants. *Plant J.* **4,** 215-223.
35. 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) Does proline accumulation play an active role in stress-induced growth reduction? *Plant J.* **31,** 699-712.
36. Schwacke, R., Grallath, S., Breitkreuz, K.E., Stransky, E., Stransky, H., Frommer, W.B. and Rentsch, D. (1999) LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. *Plant Cell* **11,** 377-392.
37. Narita, Y., Taguchi, H., Nakamura, T., Ueda, A., Shi, W. and Takabe, T. (2004) Characterization of the salt-inducible methionine synthase from barley leaves. *Plant Sci.* **167,** 1009-1016.
38. Zeh, M., Leggewie, G., Hoefgen, R. and Hesse, H. (2002) Cloning and characterization of a cDNA encoding a cobalamin-independent methionine synthase from potato (*Solanum tuberosum* L.). *Plant Mol. Biol.* **48,** 255-265.
39. Inada, M., Ueda, A., Shi, W. and Takabe, T. (2005) A stress-inducible plasma membrane protein 3 (AcPMP3) in a monocotyledonous halophyte, *Aneurolepidium chinense*, regulates cellular Na^+ and K^+ accumulation under salt stress. *Planta* **220,** 395-402.
40. Ueda, A., Kathiresan, A., Inada, M., Narita'Y., Nakamura, T., Shi, W., Takabe, T. and Bennett, J. (2004) Osmotic stress in barley regulates expression of a different set of genes than salt stress does. *J. Exp. Bot.* **55,** 2213-2218.
41. Ueda, A., Kathiresan, A., Bennett, J. and Takabe, T. (2004) Comparative expression analyses using barley cDNA microarray reveal divergent biological responses in barley and rice under salt stress. Submitted.
42. Navarre, C. and Goffeau, A. (2000) Membrane hyperpolarization and salt sensitivity induced by deletion of PMP3, a highly conserved small protein of yeast plasma membrane. *EMBO J.* **19,** 2515-2524.
43. Mitsuya, S., Taniguchi, M., Miyake, H. and Takabe, T. (2005) Disruption of *RCI2A* leads to over-accumulation of Na^+ and increased salt sensitivity in *Arabidopsis thaliana* plants. *Planta* in press.
44. Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13,** 61-72.
45. Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D. and Bohnert, H.J. (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13,** 889-905.
46. 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) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* **31,** 279-292.
47. Gale, M.D. and Devos, K.M. (1998) Comparative genetics in the grasses. *Proc. Natl. Acad. Sci. USA* **95,** 1971-1974.
48. Van Zyl, L., von Arnold, S., Bozhkov, P.V., Chen, Y., Egertsdotter, U., MacKay, J., Sederoff, R.R., Shen, J., Zelena, L. and Clapham, D.H (2002) Heterologous array analysis in Pinacea: hybridization of

Pinus taeda cDNA arrays with cDNA from needles and embryogenic cultures of *P. taeda, P. sylvestris* or *Picea abies. Comp Funct Genomics* **3,** 306-318.
49. Aharon, R., Shahak, Y., Wininger, S., Bendov, R., Kapulnik, Y. and Galili, G. (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. *Plant Cell* **15,** 439-447.
50. Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu. Rev. Plant Physiol. *Plant Mol. Biol*. **44,** 357-384.
51. Nakamura, T., Nomura, M., Mori, H., Jagendorf, A.T., Ueda, A. and Takabe, T. (2001) An isozyme of betaine aldehyde dehydrogenase in barley. *Plant Cell Physiol*. **42,** 1088-1092.

3. PHOSPHORYLATION OF RNA POLYMERASE II C-TERMINAL DOMAIN AND PLANT OSMOTIC-STRESS RESPONSES

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Abstract. Acclimation of plants to various environmental stresses involves activation of various signal transduction cascades that activate the expression of genes encoding stress tolerance-determinants. The established paradigm of this regulation is that the environmental signals are perceived by sensor molecules that trigger cellular stress signaling pathway regulating stress-specific transcription factors. However, number of recent studies in higher eukaryotic system indicated the downstream regulatory steps. Co-transcriptional regulation of RNA polymerase II (RNAPII) complex controls activity of RNAP II during transcription elongation. C-terminal domain of RNAPII largest subunit is a focal regulatory target containing heptad repeats that are reversibly phosphorylated by various kinases and phosphatases. The regulation of plant gene expression at the level of transcription elongation has recently been implied because of the CTD-phosphatase family mutants in Arabidopsis that alters stress inducible gene expression. The functional analysis of this gene family started to reveal common and unique mechanisms of plant gene expression among eukaryotes.

1. INTRODUCTION

The concerted function of transcription factors is essential in the regulation of gene suites (i.e., transcriptome) that encode determinants of multigene processes like plant growth and development, biosynthesis of complex secondary products, and responses to abiotic and biotic stresses [1-5]. In these processes, developmental programs or environmental stimuli activate "master-switch" transcription factors that control the output of the pathway. However, a cascade output is controlled by a composite series of positive and negative control points that 'finely tunes' the signature of the signal. Recent evidence indicates that a high level of gene expression control is imposed during message production or translation [6, 7]. These represent control points that modulate gene expression after the stimulus-induced promoter activation by transcription factors or alter the steady-state gene expression. Substantial information is available about global gene expression regulation that is due to the translational control [6]. Gene regulation at the level of transcript initiation and elongation is an emerging topic in biology [8-11]. Proteins with similar function in the transcription elongation complex mediate substantially

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different human diseases [10]. Such phenotypic differences likely are mediated by the preferential elongation of specific transcripts, however, with the exception of a few notable examples in animals, confirmatory comprehensive data have not yet been reported [12]. Furthermore, although positive and negative regulators of transcription elongation have been identified in yeast and animals, little is known about their identities and functions in plants. In this chapter, we will overview the current understanding of transcriptional regulation by phosphorylation of RNA polymerase II (RNAP II) C-terminal domain and their potential involvement in plant stress response.

2. CTD PHOSPHORYLATION IN TRANSCRIPTION INITIATION AND ELONGATION

Eukaryotic mRNA synthesis is catalyzed by RNAP II in a series of processes designated as pre-initiation, initiation, elongation, and termination [13] (Fig. 1).

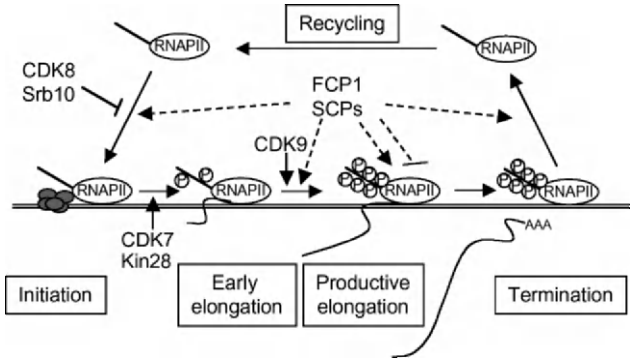

Figure 1. Phosphorylation and dephosphorylation of RNA polymerase II during transcription cycle. At initiation phase, dephosphorylated, free RNAP II is incorporated into initiation complex assembled at promoter region of a gene. The incorporated RNAP II is then phosphorylated by CDK7/Kin28 (TFIIH subunit) at CTD Ser^5. During initiation, CTD Ser^5 is predominantly phosphorylated, and this recruits mRNA capping enzyme. CDK9 phosphorylates CTD Ser^2 and promotes transition from early elongation to productive elongation phase. During productive elongation phase, CTD is predominantly phosphorylated at Ser2. At termination phase, CTD will be dephosphorylated and RNAP II will be dissociated from the template DNA and recycled. CTD phosphorylation of free RNAP II inhibits RNAP II to be incorporated to initiation complex.

During the transcription elongation step, RNAP II extends a nascent transcript to produce a full-length transcript. This step is often coupled to mRNA maturation processes such as capping, splicing, and polyadenylation [8, 14]. The factors that modulate transcription elongation alter the chromatin structure or target RNAP II in order to affect the pausing/arrest or the continuation of the transcription [15]. The carboxy-terminal domain (CTD) of the largest RNAP II subunit is the key regulatory

target [14]. The CTD contains heptapeptide repeat sequence (YSPTSPS), in which Ser residues at the second and the fifth positions are targets of CTD kinases and phosphatases [16]. The CTD repeats are found only in RNAP II and not in closely related RNAP I and III. Both the number of CTD repeat and non-repeat region of CTD contribute to normal function of RNAP II complex. Phosphorylation status of CTD determines two major forms of RNAP II. The CTD of RNAP IIO is hyperphosphorylated and exhibits lower mobility in SDS-PAGE gels, whereas the CTD of RNAP IIA is hypophosphorylated and exhibit higher mobility than RNAP IIO. Several intermediate form of animal RNAP II include RNAP IIm, which appears when animal cells were exposed to osmotic stress, and RNAP IIe in embryonic cells. The differential phosphorylation that occurs during the transcription cycle determines the functionality of RNAP II and controls the specific interaction between RNAP II CTD and various RNA processing factors.

3. CTD-KINASES

Several CTD kinases with different function have been identified in yeast and metazoans (Fig. 1, Table I). Currently, most of them belong to a cyclin-dependent kinase family and have a regulatory cyclin subunit. Phosphorylation of the CTD of the free RNAP II by Srb10 inhibits its recruitment to the preinitiation complex [17]. However, at promoters, Ser^5 in the CTD repeats is phosphorylated by TFIIH and is most strongly phosphorylated at the initiation and the early elongation stage [18]. The CTD phosphorylated at Ser^5 facilitates recruitment and activation of the capping enzyme [19]. The phosphorylation of Ser^2 by PTEF-b orthologs that occurs in the early elongation complex stimulates the transcription elongation [18]. Ser^2 of CTD remains phosphorylated during transcription elongation. CTD kinases often play a critical role in specific transcription regulation, such as, for example, transcription of HIV genome [20-22], MIHCII (major histocompatibility complex class II) [23], and heat shock induction of HSP70 (heat shock protein 70) [12].

Table I. CTD-kinase family proteins in human, Saccharomyces cerevisiae.

Human	*S. cerevisiae*	Specificity and function
TFIIH (CDK7/Cyclin H)	Kin28/	Ser5 phosphorylation at promoter
PTEF-b (CDK9/Cyclin T)	BurI/BurII Ctk1/Ctk2	Phosphorylate Ser2. Promote transition from early elongation complex to productive elongation complex
CDK8/Cyclin C	Srb10/Srb11	Ser5 phosphorylation of free RNAP II and inhibit recruitment to the promoter
CDC2		unknown
MAPK (ERK1/2)		Ser5 phosphorylation upon heat shock, osmotic stress, and in embryonic tissue

4. CTD PHOSPHATASES

The current understanding of how specific dephosphorylation of the CTD controls these processes is still rudimentary (Fig. 1). FCP1 (TFII*F* interacting *C*TD *p*hosphatase) and SCP1 (*s*mall *C*TD-*p*hosphatase) orthologs represent two classes of known CTD phosphatases that have been characterized in the non-plant systems. FCP1 orthologs and SCPs dephosphorylate Ser^2 and Ser^5 of the CTD, but FCP1 preferentially phosphorylates Ser^2 whereas SCP1 is more biased toward Ser^5 of the CTD as its substrate [24-27]. FCP1 promotes elongation activity of the ternary RNAP II complex [28], and recycling of RNAP II [26, 29]. Inactivation of yeast temperature-sensitive FCP1 resulted in overall decline of mRNA synthesis [30]. On the other hand, human FCP1 negatively regulate the transcription of the HIV-1 genome [31, 32] and heat shock gene (hsp70) expression [33]. SCP1 is a negative regulator of the transcription [27]. Interestingly, inactivation of SCP1 *in vivo* did not affect the transcription of the constitutively expressed genes, but activated the transcription of various inducible promoters such as the dexamethasone-stimulated glucocorticoid receptor activity on the GRE-TK-LUC reporter gene [27]. Functionality of CTD phosphatases is regulated by multiple protein-protein interactions. The activation of FCP1 by RAP74 subunit of the TFIIF complex are essential for FCP1 to dephosphorylate the RNAP II CTD [34-36]. TFIIF is an integral component of the transcription preinitiation complex and the transcribing RNAP II [37-41]. A direct interaction between FCP1 and Rbp4 subunit of the RNAP II has been reported as well [42].

5. MULTIPLE CTD-PHOSPHATASE-LIKE GENES IN *ARABIDOPSIS THALIANA*

Several genes in the Arabidopsis genome encode polypeptides homologous to the known CTD phosphatases (Table 2, Fig. 2). Based on the presence of distinct domains, they can be categorized into three groups (CPL: *C*TD *p*hosphatase-*l*ike).

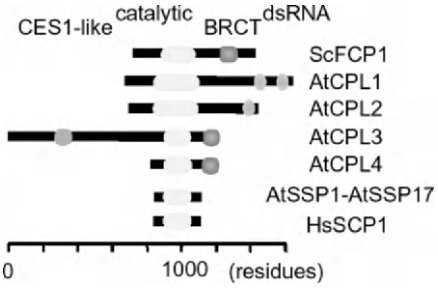

Figure 2. CTD phosphatase family proteins have different domain organization. Indicated are representative Arabidopsis (At), Saccharomyces cerevisiae (Sc) and Human (Hs) CTD phosphatase families.

Table 2. CTD phosphatase gene family in Arabidopsis thaliana.

Name	Gene	Group
CPL1	At4g21670	1
CPL2	At5g01270	1
CPL3	At2g33540	2
CPL4	At5g58000	2
SSP1	At1g29780	3
SSP2	At1g29770	3
SSP3	At5g45700	3
SSP4	At5g46410	3
SSP5	At5g11860	3
SSP6	At3g55960	3
SSP7	At3g19600-1	3
SSP8**	At3g19600-2	3
SSP9	At3g17550	3
SSP10	At2g04930	3
SSP11	At2g02290	3
SSP12	At5g23470	3
SSP13	At5g54210	3
SSP14	At1g20320	3
SSP15	At3g15330	3
SSP16	At1g43600	3
SSP17	At1g43610	3

**Fused ORFs

Group 1 CPLs (CPL1 and CPL2) have an FCP1-like catalytic domain and dsRNA binding motif(s) (DRM). Group 2 CPLs (CPL3 and CPL4) have an FCP1-like catalytic domain and a BRCT (*BR*CA1 *C-t*erminal) domain. Group 3 CPLs (SSP1~SSP17) have only a FCP1-like catalytic domain. The BRCT domain in CPL3 and CPL4 makes group 2 CPLs resemble the prototypical FCP1 CTD phosphatases that bind to TFIIF in the RNAP II complex. Group 1 CPLs are the only known examples of peptides containing both DRM and phosphatase domains. Homologs of group 1 CPLs are present in rice (GenBank accession # BAB63701), tobacco (EST# 6128f1 at http://mrg.psc.riken.go.jp/strc/BY-2%20EST.htm), and *Zinia elegans* (EST# Z713f1 at http://mrg.psc.riken.go.jp/PRIDE/index.html) indicating group 1 CPLs are ubiquitous in plants. DRM can function as a dsRNA-binding and/or a protein-protein interaction domain, and could be a target of regulatory RNA molecules and RNA binding proteins, which often play critical roles in transcription [43-46]. CTD kinase activity of PTEF-b is regulated by 7SK snRNA and by HIV tat protein that binds to tar-RNA of HIV genome [45-49]. Also, human negative elongation factor NELF contains RNA binding subunit [44]. By analogy, CPL1 and CPL2 may bind to the elongating nascent mRNAs, regulatory RNAs, or regulatory peptides through their DRMs, and may be incorporated into the transcription elongation complex. The sequence of group 3 CPLs (SSP1-SSP17) resembles SCP1. As SCP1 preferentially dephosphorylates CTD at Ser^5 and affect expression of inducible promoters, the presence of relatively large number of SSP family genes in

the Arabidopsis genome implies that promoter-specific regulation by Ser[5] phosphorylation of the CTD may be facilitated by the individual SSP isoforms.

6. MUTATIONS IN GROUP 1 AND GROUP 2 CPL GENES (*CPL1, CPL2*, *CPL3*) CAUSE UNIQUE PHENOTYPES

Insertion and point mutations in CPL1 (*cpl1/ fry2)* and CPL3 (*cpl3*) (Fig. 3) were identified through a forward genetic screening for altered regulation of stress-inducible *RD29a* promoter expression after cold, ABA, or NaCl treatment [50, 51]. Interestingly, *cpl1/fry2* and *cpl3* mutations, which inactivate isoforms of group 1 and group 2 CPLs, exhibit distinct *RD29a* expression, growth, stress response and developmental phenotypes [50, 51]. In *cpl1/fry2* mutant alleles, hyperinduction of *RD29a* occurs in response to cold-, ABA- or salt-stress. In contrast, in the *cpl3-1* mutant, hyperinduction of the *RD29a* promoter was ABA specific. The *cpl1-1* plants grow more vigorously and flower later than wild type, and the growth of *fry2-1* was hyper-sensitive to cold and ABA, whereas *cpl3-1* plants exhibit a reduced rate of fresh weight gain and early flowering. We also identified *cpl2* mutants using a reverse genetic strategy (Fig. 3). Preliminary reciprocal cross analysis between *fry2-1* and *fry2-1/+ cpl2-2/+* plants indicated that *CPL2* and *CPL1* are partially redundant, as *fry2-1 cpl2-2* male but not female double mutant gamete exhibited synthetic lethality (Koiwa et al., submitted). Analysis of *cpl2* single mutants is in progress. Apparently, CPLs have both unique and overlapping regulatory functions, perhaps through differential regulation of distinct and common gene sets.

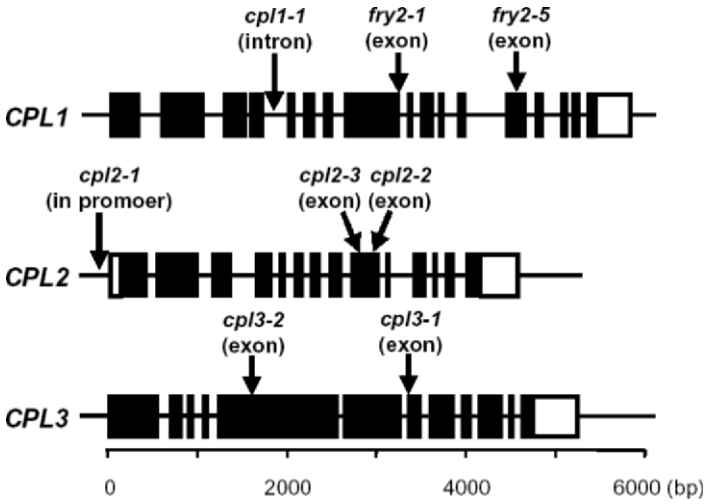

Figure 3. Alleles of cpl mutations identified in this project. Exons (■) were deduced from the cDNA sequence corresponding to CPLs. The open boxes represent untranslated regions. fry2-1 mutation is a single base substitution whereas the rest are T-DNA insertion mutations.

7. ARABIDOPSIS CPL1 AND CPL2 ARE SER^5-SPECIFIC CTD PHOSPHATASES

In order to determine the CTD phosphatase activity of CPLs, a recombinant protein for each isoform was expressed in *E. coli* and purified by Ni^{2+} affinity column. These fractions exhibited phosphatase activity toward *p*-nitrophenylphosphate (pNPP) substrate with a pH optimum between 5.5-6.0. CTD phosphatase activity of CPL1 and CPL2 was demonstrated using CTD phosphopeptides composed of 4 tandem repeats of the YSPTSPS sequence phosphorylated at Ser^2 or Ser^5 of each repeat [24]. Incubation of CTD Ser^5-PO_4 but not CTD Ser^2-PO_4 with CPL1 or CPL2 resulted in dephosphorylation of CTD (Koiwa et al, submitted). The Ser^5 CTD-phosphatase activity of CPL1 and CPL2 had an acidic pH optimum and required specific divalent cations such as Mg^{2+}, Mn^{2+}, Co^{2+} but not Ca^{2+}, Zn^{2+}, or Cu^{2+}. No detectable activity was observed with similarly purified NusA protein in any of the above assays. The functionally null *fry2-1* allele of *CPL1* encodes the N-terminal 676-amino-acid CPL1 fragment and lacks the C-terminal 292 amino acid containing the DRMs. In order to test the importance of the DRM in catalytic function of CPL1, a series of CPL1 truncation variant proteins were tested for CTD phosphatase activity. The CPL1 variants, whose C-terminus was shortened up to 467 amino acids, still retained a detectable Ser^5-specific CTD phosphatase activity, indicating the DRMs are not essential for its catalytic function. These results established that the group1 CPLs are unique, highly specific CTD Ser^5 phosphatases, and implied an essential, non-catalytic function of DRMs in CPL1.

8. CPL3 AND CPL4 ARE FUNCTIONAL PHOSPHATASES THAT INTERACT WITH THE RAP74 SUBUNIT OF TFIIF

Phosphatase activity of recombinant CPL3 has been shown using CDP-star as a chemiluminescent substrate [50]. The cDNA encoding CPL4 was isolated using RT-PCR and was subcloned into pET44a to produce recombinant NusA-CPL4 fusion protein. Activity of the affinity-purified CPL4 toward pNPP was significantly lower than that of CPL1 and CPL2, requiring longer incubation time. NusA-CPL4 fusion protein did not dephosphorylate any CTD-PO_4 (Koiwa et al., unpublished). Similar results were obtained with NusA-CPL3. The low activity toward synthetic substrates and lack of activity toward CTD-PO_4 substrate observed with CPL3 and CPL4 are similar to the results with human and *S. cerevisiae* FCP1 [25, 30, 34, 35]. It appears that the group 2 CPLs require the native RNAP II holoenzyme as their substrate like known human and yeast FCP1 orthologs. Another characteristic of FCP1 orthologs is the interaction with RAP74 (TFIIF large subunit), which likely provides a docking site for FCP1 to associate with the RNAP II holoenzyme. One RAP74 homolog was identified in the Arabidopsis genome (AtRAP74: At4g12610). The C-terminal interaction domain of AtRAP74 is homologous to both human and Drosophila RAP74s. When expressed as *in vitro*-translated peptides, the BRCT domain of both CPL3 and CPL4 interacted with GST-RAP74 fusion protein but not with GST by itself. It appears that CPL3 and CPL4 function as CTD phosphatases in similar

manner to human and *S. cerevisiae* FCP1, and provided a basis for further biochemical characterization of the group 2 CPLs.

9. CPL1, 3, 4 AND ATRAP74 BUT NOT CPL2 LOCALIZE EXCLUSIVELY IN ARABIDOPSIS NUCLEI, AND THE CPL1 C-TERMINAL REGION IS SUFFICIENT FOR THE NUCLEAR LOCALIZATION

To further confirm that CPLs function as nuclear transcriptional regulators, subcellular localization of CPL1, 2, 3, 4 and the Arabidopsis RAP74 homolog were determined. The cDNA fragments encoding these proteins were fused to the C-terminus of TAP-GFP-tag [52, 53]. The expression of the fusion protein was driven by a constitutive synthetic promoter [54]. These plasmids were introduced into Arabidopsis protoplasts by a polyethylene glycol (PEG) mediated transformation [55]. All proteins but CPL2 localized exclusively in the nuclei, which were labeled simultaneously by DsRed protein fused to a nuclear localization signal from SV40 T-antigen (NLS_{SV40}:DsRed). The further subcellular localization analysis of truncated CPL1 fragments indicated that the C-terminal 327-amino-acids fragment of CPL1 (CPL1(640-967)) was sufficient for the nuclear localization of the fusion protein (Koiwa et al., submitted). These results supported the hypothesis that CPL gene families function as nuclear transcriptional regulators.

10. FUTURE PERSPECTIVES

The recent identification of Arabidopsis CTD phosphatases, as regulators of stress-inducible transcription indicated that plant gene expression during stress responses is modulated at the level of RNAP II CTD phosphorylation. CPL1 and CPL2 are novel class of CTD phosphatases containing DRMs. This implicates that RNA molecules may control dephosphorylation of RNAP II transcribing a specific gene or a specific suite of genes. Several mutants that exhibit aberrant osmotic stress and ABA responses are due to the defects of RNA metabolism proteins, such as Sm protein involved in splicing (SAD1) [56], CAP binding protein (ABH1) [57], HYL1 protein that binds to dsRNA and regulates production of micro RNA in plants. These factors may function coordinately as a part of large transcription complex that regulates the osmotic stress signaling. Indeed, a recent study demonstrated that human FCP1 associates with the snRNP complex that contains Sm protein [58]. Identification of CPL1-interacting proteins is an essential component of future investigation. In addition to 4 CPL proteins, Arabidopsis genome encodes 17 small CTD phosphatase-like (SSP) proteins. The biochemical and *in vivo* function of SSP are currently unknown. It is tempting to speculate that each SSP specifically regulate the expression of unique suite of genes. The biochemical and mutational analysis of each SSP gene along with further characterization of CPL genes will allow us to assess precise functions of these genes, and expand our understanding of transcription elongation in plants.

REFERENCES

1. Cao, H., Glazebrook, J., Clarke, J., Volko, S. and Dong, X. (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88,** 57-63.
2. Stockinger, E., Gilmour, S.J. and Thomashow, M.F. (1997) Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA* **94,** 1035-1040.
3. Jaglo-Ottosen, K.R., Gilmour, S.J, Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280,** 104-106.
4. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S. and Yamaguchi-Shinozaki and K. Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **10,** 1391-1406.
5. Menke, F.L., Champion, A., Kijne, J.W. and Memelink, J. (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J.* **18,** 4455-4463.
6. Harding, H.P., Calfon, M., Urano, F., Novoa, I. and Ron, D. (2002) Transcriptional and translational control in the Mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.* **18,** 575-599.
7. Lee, T.I. and Young, R.A. (2000) Transcription of eukaryotic protein-coding genes. *Annu. Rev. Genet*. **34,** 77-137.
8. Shilatifard, A., Conaway, R.C. and Conaway, J.W. (2003) The RNA polymerase II elongation complex. *Annu. Rev. Biochem*. **72,** 693-715.
9. Reines, D., Conaway, R.C. and Conaway, J.W. (1999) Mechanism and regulation of transcriptional elongation by RNA polymerase II. *Curr. Opin. Cell Biol*. **11,** 342-346.
10. Conaway, J.W. and Conaway, R.C. (1999) Transcription elongation and human disease. *Annu. Rev. Biochem*. **68,** 301-319.
11. Conaway, J.W., Shilatifard, A., Dvir, A. and Conaway, R.C. (2000) Control of elongation by RNA polymerase II. *Trends Biochem. Sci*. **25,** 375-380.
12. Dubois, M.F. and Bensaude, O. (1998) Phosphorylation of RNA polymerase II C-terminal domain (CTD): a new control for heat shock gene expression? *Cell Stress & Chaperones* **3,** 147-151.
13. Hirose, Y. and Manley, J.L. (2000) RNA polymerase II and the integration of nuclear events. *Genes Dev*. **14,** 1415-1429.
14. Neugebauer, K.M. (2002) On the importance of being co-transcriptional. *J. Cell Sci.* **115,** 3865-3871
15. Hartzog, G.A., Speer, J.L. and Lindstrom, D.L. (2002) Transcript elongation on a nucleoprotein template. *Biochim. Biophys. Acta* **1577,** 276-286.
16. Dahmus, M.E. (1996) Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J. Biol. Chem.* **271,** 19009-19012.
17. Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S. and Young, R.A. (1998) Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol. Cell* **2,** 43-53.
18. Komarnitsky, P., Cho, E.J. and Buratowski, S. (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes Dev*. **14,** 2452-2460.
19. Ho, C.K. and Shuman, S. (1999) Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. *Mol. Cell* **3,** 405-411.
20. Cujec, T.P., Okamoto, H., Fujinaga, K., Meyer, K., Chamberlin, H., Morgan, D.O. and Peterlin, B.M. (1997) The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. *Genes Dev*. **11,** 2645-2657.
21. Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M.B. and Price, D.H. (1997) Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation *in vitro*. *Genes Dev*. **11,** 2622-2632.
22. Mancebo, H.S., Lee, G., Flygate, J., Tomassini, J., Luu, P., Zhu, Y., Peng, J., Blau, C., Hazuda, D. Price, D. and Flores, O. (1997) P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. *Genes Dev*. **11,** 2633-2644.

23. Kanazawa, S., Okamoto, T. and Peterlin, B.M. (2000) Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. *Immunity* **12,** 61-70.
24. Hausmann S. and Shuman, S. (2002) Characterization of the CTD phosphatase Fcp1 from fission yeast. Preferential dephosphorylation of serine 2 versus serine 5. *J. Biol. Chem*. **277,** 21213-21220.
25. Lin, P.S., Dubois, M.F. and Dahmus, M.E. (2002) TFIIF-associating carboxyl-terminal domain phosphatase dephosphorylates phosphoserines 2 and 5 of RNA polymerase II. *J. Biol. Chem*. **277,** 45949-4556.
26. Cho, E.J., Kobor, M., Kim, M., Greenblatt, J. and Buratowski, S. (2001) Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. *Genes Dev*. **15,** 3319-3329.
27. Yeo, M., Lin, P.S., Dahmus, M.E. and Gill, G.N. (2003) A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. *J. Biol. Chem*. **278,** 26078-26085.
28. Mandal, S.S., Cho, H., Kim, S., Cabane, K. and Reinberg, D. (2002) FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation. *Mol. Cell Biol*. **22,** 7543-7552.
29. Cho, H., Kim, T.K., Mancebo, H., Lane, W.S., Flores, O. and Reinberg, D. (1999) A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev*. **13,** 1540-1552.
30. Kobor, M.S., Archambault, J., Lester, W., Holstege, F.C.P., Gileadi, O., Jansma, D.B., Jennings, E.G., Kouyoumdjian, F., Davidson, A.R., Young, R.A. and Greenblatt, J. (1999) An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in S. cerevisiae. *Mol. Cell* **4,** 55-62.
31. Marshall, N.F., Dahmus, G.K. and Dahmus, M.E. (1998) Regulation of carboxyl-terminal domain phosphatase by HIV-1 tat protein. *J. Biol. Chem*. **273,** 31726-31730.
32. Licciardo, P., Napolitano, G., Majello, B. and Lania, L. (2001) Inhibition of Tat transactivation by the RNA polymerase II CTD-phosphatase FCP1. *AIDS* **15,** 301-307.
33. Dubois, M.F., Marshall, N.F., Nguyen, V.T., Dahmus, G.K, Bonnet, F., Dahmus, M.E. and Bensaude, O. (1999) Heat shock of HeLa cells inactivates a nuclear protein phosphatase specific for dephosphorylation of the C-terminal domain of RNA polymerase II. *Nuc. Acids Res*. **27,** 1338-1344.
34. Archambault, J., Pan, G., Dahmus, G.K., Cartier, M., Marshall, N., Zhang, S., Dahmus, M.E. and Greenblatt, J. (1998) FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase IIO. *J. Biol. Chem.* **273,** 27593-27601.
35. Archambault, J., Chambers, R., Kobor, M.S., Ho, Y., Bolotin, D., Andrews, B., Kane, C.M. and Greenblatt, J. (1997) An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **94,** 14300-14305.
36. Kamada, K., Roeder, R.G. and Burley, S.K. (2003) Molecular mechanism of recruitment of TFIIF-associating RNA polymerase C-terminal domain phosphatase (FCP1) by transcription factor IIF. *Proc. Natl. Acad. Sci. USA* **100,** 2296-2299.
37. Price, D.H., Sluder, A.E. and Greenleaf, A. (1989) Dynamic interaction between a Drosophila transcription factor and RNA polymerase II. *Mol. Cell Biol*. **9,** 1465-1475.
38. Bengal, E., Flores, O., Krauskopf, A., Reinberg, D. and Aloni, Y. (1991) Role of the mammalian transcription factors IIF, IIS, and IIX during elongation by RNA polymerase II. *Mol. Cell Biol*. **11,** 1195-1206.
39. Burton, Z.F., Kileen, M., Sopta, M., Ortolan, L.G. and Greenblatt, J. (1989) RAP30/74: a general initiation factor that binds to RNA polymerase II. *Mol. Cell Biol*. **8,** 1602-1613.
40. Flores, O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z.F. and Reinburg, D. (1991) The small subunit of transcription factor IIF recruits RNA polymerase II into the preinitiation complex. *Proc. Natl. Acad. Sci. USA* **88,** 9999-10003.
41. Killeen, M., Coulombe, B. and Greenblatt, J. (1992) Recombinant TBP, transcription factor IIB, and RAP30 are sufficient for promoter recognition by mammalian RNA polymerase II. *J. Biol. Chem*. **267,** 9463-9466.
42. Kimura, M., Suzuki, H. and Ishihama, A. (2002) Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in Schizosaccharomyces pombe involves direct interaction between Fcp1 and the Rpb4 subunit of pol II. *Mol. Cell Biol*. **22,** 1577-1588.
43. Isel, C. and Karn, J. (1999) Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation. *J. Mol. Biol*. **290,** 929-941.

44. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa., J. and H. Handa, H. (1999) NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. *Cell* **97,** 41-51.
45. Nguyen, V.T., Kiss, T., Michels, A.A. and Bensaude, O. (2001) 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature* **414,** 322-325.
46. Yang, Z., Zhu, Q., Luo, K. and Zhou, O. (2001) The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature* **414,** 317-322.
47. Sano, M., Abdellatif, M., Oh, H., Xie, M., Bagella, L., Giordano, A., Michael, L.H., DeMayo, F.J. and Schneider, M.D. (2002) Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle-cell hypertrophy. *Nat. Med*. **8,** 1310-1317.
48. Michels, A.A., Nguyen, V.T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L. and Bensaude, O. (2003) MAQ1 and 7SK RNA interact with CDK9/cyclin T complexes in a transcription-dependent manner. *Mol. Cell Biol.* **23,** 4859-4869.
49. Kobor, M.S. and Greenblatt, J. (2002) Regulation of transcription elongation by phosphorylation. *Biochim. Biophys. Acta* **1577,** 261-275.
50. Koiwa, H., Barb, A.W., Xiong, L., Li, F., McCully, M.G., Lee, B.H., Sokolchik, I., Zhu, J., Gong, Z., Reddy, M., Sharkhuu, A., Manabe, Y., Yokoi, S., Zhu, J.K., Bressan, R.A. and Hasegawa, P.M. (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate Arabidopsis thaliana abiotic stress signaling, growth, and development. *Proc. Natl. Acad. Sci. USA* **99,** 10893-10898.
51. Xiong, L., Lee, H., Ishitani, M., Tanaka, Y., Stevenson, B., Koiwa, H., Bressan, R.A., Hasegawa, P.M. and Zhu, J.K. (2002) Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **99,** 10899-10904.
52. Koiwa, H., Hausmann, S., Bang, W.Y., Ueda, A., Kondo, N., Hiraguri, A., Fukuhara, T., Bahk, J.D., Yun, D.J., Bressan, R.A., Hasegawa, P.M. and Shuman, S. (2004) Arabidopsis C-terminal domain phosphatase-like 1 and 2 are essential Ser-5-specific C-terminal domain phosphatases. *Proc. Natl. Acad. Sci. USA* **101,** 14539-14544.
53. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol*. **17,** 1030-1032.
54. Chiu, W.L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr. Biol*. **6,** (1996) 325-330.
55. Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E. and Gelvin, S.B. (1995) Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. *Plant J*. **7,** 661-676.
56. Abel, S. and Theologis, A. (1994) Transient transformation of *Arabidopsis* leaf protoplasts: a versatile experimental system to study gene expression. *Plant J* **5,** 421-427.
57. Xiong, L., Gong, Z., Rock, C.D., Subramanian, S., Guo, Y., Xu, W., Galbraith, D. and Zhu, J.K. (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in Arabidopsis. *Dev. Cell* **1,** 771-781.
58. Hugouvieux, V., Kwak, J.M. and Schroeder, J.I. (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. *Cell* **106,** 477-487.
59. Licciardo, P., Amente, S., Ruggiero, L., Monti, M., Pucci, P., Lania, L. and Majello, B. (2003) The FCP1 phosphatase interacts with RNA polymerase II and with MEP50 a component of the methylosome complex involved in the assembly of snRNP. *Nucleic. Acids Res*. **31,** 999-1005.

SECTION II

TEMPERATURE STRESS

4. TRIENOIC FATTY ACIDS AND TEMPERATURE TOLERANCE OF HIGHER PLANTS

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Abstract. The biomembrane of plant cells contains a high content of highly unsaturated fatty acids (polyunsaturated fatty acids) referred to as trienoic fatty acids. Although the amount of trienoic fatty acids varies according to the living environment of the plant, plants having a high ability to tolerate low temperatures, such as wheat, increase the amount of trienoic fatty acids to account for more than 80% of all fatty acids contained in the biomembrane when acclimated to low temperatures. On the other hand, some plants that thrive in deserts and other hot, dry regions demonstrate a remarkable decrease in the amount of trienoic fatty acids in high-temperature environments. On the basis of these findings, trienoic fatty acids are considered to be intimately involved in plant temperature tolerance. As reported in this study, we have created plants, using genetic engineering techniques, with an excellent temperature tolerance by inhibiting the activity of ω-3 fatty acid desaturase, an enzyme which synthesizes trienoic fatty acids.

1. INTRODUCTION

Climate changes have been predicted for the 21st century that will occur on a global scale as the mean air temperature rises due to increasing concentrations of carbon dioxide and other trace greenhouse-effect gases in the atmosphere [1]. Since agriculture and forestry are industries that are engaged in production through utilization of the natural environment, productivity is highly susceptible to climate changes. Consequently, there are concerns over the effects of climate changes brought about by greenhouse effects. In consideration of these circumstances, although considerable research has been conducted to evaluate the effects of global warming on agriculture [2-5], efforts are being made to search for specific and practical approaches to enhancing plant tolerance to high-temperature environments. This report provides an introduction to a novel genetic engineering approach, that we recently developed, for imparting high-temperature tolerance to plants [6,7]. The technique introduced here generates an efficacy that is not transient, but rather persists during plant growth. This efficacy is achieved through the direct control of intrinsic gene expression without introducing bacterial or other extrinsic genes. From these advantages, the techniques described here have the potential to evolve into a practical technology.

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2. THE RELATION BETWEEN POLYUNSATURATED FATTY ACIDS AND PLANT LOW-TEMPERATURE TOLERANCE

Lipids, which are among the basic constituents of biomembranes, have been a focus of attention since the 1960s as one of the factors affecting temperature sensitivity in plants [8]. For example, the physiochemical characteristics displayed by lipid bilayers at different temperatures differ with the species of the lipid head group or their esterified fatty acids, and the their lipid constituents and fatty acid constituents change depending on the environmental growth temperature [9-11]. There has also been an interest in the relationship of low temperature tolerance to the biosynthesis and rearrangement of biomembranes in response to temperature. Since the late 1980s, a series of mutant strains have been isolated in *Arabidopsis* with altered fatty acid constituents in their biomembranes, providing a breakthrough in the clarification of the pathways by which the polyunsaturated fatty acids contained in biomembrane lipids are generated [12]. The analysis of these mutant strains has shown that the degree of unsaturation of fatty acids of the biomembrane lipids is related to the low-temperature tolerance of a plant [13,14]. *fad5* is a mutant strain deficient in 16:0 fatty acid desaturase localized in chloroplasts, while *fad6* is a strain deficient in an enzyme similarly localized in chloroplasts that desaturates 16:1 and 18:1 fatty acids (Fig. 1). In addition, *fad2* is a mutant strain deficient in 18:1 fatty acid desaturase localized in the endoplasmic reticulum (Fig. 1). The cellular content of polyunsaturated fatty acids, including trienoic fatty acids, are decreased in these mutant strains. Chlorosis (whitening phenomenon of the leaves) and inhibition of growth are observed in these mutants, but not in the wild type, when they are subjected to low-temperature treatment. These findings indicate that polyunsaturated fatty acids are important for the tolerance of plants to low temperatures.

3. AN OVERVIEW ON STRESS SIGNAL TRANSDUCTION CLONING OF THE ω-3 FATTY ACID DESATURASE GENE

In general, trienoic fatty acids are present in the greatest amounts among fatty acids contained in plant membrane lipids. Trienoic fatty acids are polyunsaturated fatty acids that have three double bonds, and their content varies considerably according to the plant species and the living environment. Trienoic fatty acids are formed from dienoic fatty acids (having two double bonds) through the activity of ω-3 fatty acid desaturase. Due to the difficulty of characterization by conventional biochemical methods, the cloning of the ω-3 fatty acid desaturase gene has been performed by genetic techniques, namely by map-based cloning methods, using mutant strains of *Arabidopsis* [15,16]. ω-3 fatty acid desaturase genes cloned thus far are divided into two types consisting of a type localized in chloroplasts (*FAD7* and *FAD8*) and a type localized in the endoplasmic reticulum (*FAD3*). The expression of the *FAD8* gene is temperature-dependent, and is switched on and off by a difference of as little as a few °C, bordering on 25°C [17].

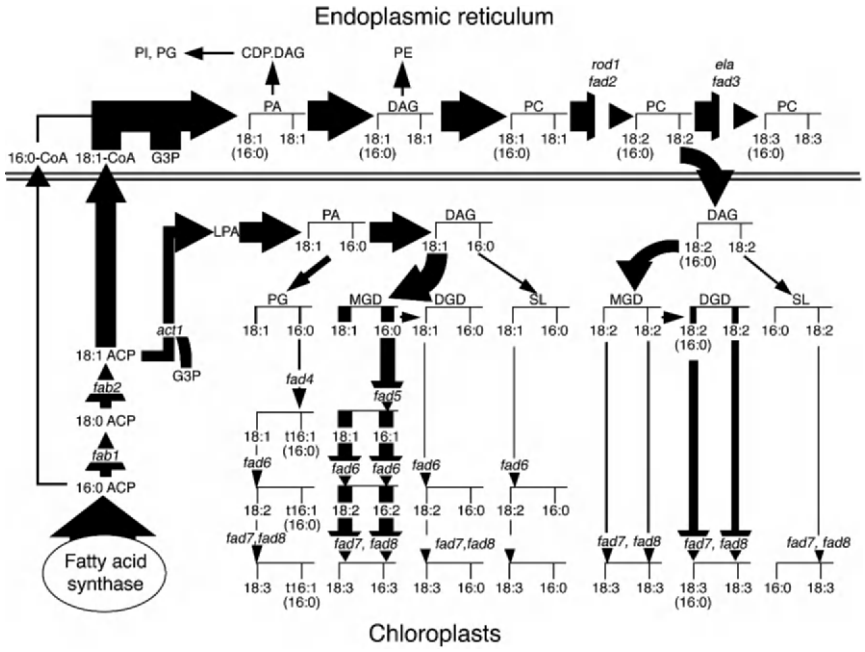

Figure 1. Biosynthetic pathways of major glyceroglycolipids and phospholipids in leaf cells of Arabidopsis and desaturase-deficient mutants. Interruption of the pathway represents the location of Arabidopsis mutations.

4. TRIENOIC FATTY ACIDS AND PLANT LOW-TEMPERATURE TOLERANCE

During the initial cloning of the *FAD* genes, our objective was to develop a plant exhibiting a high tolerance to low temperatures. Previous reports had suggested that increasing the degree of fatty acid unsaturation in biomembrane lipids was important in developing tolerance for low temperatures. When the two types of genes described above were respectively and forcibly expressed by their insertion into tobacco plants, the content of trienoic fatty acids increased in leaf tissue from the insertion of *FAD7*, localized to chloroplasts [18], while the trienoic fatty acid content increased in root tissue by insertion of *FAD3*, which is localized to the endoplasmic reticulum [19]. While no difference was observed in low temperature tolerance between plants carrying *FAD3* and wild-type plants [19], low temperature tolerance was observed to be improved in the case of plants carrying *FAD7* [18]. This difference, however, was only observed under specific and limited conditions. For example, subjecting a wild-type tocacco plant that thrives at 25°C to low temperature treatment for 7 days at 1°C without going through an acclimation process, and subsequently returning the plant to the original temperature environment,

leads to growth inhibition and chlorosis in young leaves prier to beginning development. This reaction to the exposure to low temperature was not observed in the transgenic tobacco plants carrying *FAD7*. Although this result suggests that trienoic fatty acids enhance tolerance to low temperatures, the effect may be limited to specific plant tissues or growth processes.

5. TRIENOIC FATTY ACIDS AND PLANT HIGH-TEMPERATURE TOLERANCE

Although the increase in trienoic fatty acids did not produce as great an improvement in low-temperature tolerance as was expected, we examined the reverse concept, that a decrease in the trienoic fatty acid content of the biomembrane may increase the high-temperature tolerance of plants. In the study described above, the ω-3 fatty acid desaturase gene was linked to a potent expression promoter, such as the cauliflower mosaic virus 35S promoter, to increase the amount of enzyme produced within the plant. However, trangenic lines were found in which the expression of the intrinsic ω-3 fatty acid desaturase gene was co-suppressed by gene silencing.

The correlation between the trienoic fatty acid content of the biomembrane and the ability of the plant to tolerate high temperatures was analyzed using two transgenic tobacco lines, T15 and T23, in which the activity of chloroplast-localized ω-3 fatty acid desaturase was decreased by gene silencing. Although trienoic fatty acids in the chloroplast membrane lipids of these transgenic tobacco lines were held to an extremely low level, the level of dienoic acids increased in a manner corresponding to the amount of decrease of trienoic fatty acids (Table 1). In addition, few changes were detected in the lipid molecular species of biomembranes, other than in the chloroplast membrane. Although there was no difference observed in growth between the transgenic tobacco plant and the wild-type plant over the range of low temperatures to the normal growth temperature, at high temperatures, clear differences in growth occurred. For example, in plants cultivated at 30°C for 45 days after germination, the fresh weight of the aerial parts of the T15, T23, and wild-type plants was 492 ± 81 mg, 445 ± 62 mg, and 399 ± 69 mg ($n = 5$), respectively. At a higher temperature (36°C), marked differences in the growth of the transgenic tobacco lines and the wild type were seen (Fig. 2A). After cultivating plants at 36°C for 45 days, fresh weight of the aerial parts of the T15 and T23 lines and the wild type was 124 ± 49 mg, 123 ± 23 mg, and 13 ± 6 mg ($n = 5$), respectively. Since growth of the transgenic lines continued to be uninhibited beyond 45 days at 36°C, the observed improvement in high-temperature tolerance has been suggested to be different from that resulting from the induction of heat shock protein [20].

When the plants were exposed to a considerably higher temperature (47°C), the leaves of the wild type withered within 2 days, and the plant bodies exhibited chlorosis after 3 days that resulted in death (Fig. 2B). In contrast, although the growth of the T15 and T23 plants was suppressed, damage due to high temperature was avoided by the plant body (Fig. 2B), and, when returned to a temperature suitable for growth (25°C), the plants continued to grow.

Table 1. Fatty acid composition of individual membrane lipids from leaves of wild-type (WT) and gene-silenced tobacco (T15, T23) plants. The major classes of membrane lipids were isolated from the total lipid extracted from mature leaves, and the fatty acid composition was determined. Each value represents the mean of two independent experiments. Dash (-) indicates trace amounts (<1.0%).

Lipid class	Percent of total polar lipids(%)	Fatty acid							
		16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Monogalactosyldiacyglycerol									
WT	46.2	2.0	-	-	12.1	-	-	2.5	82.0
T15	48.3	2.7	1.6	10.2	2.2	-	2.1	53.7	27.1
T23	45.3	3.9	2.8	10.5	4.1	-	2.2	38.6	37.4
Digalactosyldiacyglycerol									
WT	35.2	33.0	1.1	-	-	2.5	2.7	6.4	51.9
T15	36.3	21.8	4.4	1.3	-	1.7	4.1	42.8	23.3
T23	39.0	31.2	-	-	-	4.1	6.0	34.7	21.9
Phosphatidylglycerol									
WT	7.3	26.6	36.9	-	-	2.3	7.4	11.1	15.8
T15	7.0	25.2	34.3	-	-	4.5	10.9	22.5	2.2
T23	6.6	24.8	33.6	-	-	4.6	13.4	19.8	3.0
Phosphatidylcholine									
WT	8.9	26.0	1.5	-	-	4.1	4.1	41.6	23.0
T15	6.7	24.5	1.1	-	-	5.2	5.2	52.5	11.7
T23	7.3	21.3	1.3	-	-	5.9	5.9	53.2	12.9
Phosphatidylethanolamine									
WT	2.5	31.5	1.8	-	-	7.0	2.7	42.2	14.4
T15	2.4	25.3	-	-	-	2.6	2.7	56.0	12.1
T23	3.6	31.6	-	-	-	4.9	3.7	47.0	11.9

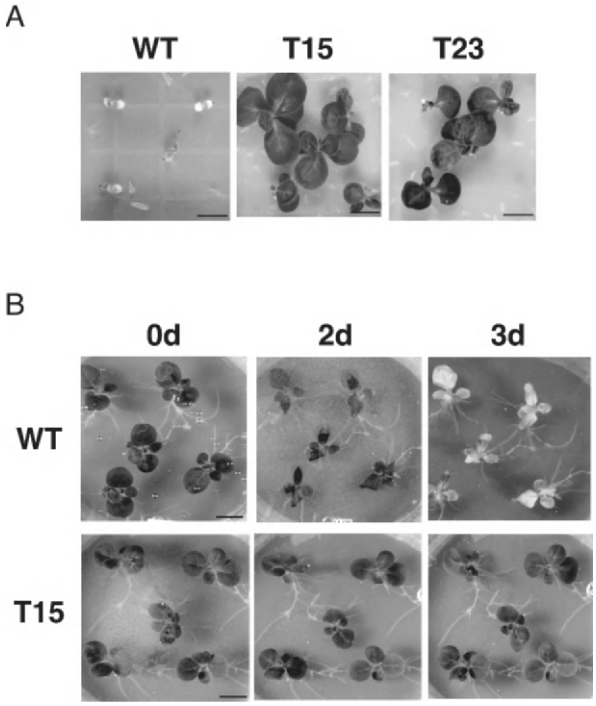

Figure 2. Comparison of the high-temperature tolerance of wild type tobacco (WT) and gene-silenced tobacco having decreased levels of trienoic fatty acids (T15, T23) (reprinted with permission from Science 287, 476-479, 2000). (A) Exposed to long-term growth at 36°C (60 days). (B) Exposed to 47°C for 0, 2 and 3 days. Scale bar, 1 cm.

Photosynthesis is one of the most heat-sensitive functions of plant cells. Temperatures in the range of 35°C to 45°C tend to inhibit photosynthesis. In order to assess the effect of high temperatures on the photosynthetic machinery, intact leaves from transgenic tobacco and *Arabidopsis* mutant were pretreated at various temperatures between 25°C and 55°C, and the level of photosynthetic activity was measured using O_2 evolution as the index of activity. At 40°C, the photosynthetic activity of the wild-type tobacco plants was significantly diminished, whereas the activity of the transgenic T15 and T23 plants was higher than that at the normal growing temperature of 25°C (Fig 3A). Moreover, a similar improvement was observed in the high-temperature tolerance of photosynthetic activity in mutant strains of *Arabidopsis* deficient in the chloroplast-localized ω-3 fatty acid desaturase gene (*fad7/8*) (Fig 3B). When these results and the various conditions of high-temperature treatment (including factors such as temperature region and treatment time) were examined comprehensively, they suggest that a decrease in the trienoic fatty acid content of chloroplast membrane lipids improves the ability to tolerate high temperatures, regardless of interspecies physiological properties.

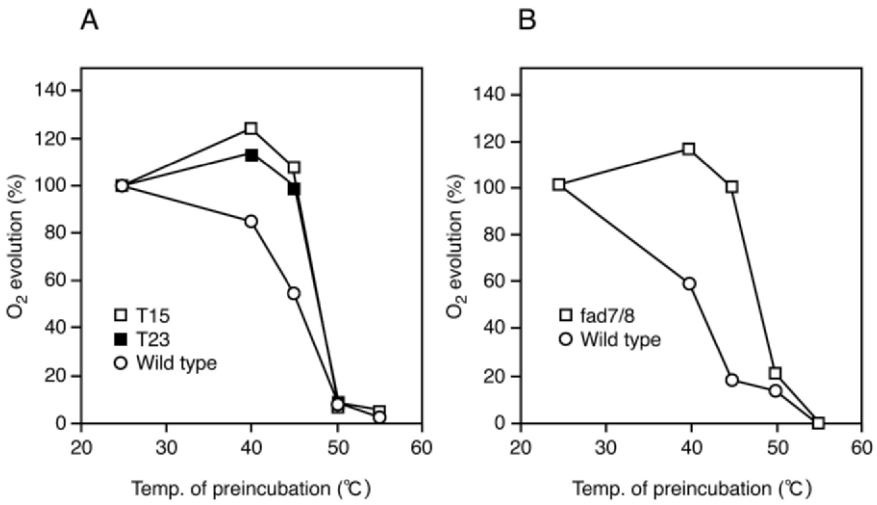

Figure 3. Oxygen evolution from transgenic tobacco and mutant Arabidopsis leaves that had been preincubated at various temperatures for 5 min. (A) T15, T23, and wild-type tobacco. (B) fad7fad8 double mutant and wild-type Arabidopsis. For each plant line, the O_2 evolution at 25°C was set at 100%. The oxygen evolution at 25°C was 2.4, 2.5, and 2.5 mmol O_2 m^{-2} s^{-1} in T15, T23, and WT tobacco, respectively, in (A); 1.4 and 1.7 mmol O_2 m^{-2} s^{-1} in fad7fad8 and wild-type Arabidopsis, respectively, in (B). Each data point represents the mean value from four independent experiments.

6. FUTURE OUTLOOK

Currently, there is considerable discussion on the topic of genetically-altered crops. For example, agricultural corps containing large amounts of the product of an extrinsic gene obtained from bacteria raise questions concerning the safety of those corps when consumed as food. In this study, plants having a high tolerance to high temperatures were able to be produced by manipulating genes common to nearly all plant species. Our results suggest the ability to develop useful plants while minimizing the risk of an accompanying gene recombination by skillfully utilizing the plant's mechanism of environmental adaptation. For example, trees distributed throughout sub-tropical and temperate regions tend to decrease the amount of trienoic fatty acids during an accompanying rise in the growth temperature. On the other hand, trees that are distributed in cold regions and are unable to adapt to growth in sub-tropical and tropical regions, lack this tendency. Although the insertion of genes into trees is considered difficult, improvement in the temperature tolerance of trees by future advances in technology are possible. In addition, damage to wheat and other agricultural crops from frequent occurrences of heat waves

caused by global warming is expanding. The findings obtained in this study will be useful in the development of agricultural crops tolerant to heat.

REFERENCES

1. IPCC (2001) IPCC Third Assessment Report – Climate Change 2001. http://www.ipcc.ch/
2. Newman, J.E. (1980). Climate change impacts on the growing season of the North American corn belt. *Biometeorology* **7,** 128-142.
3. Rosenzweig, C. (1985). Potential CO_2-induced climate effects on North American wheat-producing regions. *Climate Change* **7,** 367-389.
4. Smit, B., Ludlow, L. and Brklacich, M. (1988). Implication of a global climate warming for agriculture: a review and appraisal. *J. Environ. Quart.* **17,** 519-527.
5. Rosenzweig, C. and Parry, M.L. (1994). Potential impact of climate change on world food supply. *Nature* **367,** 133-138.
6. Murakami, Y., Tsuyama, M., Kobayashi, Y., Kodama, H. and Iba, K. (2000). Trienoic fatty acids and plant tolerance of high temperature. *Science* **287,** 476-479.
7. Iba, K. (2002). Acclimative response to temperature stress in higher plants: Approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **53,** 225-245.
8. Quinn, P.J. and Williams, W.P. (1993). The structural role of lipids in photosynthetic membranes. *Biochim. Biophys. Acta* **737,** 223-266.
9. Matsuda, O., Sakamoto, H., Hashimoto, T. and Iba, K. (2005). A temperature-sensitive mechanism that regulates post-translational stability of a plastidial w-3 fatty acid desaturase (FAD8) in *Arabidopsis* leaf tissues. *J. Biol. Chem*. **280,** 3597-3604.
10. Sharkey, T.D. (2000). Some like it hot. *Science* **287,** 435-437.
11. Thomas, P.G., Dominy, P.J., Vigh, L., Mansourian, A.R. and Quinn, P.J. (1986). Increased thermal stability of pigment-protein complexes of pea thylakoids following catalytic hydrogenation of membrane lipids. *Biochim. Biophys. Acta* **849,** 131-140.
12. Shimada, T., Wakita, Y., Otani, M. and Iba, K. (2000). Modification of fatty acid composition in rice plants by transformation with a tobacco microsomal w-3 fatty acid desaturase gene (*NtFAD3*). *Plant Biotech*. **17,** 43-48.
13. Hugly, S. and Somerville, C. (1992). A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiol*. **99,** 197-202.
14. Miquel, M., James, D.Jr., Dooner, H. and Browse, J. (1993). *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. *Proc. Natl. Acad. Sci. USA* **90,** 6208-6212.
15. Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M. and Somerville, C. (1992). Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science* **258,** 1353-1354.
16. Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V., Hugly, S. and Somerville, C. (1993). A gene encoding a chloroplast w-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the *fad7* mutant of *Arabidopsis thaliana*. *J. Biol. Chem*. **268,** 24099-24105.
17. Gibson, S., Arondel, V., Iba, K. and Somerville, C. (1994). Cloning of a temperature-regulated gene encoding a chloroplast w-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol.* **106,** 1615-1621.
18. Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M. and Iba, K. (1994). Genetic enhancement of cold tolerance by expression of a gene for chloroplast w-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol*. **105,** 601-605.
19. Hamada, T., Kodama, H., Takeshita, K., Utsumi, H. and Iba, K. (1998). Characterization of transgenic tobacco with an increased w-linolenic acid level. *Plant Physiol*. **118,** 591-598.
20. Lee, J.H., Hubel, A. and Schoffl, F. (1995). Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic *Arabidopsis*. *Plant J*. **8,** 603-612.

SECTION III

OXIDATIVE STRESSES

5. NITRIC OXIDE RESEARCH IN AGRICULTURE:

bridging the plant and bacterial realms

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Abstract. Organisms produce and respond to membrane-diffusible nitric oxide (NO) for a multitude of regulatory and defensive purposes. Bacteria establish intimate associations with plants and therefore NO metabolism by one organism can influence the physiology of the other. In plants, NO increases the bioavailability of iron and stimulates systemic pathogen defense pathways. Evidence provided by recent studies and reinterpretation of older published results implicates bacterial-derived NO in the modulation of these plant activities. Exploiting NO to benefit crop plants through manipulation of plant-associated bacterial communities presents agriculturalists with an ecologically-based alternative to genetic engineering of NO networks in plants.

1. INTRODUCTION

Abiotic and biotic stimuli beyond the optimal range for survival act as "stressors" on living organisms, causing metabolic dysfunction that can lead to death. A growing number of reports demonstrating higher stress tolerance in transgenic over wild-type plants provide strong evidence for verifying specific gene(s) in stress tolerance mechanisms [1-5]. Plants are distinguished from animals by the close degree to which their growth and development is tied to the surrounding environment [6]. A major difficulty in screening for stress tolerant plants under controlled environmental conditions is replicating the multiplicity of biotic and abiotic elements experienced in the natural environment that may modulate or depress the plant response. In our view, interactions between plants and the surrounding environment have been overlooked or underestimated in breeding programs, be they conventional or molecular-based.

Recent progress on stress physiology in medical sciences has implicated nitric oxide (NO) in fundamental stress response roles. NO is a small gaseous radical molecule previously studied primarily as an air pollutant (NO_X = NO + NO_2) and metabolic product of certain bacteria. It is now evident that NO is enzymatically produced for regulatory purposes in plants, fungi, protozoa and bacteria. Thus, understanding pathogenic and mutualistic interactions in terms of NO has become a

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fruitful area for research. However, studies of NO in plant biology have just begun and, though NO research holds enormous potential applications, our current state of understanding is quite limited. In this chapter, following a brief overview of plant stress responses, we review our current understanding of NO biology with emphasis on plant-bacterial interactions and possible applications for agriculture.

2. NO, A NEWLY IDENTIFIED PLAYER IN STRESS RESPONSES

Stress responses share similar characteristics among all living organisms. Dysfunctional metabolism and oxidative destruction of cells are typical results of stress-induced damage. The term "stress" was first applied to biological systems by Hans Selye [7-9] who recognized general nonspecific responses to diverse stressors [10]. For example, plants display similar responses when exposed to high temperature, high light, high salinity or drought. At a molecular level we now know that cellular production of reactive oxygen species (ROS) is a common early event in biological stress responses. Superoxide ($O_2^{\bullet-}$) is the most commonly produced ROS, and hydrogen peroxide (H_2O_2), the only diffusive molecule among ROS, is produced secondarily. Highly toxic hydroxyl radicals (•OH) or singlet oxygen ($^1O_2$) can also form under stress conditions [11].

Plants cope with stressful conditions through multiple detoxification and repair mechanisms. In plants, oxygenic electron transport in chloroplasts is the major source of ROS (Fig. 1) and prompt scavenging of these harmful molecules is essential. As long as antioxidant systems (scavenging mechanisms) are functional, ROS accumulation is usually not observed in favorable conditions.

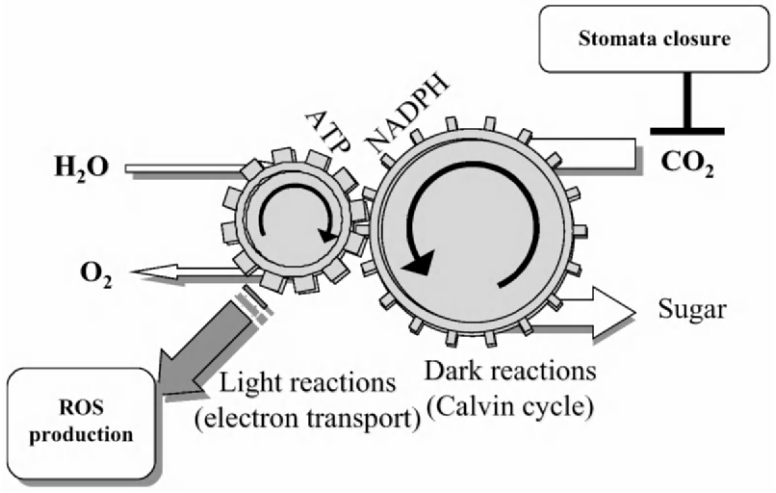

Figure 1. Schematic representation of plant photosynthetic metabolism illustrating the light reactions of chloroplasts as the primary source of reactive oxygen species.

Under severe stress conditions, however, antioxidant systems may not be able to destroy all ROS produced, a situation where oxidative damage of proteins, lipids and DNA leads to metabolic dysfunction, cell destruction and mutation. Thus, strategies for molecular breeding of stress tolerant plants are best grounded on an understanding of ROS scavenging mechanisms.

Recent biochemical investigations have demonstrated that inorganic nitrogen can be converted to toxic molecules whose characteristics are very similar to those of ROS; they are collectively referred to as reactive nitrogen species (RNS) and include the free-radical NO. The combination of a gaseous nature, membrane diffusibility and short half-life clearly differentiate NO from any ROS, and permit its utility as a comprehensive bio-signal, transmissible among diverse organisms that share a given habitat.

3. NO LESSON FROM APPLE ORCHARDS: SOLVING ONE PROBLEM CAN LEAD TO ANOTHER

In the middle of the last century breeders developed several varieties of apple rootstocks on which grafted scions grow to a much smaller size than normal. Their work provides a cautionary tale for applied agricultural researchers on altering a pleiotrophic phenotype for a single-minded purpose. The efficiency gains resulting from higher permissible planting densities and ease of harvest quickly led to the dominance of dwarfing rootstocks in commercial orchards [12]. Not noticed in the transition, however, was the relatively low tolerance of these rootstocks to the fungus *Rhizoctonia solani*, a major cause of "replant disease" of apple (Fig. 2) [13]. Replant disease syndromes occur when pathogens resident to the root system of a harvested crop attack new plantings [14], oftentimes resulting in devastating losses to crop productivity. For many years problems arising from the increased disease susceptibility of dwarfing rootstocks were avoided by establishing orchards on readily available virgin soils or the pre-plant application of broad spectrum fumigants including methyl bromide.

As we enter the 21st-century, virgin soils suitable for apple are becoming rare and the use of methyl bromide, which also is an ozone-depleting chemical, is destined by international agreement to be phased out [15]. Thus, there is now a need to breed new dwarfing rootstocks resistant to replant pathogens or to find alternative methods of cultivation that obviate the problem.

Our search for microbial-mediated mechanisms of disease control has led us to investigate the potential influence of bacterially emitted NO on plant disease resistance. Following findings in medical science, the importance of NO in plant physiology has only recently come to be appreciated. NO has signaling roles in plant defenses against pathogens [16-20], seed germination [21], root development [22], legume nodule formation (presumptively) [23] as well as in adaptive responses to the stresses of desiccation [24] and UV-B light [25] in leaves and O_2 deficiency in roots [26,27] . Undoubtedly programs will be undertaken to genetically alter the NO production or response pathways of plants for commercial purposes. However, the multivariate functions of NO in plants make the chances of achieving specificity

unlikely. A more feasible approach to harnessing the power of NO for agriculture may be in the biorational design of cultivation systems that seek to enhance ecologically sound associations between bacteria and crop plants.

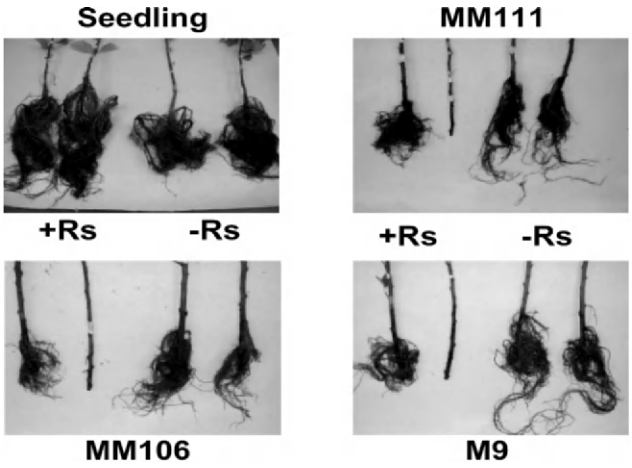

Figure 2. Apple rootstocks display differential tolerance/resistance toward the fungal pathogen Rhizoctonia solani. Shown is the range of root biomass on apple rootstocks harvested from orchard soil infested (+Rs) or not infested (-Rs) with R. solani AG-5. Seedling is a non-dwarfing rootstock whereas M9, MM106 and MM111 are dwarfing rootstocks.

4. PLANT-BENEFICIAL *STREPTOMYCES*

Sustainable means to prevent replant disease have been developed through the pre-plant incorporation of *Brassica napus* (rape) seed meals (RSM) into orchard soils. Compared to trees grown in untreated replant soils, trees established in RSM-amended soils display increased tree growth and fruit yield along with lower incidences of disease, which are equivalent to that conferred by soil fumigation [28,29]. Split root experiments have demonstrated that roots grown in RSM-amended soil are stimulated to systemically transmit protection to other roots of the same plant challenged with *R. solani* in soil that does not contain the seed meal [30].

The substantial suppression of root rot disease in RSM-amended soils is consistently associated with higher rates of apple root colonization by *Streptomyces* [30,31], a group of ubiquitous spore-forming, filamentous Gram-positive bacteria, many of which can establish endophytic root association with a variety of plant species [32-35]. Disease suppressiveness of RSM-amended soil is eliminated by pasteurization, but can be restored to these soils through the addition of individual *Streptomyces* sp. isolates [36]. Rootstocks that respond most positively to RSM amendment generally have the highest rate of colonization by *Streptomyces* spp. [37]. These findings implicate *Streptomyces* as the agents of disease-suppressiveness conferred by RSM soil amendment.

Many *Streptomyces* produce antibiotics and the documented plant growth promoting effects of both introduced and resident populations [38] of these bacteria are generally attributed to their antagonistic capacity toward soilborne pathogens. For instance, *Streptomyces lydicus* strain WYEC108 produces an antibiotic with strong in vitro activity against the oomycete phytopathogen *Pythium ultimum* [39] and coating pea seeds with spores of this strain substantially reduces invasion by *P. ultimum* in an oomycete-enrinched soil [40].

Though logical to posit a role for *Streptomyces*-derived antibiotics in disease suppression, several lines of evidence indicate that means other than or in addition to antibiosis are involved. Soil-borne *Streptomyces grieseovirdis* (Mycostop®) has been reported to reduce foliar infection of wheat and lettuce by the phytopathogens *Blumeria graminis* and *Botrytis cinerea*, respectively [41], implying that the bacterium may somehow stimulate plant systemic immunity. Antibiotics produced by *Streptomyces* sp. R-5 that inhibited the rhododendron pathogen *Pestalotiopsis sydowiana* in vitro did not inhibit mycelial growth in plants infused with these antibiotics; inhibition of the pathogen in planta occurred only with the concomitant presence of strain R-5 [42]. Coombs et al. [43] found that protection against *Gaeumannomyces graminis* var. *tritici* infection conferred to wheat by some endophytic *Streptomyces* strains did not consistently correlate with in vitro antagonism, suggesting that "antibiosis may be only one of the contributory factors for biocontrol capability." It is possible that some of the inconsistency could be due to the limitations of typical in vitro antibiosis screens, which have difficulty in detecting antimicrobial activity by slow-diffusing hydrophobic compounds. Furthermore, at least one streptomycete metabolite, fistupyrone, inhibits in vivo infection by *Alternaria brassicicola* without displaying in vitro fungicidal activity [44].

Even in the absence of pathogen pressure *Streptomyces* colonization can bring about substantial benefits to plant growth and production. Legumes colonized by *S. lydicus* WYEC108 exhibit dramatic increases in growth rate, root nodulation frequency, and nitrogen fixing activity [35,45,46], phenotypes known to be positively affected by iron availability. *S. lydicus* WYEC108-colonized pea plants were found to have a significantly greater content of iron in their nodules and much of this iron was probably incorporated in the nitrogenase enzymes of N_2-fixing bacteroids, which appeared healthier in *Streptomyces*-colonized nodules [35]. A survey of six strains showed that beneficial *Streptomyces* produce significantly more iron-chelating siderophores than do strains that confer little or no benefit to the host [47].

5. THE NO-IRON BRIDGE

We postulate that *Streptomyces*-derived NO in concert with NO endogenously produced in roots may have a role in increasing iron bioavailability in plants. Many *Streptomyces* spp. posses a nitric oxide synthase (NOS) gene and have been shown to produce NO in culture [30,48], including *S. griseovirdis* and *S. lydicus* strain WYEC108 (unpublished results). Bacterial NOS, found among several gram

positive genera, are homologous to the oxidase domain of animal NOS and form NO through the oxidation of L-arginine (Fig. 3). Supplementation of plants with NO can reverse chlorosis under iron-limiting conditions [49]. Furthermore, following exposure to compounds that lower levels of endogenous NO, iron-replete plants develop intraveinal chlorosis [49]. NO was not found to increase the total iron content of plants but, by reducing Fe(III) to Fe(II) and forming iron-nitrosyl complexes, NO may enable passage of iron from the apoplast across cell membranes and thereby increase the availability of the metal for metabolic processes [50,51].

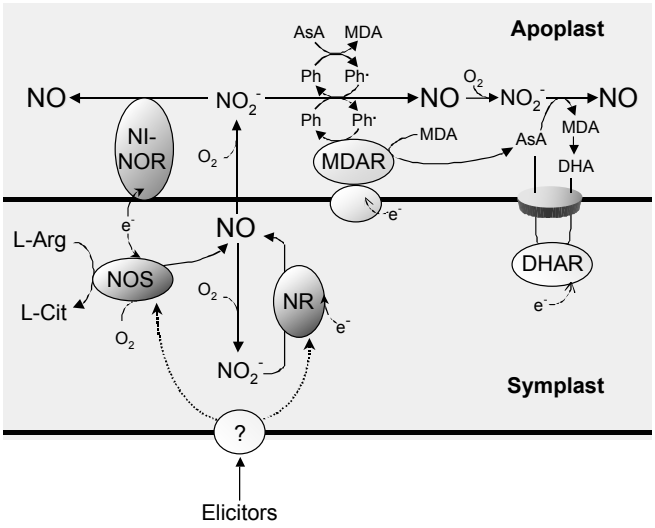

Figure 3. A model for generation and propagation of NO signal in response to pathogen elicitation by co-induction of the L-Arg → L-Cit pathway, catalyzed by NOS, and the nitrite → NO pathway, catalyzed by nitrate reductase (NR) in the symplast and by phenolic (Ph) or ascorbic acid (AsA) mediated reduction in the apoplast. Carriers of electrons (e^-) are omitted for simplicity.

Several reports have linked nitrate reductase (NR) activity with iron capture in plants and algae [52-54]. It remains to be determined whether this correspondence is related to NR's ability to form NO by the reduction of nitrite (Fig. 3) [55-58].

The redox state of NO appears to be an important factor in determining its influence as a signal molecule. Levels of ferritin were found to increase following exposure of *A. thalania* leaves and suspension cultured cells to the NO^+ donor SNP but not to the NO· donors, SNAP, GSNO or NOC-18; SNAP actually prevented iron-induced ferritin accumulation [59,60]. Since NO can be oxidized by Fe(III), the redox state of NO may provide information to cells useful for maintaining iron homeostasis and metabolism [51]. Reduction to Fe(II) is necessary for incorporation of iron into ferritin where it is then oxidized back to Fe(III) for safe storage [51,61].

Ferritin levels change during the course of nodule development and senescence. As nodules form, ferritins act as an iron reservoir, with each protein able to store

thousands of iron atoms [62]. During nodule maturation, ferritin levels decline and the released iron is incorporated into the increasingly numerous nitrogenase enzymes [63]. The reverse process occurs during senesence, with declines in nitrogenase being paralleled by increases in ferritin, thus preventing Fenton reactions and the runaway oxidative damage that would ensue were free iron atoms to be released [64]. Hérourt et al. [23] speculated that NO might have a regulatory role in nodule formation and function based on the finding of NOS-like activity in the roots and nodules of *Luinus albus*, along with observations in soybean nodules of a plentiful supply of arginine substrate [65], and presence of NO-leghemoglobin complexes [66]. They alluded to enticing unpublished pharmacological data indicating "that the modulation of NO levels leads to the modification of the number of nodules per root" but to our knowledge these results remain unpublished.

6. NO RELATIONS TO SOME OTHER SIGNAL MOLECULES

An inverse relationship between NO and ethylene emission rates has been observed in legumes as well as other plants [67]. Nodulation is negatively regulated by ethylene and, therefore, one possible mechanism whereby *Streptomyces* enhance nodulation may be through their release of NO. Rhizobia hyperinfection occurs in an ethylene-insensitive mutant of *Medicago trunculata* [68]. Some rhizobia enhance legume nodulation by inhibiting ethylene production in the plant host, thereby decreasing the frequency of infection thread abortion [69-71]. Based on this background, it would be worthwhile to survey nodulation-enhancing *Streptomyces* for NO production and to determine if mutation of the NOS gene diminishes the capacity of a given strain to enhance nodulation.

Products of rhizosphere bacteria, including salicylic acid (SA) and lipopolysaccharide (LPS), are known to elicit systemic resistance against pathogens [72]. Plants commonly make SA in response to infection by a pathogen. In this process, termed the oxidative burst, NO and H_2O_2 formed at the site of ingression stimulate the synthesis of SA which can travel throughout the plant to activate systemic acquired resistance mechanisms [16,18,73]. Constitutive production of SA by rhizobacteria is thought to prime this defense pathway and thereby allow a more rapid response to pathogen infection [72].

Although untested, it is an intriguing possibility that NO-emitting *Streptomyces* and other bacteria may prime the same resistance pathway by stimulating plant SA production in the absence of a pathogen. The NO-inducible α-*DOX-1* of *Arabidopsis thaliana* encodes for a lipid dioxygenase that converts fatty acids into oxylipins known to have antimicrobial and antioxidant properties [19]. Plants sown into soil were found to exhibit significantly higher levels of α-*DOX-1* expression in their roots [19], perhaps as a consequence of NO release by soil microbes.

Recent evidence implicates NO as a transducer of the LPS modulated signal in *A. thaliana*. In response to LPS, peels of wild-type plants displayed an NO burst, which was reduced by 80% in an AtNOS1 mutant [74]. To date AtNOS1, a homolog of the NO-synthesizing br-1 protein of the snail *H. pomatia*, is the only protein identified in plants to have NOS-like activity [24]. (A prominent report of an

"iNOS" in plants has recently been retracted [75]). During infection with *Pseudomonas syringae* pv tomato*, A. thaliana* deploys an alternative oxidase (AOX) [76] which, unlike cytochrome *c*, is tolerant of NO. Operation of AOX therefore permits continued oxidative respiration, albeit of lower ATP yield, without generating excess toxic reactive oxygen species that would otherwise form under cytochrome *c*-inhibiting conditions [77]. Exposure to exogenously supplied NO was shown by Northern and microarray analyses to induce transcription of AOX in *A. thaliana* [78]. Therefore, one might expect that LPS, as an inducer of AtNOS1, would stimulate expression of AOX. However, as determined by microarray analysis, AOX expression was not induced by exposure to LPS [74]. These disparate results cannot be resolved with our present state of knowledge. Reproducibility is a problem with microarray data [79] and thus the lack of AOX induction by LPS should be confirmed by Northern analysis. Also, care must be taken not to over-generalize from studies of *A. thaliana*, which differs significantly from other plants (even other Brassicacea) in pathogen-induced signal transduction [72].

7. ANTIMICROBIAL CONSEQUENCES OF NO RELEASE DURING THE OXIDATIVE BURST

In conjunction with antimicrobial activities that are activated in part by NO, including phytoalexin release, crosslinking reactions [80], host cell death [17] and oxylipin formation [19], nitrosative stress at an infection site probably contributes to inhibition of potential invaders. Within hours of exposure to an avirulent *Pseudomonas syringae* strain, soybean cell suspensions accumulate 10 μM H_2O_2 and 2 μM NO, levels which are substantially higher than those induced by a virulent strain [16]. NO concentrations of ≤ 2 μM induce the activity of the flavohemoglobin NO dioxygenase (NOD) in growing *Escherichia coli* cells and can kill resting late stationary phase cells [81]. Under aerobic conditions expression of NOD lowers cellular NO toxicity by protecting aconitase from inactivation by NO [81]. Strains of *Erwinia chrysanthemi* with mutations in the HmpX flavohemoglobin gene are substantially compromised in their ability to cause disease [82] indicating that bacterial mechanisms for eliminating NO may be phytopathogenicity factors. Experiments utilizing transgenic expression of bacterial Hmp in *Arabidopsis* and in avirulent *P. syringae* have provided evidence that confirms and further defines the role of NO in initiating plant responses to infection. The presence of Hmp, whether expressed by the bacterium or plant, causes a significant reduction in tissue H_2O_2 accumulation due to increased H_2O_2-degrading activity [20]. The authors speculate that removal of NO inhibition on catalase may account for the higher H_2O_2-degrading activity. Hmp plants displayed hypersensitivity symptoms after exposure to avirulent *P. syringae* that were even more severe when the infecting bacterium also expressed Hmp [20]. Most dramatic among the effects of Hmp on gene expression was the highly diminished accumulation of *pal* transcripts [20], confirming previous pharmacological-based studies [16]. Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway that leads to biosynthesis of SA as well as flavonoid phytoalexins [80].

Another source of nitrosative stress at infection sites is $ONOO^-$, produced in the reaction of NO with $O_2^{\cdot-}$. Enzymatic generation of $O_2^{\cdot-}$ can occur through the activity of NADPH oxidase [83] or nitrate reductase [11]. In mammalian phagocytes $ONOO^-$ is thought to function in the killing of microorganisms within the phagosome [84].

8. A MODEL FOR PLANT NO SYNTHESIS AND PROPAGATION

The model of NO synthesis depicted in Figure 3 was designed to reconcile observations of Yamamoto et al. [85] and Zeidler et al. [74] who reported increases in NR transcript accumulation and NOS activity, respectively, in response to pathogen elicitation. In an *Arabidopsis AtNOS* mutant induction of NO synthesis by LPS was reduced by approximately 80% relative to the wild-type plant [74], an observation that indicates a necessary role for AtNOS in pathogen-inducible NO synthesis but does not necessarily limit the role of NR to simply filling in the remaining 20%. Spontaneous oxidation of NO to nitrite would limit the intensity of the NO signal were it not for the presence of mechanisms to recycle nitrite back to NO. The increase in NR transcripts in potato [85] and NR protein in a *Brassica* sp. hybrid [86] in response to pathogens might indicate a role for NR in reduction of nitrite to NO in the symplasm. Similarly, pathogen elicitation stimulates accumulation of apoplastic phenolics [80] able to reduce nitrite to NO under the acidic conditions found in the apoplast [87]. Monodehydroascorbate reductase (MDAR) may be important for propagation of NO through the apoplast since it can reduce phenoxyl radicals to their parental phenolic forms [88] and reduce monodehydroascorbate to ascorbate, which itself is able to reduce nitrite to NO [89]. Analysis of NR and MDAR mutants should better define the role of these enzymes in maintaining the NO signal in plants.

9. THE OFT-IGNORED ENDOPHYTES

The external surfaces and interstitial spaces inside plants are rife with bacteria [90-92] and fungi [93]. Even vigorous treatment of seeds with disinfectants brings no guarantee of yielding a sterile plant.

We have reported on the isolation of a leaf endophyte *Rhodococcus* strain APG1 that produces NO via NOS [94]. NO synthesis is apparently involved in the bacterium's acclimation to harsh oxidative conditions found on leaves. In bacteria NO activates OxyR and SoxR, global regulators of genes involved in oxidative stress tolerance, including catalase and superoxide dismutase genes [95,96]. We observed substantial concomitant increases in NOS and catalase activity in *Rhodococcus* APG1 cells provided with sucrose, the primary source of available carbon in plant leaves [94].

We have also identified NOS activity in the *Bacillus mycoidies* strain PVL2, isolated from surface sterilized seeds of *Phaseolus vulgaris*. *Bacillus* spp. are commonly found as plant endophytes and genomic sequencing has revealed the presence of *nos* genes in many species. Interestingly, several *Bacillus* spp. also

possess *AtNos* homologs [97] although confirmation of NOS activity by the expressed products remains to be determined.

The nearly ubiquitous presence of bacteria, many of which contain NOS, in plant tissues should be taken into account in studies of NO synthesis in plants. The lack of responsiveness of intact bacterial cells to certain NOS inhibitors [94,98,99] may bear relevance on some observations of inhibitor-resistant NOS activity in plant preparations.

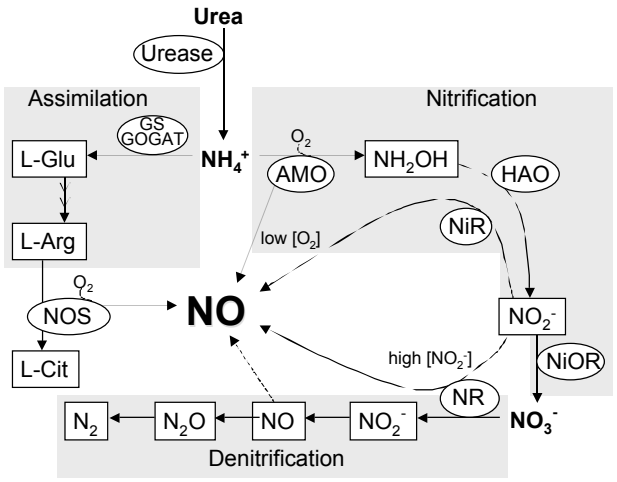

Figure 4. Bacterial routes for production of NO from nitrogen provided in urea, ammonia and nitrate fertilizer (bold). Enzymes of autotrophic nitrification by Nitrosomonas spp. are ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO) and nitrite reductase (NiR) [106]; Nitrobacter spp. can convert nitrite to nitrate via nitrite oxidoreducatse (NiOR). At low pH (<5.5) nitrite can dismutate into NO via nitrous acid. NO can be formed from nitrite by nitrite:NO oxidoreductase (NI-NOR) in some nondenitrifying bacteria [128] and, at high nitrate concentrations, by nitrate reductase (NR) [129,130]. The dashed line indicates steps where NO may be lost as a free intermediate denitrification [131]. Assimilation of ammonia via the glutamine synthetase (GS) glutamate synthase (GOGAT) pathway leads, after multiple steps, to the generation of L-arginine, the substrate for nitric oxide synthase (NOS). Most non-nitrogenous reactants, coenzymes and products are omitted for simplicity.

10. PLANT EXPOSURE TO NO RELEASED FROM MICROBIAL ENERGETIC METABOLISM

The full breadth of known biological mechanisms for synthesizing NO are found in the bacterial domain (Fig. 4). From an agricultural perspective the conversion of N in fertilizers to NO and other gaseous N compounds is generally viewed as a wasteful loss of nutrients. However, our growing understanding of NO as a signal

implies that NO formed by microbial processes may influence plant physiology, perhaps, as discussed above, to the benefit of the plant in some cases.

The major source of NO in most agricultural soils is nitrification [100-104] (Fig. 5) . Autotrophic nitrifiers that rely on ammonia as a source of high energy electrons are the predominant sources of nitrification activity [105]. Under conditions common in soil microenvironments where O_2 is not plentiful, nitrifiers will reduce endogenously formed nitrite to NO instead of utilizing O_2 as an electron sink [106,107]. The soil depicted in Figure 5 had not previously received nitrogenous fertilizer and thus there is a substantial delay in the onset of peak NO production activity. In an annually fertilized orchard soil the peak NO release rate is reached at approximately one week following amendment with RSM (Cohen & Mazzola, unpublished results).

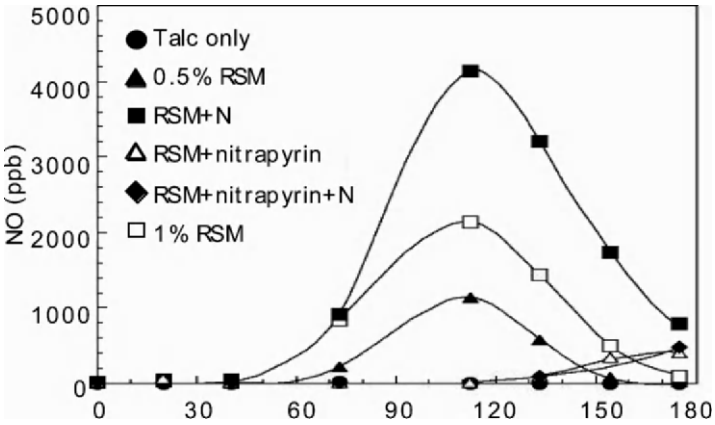

Figure 5. Nitrification-dependent NO production in response to incorporation of powdered Brassica napus var. Dwarf Essex seed meal into previously uncultivated forest soil. The soils were assayed as previously described [30]. Treatment with the nitrification inhibitor nitrapyrin was carried out by mixing 2 g of a 5 mg nitrapyrin g^{-1} talc suspension into 0.5 L soil. To all other soils talc without nitrapyrin was added at the same rate. Some soils were supplemented with N by mixing 575 mg NH_4Cl into the talc suspension (approximately equivalent to the amount of N added in the 0.5% RSM-amended soils). Assays preformed by Emi Yamamoto and Nao Arita, University of the Ryukyus, Okinawa.

In the 1960's a correlation was noted between the capacity of a soil amendment to stimulate nitrification and its ability to suppress infection by some fungal pathogens [108]. That nitrification-derived NO may be involved in activating plant defenses is an intriguing possibility we are presently investigating. Preliminary evidence indicates that initial RSM-mediated protection of apple seedling roots against *R. solani* infection requires nitrification activity.

In low pH and/or flooded environments, such as in rice paddies, denitrification serves as the primary source of NO [100,109]. Production of gaseous N by

denitrification had been thought to be exclusive to the bacteria but is now documented to occur among the fungi [110,111]. Under anoxic conditions, mammalian [112] and algal [113] mitochondria have also been shown to be capable of producing NO. In mammals this production of NO is an important adaptive response that functions to increase the supply of O_2 to tissues by stimulating blood vessel dilation. In plant roots under anoxic conditions NO may have an analogous role in increasing the availability of oxygen by promoting aerenchyma formation [26].

Considering the multiplicity of mechanisms in roots for converting nitrite to NO [22], the formation of nitrite from nitrate by rhizosphere bacteria may impact root NO status. *Azospirillum* spp. strains that carry out only the single-step reduction of nitrate to nitrite are much more likely to form associations with plant hosts than are those that have a complete denitrification pathway [114]. Zimmer et al. [115] provided evidence that some unknown metabolite of *Azospirillum*-derived nitrite acts as a root growth-promoting auxin. It is conceivable that NO may be this metabolite since NO is known to have root growth-promoting effects [22].

11. WHY NOS IN BACTERIA?

Unlike nitrification or denitrification that release NO as a byproduct of energy metabolism, the reaction catalyzed by NOS is energy consuming and a functional NOS enzyme is probably not essential for the survival of any bacterium, thus raising the question of what selective advantage possession of NOS confers. We have already mentioned evidence of a role for bacterial NOS in regulation of oxidative stress genes but roles for NO in interactions with other species may also exert selective pressure for retention of the *nos* gene.

Ironically, though up to this point our discussions have focused on potential benefits of bacterial NO on plant physiology, the only established role for bacterial NOS-derived NO (albeit indirect) is detrimental to plants. Nitrated dipeptide phytotoxins called thaxtomins are released by some *Streptomyces* species, such as *S. turgidoscabies*, and cause scabbing of tuber crops. It has been shown that an endogenous *Streptomyces* NOS is the source of the thaxtomin nitro group and that *nos* mutants are not phytotoxic [48]. Nitro groups are found in other secondary metabolites of *Streptomyces* spp. For instance, nitration of lipopeptide arylomycins by *Streptomyces* sp. Tü 6075 is associated with increased antibacterial activity [116]. Further investigations are required to determine if NOS has a broader role in the nitration of arylomycins and other secondary metabolites. In the rhizosphere, populations of bacteria are several times higher than in bulk soil [105] and antibiotic release probably helps certain bacteria carve out a niche in the crowd.

High population densities stimulate bacteria, such as the denitrifying *Pseudomonas aeruginosa*, to convert from a motile planktonic state to an attached biofilm-competent state by intercellular signaling mechanisms that do not involve organic quorum sensing molecules [117]. Among autotrophic nitrifying bacteria, which release large amounts of NO as a metabolic end-product, NO has been demonstrated to serve as a biofilm-stimulating signal [118]. The constituent bacteria

in a biofilm share many advantages, including increased resistance to oxidative stress, chemical toxicity and protozoan grazing. Protozoa reach high densities in the rhizosphere and numerous studies have shown that protozoa substantially improve nitrogen availability for roots through consumption and metabolic processing of bacteria [119]. Assuming that recognition of NO as a biofilm-inducing signal extends beyond the nitrifying bacteria, NOS activity might help bacteria avoid ingestion by bactivorous protozoa.

Like other organisms, protozoa produce and respond to NO. In many respects, mechanisms of microbial killing by neutrophils are similar to those of amoebic protozoa such as *Naegleria* spp. (Fig. 6) [120]. NO synthesis in neutrophils is thought to function both in subjecting captured prey to nitrosative stress and in activating proteinaceous antimicrobial effectors [121] but, to our knowledge, there has been no search for equivalent functions in amoebic NO-producing protozoa. Encystment is a common differentiation alternative for protozoa under starvation stress. It has been shown that increased NOS activity in starved macroplasmodia of *Physarum polycephalum* stimulates attainment of sporulation competence [122]. Conversely, endogenous NO production in starving *Dictyostelium discoideum* amoebae inhibits cell aggregation that precedes cellular differentiation [123]. These observations of protozoan responsiveness to NO imply that the capacity to produce NO may allow bacteria, in sufficient collective numbers as in the rhizosphere, to manipulate the physiological or developmental state of their predators.

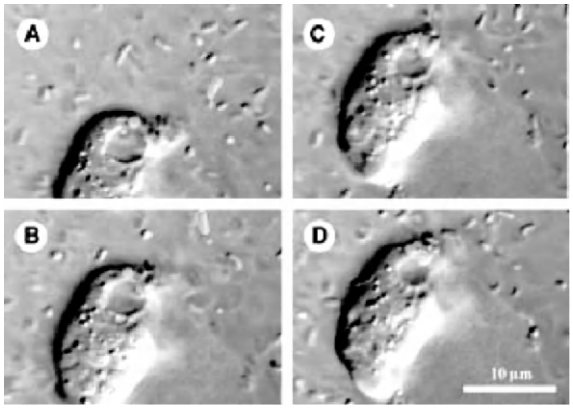

Figure 6. A four second time series from a Naegleria americana culture showing accumulation of the NO indicator DAF-FM in its triazole form (green). Nomarski images are overlain with simultaneously gathered confocal fluorescence images (Zeiss LSM 5 Pascal; argon laser 488 nm peak excitation, 515 nm emission). The field of view was moved to follow the trophozoite moving downward to the left. The protozoan, isolated from orchard soil that had been amended 7 days previous with B. napus var. Dwarf Essex seed meal, was maintained on heat-killed E. coli cells. A prior gentamicin and penicillin-streptomycin treatment regime, recommended for generating bacteria-free xenic amoeba culture [132], did not eliminate two NO-producing bacteria from the culture: Variovorax paxadoxus and Pseudomonas aureofaciens.

12. EMPLOYING BACTERIA TO SKIRT COMPLEXITY

In the field of NO research we are only beginning to elucidate the subtle influences of NO concentration, timing, cooperativity and antagonism within the layered and multifaceted regulatory mechanisms of plants [6,124]. The disparate physiological effects of the free radical and ionic forms of NO add another level of complexity to NO studies in biology. The plant kingdom is composed of organisms of widely diverse physiologies and habitats and thus we should expect differences in the actions of NO among plant species.

NO was originally viewed solely as a toxicant but now the preponderance of NO studies focus on its beneficial actions at lower physiological concentrations. Its hormetic dose-response, from beneficial to toxic with increasing concentration, describes the activity of many bioactive compounds [125]. Thus, consideration of NO dosage effects is a key in proper experimental design. With further improvements in the sophistication of technology available to researchers, results that now appear contradictory may eventually fall into place within coherent models for NO function.

Life has dealt with NO for a much longer period than O_2. Adaptation to oxic conditions is hypothesized to have occurred through the modification of NO-metabolizing enzymes [126,127]. In the language of life NO, like its English homonym "no", is simple but essential for deriving the meaning of more complex words (signals) within sentences (pathways) and paragraphs (networks). Anyone who grew up in the United States during the 1980's will recall Nancy Regan's admonition to "Just say no to drugs". Remove the word "no" and suddenly the phase is more apropos of a suggested toast at the opening of a crackhouse: Just say '*to drugs*!' Humanity would do well to keep this linguistic analogy in mind as we tinker with the communication systems of life. Genetic engineering generally results in switching genes on or off. Achieving desired alterations in the expression of fundamental regulatory modulators like NO should require more subtle technical approaches combined with a system-wide integrated knowledge base that we do not presently possess. Through the trial and error of evolutionary eons bacteria have developed a repertoire of mechanisms for manipulating plants including the release of plant-active compounds. Plants, in turn, have learned to utilize bacteria as sentinels that signal environmental changes and protect against pathogens. A better understanding of these interrelationships, in which NO plays numerous roles, will facilitate strategies that employ bacteria as interlocutors in our efforts to influence the plant world.

REFERENCES

1. Allen, R.D., Webb, R.P. and Schake, S.A. (1997) Use of transgenic plants to study antioxidant defenses. *Free Radical Biol. Med.* **23,** 473-479.
2. Kim, S., Kang, J.Y., Cho, D.I., Park, J.H. and Kim, S.Y. (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant J.* **40,** 75-87.

3. Ohara, K., Kokado, Y., Yamamoto, H., Sato, F. and Yazaki, K. (2004) Engineering of ubiquinone biosynthesis using the yeast *coq2* gene confers oxidative stress tolerance in transgenic tobacco. *Plant J.* **40,** 734-743.
4. Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S. and Soave, C. (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *Plant J.* **38,** 940-953.
5. Niggeweg, R., Michael, A.J. and Martin, C. (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nat. Biotechnol.* **22,** 746-754.
6. Yamasaki, H. (2005) The NO world for plants: achieving balance in an open system. *Plant Cell Environ.* **28,** 78-84.
7. Selye, H. (1950) Stress and the general adaptation syndrome. *Brit. Med. J.* **4667,** 1383-1392.
8. Selye, H. (1956) What is stress? *Metabolism* **5,** 525-530.
9. Selye, H. (1936) A syndrome produced by diverse nocuous agents. *Nature* **138,** 32.
10. Selye, H. (1946) The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol.* **6,** 117-230.
11. Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* **468,** 89-92.
12. Westwood, M.N. (1993) *Temperate-zone pomology: physiology and culture*, Timber Press.
13. Mazzola, M. (1997) Identification and pathogenicity of *Rhizoctonia* spp. isolated from apple roots and orchard soils. *Phytopathology* **87,** 582-587.
14. Mazzola, M. (1998) Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. *Phytopathology* **88,** 930-938.
15. Subbarao, K.V. (2002) Introduction: Methyl bromide alternatives – meeting the deadlines. *Phytopathology* 92, 1334-1336.
16. Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394,** 585-588.
17. Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Nat. Acad. Sci. USA* **98,** 13454-13459.
18. Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Nat. Acad. Sci. USA* **95,** 10328-10333.
19. Ponce de León, I., Sanz, A., Hamberg, M. and Castresana, C. (2002) Involvement of the *Arabidopsis* α-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. *Plant J.* **29,** 61-72.
20. Zeier, J., Delledonne, M., Mishina, T., Severi, E., Sonoda, M. and Lamb, C. (2004) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. *Plant Physiol.* **136,** 2875-2886.
21. Beligni, M.V. and Lamattina, L. (2001) Nitric oxide in plants: the history is just beginning. *Plant Cell Environ.* **24,** 267-278.
22. Stohr, C. and Ullrich, W.R. (2002) Generation and possible roles of NO in plant roots and their apoplastic space. *J. Exp. Bot.* **53,** 2293-2303.
23. Hérouart, D., Baudouin, E., Frendo, P., Harrison, J., Santos, R., Jamet, A., Van De Sype, G., Touati, D. and Puppo A., (2002) Reactive oxygen species, nitric oxide and glutathione: a key role in the establishment of the legume-Rhizobium symbiosis? *Plant Physiol. Biochem.* **40,** 619-624.
24. Guo, F.Q., Okamoto, M. and Crawford, N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100-103.
25. Costa, H., Gallego, S.M. and Tomaro, M.L. (2002) Effect of UV-B radiation on antioxidant defense system in sunflower cotyledons. *Plant Sci.* **162,** 939-945.
26. Dordas, C., Rivoal, J. and Hill, R.D. (2003) Plant haemoglobins, nitric oxide and hypoxic stress. *Ann. Bot.* **91** (SPEC. ISS. JAN.), 173-178.
27. Dat, J.F., Capelli, N., Folzer, H., Bourgeade, P. and Badot, P.M. (2004) Sensing and signalling during plant flooding. *Plant Physiol. Biochem.* **42,** 273-282.
28. Mazzola, M. and Mullinix, K. (2005) Comparative field efficacy of measures containing *Brassica napus* seed meal or green manure for the management of apple replant disease. *Plant Dis.* **89,** In Press.

29. Cohen, M.F. and Mazzola, M. (2004) A reason to be optimistic about biodiesel: seed meal as a valuable soil amendment. *Trends Biotechnol.* **22,** 211-212.
30. Cohen, M.F., Yamasaki, H. and Mazzola, M. (2005) *Brassica napus* seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of Rhizoctonia root rot. *Soil Biol. Biochem.* **37,** 1215-1227.
31. Mazzola, M., Granatstein, D.M., Elfving, D.C. and Mullinix, K. (2001) Suppression of specific apple root pathogens by *Brassica napus* seed meal amendment regardless of glucosinolate content. *Phytopathology* **91,** 673-679.
32. Taechowisan, T. Peberdy, J.F. and Lumyong, S. (2003) Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.* **19,** 381-385.
33. Coombs, J.T. and Franco, C.M.M. (2003) Isolation and identification of actinobacteria from surface-sterilized wheat roots. *Appl. Envrion. Microbiol.* **69,** 5603-5608.
34. Kortemaa, H., Rita, H., Haahtela, K. and Smolander, A. (1994) Root-colonization ability of antagonistic *Streptomyces griseovirdis*. *Plant Soil* 163, 77-83.
35. Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H., Deobald, L.A., Bailey, J.F. and Morra, M.J. (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl. Environ. Microbiol.* **68,** 2161-2171.
36. Cohen, M.F. and Mazzola, M. (2005) Suppression of Rhizoctonia root rot by *Streptomyces* in *Brassica napus* seed meal-amended soil. In *American Phytopathological Society Annual Meeting Phytopathology* 95:S20.
37. Mazzola, M., Cohen, M.F. and Fazio, G. (2004) Impact of soybean and rapeseed seed meal on microbial populations and growth of apple in replant orchard soils. *Phytopathology* **94,** S68.
38. Wiggins, B.E. and Kinkel, L.L. (2005) Green manures and crop sequences influence potato diseases and pathogen inhibitory activity of indigenous *Streptomycetes*. *Phytopathology* **95,** 178-185.
39. Crawford, D.L., Lynch, J.M., Whipps, J.M. and Ousley, M,A, (1993) Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl. Envrion. Microbiol.* **59,** 3899-3905.
40. Yuan, W.M. and Crawford, D.L. (1995) Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl. Envrion. Microbiol.* **61,** 3119-3128.
41 Lahdenpera, M.-L., Simon, E. and Uoti, J. (1991) Mycostop – a novel biofungicide based on *Streptomyces* bacteria. In *Biotic Interactions and Soil-Borne Ddiseases* (Beemster, A.B.R., Bollen, G.J., Gerlagh, M., Ruissen, M.A., Schippers, B. and Tempel, A. eds.), pp. 253-263, Elsevier.
42. Shimizu, M., Furumai, T., Igarashi, Y., Onaka, H., Nishimura, T., Yoshida, R. and, Kunoh, H. (2001) Association of induced disease resistance of rhododendron seedlings with inoculation of *Streptomyces* sp. R-5 and treatment with actinomycin D and amphotericin B to the tissue-culture medium. *J. Antibio.* **54,** 501-505.
43. Coombs, J.T., Michelsen, P.P. and Franco, C.M.M. (2004) Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. *tritici* in wheat. *Biol. Control* **29,** 359-366.
44. Igarashi, Y., Ogawa, M., Sato, Y., Saito, N., Yoshida, R., Kunoh, H., Onaka, H. and Furumai, T. (2000) Fistupyrone, a novel inhibitor of the infection of Chinese Cabbage by *Alternaria brassicicola*, from *Streptomyces* sp. TP-A0569. *J. Antibio.* **53,** 1117-1122.
45. Tokala, R.K. and Crawford, D.L. (2005) *Streptomyces* promote nitrogen fixation in legumes by aiding in nodular assimlation of iron. *(Personal communication)*.
46. Gregor, A.K., Klubek, B. and Varsa, E.C. (2003) Identification and use of actinomycetes for enhanced nodulation of soybean co-inoculated with *Bradyrhizobium japonicum*. *Can. J. Microbiol.* **49,** 483-491.
47. Tokala, R.K. (2004) Ph.D. Thesis. University of Idaho.
48. Kers, J.A., Wach, M.J., Krasnoff, S.B., Widom, J., Cameron, K.D., Bukhalid, R.A., Gibson, D.M., Crane, B.R. and Loria, R. (2004) Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. *Nature* **429,** 79-82.
49. Graziano, M., Beligni, M.V. and Lamattina, L. (2002) Nitric oxide improves internal iron availability in plants. *Plant Physiol.* **130,** 1852-1859.
50. Vanin, A.F., Svistunenko, D.A., Mikoyan, V.D., Serezhenkov, V.A., Fryer, M.J., Baker, N.R. and Cooper, C.E. (2004) Endogenous superoxide production and the nitrite/nitrate ratio control the concentration of bioavailable free nitric oxide in leaves. *J. Biol. Chem.* **279,** 24100-24107.
51. Graziano, M. and Lamattina, L. (2005) Nitric oxide and iron in plants: an emerging and converging story. *Trends Plant Sci.* **10,** 4-8.

52. Maldonado, M.T. and Price, N.M. (2000) Nitrate regulation of Fe reduction and transport by Fe-limited *Thalassiosira oceanica*. *Limnol. Oceanogr.* **45,** 814-826.
53. Castignetti, D. and Smarrelli, J. (1986) Siderophores, the iron nutrition of plants, and nitrate reductase. *FEBS Lett.* **209,** 147-151.
54. Smarrelli, J., John and Castignetti, D. (1986) Iron acquisition by plants: the reduction of ferrisiderophores by higher plant NADH: Nitrate reductase. *Biochim. Biophys. Acta. (BBA) - General Subjects* **882,** 337-342.
55. Rockel, P., Strube, F., Rockel, A., Wildt, J. and Kaiser, W.M. (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J. Exp. Bot.* **53,** 103-110.
56. Sakihamam, Y., Nakamura, S. and Yamasaki, H. (2002) Nitric oxide production mediated by nitrate reductase in the green alga Chlamydomonas reinhardtii: an alternative NO production pathway in photosynthetic organisms. *Plant Cell Physiol.* **43,** 290-297.
57. Yamasaki, H. (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. *Phil. Trans. R. Soc. Lon. B* **355,** 1477-1488.
58. Yamasaki, H., Sakihama,Y. and Takahashi, S. (1999) An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant. Sci.* **4,** 128-129.
59. Murgia, I., Delledonne, M. and Soave, C. (2002) Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. *Plant Journal* **30,** 521-528.
60. Murgia, I., de Pinto, M.C., Delledonne, M., Soave, C. and De Gara, L. (2004) Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. *J. Plant Physiol.* **161,** 777-783.
61. Harrison, P.M. and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta (BBA) - Bioenergetics* **1275,** 161-203.
62. Briat, J.F. and Lobreaux, S. (1997) Iron transport and storage in plants. *Trends Plant Sci.* **2,** 187-193.
63. Ragland, M. and Theil, E.C. (1993) Ferritin (mRNA, protein) and iron concentrations during soybean nodule development. *Plant. Mol. Biol.* **21,** 555-560.
64. Matamoros, M.A., Baird, L.M., Escuredo, P.R., Dalton, D.A., Minchin, F.R., Iturbe-Ormaetxe, I., Rubio, M.C., Moran, J.F., Gordon, A.J. and Becana, M. (1999) Stress-Induced legume root nodule senescence. Physiological, biochemical, and structural alterations. *Plant Physiol.* **121,** 97-112.
65. Streeter, J.G. (1987) Carbohydrate, organic acid, and amino acid composition of bacteriods and cytosol from soybean nodules. *Plant Physiol.* **85,** 768-773.
66. Mathieu, C., Moreau, S., Frendo, P., Puppo, A. and Davies, M.J. (1998) Direct detection of radicals in intact soybean nodules: Presence of nitric oxide-leghemoglobin complexes. *Free Radical Biol. Med.* **24,** 1242-1249.
67. Leshem, Y.Y., Wills, R.B.H. and Ku, V.V.V (1998) Evidence for the function of the free radical gas - nitric oxide (NO.) - as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem.* **36,** 825-833.
68. Penmetsa, R.V. and Cook, D.R. (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* **275,** 527-530.
69. Ma, W., Guinel, F.C. and Glick, B.R. (2003) *Rhizobium leguminosarum* biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. *Appl. Environ. Microbiol.* **69,** 4396-4402.
70. Ma, W., Charles, T.C. and Glick, B.R. (2004) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. *Appl. Environ. Microbiol.* **70,** 5891-5897.
71. Yuhashi, K., Ichikawa, N., Ezura, H., Akao, S., Minakawa, Y., Nukui, N., Yasuta, T. and Minamisawa, K. (2000) Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Appl. Environ. Microbiol.* **66,** 2658-2663.
72. van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36,** 453-483.
73. Klessig, D.F., Durner, J., Noad, R., Navarre, D.A., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E. and Silva, H. (2000) Nitric oxide and salicylic acid signaling in plant defense. *Proc. Nat. Acad. Sci. USA* **97,** 8849-8855.
74. Zeidler, D., Zahringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P. and Durner, J. (2004) Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc. Nat. Acad. Sci. USA* **101,** 15811-15816.
75. Travis, J. (2004) NO-making enzyme no more: *Cell, PNAS* papers retracted. *Science* **306,** 960.

76. Simons, B.H., Millenaar, F.F., Mulder, L., Van Loon, L.C. and Lambers, H. (1999) Enhanced expression and activation of the alternative oxidase during infection of Arabidopsis with *Pseudomonas syringae* pv tomato. *Plant Physiol.* **120,** 529-538.
77. Yamasaki, H., Shimoji, H., Ohshiro, Y. and Sakihama, Y. (2001) Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria. *Nitric Oxide* **5,** 261-270.
78. Huang, X., von Rad, U. and Durner, J. (2002) Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. *Planta* **215,** 914-925.
79. Marshall, E. (2004) Getting the noise out of gene arrays. *Science* 306, 630-631.
80. Cohen, M.F., Sakihama, Y. and Yamasaki H. (2001) Roles of plant flavonoids in interactions with microbes: from protection against pathogens to the mediation of mutualism. *Recent Res. Devel. Plant Physiol.* **2,** 157-173.
81. Gardner, P.R., Costantino, G. and Salzman, A.L. (1998) Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli*. Role of nitric-oxide dioxygenase in the protection of aconitase. *J. Biol. Chem.* **273,** 26528-26533.
82. Favey, S., Labesse, G., Vouille, V. and Boccara, M. (1995) Flavohaemoglobin HmpX: a new pathogenicity determinant in *Erwinia chrysanthemi* strain 3937. *Microbiology* **141,** 863-871.
83. Bolwell, G.P. (1999) Role of active oxygen species and NO in plant defense responses. *Curr. Opin. Plant Biol.* **2,** 287-294.
84. John, G.S., Brot, N., Ruan, J., Erdjument-Bromage, H., Tempst, P., Weissbach, H. and Nathan, C. (2001) Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. *Proc. Nat. Acad. Sci. USA* **98,** 9901-9906.
85. Yamamoto, A., Katou, S., Yoshioka, H., Doke, N. and Kawakita, K. (2003) Nitrate reductase, a nitric oxide-producing enzyme: induction by pathogen signals. *J. Gen. Plant Pathol.* 69, 218-229.
86. Subramanian, B., Bansal, V.K. and Kav, N.N. (2005) Proteome-level investigation of *Brassica carinata*-derived resistance to *Leptosphaeria maculans*. *J. Agric. Food Chem.* **53,** 313-324.
87. Bethke, P.C., Badger, M.R. and Jones, R.L. (2004) Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* **16,** 332-341.
88. Sakihama, Y., Mano, J., Sano, S., Asada, K. and Yamasaki, H. (2000) Reduction of phenoxyl radicals mediated by monodehyroascorbate reductase. *Biochem. Biophys. Res. Commun.* **279,** 949-954.
89. Pignocchi, C. and Foyer, C.H. (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr. Opin. Plant Biol.* **6,** 379-389.
90. Holland, M.A. and Polacco, J.C. (1994) PPFMs and other covert contaminants: Is there more to plant physiology than just plant? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 197-209.
91. Quadt-Hallmann, A., Hallmann, J., Kloepper, J.W. and Mahaffee, W.F. (1997) Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* **43,** 895-914.
92. Mundt, J.O. and Hinkle, N.F. (1976) Bacteria within ovules and seeds. *Appl. Envrion. Microbiol.* **32,** 694-698.
93. Vandenkoornhuyse, P., Baldauf, S.L., Leyval, C., Straczek, J. and Young, J.P. (2002) Extensive fungal diversity in plant roots. *Science* **295,** 2051.
94. Cohen, M.F. and Yamasaki, H. (2003) Involvement of nitric oxide synthase in sucrose-enhanced hydrogen peroxide tolerance of *Rhodococcus* sp. strain APG1, a plant-colonizing bacterium. *Nitric Oxide* **9,** 1-9.
95. Demple, B. (2002) Signal transduction by nitric oxide in cellular stress responses. *Mol. Cell. Biochem.* **234, 11**-18.
96. Kim, S.O., Merchant, K., Nudelman, R., Beyer, W.F.J., Keng, T., DeAngelo, J., Hausladen, A. and Stamler, J.S. (2002) OxyR: A molecular code for redox-related signaling. *Cell* **109 ,** 383-396.
97. Zemojtel, T., Penzkofer, T., Dandekar, T. and Schultz, J. (2004) A novel conserved family of nitric oxide synthase? *Trends Biochem. Sci.* **29,** 224-226.
98. Choi, W.S., Seo, D.W., Chang, M.S., Han, J.W., Hong, S.Y., Paik, W.K. and Lee, H.W. (1998) Methylesters of L-arginine and N-nitro-L-arginine induce nitric oxide synthase in *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* **246,** 431-435.
99. Wach, M.J., Kers, J.A., Krasnoff, S.B., Loria, R. and Gibson, D.M. (2005) Nitric oxide synthase inhibitors and nitric oxide donors modulate the biosynthesis of thaxtomin A, a nitrated phytotoxin produced by Streptomyces spp. *Nitric Oxide* **12,** 46-53.

100. Godde, M. and Conrad, R. (2000) Influence of soil properties on the turnover of nitric oxide and nitrous oxide by nitrification and denitrification at constant temperature and moisture. *Biol. Fertil. Soils* **32,** 120-128.
101. Harrison, R. and Webb, J. (2001) A review of the effect of N fertilizer type on gaseous emissions. *Adv. Agron.* **73,** 65-108.
102. Veldkamp, E. and Keller, M. (1997) Fertilizer-induced nitric oxide emissions from agricultural soils. *Nutr. Cycl. Agroecosyst.* 48, 69-77.
103. Jousset, S., Tabachow, R.M. and Peirce, J.J. (2001) Soil nitric oxide emissions from nitrification and denitrification. *J. Environ. Eng.-ASCE* **127,** 322-328.
104. Dunfield, P.F. and Knowles, R. (1999) Nitrogen monoxide production and consumption in an organic soil. *Biol. Fertil. Soils* **30,** 153-159.
105. Atlas, R.M. and Bartha, R. (1993) *Microbial ecology: fundamental and applications*, Benjamin/Cummings Inc.
106. Jetten, M.S.M. (2001) New pathways for ammonia conversion in soil and aquatic systems. *Plant Soil* **230,** 9-19.
107. Whittaker, M., Bergmann, D., Arciero, D. and Hooper, A.B. (2000) Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium Nitrosomonas europaea. *Biochim. Biophys. Acta-Bioenerg.* **1459,** 346-355.
108. Huber, D.M., Watson, R.D. and Steiner, G.W (1965) Crop residues, nitrogen, and plant disease. *Soil Sci.* **100,** 302-308.
109. Anderson, I.C., Poth, M., Homstead, J. and Burdige, D. (1993) A comparison of NO and N2O production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrophic nitrifier *Alcaligenes faecalis*. *Appl. Environ. Microbiol.* **59,** 3525-3533.
110. Tsuruta, S., Takaya, N., Zhang, L., Shoun, H., Kimura, K., Hamamoto, M. and Nakase, T. (1998) Denitrification by yeasts and occurrence of cytochrome P450 nor in *Trichosporon cutaneum*. *FEMS Microbiol. Lett.* **168,** 105-110.
111. Takaya, N. (2002) Dissimilatory nitrate reduction metabolisms and their control in fungi. *J. Biosci. Bioeng.* **94,** 506-510.
112. Kozlov, A.V., Staniek, K. and Nohl, H. (1999) Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett.* **454,** 127-130.
113. Tischner, R., Planchet, E. and Kaiser, W.M. (2004) Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga Chlorella sorokiniana. *FEBS Lett.* **576,** 151-155.
114. Baldani, V.L.D. and Döbereiner, J. (1980) Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* **12,** 433-439.
115. Zimmer, W., Roeben, K. and Bothe, H. (1988) An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*. *Planta* **176,** 333-342.
116. Schimana, J., Gebhardt, K., Holtzel, A., Schmid, D.G., Sussmuth, R., Muller, J., Pukall, R. and Fiedler, H.P. (2002) Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by *Streptomyces* sp Tu 6075 - I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.* **55,** 565-570.
117. Boles, B.R., Thoendel, M. and Singh, P.K. (2004) Self-generated diversity produces "insurance effects" in biofilm communities. *Proc. Nat. Acad. Sci. USA* **101,** 16630-16635.
118. Schmidt, I., Steenbakkers, P.J., op den Camp, H.J., Schmidt, K. and Jetten, M.S. (2004) Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. *J. Bacteriol.* **186,** 2781-2788.
119. Zwart, K.B., Kuikman, K.B. and van Veen, J.A. (1994) Rhizosphere protozoa: Their significance in nutrient dynamics. In *Soil protozoa* (Darbyshire, J.F., ed.), pp. 209, CAB International
120. Herbst, R., Ott, C., Jacobs, T., Marti, T., Marciano-Cabral, F. and Leippe, M. (2002) Pore-forming polypeptides of the pathogenic protozoon *Naegleria fowleri*. *J. Biol. Chem.* **277,** 22353-22360
121. Nathan, C. and Shiloh, M.U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Nat. Acad. Sci. USA* **97,** 8841-8848
122. Golderer, G., Werner, E.R., Leitner, S., Grobner, P. and Werner-Felmayer G. (2001) Nitric oxide synthase is induced in sporulation of *Physarum polycephalum*. *Genes Dev.* **15,** 1299-1309
123. Tao YP, TP Misko TP, Howlett AC, Klein C (1997) Nitric oxide, an endogenous regulator of *Dictyostelium discoideum* differentiation. *Development* **124,** 3587-3595
124. Steven, J.N., Radhika, D. and John, T.H. (2003) Nitric oxide signalling in plants. *New Phytol.* **159,** 11-35

125. Calabrese, E.J. and Baldwin, L.A. (2003) Toxicology rethinks its central belief. *Nature* **421,** 691-692
126. Castresana, C. and Saraste, M. (1995) Evolution of energetic metabolism: the respiration early hypothesis. *Trends Biol. Sci.* **20,** 443-448
127. Saraste, M. and Castresana, J. (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.* **34,** 1-4
128. Toffanin, A., Wu, Q., Maskus, M., Caselia, S., Abruna, H.D. and Shapleigh, J.P. (1996) Characterization of the gene encoding nitrite reductase and the physiological consequences of its expression in the nondenitrifying *Rhizobium "hedysari"* strain HCNT1. *Appl. Environ. Microbiol.* **62,** 4019-4025
129. Kaldorf, M., Linne von Berg, K.H., Meier, U., Servos, U. and Bothe, H. (1993) The reduction of nitrous oxide to dinitrogen by *Escherichia coli*. *Arch. Microbiol.* **160,** 432-439
130. Ji, X.B. and Hollocher, T.C. (1988) Reduction of nitrite to nitric oxide by enteric bacteria. *Biochem. Biophys. Res. Commun.* **157,** 106-108
131. Watmough, N.J., Butl, G., Cheesman, M.R., Moir, J.W., Richardson, D.J. and Spiro, S. (1999) Nitric oxide in bacteria: synthesis and consumption. *Biochim. Biophys. Acta-Bioenerg.* **1411,** 456-474
132. Schuster, F.L. (2002) Cultivation of pathogenic and opportunistic free-living amebas. *Clin. Microbiol. Rev.* **15,** 342-354

6. ULTRAVIOLET RADIATION STRESS:

Molecular and physiological adaptations in trees

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Abstract. The ozone layer acts like a giant sunshade, protecting forests and other life forms on the Earth's surface from much of the Sun's harmful ultraviolet radiation. The depletion in stratospheric ozone layer due to anthropogenically released pollutants such as CFC_s during the last few decades has resulted in increased UV radiation at ground level. UV radiation (100-400 nm) consists of UV-C (100-280 nm), UV-B (280-315 nm) and UV-A (315-400 nm) radiations. Out of these three, UV-B radiation has a disproportionately large photobiological effect on forest tree species. Enhanced UV radiation affects the tree species by modifying both their biological and biochemical environment. Damage may occur in a number of ways including the direct destruction of genetic material DNA at molecular level, disruption of membranes and other cell structure and generation of highly reactive chemicals known as "free radicals". It also inhibits various physiological processes including photosynthesis, nutrient assimilation, chlorophyll and protein synthesis, which results into reduced growth and development of the tree as a whole. Enhanced UV radiation especially UV-B radiation leads to several biochemical changes in the plants and synthesize secondary metabolites (condensed tannins, phenolics, flavonoids etc.) in the leaves and other parts of the plant. These secondary metabolites are synthesized as an adaptive mechanism in trees against enhanced UV radiation and have UV-absorbing properties. It reduces the insect herbivory, several viral and fungal diseases of a tree. Elevated CO_2 concentration ameliorates the damaging effects of UV radiation, whereas, drought in combination to enhanced UV radiation has inhibitory effect on plant growth and development. The inhibitory effects of enhanced UV radiation on the tree growth and development are more pronounced in the trees grown at the higher altitudes and at the equator as compared to the trees grown at lower altitude and at higher latitude. Trees undergo several changes to overcome the damaging effects of increased UV radiation by expressing new genes, synthesizing UV-absorbing compounds (secondary metabolites) and reactive oxygen scavenging enzymes. These processes in combination lead to morphological changes and thereby trees are able to tolerate UV radiation stress.

1. INTRODUCTION

The Earth receives the Sun's energy in the form of electromagnetic radiation. Humans are able to see only a small portion of this radiation, i.e., visible light (400-700 nm). The vast majority of sunlight is beyond human perception. Ultraviolet rays are just outside the realm of visible light and are characterized by wavelengths

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shorter than the last visible light ray, violet, hence "ultraviolet." UV-radiation that reaches Earth's surface is in the wavelengths between 280-400 nm. It is the part of the electromagnetic spectrum that can reach a high enough level on Earth to damage plants ecosystem. Ultraviolet radiation has been arbitrarily divided into three wave bands termed UV-A, UV-B and UV-C [1].

UV-C (100 to 280 nm) can be found in the artificial sources such as mercury arc lamps and germicidal lamps. UV-C is completely absorbed by the ozone layer and oxygen.

UV-B (280 to 315 nm) is the most intense usually between the hours of 10:00 am to 3:00 pm when the sunlight is brightest. It is much intense in the summer months accounting for about 60-70% of a trees yearly UV-B dose. UV-B does not penetrate the glasses. UV-B is mostly absorbed by the ozone layer, but some does reach the Earth's surface.

UV-A (315 to 400 nm) radiation is more constant than UV-B without any variation during the day and throughout the year. UV-A is neither filtered by glass nor absorbed by the ozone layer.

Shorter the wavelength, more energetic is the radiation causing damages to the trees. UV-C can do great damage but fortunately poses no risk to life on Earth. It is completely filtered out by oxygen and ozone in the stratosphere. Ozone also plays a vital role in filtering UV-B, the ultraviolet radiation that is the greatest threat to life on Earth [2]. However, even a fully functioning ozone layer does not absorb all the UV-B rays. UV-A surpasses the stratosphere virtually unfiltered, but compared to the shorter wavelengths, UV-A causes little harm. If the ozone layer thins further, the increased UV penetration will have severe consequences for trees life and other life forms as well. Substantial reductions of upto 50% in the ozone column have been observed over Antarctica [3]. In the tropics, due to small solar zenith angle and thin ozone layer, plants encounter much higher level of UV-radiation than at higher latitude [4]. Increased UV radiation could inhibit tree growth and development, photosynthesis, stunting plant life and agriculture. On a clear day, the level of UV radiation reaching the ground depends on how direct the sunrays are. Rays hitting the Earth directly, naturally, have more energy than less direct rays. They move through less energy-filtering atmosphere than lower angled rays.

On Long Island in ideal conditions, the greatest intensity of UV radiation will reach the area in summer solstice during the solar noon when the Sun is at its highest point in the sky for the entire year. This is the moment in time when the sunrays are most direct. Before and after this date and time, the sun hits the area at lower angles, less directly, decreasing the radiation. In general, the closer one moves towards the equator, more direct the sunrays will be. That area will receive, on average, high levels of UV radiation for the year. Atmosphere is rarely completely clear, and even it is clear, it contains various materials that will limit radiation. Dust particles and air molecules floating in the air absorb radiation and also reflect it scattering and deflecting it back into space. The higher the elevation the more unfiltered radiation that area will receive. A high elevation simply lies above many of the dust particles and air molecules preventing the radiation's passage. In addition, if an area is high enough, it will also perch above another factor limiting radiation, clouds.

2. FACTORS AFFECTING UV RADIATION

The level of UV radiation reaching on the Earth surface depends upon a number of factors and UV- radiation at the earth's surface is modified by temporal, geographical and meteorological factors [5]. Following factors influence the risk of UV radiation exposure and its consequences on tree health and life [1]:

Ozone Layer- The ozone layer absorbs most of the sun's UV rays, but the amount of absorption varies depending on the time of the year and other natural phenomena. The absorption of UV radiation has decreased, as the ozone layer has become thinner due to the release of ozone-depleting substances that have been widely used in industry viz., CFCs, (green house gases) etc.

Time of the Day- When the sun is at its highest in the sky around noon, sun rays have the least distance to travel through the atmosphere and UV-B levels are at the highest. During early morning and late afternoon, sunrays pass through the atmosphere at an angle and travel longer distance and their intensity is reduced.

Time of the Year- The sun angle varies with the season and causes the intensity of UV rays to change. During summer months, UV intensity tends to be the highest.

Cloud Cover- Clouds play a big role in allowing the amount of UV radiation reaching the Earth's surface. On a cloudy day, depending on the shape and thickness of the clouds, they can absorb and reflect 35-80% of the Sun's radiant energy and along with the other effects prevents all but a negligible amount of radiation from reaching the ground. Even with heavy cloud cover the scattered UV component of sunlight is seldom less than 10% of that under clear sky [6].

Latitude- At equator sunrays are the strongest, where it is most directly overhead and UV rays have to travel the least distance through the atmosphere. Ozone layer is usually thinner in the tropics compared to the mid- and high-latitudes, so there is less ozone to absorb the UV radiation, and hence more UV radiation passes through the atmosphere. At higher latitudes, the Sun is lower in the sky, so UV rays must travel a long distance through ozone-rich portions of the atmosphere and, in turn, trees grown at these latitudes are less exposed to UV radiation.

Altitude- At higher altitude the UV intensity increases because there are fewer atmospheres to absorb the damaging rays. In general, each 1 km increase in altitude increases the UV flux by about 6% [7]. The trees grown at higher altitudes have higher risk of overexposure to UV radiation.

Other Factors- Additional factors limiting UV radiation include haze, smog, surface reflection and other air pollution [8, 9]. All these factors are taken into account in a scale, called the UV index, measuring the amount of UV radiation reaching the ground and its relative danger to plant life.

3. IMPACT OF INCREASED UV RADIATION ON TREES

Forests occupy about 31 percent of the Earth's land area and make up over 90 percent of the Earth's biomass. Forests account for two-third of the carbon that is "fixed" or withdrawn from the atmosphere. Forests thus play a major role in how much carbon is free in the atmosphere, which in turn affects the magnitude of the "greenhouse effect." A little information exists on the effects of UV-B radiation on

forest tree species [10]. Tropical forests, though representing nearly one-half of the global productivity and much of the total tree species diversity, have received very little attention with respect to the ozone reduction problem. Although little, or no, ozone reduction has thus far occurred in the tropics, only a small decrease of ozone at these latitudes would result in a very sizeable increase of UV-B radiation since solar UV-B radiation is already very intense in these regions. Study has shown that excluding existing solar UV-B radiation with filters can result in increased growth of four broadleaf species [11]. Otherwise, the effects of UV-B radiation on tropical tree species have not received much attention. The perception of increased UV-B radiation has been diverted by global climate change; the problem has not gone away. In fact, even if ozone-depleting emissions were halted immediately, the detrimental gases already in the stratosphere break down slowly. Studies on agricultural species have shown that about 60 percent are at least moderately sensitive to high levels of UV-B radiation.

One of the primary interests of a tree physiologist is what effect UV-B has on ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO), the most abundant protein on Earth. It is believed that increased carbon dioxide and global warming will offer a buffer against UV-B damage to a certain extent. Increased carbon dioxide can enhance plant growth, but when combined with the negative effect of UV-B radiation on photosynthetic enzymes in C_3 and C_4 plants [12, 13], the result is far from certain. Physiologists are worried about whether the increased UV-B radiation will change carbon allocations within trees. They may have to put much of their photosynthetic products into protective mechanisms at the expense of growth. Growth processes uses photosynthetic assimilate and are controlled by content and activity of hormones and inhibitors. In most cases the treatment of plants with UV leads to the shift of hormone inhibitor balance towards inhibitor predominance [14]. There could be more severe direct effects also, but considering the role of trees in regulating atmospheric carbon, even small effects could in turn have large effects on climate change.

UV-B affects trees by modifying both biological and chemical environment. The most important of these are the nucleic acids and to a much lesser extent the proteins and other molecules [15]. Damage may occur in a number of ways, including the direct destruction of the genetic material DNA, deactivation of enzymes, disruption of membranes and other cell structures and the generation of highly reactive chemical agents known as free radicals. Elevated UV exposure can cause temporary or irreversible damage to photosynthetic apparatus (including the bleaching of the pigments) [16], loss of electron transport efficiency [17] to processes of cell division and growth regulation, and the composition and replication of genetic material. Consequences include a reduction in growth yield, changes in levels and effects of plant hormones [14] and alteration of periods of dormancy, flowering, etc. Biological repair mechanisms exist [18], mutations may remain as errors in the repair processes. In addition, the repair mechanisms themselves may be deactivated by high UV doses. The interaction of all these processes can lead to a variety of adverse effects on plants. Many effects are sub-lethal, may interact with other factors and therefore, very difficult to attribute to UV-enhancement specifically.

4. MOLECULAR AND CELLULAR CONSTITUTION

DNA readily absorbs UV-B radiation, which commonly changes the shape of the molecule in one of several ways. Changes in the DNA molecule often mean that protein-building enzymes cannot read the DNA code at that point on the molecule. As a result, distorted proteins can be made, or cells can die. However, living cells are "smart." Over millions of years of evolving in the presence of UV-B radiation, cells have developed the ability to repair DNA. Enzymes such as, BER (base excision repair), NER (nucleotide excision repair) and photolyase arrive at the damage site, remove the damaged section of DNA, and repair it with the proper components (based on information elsewhere on the DNA molecule). This makes DNA somewhat resilient to damage by UV-B.

Exposure of plants to UV-B radiation results in changes in expression of a large number of genes. Before UV-B radiation or light of other wavelengths can give rise to a cellular response, it has to be perceived by some kind of receptor, and the information transduced *via* a signalling pathway to the target molecules, be it proteins in the cytoplasm or the genetic material in the nucleus. Elevated solar UV-B doses increase the frequency of somatic homologous DNA rearrangements in plants. Increases in recombination are accompanied by a strong induction of photolyase and *Rad51* gene expression. These genes are putatively involved in major DNA repair pathways, photoreactivation and recombination repair. In plants that are deficient in photoreactivating ultraviolet-induced cyclobutane pyrimidine dimers, recombination under elevated UV-B regimes greatly exceeds wild-type levels. The results showed that homologous recombination repair pathways might be involved in eliminating UV-B-induced DNA lesions in plants. Increased solar UV-B radiation is forecasted for the early 21st century may affect genome stability in plants [19].

Scots pine seedlings were exposed in a phytotron to simulated outdoor climates with ambient or twice-ambient levels of ozone (26-96 and 52-192 nL L^{-1}, respectively) combined with zero or low ambient levels of UV-B. Needles and cotyledons were evaluated at eight dates up to 44 days of treatment. Cinnamyl-alcohol dehydrogenase (CAD) and stilbene synthase (STS) were induced by ozone. The mRNAs of these stress-related enzymes were induced only under twice-ambient ozone concentrations. Ozone-induced transient STS transcript levels reached their maximal values between day 1 and day 5, and were more pronounced in the presence of UV-B. CAD mRNA content and CAD enzyme activity increased continuously under enhanced ozone concentrations. Additional UV-B resulted in suppressed transcript level and enzymic activity of CAD. The results indicated that at the level of gene expression, there is an interaction between responses to UV-B and ozone [20].

Suesslin *et al.* [21] have observed that ultraviolet-B radiation triggers the activation of protection mechanisms and various photomorphogenic responses. One of the *Arabidopsis* mutants, uli3, shows a reduced sensitivity of UV-B induced gene expression events. The *ULI3* gene encodes an 80 kDa protein with potential domains for heme- and diacylglycerol-binding. In transiently transfected protoplasts, the ULI3: GFP fusion protein is localized in the cytoplasm but also adjacent to membranes. In etiolated seedlings irradiated by UV-B, *ULI3* mRNA expression is

strongly up regulated. This is caused by elevated transcription as demonstrated using stable transformants where a GUS-reporter is driven by the *ULI3*-promoter. *ULI3* is preferentially expressed in the outer cell layers in leaves, stems and flowers, but not in roots. There is evidence that ULI3 represents a specific component involved in UV-B-mediated signal transduction in higher plants.

The perception of low levels of UV-B probably occurs via a UV-B photoreceptor, followed by several different signalling pathways. These pathways include second messengers such as calcium, kinases and the catalytic formation of reactive oxygen species. On other hand, high levels of UV-B probably cause cellular damage and oxidative stress, thus activating a general stress signal transduction pathway, which leads to a response similar to that, which occurs after pathogen attack, and other stresses. Some of the genes identified so far as being regulated by UV-B encode proteins involved in the biosynthesis of protective pigments, DNA repair and antioxidative enzymes, photosynthetic genes, cell cycle genes, and stress genes induced by other types of stimuli i.e., pathogenesis-related proteins and senescence-induced genes [22].

UV-B radiation damages a variety of cellular components like the membrane of leaf cells, particularly the structure of chloroplast membrane. Under UV-B radiation, the plasma membrane in *Schefflera octophylla* was contracted and corrugated, and separated from the cell wall. The chloroplast in *S. octophylla* and *Pterospermum heterophyllum* was dilated and even broken and the thylakoid dilated and adhered. In *Archidendron clypearia*, the mitochondria appeared to be cavitated. Starch grains in chloroplast in *A. clypearia* and *P. heterophyllum* were increased, and the black particles in chloroplast in *S. octophylla* and *Sapium discolor* were also increased, and appeared on the mitochondrial membrane of *S. octophylla* [23].

A tree's ability to resist the effects of UV-radiation is a product of its ability to reduce exposure (through the optimisation of growth pattern and the production of UV absorbing pigments) and to repair or replace damaged molecules. The expression of these UV resistance mechanisms is sensitive to the quantity and quality of ambient light. The significance of DNA repair as a UV resistance mechanism was investigated by comparing the effects of solar UV on the growth of a variety of repair-proficient and -deficient *Arabidopsis* lines grown under natural light, focusing on the effects of UV on the growth of well-established plants. UV had an inhibitory effect on the growth of wild-type plants, and this effect was enhanced in repair-defective lines. Plants defective in the cyclobutane dimer photolyase are most sensitive to the effects of UV-B on plant height and rosette diameter growth, indicating that the enzyme plays an important role in UV resistance. A mutant defective in nucleotide excision repair (NER), a non-specific repair pathway, also displayed a significant, though less sensitivity to solar UV-B, suggesting that either the transcription-coupled repair of UV-induced dimers, or the repair of some other UV-induced lesion was also important for optimal growth [24].

5. BIOCHEMICAL CONSTITUTION

Increased UV-B radiation exposure to the plants leads to biochemical changes in the leaves, such as secondary compounds, which are having UV-absorbing properties like condensed tannins, several flavonoids, and some hydrocinnamic acid contents are increased. The solar UV-B radiation plays an important role in the formation of secondary metabolites in the leaves of birch seedlings [25] scot pine seedlings [26] and leaves of woody tree species [27]. Seasonal variation in accumulation of UV-B absorbing compounds noticed is more correlated to the developmental processes than to the seasonal fluctuations of the UV-B radiation [28].

Secondary compounds, such as lignin, are important as structural materials. These are related to phenolic compounds and may change in composition with elevated UV-B radiation [29]. If the ratio of lignin to cellulose in plant tissues changes, it can alter the rate of decomposition. The seedlings of *Acer saccharum* and *Acer platanoids* exposed to UV-A radiation accumulated higher concentrations of anthocyanins and other flavonoids. The roots synthesized more UV-absorbing compounds than shoots; coumarin, aesculetin, scoparone and various phenolic acids were identified in the root extracts [30].

The prolonged exposure of enhanced UV-B radiation to seven-year-old Norway spruce seedlings caused significant decrease in chlorophyll *a* and *b* content. This decrease was mainly caused by the decrease in chlorophyll *a* content (up to 21%). This decrease in photosynthetic pigment caused reduced photosynthetic gas exchange. The antenna size also reduced due to long-term exposure to UV-B radiation [31]. Concentration of foliar phenolics increased in populus when exposed to enhanced UV-B radiation [32]. Exposure of *Leucadendron laureolum* seeds to UV-B radiation resulted in decreased seed germination, which was associated with decreased foliar chlorophyll *a* and carotenoid levels in the seedlings [33].

One important conifer species, loblolly pine has been shown to be particularly sensitive to UV-B radiation. Loblolly pine responded to UV-B radiation under a full solar spectrum by increasing needle flavonoid content [10]. Two-year-old tissue had higher foliar flavonoids content under enhanced UV-B radiation in *Quercus rubra, Pinus ponderosa* and *Psedotsuga menziesii* under field condition [32].

Plant parts in addition to leaves, accumulate specific compounds in response to UV- radiation exposure [34]. Concentration of sucrose, raffinose and glucose in the bark of silver birch were higher in UV treated saplings than in saplings grown in ambient radiations. Saplings grown in the elevated UV-A + UV-B radiations had significantly increased concentration of certain UV-B absorbing phenolics, such as salidroside, 3,4 -dihydroxypropiohenone-3-glucoside, (+)- catechin and (-)-epicatechin compared with saplings grown in ambient radiation.

6. GROWTH AND DEVELOPMENT

The timing of life phases of plants is a combination of response to environmental factors and the genetic constitution of the plant. UV-B exposure can alter the timing of events such as flowering, entering and breaking of dormancy, and even senescence is important not only to the individual plant, but to also how plants

interact with other plants. For example, a shift in the timing of flowering can mean that a plant species may not have sufficient insect pollinators available at the new time of flowering because either the insects are not present or other plant species are attracting these pollinators. Such changes could also conceivably be important in agricultural systems, but intervention with management options may make these changes less important. Increased UV-B may advance or delay (depending on species) the time of flowering in tree species. There is little work at present on flowering responses and virtually nothing on other potential effects of UV-B on life phase timing of tree species.

Exposure of *Populus* clones to near ambient UV-B radiation caused shift in allocation of carbon from leaf development to other pools probably to the UV absorbing compounds (flavonoids derivatives). Such reallocation curtails leaf development and reduces photosynthetic capacity compared to subambient levels of UV-B radiation and affect growth over long duration [35]. In *Populus deltroids* trees when grown under enhanced UV-B radiation, height, diameter and biomass reduced significantly, but shoot to root ratio increased [36].

Five-year-old European beech trees were exposed to increased UV-B radiation and ambient UV-B radiations. The increased level of UV-B had adverse effect on net photosynthesis (Pn), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm) and accelerated senescence measured as yellowing of leaves [37]. Significant reduction in plant height, leaf area and shoot dry weight was observed when *Salvia splendens* plants were grown under enhanced UV-B radiation level. Reduction in leaf area ratio and simultaneous increase in specific leaf area was also observed due to enhanced UV-B radiation, which indicated that UV-treatment might have induced significant accumulation of photosynthates in leaf blades [38].

Leaf anatomical changes are important in determining the degree of injury sustained by plants exposed to natural and enhanced levels of UV-B radiation. The degree to which leaf anatomy changes due to increased UV levels is poorly understood in most of the tree species. Four tree species were exposed to enhanced UV- levels and examined for change in leaf anatomy. *Populus tricocarpa,* an intermediate broad leaf species, showed significantly thicker palishade parenchyma in recently matured leaves and increased leaf area. *Quarcus rubra,* a semi determinate broad leaf species, exhibited significantly thicker palisade parenchyma as compared to control. *Psuedotisuya menziesii* a coniferous species with flattened needles, and *Pinus ponderosa,* an evergreen coniferous species with a complete hypodermis, showed no significant changes in the leaf area or specific leaf weight under enhanced UV-B radiation [39].

Loblolly pine is the leading commercial species in the south-eastern United States and accounts for a large portion of pulp producing capacity. Loblolly pine is one of the most susceptible species to UV-B radiation. When loblolly pine seedlings were exposed to enhanced UV-B radiation a reduction of 40 and 16%, respectively was observed in biomass and height [10, 40]. In Douglaus fir seedlings, increased lateral branching was noticed [41]. Studies have demonstrated deleterious effects of UV-B radiations on tree growth and physiology [42]. Shift in dry matter allocation towards roots contributed to lower shoot / root and leaf area ratio in UV-B treated silver birch seedlings [43]. However, UV-B radiation inhibited the growth of main

roots and stems, decreased number of lateral roots and leaf number as well as leaf area in *Psychotria rubra, Schefflera octophylla, Archidendron clypearia, Pterospermum heterophyllum, Sapium discolor* and *Albizia lebbeck* seedlings [23].

7. MINERAL NUTRITION

Uptake and translocation of mineral nutrients within the plant can be affected by elevated UV-B radiation. Consequently, the mineral nutrient status of plants can affect plant responsiveness to UV-B radiation [44, 45, 46]. Nitrogen concentration in plant tissues can increase under elevated UV-B, which is linked with reduced insect herbivory [47, 48].

Growth and pigment accumulation of plants exposed to UV-radiation are dependent upon the availability of mineral nutrients. Young scot pine seedlings grown at different nutrient levels were exposed to UV-A and UV-B radiation. The growth and the accumulation of photosynthetic pigments were positively affected by the availability of nutrients, while the accumulations of condensed tannins and its precursor, (+)- catechin decreased significantly under high nutrient level. Nutrient level did not markedly affect the total accumulation of flavonoid [49]. Silver birch seedlings when exposed to enhanced UV-B radiations, a marked reduction in nitrogen allocation to leaves and increased allocation of phosphorus to roots was noticed [43].

Tosserams *et al.* [50] reported that an increase in carbon accumulation in nutrient stressed plants led to a reduction in UV-B induced damages because of increased foliar UV-B absorbance by enhanced accumulation of phenolic compounds and leaf thickening. Total biomass production of plants at low nutrient supply was 50% lower as compared to plants grown at high nutrient supply, while net photosynthesis decreased only by 12%. Increased levels of UV-B reduced biomass production under non-limiting nutrition conditions only and there was no effect on the plants grown under limiting nutrient condition. This response was associated with accumulation of carbohydrates under nutrient limited nutrition condition.

8. PHOTOSYNTHESIS

Effects of UV radiation, particularly those of the biologically more active UV-B radiation on trees [51, 52] and specifically on photosynthesis [53, 54] have been extensively investigated. Studies with artificial UV-B sources have shown that UV-B radiation may strongly affect PSII, whereas PSI appears to be relatively insensitive [53]. Reductions in photosynthetic rate and capacity have been described for a number of tree species. Experiments have shown that as little as 16 per cent decrease in current ozone level could result in 40 per cent reduction in the productivity of Loblolly pine. Similar effects are reported for jackpine, spruce, sycamore, birch and ash; however, the effects are normally more severe in conifers than in deciduous species. Absorption of light in excess of photosynthetic utilization by green plant leaves may lead to a reduction in the potential efficiency of PSII, which persists in low light or darkness and is regarded as the major cause of

photoinhibition of photosynthesis [55]. It has been shown for many plant species that photoinhibition of photosynthesis does occur under natural conditions [56]. In studies with tropical plants, substantial reductions in the potential efficiency of PSII, indicated by a decline in the "dark-adapted" ratio of variable to maximum chlorophyll (Chl) *a* fluorescence (F_v/F_m) have been observed upon direct exposure to the solar beam of outer crown leaves of mature forest trees [57] and of plants situated in treefall gaps within the tropical forest [58, 59].

UV-B photons inactivate PSII via non–interacting mechanism, which affects different target sites. When UV-B radiation is accompanied by low intensity visible light, which provides protection against photodamage results in synergistically enhanced protein repair capacity and protects PS- II damage. However, this ameliorating effect becomes insignificant at high light intensities characteristic of direct sunlight [60].

UV-B radiation inhibited CO_2 assimilation when seedlings of *Anacardium exculsum, Virola surinamensis* and *Colophyllum longifalium* were exposed to direct sunlight as compared to UV-B filtered sunlight. The rate of photosynthetic uptake by the leaves was affected which occurred due to inhibition of potential PSII efficiency. The exposure to UV-B radiation also decreased the efficiency of P 700 photooxidation [61]. However, electron transport, RuBisCO activity and enzymes from the Calvin cycle were highly sensitive to UV-B radiation along with low temperature stress in Norway spruce trees, which affected the CO_2 fixation level adversely [62].

The UV-A component of sunlight is also a significant damaging factor for photosynthesis, which targets PSII complex. UV-A radiation results in rapid inhibition of oxygen evolution accompanied by the loss of signal from the S2 state of the water-oxidizing complex. UV-A irradiance also affects the relaxation of flash induced variable chlorophyll fluorescence. The primary damage site of UV-A irradiance is the catalytic manganese cluster of the water-oxidizing complex, where electron transfer to Tyr-Z^0 and $P680^+$ is inhibited. The damaging mechanism of UV-A radiation is very similar to that induced by the shorter wavelength UV-B radiation, but different from photosynthetically active longer wavelength radiation (400-700 nm) [63]. Primary damaging site of UV-B is the water-oxidizing complex. It also induces degradation of D1 and D2 protein subunits of PSII reaction center [62].

Sun facing leaves from the outer crown of the mature trees are less susceptible to UV- stress in comparison to leaves in shade [64]. Photoinhibition of PSII occurred when shade leaves of tropical trees were exposed to UV- light. Substantially less inhibition of PSII occurred when UV-B radiation was excluded and was further reduced when both UV-B and UV-A were excluded [65]. The effects of long-term UV-B radiation exposure on the growth and morphology of woody perennials, the gas exchange and photosynthesis of five common deciduous tree species (*Fraxinus excelsior, Betula pendula, Tilia cordata, Quercus robur* and *Acer pseudoplatanus*) were measured. All five tree species had been exposed to UV-B radiation for 5 years in the field at an enhancement level equivalent to 18% ozone depletion. Measurements made during the fifth year of UV-B irradiation recorded reductions in light-saturated photosynthesis, transpiration and water use efficiencies. These

changes were accompanied by marked reductions in individual leaf area, stomatal density and conductance and carboxylation efficiency. There were no significant changes in the maximum variable fluorescence ratio, the quantum requirement of oxygen evolution, or light-saturated O_2 production. Analysis of the response of net carbon assimilation to changing intercellular CO_2 concentration (A/ci response) demonstrated no significant change in stomatal limitation. Reductions in photosynthesis were consistent with decreased carboxylation efficiency [66].

Krause *et al.* [64] studied the effect of UV-A and UV-B on the leaves of tropical trees. The ambient UV-B and UV-A radiation, observed upon exposure to full, direct sunlight contributed to the reversible decline in potential PSII efficiency. Sensitivity of PSII to natural UV-B light depended upon the acclimation status and developmental stages of the leaves and tended to decline with increased levels of vacuolar UV-absorbing compounds. In shade-grown tree seedlings periodically exposed to full sunlight, protein damage was indicated by the strong effects of ambient PAR and UV light on F_v/F_m and fluorescence induction kinetics, and by the extremely slow recovery. Leaves of the crown of mature trees that are acclimated to full sunlight may achieve full protection against adverse effects of UV on PSII. But at certain stages of development, or depending on the prior light environment (incomplete acclimation), ambient UV light may cause temporary losses in PSII efficiency. Although tropical plants are capable of effectively protecting their leaves against UV stress, an anthropogenic rise in ambient UV-B radiation in the tropics may markedly affect their PSII efficiency.

9. UV RADIATION AND OTHER STRESS FACTORS

Plants and other organisms in nature are seldom affected by only a single stress factor, such as UV-B radiation. Instead, they typically respond to several factors acting in concert, such as water stress, increased atmospheric CO_2, mineral nutrient availability, heavy metals, tropospheric air pollutants and temperature. Therefore, it is important to keep in mind that the effectiveness of UV-B radiation can be greatly increased or decreased by such factors. Visible radiation is an important ameliorating factor and thus, natural levels as possible should be applied in laboratory experiments for attaining realistic results.

10. WATER STRESS

Among the most common of factors in nature is water stress. Interactions between UV-B radiation and water status of plants also occur. Elevated UV-B radiation in field experiments tended to alleviate drought symptoms in two Mediterranean pine species [67, 68]. UV-B radiation acts as an environmental signal to induce tolerance to high light drought stress. Douglas-fir seedlings grown with 6 kJm^{-2} day^{-1} of biologically effective UV-B, which is ambient for the inter mountain regions of Idaho, USA, have higher light saturated carbon assimilation rates than seedlings grown without UV-B radiation. UV-B radiation along with drought stress

(withholding water for 15 days) had 50% high light saturated carbon assimilation rate and 40% high seedlings water potential [69].

11. INCREASED CO_2 CONCENTRATION

Increases of atmospheric CO_2 are a certain element of global climate change and atmospheric CO_2 concentration will likely double by the middle of the next century [70]. Many experiments with elevated CO_2 employ a twice-ambient CO_2 concentration as a treatment condition. Such a doubling often results in more pronounced plant responses than are evident in many elevated UV-B radiation lamp experiments designed to simulate up to 20% ozone column reduction under field conditions. However, responses to CO_2 are small in semi-natural ecosystems where nutrient or water availability may strongly constrain plant growth. For example, Gwynn-Jones *et al.* [71] showed that growth responses to elevated CO_2 and enhanced UV-B (both alone and in combination) were small during the first three years of experimentation in a sub-arctic heath. Also, most ecosystem-level effects of elevated CO_2 are mediated through changes in plant tissues. When studied independently, plant growth responses to changes in UV-B radiation and atmospheric CO_2 concentration generally are thought to be in opposite directions. Usually, in most experiments employing both elevated CO_2 and UV-B radiation, these factors do not yield interactions, with some exceptions [72, 73]. Elevated CO_2 sometimes appears to provide some protection against elevated UV-B radiation for some species; yet, elevated UV-B radiation can limit the ability of some species to take advantage of elevated CO_2 in photosynthesis. Allocation of biomass in plants can also change in a complicated fashion with the combination of CO_2 and UV-B radiation treatments [73]. Increased temperature is also a predicted element of global climate change. In a study combining two levels of UV-B radiation with two levels of CO_2 and two temperatures, the results indicated that either elevated CO_2 or somewhat high temperature had similar effects in reducing the growth-inhibiting effects of elevated UV-B radiation [74].

In trees, the allocation of carbon to secondary metabolites has been shown to be determined by both the availability of resources (e.g., CO_2 concentration) and by specific stress factors (e.g., UV radiation). In combination, CO_2 and UV-B radiation may differentially affect tree growth and morphogenic parameters, and elevated CO_2 may ameliorate the effects of UV-B radiation. The effects of increased atmospheric CO_2 concentration and UV-B radiation on growth and the accumulation of different types of secondary metabolites were studied in silver birch (*Betula pendula*). Seedlings were exposed to 350 and 700 μmol mol^{-1} of CO_2 in a greenhouse. At both CO_2 levels, additional UV-B was either present (8.16 kJ m^{-2} day^{-1} of biologically effective UV-B irradiance) or absent. The time course of accumulation of individual secondary compounds and the shifts in allocation of carbon between biomass and the secondary metabolites (phenolic acids, flavonoids, condensed tannins) were studied during a month-long exposure. The activities of enzymes (L-phenylalanine ammonia-lyase [PAL], EC 4.3.1.5; peroxidase, EC 1.11.1.7; polyphenol oxidase, EC 1.10.3.1) were also determined for leaves. UV-B radiation significantly increased biomass, PAL activity, and the accumulation of phenolic acids and flavonoids in

seedlings. Elevated CO_2 concentration increased the activities of all the enzymes and the accumulation of condensed tannins in leaves, especially with UV-B radiation. Because the observed UV-B induction of flavonoids was small under a high CO_2 concentration, it was suggested that the excess of carbon in the atmosphere might moderate the effect of UV-B by increasing the metabolic activity of leaves (high enzyme activities) and changing the allocation of internal carbon between different primary and secondary metabolites in the plant [75].

12. OXIDATIVE STRESS

The nature and origin of the reactive oxygen species (ROS) are involved in the early part of UV-B-induced signalling pathway. The increase in PR-1 transcript and decrease in Lhcb transcript in response to UV-B is mediated through pathways involving hydrogen peroxide derived from superoxide (O^{-2}). The origins of the ROS were also shown to be distinct and to involve NADPH oxidase and peroxidase(s). The up-regulation of Chs by UV-B was not affected by ROS scavengers, but was reduced by inhibitors of nitric oxide synthase (NOS) or NO scavengers. This suggests that UV-B exposure leads to the generation of ROS, from multiple sources, and NO through increased NOS activity, giving rise to parallel signalling pathways mediating responses of specific genes to UV-B radiation [76].

Four-year-old Scotts pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) seedlings potted in natural soil were exposed in phytochambers to fluctuating O_3 concentrations and UV-B radiation. After one growing season, current-year needles were collected and analysed for activities of antioxidative enzymes (superoxide dismutase, SOD, EC 1.15.1.1; catalase, CAT, EC 1.11.1.6; guaiacol peroxidase, POD, EC 1.11.1.7) and soluble antioxidants (ascorbate and glutathione). CAT, POD, ascorbate and glutathione, but not SOD, were increased in needles of both species in response to twice-ambient O_3 levels. UV-B radiation in the presence of ambient O_3 caused an increase in total SOD activity in spruce but had no effect on antioxidants in pine. Twice ambient O_3 levels together with low UV-B radiation counteracted the O_3 induced increases in ascorbate and CAT in pine but not in spruce. Under these conditions, spruce needles showed the highest antioxidative protection and revealed no indication of lipid peroxidation. Pine needles exposed to UV-B and elevated O_3 levels showed elevated lipid peroxidations and a 5-fold increase in dehydro-ascorbate, suggesting that this species was less protected and suffered higher oxidative stress then spruce [77].

Active oxygen formation (AOS) increased in the embryos of *Fucus spiralis* exposed to enhanced UV-B. Two photoinhibition responses were recognized: (i) a rapid decline of the PSII yield due to the violoxanthin-zeaxanthin cycle (photoprotection), and (ii) a slower second-phase decline, correlated with active oxygen production. The electron transport rate increased with embryo age, and was correlated with AOS production. Enhanced AOS production was associated with slow recovery of the PSII yield, with increased effective UV dose [78].

13. INSECTS

The extent to which plant tissues are consumed by insects or the degree to which pathogens attack plants is regulated by several properties of the plant host tissues. Experiments show that present-day solar UV-B radiation can substantially reduce insect herbivory of agricultural and native plant foliage [48, 79]. Field studies involving supplementation of solar UV-B radiation with lamp systems indicated a substantial reduction in populations of a herbivorous insect on a heathland plant [80]. The reasons for these changes are not always clear, but they may be mediated through changes in plant secondary chemistry or alterations in plant nitrogen or sugar content. Studies involving UV lamps indicated decreased herbivory by a moth caterpillar under elevated UV-B radiation and this was attributed to increases of host pea plant tissue nitrogen content [47]. Mulberry plants previously irradiated with UV from lamps suffered less herbivory by silkworms (*Bombyx mori*) and the low consumption was attributed to low sucrose content of the foliage [81]. In laboratory, studies McCloud and Berenbaum [82] have shown that UV-B radiation can increase furanocoumarin content of plant tissue, which, in turn, results in slow development of certain insect larvae during early life stages of the larvae. Thus, the foregoing would suggest that insect herbivory might always be decreased by UV-B radiation. The results of most of these studies indicate that the effects on insect herbivory are all due to biochemical changes in the host plant tissues. However, there are some indications that some insects may respond directly to solar UV-B radiation.

14. PLANT PATHOGEN

Plant fungal and viral diseases react in several different ways to UV-B radiation in various experiments, conducted primarily in laboratory and greenhouse conditions. In four of ten studies, UV-B was found to counteract disease severity and in the other six studies, it promoted disease development [83]. The direction of the UV-B radiation effect on disease severity can also vary with the variety of the host. It is not clear in many of these experiments whether the changes in disease severity are due simply to changes caused by UV-B radiation in the host plant, or direct UV-B radiation effects on the fungal or viral pathogens was involved. Plants exposed to UV-B radiation prior to infection were more susceptible to subsequent infection by fungal pathogens, while UV-B irradiation after infection had no effect on disease severity [84]. Such an experiment suggests that the effect of UV-B radiation is mediated through changes in the host plant tissues. There is also evidence from solar UV-B exclusion studies that incidence of fungal disease increases when UV-B is removed [85].

Pedunculate oak (*Quarcus robur*) saplings were exposed to ambient levels of UV-A and UV-B radiation and controlled solar radiation. The effect on leaf fungi was examined weekly over a four month-period using two techniques; the spore fall and leaf impression methods, which differentiated between those fungi occurring on the upper (adaxial) and lower (abaxial) surfaces of the leaves. The abundance of *Aureobasidium pulluluns* and *Sprobololymes roseus,* two leaf yeasts which had adaxial: abaxial ratio of <1 under ambient levels of UV-B radiations was negatively

correlated with increasing ambient level of UV-B radiations and reduced significantly on adaxial leaf surfaces by the supplemental UV-B radiation. *Alternaria* and *Monographelle nivalis* showed consistent responses on adaxial surfaces to UV-A radiation applied over control + treatment array [86]. These results suggest that the current levels of shortwave radiation already influence the distribution of fungi on leaf surfaces and further increase will have more detrimental effect on leaf fungi distribution.

These changes in insect herbivory and disease severity caused by alterations of solar UV-B can be sizeable; they can operate in different directions and have very important implications for both agricultural and non-agricultural ecosystems. They may be much more important than known influences of UV-B radiation on plant production based on realistic field studies. Even roots of plants whose shoots are exposed to elevated UV-B radiation can be affected as indicated by root interactions with microorganisms. For example, the nature of microorganism assemblage associated with roots of sugar maple trees (*Acer saccharum*) was altered by exposure of the tree shoots to elevated UV-B radiation [78]. This was obviously a systemic effect of UV-B expressed in the roots of the host plant.

15. INTERACTION BETWEEN SPECIES

The primary plant productivity in forests and grasslands may not be greatly affected by ozone reduction even if the growth of some plants is diminished. The plant species differ greatly in growth responsively to UV-B. It is anticipated that a productivity reduction of one species will probably lead to increased productivity of another relatively more UV-tolerant species. This is likely because more resources will be available to the tolerant species. Thus, the overall productivity of the system may well remain about the same, while species composition may change. However, a change in the balance of species could have far-reaching consequences for the character of many ecosystems.

Another mechanism whereby the competitive balance of plant species can be changed by increased UV-B is through changes in plant form. Even if plant production *per se* is not affected by increased UV-B, changes in plant form can result in changes in which species can more effectively compete for sunlight. A quantitative analysis of competition for sunlight in the mixed stands with and without supplemental UV-B showed that subtle changes in plant form of the two species were sufficient to change the balance of competition for sunlight that is necessary for photosynthesis [87]. Therefore, one species can achieve some advantage over the other because one captures more sunlight for photosynthesis. In these experiments, the wheat benefited from increased UV-B and weed suffered. However, in other mixtures of crop and weeds, the situation might be reversed. Also, other changes in plant form, such as greater allocation of biomass to roots might change competitive effectiveness of individual species for soil moisture and nutrients. In grasslands and forests that are not managed intensively, similar changes in species composition may be experienced. Ecosystem-level experiments with nonagricultural systems are only beginning. Early reports of one experiment in a

subarctic heath ecosystem suggest that species composition changes may result from UV-B supplementation [88].

16. IMPACT OF UV-B AT DIFFERENT LATITUDES AND ALTITUDES

UV-B irradiance is naturally very low in high-latitude ecosystems, such as tundra and sub arctic areas. There is experimental evidence that the plants in such systems react to increased UV-B associated with realistic levels of ozone depletion. Some plant species exhibit growth inhibitions and others do not, thus, eventually altered community composition is expected [48, 88, 89]. Long-term observations of species composition are being pursued in high-latitude subarctic systems in Sweden, a high arctic site on Spitzbergen Island and in southernmost Argentina. In the latter system, attenuating the naturally occurring solar UV-B radiation increased insect herbivory, decreased plant tissue nitrogen concentrations and increased populations of some microfauna [48, 89]. The subarctic studies in Sweden have been underway for years and show various effects including decreased litter decomposition [29], increased fruit formation and greatly increased insect herbivory [71].

Although terrestrial ecosystems at high latitudes are not highly productive for grazing, timber production, etc., the influence of ozone reduction on these systems is important for several reasons. Carbon sequestration is generally quite high in these ecosystems, including the extensive peat formations that are also being studied in the Swedish subarctic and southern Argentinean systems. Compared with other locations, these ecosystems are under the greatest ozone depletion, especially in the Southern Hemisphere, and they also experience the greatest warming as the global greenhouse effect intensifies. Thus, they are sensitive indicators of several features of climate change.

In British Columbia and Canada, greenhouse and growth chamber experiments were conducted to determine the responses of tree seedlings to increased UV-B levels and geographic variations and also to estimate the risk of damage to various species. Seedlings grown with increased UV-B levels showed pronounced changes in morphology, growth, and stress tolerance. UV-B exposure was more effective on low- than high-elevation seed sources. The relative ranking, most sensitive to most resistant, of the seedlings based on the effects of increased UV-B radiation was: *Betula papyrifera* > *Larix occidentalis* > *Tsuga heterophylla* > *Picea engelmannii* > *Pseudotsuga menziesii* var. menziesii > *Thuja plicata* > *Chamaecyparis nootkatensis* > *Picea sitchensis* > *Picea glauca* x engelmannii > *Abies grandis* > *Pseudotsuga menziesii* var. glauca and > *Pinus contorta* var. latifolia [90].

REFERENCES

1. Diffey, B.L. (1991) Solar ultraviolet radiation effect on biological systems. Review in *Phys. Med. Biol.* **36:** pp 299-238.
2. Fredrick, J.E. (1990) Trends in atmospheric ozone and ultraviolet radiation: mechanisms and observations for the Northern Hemisphere. *Photochem. Photobiol.* **51,** 757-763.
3. Farman, J.C. Gardiner, B.G. and Shaklin, J.D. (1985) Large losses of total ozone in Antactica reveal seasonal ClOxNOx interaction. *Nature* **315,** 207-210.

4. Madronich, S. McKenzie, R.L., Caldwell, M.M. and Björn, L.O. (1995) Changes in ultraviolet reaching the earth's surface. *Ambio*. **24,** 143-152.
5. Fredrick, J.E., Snell, H.E. and Haywood, E.K. (1989) Solar ultraviolet radiation at the earth's surface. *Photochem. Photobiol.* **50,** 443-450.
6. Paltridge, G.W. and Barton, I.J. (1978) Erythemal ultraviolet distribution over Australia-the calculations, detailed results and input data. *Division of Atmospheric Physics Technical Paper* 33 (Austalia: Commonwealth Scientific and Industrial Research Organisation).
7. Cutchis, (1980) A formula for comparing annual damaging ultravilet (DUV) radiation doses at tropical and mid-latitude sites. *Final Report* FAA-EE 80-21 (Washington, DC: US Department of Transportation, Federal Aviation Administration Office of Environment and Energy).
8. Doda, D.D. and Green, A.E.S. (1980) Surface reflection measurements in the UV from airborne plateform. Part 1 *Appl. Opt.* **19,** 2140-2145.
9. Doda, D.D. and Green, A.E.S. (1980) Surface reflection measurements in the UV from airborne plateform. Part 2 *Appl. Opt.* **20,** 636-642.
10. Teramura, A.H. and Sullivan, J.H. (1991) Potential impacts of increased solar UV-B on global plant productivity. In *Photobiology* (Riklis, E. ed). New York: Plenum Press, pp. 625-634.
11. Kaufmann, M. R. (1978) The effect of ultraviolet (UV-B) radiation on Engelmann spruce and lodgepole pine seedlings. UV-B Biological and Climatic Effects Research (BACER). *Final Report* EPA-IAG-D6-0168. EPA, Washington, DC.
12. Vu, C.V., Allen, L.H. and Garrad, L.A. (1982) Effect of supplemental UV-B radiation on primary photosynthetic carboxilating enzyme and soluble proteins in leaves of C_3 and C_4 crop plants. *Physiol. Plant* **55,** 11-16
13. Vu, C.V., Allen, L.H. and Garrad, L.A. (1983) Effect of enhanced UV-B radiation (280-320 nm) on ribulose-1,5-bisphosphate carboxilase in pea and soybean. *Environ. Exp. Bot.* **24.** 131-143.
14. Shomansurov, S. (1981) Natural regulators in bean growing at high mountain conditions. *Sov. Plant Physiol.* **28,** 122-125.
15. Harm, W. (1980) *Biological Effects of Ultraviolet Radiation* (Cambridge: Cambridge University Press) pp 687-712.
16. Kulandaivelu, G. and Nooruden, A.M. (1983) Comparative study of the action of ultraviolet-C and ultraviolet-B radiation on photosynthetic electron transports. *Phsiol. Plant* **58,** 389-394.
17. White, A.L. and Jahnke, L.S. (2002) Contrasting effects of UV-B on photosynthesis and photoprotection of β-carotene in two *Dunaliella* spp. *Plant Cell Env.***43,** 877-884.
18. Freifelder, D. (1987) *Moleculer Biology* 2nd edn (Boston: Jones and Bartlett) pp. 277-292.
19. Ries, G., Heller, W., Puchta, H ultraviolet., Sandermann, H., Seldlitz, H.K. and Hohn, B. (2000) Elevated UV-B radiation reduces genome stability in plants. *Nature* **406,** 98-101.
20. Zinser, C., Jungblut, T., Heller, W., Seidlitz, H.K., Schnitzler, J.P., Ernst, D. and Sandermann, H. (2000) The effect of ozone in Scots pine (*Pinus sylvestris* L.): Gene expression, biochemical changes and interactions with UV-B radiation. *Plant Cell Env*. **23,** 975-982.
21. Suesslin, C. and Frohnmeyer, H. (2003) An *Arabidopsis* mutant defective in UV-B light-mediated responses. *Plant J.* **33,** 591-601.
22. Brosche, M. and Strid, A. (2003) Molecular events following perception of ultraviolet-B radiation by plants. *Physiol. Plant.* **117,** 1-10.
23. Zhu, S.Q., Xu, X.M., Chen, Z.H., Li, S.S. and Zhang, D.M. (2002) Effect of UV-B radiation on seedling growth and chloroplast ultrastructure changes in some woody plants *J. Trop. Subtrop. Bot*. **10,** 235-244.
24. Britt, A. and Fiscus, E.L. (2003) Growth responses of Arabidopsis DNA repair mutants to solar irradiation. *Physiol. Plant*. **118,** 183-192.
25. Lavola, A., Aphalo, P.J., Lahti, M. and Tiitto, R.J. (1998) Nutrient availability and the effect of increasing UV-B radiation on Scots pine. *Env. Exp. Bot*. **94,** 49-60.
26. Jungblut, T.P., Schnitzler, J.P., Heller, W., Hertkorn, N., Sandermann, H. and Brouillard, R. (1994) Polyphenols 94, 17th Intrnational Confreance, Palma de Mallorea, Spain, Eds. M. Jay and A. Scalbert 411-412.
27. de-la Rosa, T.M., Julkunen-Tiitto, R., Lehto, T. and Aphalo, P.J. (2001) Secondary metabolites and nutrient concentrations in silver birch seedlings under five levels of daily UV-B exposure and two relative nutrient addition rates. *New Phytol*. **150,** 121-131.

28. Liakoura, V., Manetas, Y. and Karabourniotis, G. (2001) Seasonal fluctuations in the concentration of UV-absorbing compounds in the leaves of some Mediterranean plants under field conditions. *Physiol. Plant*. **111,** 491-500.
29. Gehrke, C., Johanson, U., Callaghan, T., Chadwick, D. and Robinson, C.H. (1995) The impact of enhanced ultraviolet-B radiation on litter quality and decomposition processes in *Vaccinium* leaves from the Subarctic. *Oikos*. **72,** 213-222.
30. Flint, S.D. and Caldwell, M.M. (1998) Solar UV-B and visible radiation in tropical forest gaps: measurements partitioning direct and diffuse radiation. *Global Change Biol*. **4,** 863-870.
31. Sprtova, M., Marek, M.V., Nedbal, L., Prasil, O. and Kalina, J. (1999) Seasonal changes of photosynthetic assimilation of Norway spruce under the impact of enhanced UV-B radiation. *Plant Sci.* **142,** 37-45.
32. Warren, J.M., Bassman, J.H., Mattinson, D.S., Fellman, J.K., Edwards, G.E. and Robberecht, R. (2002) Alteration of foliar flavonoid chemistry induced by enhanced UV-B radiation in field-grown Pinus ponderosa, Quercus rubra and Pseudotsuga menziesii. *J. Photochem. Photobiol*. **66,** 125-133.
33. Musil, C.F. and Wand, S.J.E. (1994) Differential stimulation of an arid-environment winter ephemeral *Dimorphotheca pluvialis* (L.) Moench by ultraviolet-B radiation under nutrient limitation. *Plant Cell Env.* **17,** 245-255.
34. Tegelberg, R., Aphalo, P.J. and Julkunen-Tiitto, R. (2002) Effects of long-term, elevated ultraviolet-B radiation on phytochemicals in the bark of silver birch (Betula pendula). *Tree Physiol*. **22,** 1257-1263.
35. Schumaker, M.A., Bassman, J.H., Robberecht, R. and Radamaker, G.K. (1997) Growth, leaf anatomy, and physiology of Populus clones in response to solar ultraviolet-B radiation. *Tree Physiol*. **17,** 617-626.
36. Bassman, J.H., Robberecht, R. and Edwards, G.E. (2001) Effects of enhanced UV-B radiation on growth and gas exchange in *Populus deltoides* Bartr. ex Marsh. *Intl. J. Plant Sci*. **162,** 103-110.
37. Zeuthen, J., Mikkelsen, T.N., Muller, G.P. and Ro Poulsen, H. (1997) Effects of increased UVB radiation and elevated levels of tropospheric ozone on physiological processes in European beech (*Fagus sylvadca*). *Physiol. Plant*. **100,** 281-290.
38. Lercari, B., Caruso, E., Granmm, A., Pardossi, A. and Bertram, L. (1989) The use of UV radiation to control the architecture of Salvia splendens plants. III influence of the time of the year on plant sensitivity to UV treatments. *Agr. Medit*. **128,** 266-272.
39. Nagel, L.M., Bassman, J.H., Edwards, G.E., Robberecht, R. and Franceshi V.R. (1998) Leaf anatomical changes in Populus trichocarpa, Quercus rubra, Pseudotsuga menziesii and Pinus ponderosa exposed to enhanced ultraviolet radiation. *Physiol. Plant*. **104,** (1998) 385-396.
40. Walker, L.C. (1980) *Regional Silviculture of the United States* (Barrett, J.W. ed) Washington DC, John Wiley and Sons, pp. 5645-5646.
41. Bassman, J.H., Edwards, G.E. and Robberecht, R. (2002) Long-term exposure to enhanced UV-B radiation is not detrimental to growth and photosynthesis in Douglas-fir. *New Phytol*. **154,** 107-120.
42. Sullivan, J.H. and Teramura, A.H. (1989) The effects of ultraviolet-B radiation on loblolly pine: 1. Growth, photosynthesis and pigment production in greenhouse-grown saplings. *Physiol. Plant*. **77,** 202-207.
43. de la Rosa, T.M., Aphalo, P.J. and Lehto, T. (2003) Effects of ultraviolet-B radiation on growth, mycorrhizas and mineral nutrition of silver birch (*Betula pendula* Roth) seedlings grown in low-nutrient conditions *Global Change Biol*. **9,** 65-73.
44. Murali, N.S. and Teramura, A.H. (1985) Effects of ultraviolet-B irradiance on soybean. VII. Biomass and concentration and uptake of nutrients at varying P supply. *J. Plant Nutrition* **8,** 177-192.
45. Ros, J. (1995) First European Symposium on the Effects of Environmental UV-B Radiation, Eds. H. Bauer and C. Nolan, Brussels, Luxembourg, 245-252.
46. Musil, C.F. and Wand, S.J.E. (1994) Differential stimulation of an arid-environment winter ephemeral *Dimorphotheca pluvialis* (L.) Moench by ultraviolet-B radiation under nutrient limitation. *Plant Cell Environ*. **17,** 245-255.
47. Hatcher, P.E. and Paul, N.D. (1994) The effect of elevated UV-B radiation on herbivory of pea by *Autographa gamma*. *Entomol. Exp. Applicata* **71,** 227-233.
48. Rousseaux, M.C., Ballaré, C.L., Scopel, A.L., Searles, P.S. and Caldwell, M.M. (1998) Solar ultraviolet-B radiation affects plant-insect interactions in a natural ecosystem of Tierra del Fuego (southern Argentina). *Oecologia* 228-235.

49. Lavola, A., Aphalo, P.J., Lahti, M. and Tiitto**,** R.J. (2003) Nutrient availability and the effect of increasing UV-B radiation on Scot pine. *Env. Exp. Bot.* **49,** 49-60.
50. Tosserams, M., Smet, J., Magendans, E., Rozema, J. and Rozema, J. (2001) Responses of plants to UV-B radiation, *Adv. Vegetation Science*, **18,** 159-168.
51. Rozema, J., Van de Staaij, J., Björn, L.O. and Caldwell, M. (1997) UV-B as an environmental factor in plant life: Stress and regulation. *Trends Ecol. Evol*. **12,** 22-28.
52. Jansen, M.A.K., Gaba, V. and Greenberg, B.M. (1998) Higher plants and UV-B radiation: balancing damage, repair and acclimation *Trends Plant Sci*. **3,** 131-135.
53. Teramura, A.H. and Ziska, L.H. (1996) Ultraviolet-B radiation and photosynthesis: Photosynthesis and the Environment, Ed. N.R. Baker, Kluwer Academic Publishers, Dordrecht, The Netherlands, 435-450.
54. Allen, D.J., Nogués, S. and Baker, N.R. (1998) Ozone depletion and increased UV-B radiation: Is there a real threat to photosynthesis? *J. Exp. Bot*. **49,** 1775-1788.
55. Baker, N.T. and Bowyer, J.R. (1994) *Photoinhibition of photosynthesis*. *From Molecular Mechanisms to the Field*, Oxford: Bios Scientific Publishers, pp. 239-254.
56. Long, S.P., Humphries, S. and Falkowski, P.G. (1994) Photoinhibition of photosynthesis in nature. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 633-662.
57. Krause, G.H., Virgo, A. and Winter, K. (1995) High susceptibility to photoinhibition of young leaves of tropical forest trees. *Planta* **197,** 583-591.
58. Krause. G.H. and Winter, K. (1996) Photoinhibition of photosynthesis in plants growing in natural tropical forest gaps. A chlorophyll fluorescence study. *Bot. Acta* **109,** 456-462.
59. Thiele, A., Krause, G.H. and Winter, K. (1998) *In situ* study of photoinhibition of photosynthesis and xanthophyll cycle activity in plants growing in natural gaps of the tropical forest. *Aust. J. Plant Physiol*. **25,** 189-195.
60. Sicora, C., Mate, Z. and Vass, I. (2003) The interaction of visible and UV-B light during photodamage and repair of Photosystem II. *Photosynth.. Res.* **75,** 127-137.
61. Krause, G.H., Grube, E., Virgo, A. and Winter, K. (2003) Sudden exposure to solar UV-B radiation reduces net CO2 uptake and photosystem I efficiency in shade-acclimated tropical tree seedlings *Plant Physiol*. **131,** 745-752.
62. Urban, O., Sprtova, M. and Marek, M.V. (1999) The effects enhanced UV-B radiation on the photosynthetic characteristics of Norway spruce (Picea abies (L.) Karst.)trees growing in field conditions. *J. Forest Sci*. **45,** 223-229.
63. Vass, I., Turcsanyi, E., Touloupakis, E., Ghanotakis, D. and Petrouleas, V. (2002) The mechanism of UV-A radiation-induced inhibition of photosystem II electron transport studied by EPR and chlorophyll fluorescence. *Biochemistry* **41,** 10200-10208.
64. Krause, G.H.K., Schmude, C., Garden, H., Koroleva, O.Y. and Winter, K. (1999) Effects of solar ultraviolet radiation on the potential efficiency of photosystem II in leaves of tropical plants. *Plant Physiol*. **121,** 1349-1358.
65. Krause, G.H., Garden, H., Schmude, C., Koroleva, O.Y., Winter, K. and Garab, G. (1998) In *Photosynthesis: Mechanisms and Effects*. Proc. XIth International Cong. Photosynthesis, Budapest, Hungary: Volume III, pp. 2409-2412.
66. Keiller, D.R. and Holmes, M.G. (2001) Effects of long-term exposure to elevated UV-B radiation on the photosynthetic performance of five broad-leaved tree species. *Photosynthesis Res*. **67,** 229-240.
67. Petropoulou, Y., Kyparissis, A., Nikolopoulos, D. and Manetas, Y. (1995) Enhanced UV-B radiation alleviates the adverse effects of summer drought in two Medieterranean pines under field conditions. *Physiol. Plant.* **94,** 37-44.
68. Manetas, Y., Petropoulou, Y., Stamatakis, K., Nikolopoulos, D., Levizou, E., Psaras, G. and Karabournioutis, G. (1997) Beneficial effects of enhanced UV-B under field conditions: improvement of needle water relations and surviving capacity of *Pinus pinea* L. seedlings during the dry Mediterranean summer. *Plant Ecol*. **128,** 100-108.
69. Poulson, M.E., Donahue, R.A., Konvalinka, J. and Boeger, M.R.T. (2002) Enhanced tolerance of photosynthesis to high-light and drought stress in Pseudotsuga menziesii seedlings grown in ultraviolet-B radiation. *Tree Physiol.* **22,** 829-838.
70. Mitchell, J.F.B., Mananbe, S., Meleshko, V. and Tokioka, T. (1990) *Climate Change*, (Houghton, J.T., Jenkins, G.J. and Ephraums, J.J., eds). Cambridge: Cambridge University Press, pp. 92-130.

71. Gwynn-Jones, D., Lee, J.A. and Callaghan, T.V. (1997) Effects of enhanced UV-B radiation and elevated carbon dioxide concentrations on a sub-arctic forest heath ecosystem. *Plant Ecol*. **128,** 242-249.
72. Björn, L.O., Callaghan, T.V., Johnsen, I., Lee, J.A., Manetas, Y., Paul, N.D., Sonesson, M., Wellburn, A.R., Coop, D, Heide-Jørgensen, H.S., Gehrke, C., Gwynn-Jones, D., Johanson, U., Kyparissis, A., Levizou, E., Nikolopoulos, D., Petropoulou, Y. and. Stephanou, M. (1997) The effects of UV-B radiation on European heathland species. *Plant Ecol*. **128,** 252-264.
73. Sullivan, J.H. (1997) Effects of increasing UV-B radiation and atmospheric CO_2 on photosynthesis and growth: implications for terrestrial ecosystems. *Plant Ecol*. **128,** 194-206.
74. Mark, U. and Tevini, M. (1997) Effects of solar ultraviolet-B radiation, temperature and CO_2 on growth and physiology of sunflower and maize seedlings. *Plant Ecol*. **128,** 224-234.
75. Lavola, A., Julkumen-Tiitto, R., de-la Rosa, T.M., Lehto, T. and Aphalo, P.J. (2000) Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO_2 exposure. *Physiol. Plant.* **109,** 260-267.
76. Mackerness, S.A.H., John, C.F., Jordan, B. and Thomas, B. (2001) Early signaling components in ultraviolet-B responses: Distinct roles for different reactive oxygen species and nitric oxide *FEBS Lett*. **489,** 237-242.
77. Baumbusch, L.O., Eiblmeier, M., Schnitzler, J.P., Heller, W., Sandermann Jr., H. and Polle, A. (1998) Interactive effects of ozone and low UV-B radiation on antioxidants in spruce (Picea abies) and pine (Pinus sylvestris) needles. *Physiol. Plant*. **104,** 248-254.
78. Coelho, S., Rijstenbil, J.W., Sousa, I.P. and Brown, M.T. (2001) Cellular responses to elevated light levels in *Fucus spiralis* embryos during the first days after fertilization. *Plant Cell Env.* **24,** 801-810.
79. Ballare, C.L., Scopel, A.L., Stapleton, A.E. and Yanovsky, M.J. (1996) Solar ultraviolet-B radiation affects seedling emergence, DNA integrity, plant morphology, growth rate, and attractiveness to herbivore insects in *Datura ferox. Plant Physiol*. **112,** 161-170.
80. Salt, D.T., Moody, S.A., Whittaker, J.B. and Paul, N.D. (1998) Effects of enhanced UVB on populations of the phloem feeding insect *Strophingia ericae* (Homoptera: Psylloidea) on heather (*Calluna vulgaris*). *Global Change Biol*. **4,** 91-96.
81. Yazawa, M., Shimizu, T. and Hirao, T. (1992) Feeding response of the silkworm, *Bombyx mori*, to UV irradiation of mulberry leaves. *J. Chem. Ecol.* **18, 561**-569.
82. McCloud, E.S. and Berenbaum, M.R. (1994) Stratospheric ozone depletion and plant-insect interactions: Effects of UVB radiation on foliage quality of *Citrus jambhiri* for *Trichoplusia ni*. *J. Chem. Ecol.* **20,** 525-539.
83. Manning, W.J. and Tiedemann, A.V. (1995) Climate change: Potential effects of increased atmospheric carbon dioxide (CO2), ozone (O3), and ultraviolet-B (UV-B) radiation on plant diseases. *Env. Pollution*, **88,** 219-245.
84. Orth, A.B., Teramura, A.H. and Sisler, H.D. (1990) Effects of ultraviolet-B radiation on fungal disease development in *Cucumis sativus*. *Am. J. Bot*. **77,** 1188-1192.
85. Gunasekera, T.S., Paul, N.D. and Ayres, P.G. (1997) The effects of ultraviolet-B (UV-B: 290-320 nm) radiation on blister blight disease of tea (*Camellia sinensis*). *Plant Pathol*. **46,** 179-185.
86. Newsham, K.K., Low, M.N.R., McLeod, A.R., Greenslade, P.D. and Emmett, B.A. (1997) Ultraviolet-B radiation influences the abundance and distribution of phylloplane fungi on pedunculate oak (*Quercus robur*). *New Physiol*. **136,** 287-297.
87. Barnes, P.W., Flint, S.D. and Caldwell, M.M. (1995) Early-season effects of supplemented solar UV-B radiation on seedling emergence, canopy structure, simulated stand photosynthesis and competition for light. *Global Change Biol.* **1,** 43-53.
88. Johanson, U., Gehrke, C., Björn, L.O., Callaghan, T.V. and Sonesson, M. (1995) The effects of enhanced UV-B radiation on a subarctic heath system. *Ambio* **24,** 106-111.
89. Searles, P.S., Caldwell, M.M. and Winter, K. (1995) Polyphasic chlorophyll and fluorescence transient in plants and cyanobacteria. *Photochem. Photobiol*. **61,** 32-38.
90. L'Hirondelle, S.J. and Binder, W.D. (2002) Land Management Handbook Ministry of Forests, British Columbia, 34.

7. INVOLVEMENT OF ALDEHYDE DEHYDROGENASE IN ALLEVIATION OF POST-ANOXIC INJURY IN RICE

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Abstract. Oxygen deprivation induced by submergence, flooding and waterlogging is an environmental stress that affects the growth of plants and production of crops. Plants undergo metabolic and morphological changes to avoid or alleviate the stresses arising from low oxygen conditions. One well-known metabolic change is activation of the glycolytic and fermentation pathways, which are important for ATP production under anaerobic conditions. In some plant species, morphological changes include elongation of internodes or petioles, aerenchyma formation and formation of a barrier to radial oxygen loss. Under post-anoxic conditions, plants suffer from injurious substances, reactive oxygen species and acetaldehyde. Plants have various mechanisms for metabolizing these harmful molecules to prevent or alleviate post-anoxic injuries. This paper reviews the current understanding of adaptation and tolerance mechanisms of plants that are activated under low oxygen conditions and following re-aeration.

1. INTRODUCTION

Rapid environmental changes subject plants to various abiotic stresses that adversely affect their growth and development. Plants can be deprived of oxygen by winter ice encasement, spring floods and heavy rainfall [1]. For example, in Australia, losses of wheat due to waterlogging amount to about 300 million Australian dollars per year [2]. Because plants are sessile, their ability to respond quickly to stressful environmental conditions such as submergence and waterlogging is crucial for adaptation and survival. Thus, it is important to understand how plants adapt to and survive oxygen deprivation. In this article, we focus on the metabolic and morphological changes that occur in plants under submergence and waterlogging and on how plants recover from post-anoxic injury.

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2. METABOLIC CHANGES UNDER SUBMERGENCE AND WATERLOGGING

Plants are injured by oxygen deprivation as a result of submergence and waterlogging. This type of injury is referred to as anoxic injury (or hypoxic injury). Anoxic injury and hypoxic injury may be caused by a decrease in ATP production, an accumulation of toxic end products of anaerobic metabolism, and cytoplasmic acidosis during extended anaerobiosis [3]. To alleviate this injury, plants make some metabolic changes. Under anaerobic conditions, ATP production is shifted from oxidative phosphorylation to the less efficient glycolysis and fermentation (Figure 1).

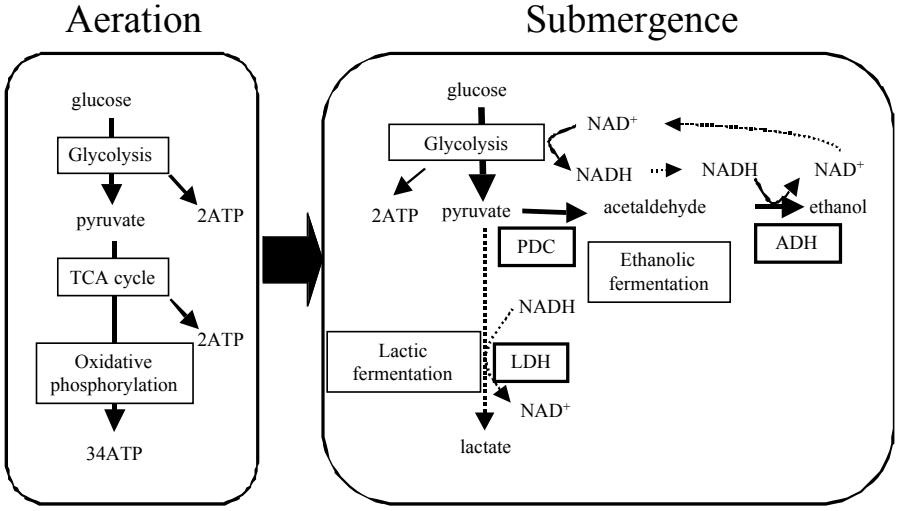

Figure 1. Changes in metabolism that occur in plants as a result of submergence. During aerobic conditions (left), ATP is produced efficiently through glycolysis, the TCA cycle and oxidative phosphorylation. Under submerged conditions (right), ATP production mainly depends on glycolysis. Ethanolic and lactic fermentations are activated to provide the NAD^+ that is required for glycolysis. Ethanolic fermentation is driven by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). Lactic fermentation is driven by lactate dehydrogenase (LDH).

Expressions of genes encoding enzymes in these pathways are induced in response to oxygen deprivation [4]. As NAD^+ is needed to sustain glycolysis, fermentation pathways play a role in providing NAD^+ to the glycolytic pathway. Three main fermentation pathways, whose end products are ethanol, lactate and alanine, are enhanced during anaerobic conditions [2]. Among the three fermentation pathways, ethanolic and lactic fermentations can regenerate NAD^+ from NADH (Figure 1). It has been suggested that cytosolic pH controls the ratio between ethanolic fermentation and lactic fermentation [5,6,7], *i.e.*, a decrease in cytosolic pH increases ethanolic fermentation and decreases lactic fermentation. Lactate, an end product of lactic fermentation, could cause a decrease of cytosolic pH. In addition,

H^+-ATPase pumps could be responsible for the decrease of cytosolic pH [8,9]. Because low pH inhibits the activity of lactate dehydrogenase (LDH) and increases the activity of pyruvate decarboxylase (PDC) (Figure 1), the main fermentation pathway shifts from lactic fermentation to ethanolic fermentation [10, 11].

In rice, the anoxia-tolerant cultivars FR13A and Calrose have higher rates of ethanolic fermentation than the anoxia-intolerant cultivars IR22 and IR42 [12,13]. An increase of the rate of ethanolic fermentation in rice leads to a corresponding increase in survival of rice under submerged conditions [14]. By contrast, a rice mutant with reduced ADH activity was more vulnerable to submergence than the wild type of rice [15]. Taken together, these results indicate that ethanolic fermentation plays an important role in the adaptation of plants to low oxygen conditions.

3. MORPHOLOGICAL CHANGES UNDER SUBMERGENCE AND WATERLOGGING

In order to avoid hypoxia stress, some plants undergo morphological changes such as (1) elongation of internodes or petioles, (2) aerenchyma formation, and (3) formation of a barrier to radial oxygen loss (ROL). Each of these changes is described below.

3.1. Elongation of internodes or petioles

Deepwater rice and *Rumex palustris* have an ability to elongate their internodes and petioles, respectively, under submerged conditions [16,17]. The elongation of these organs allows the plants to obtain oxygen above the surface of the water. As the diffusion of gas in water is 10,000 times slower than in air, gases (*e.g.*, ethylene) produced in plants cannot be discharged in water [18]. As a result, the amount of ethylene increases in the internodal air spaces of deepwater rice and in the petioles of *R. palustris* soon after submergence [17,19]. The increased ethylene can generally cause the decrease of abscisic acid (ABA) and the increase of gibberellin (GA), both of which promote the elongation of internodes or petioles. Even under submerged conditions, the synthesis of ethylene is maintained by activation of the expressions of genes for enzymes involved in ethylene biosynthesis, such as ACC synthase and ACC oxidase [16,17]. In plants, ethylene is synthesized from *S*-adenosyl-L-methionine (SAM) via two reactions catalyzed by ACC synthase and ACC oxidase [20]. In *R. palustris*, expressions of *RP-ACS1*, an ACC synthase gene, and *RP-ACO1*, an ACC oxidase gene, are up-regulated under submerged conditions [17,21]. In deepwater rice, the induction of *Os-ACS1*, *Os-ACS5* and *Os-ACO1* are observed in the intercalary meristem under submergence [22, 23, 24]. Significant increases in the activities of ACC synthase and ACC oxidase were also observed in deepwater rice and *R. palustris* under submergence [16,17,21,23].

Other hormones related to elongation during submergence are ABA and GA. Within 1 h of submergence, the ABA level in petioles of *R. palustris* was reduced by 80% [17] and after 4 h of submergence, the ABA level in deepwater rice was

reduced by 55% [23]. Application of ABA to submerged *R. palustris* and deepwater rice significantly inhibited their elongation [17,25]. On the other hand, when deepwater rice was treated with ethylene for 3 h, the ABA level in the intercalary meristem and the cell elongation zone decreased by 75% [25]. Ethylene also decreased the level of ABA during elongation of petioles of *R. palustris* [17]. These results indicate that ethylene is responsible for the decrease of ABA contents in deepwater rice and *R. palustris* under submerged conditions. Submergence-induced elongation in plants also depends on the endogenous GA level. The level of GA_1, which is the active GA in rice, and GA_{20}, which is the immediate precursor of GA_1, increased during submergence [25]. Moreover, the inhibition of growth of internodes in deepwater rice by ABA was recovered by GA [25], and ethylene enhanced the responsiveness of deepwater rice internodes to GA [26]. These findings suggest that the rapid growth of deepwater rice internodes is controlled by increased ethylene under submergence, thereby altering the balance between the endogenous levels of ABA and GA [25].

3.2. Aerenchyma formation

The aerenchyma is an internal aeration system for the transfer of oxygen from the shoot that increases plant survival in low-oxygen soil environments. The aerenchyma occurs in two forms, schizogenous aerenchyma and lysigenous aerenchyma [27,28]. Schizogenous aerenchyma occurs when intercellular gas spaces form during tissue development without cell death. Spaces are formed by differential growth, with adjacent cells separating from one another at the middle lamella. Schizogenous aerenchyma formation is often observed in wetland species like *Rumex* [28]. Lysigenous aerenchyma formation is formed by cell death in root cortex cells and the cell death is first detected in the mid cortex [29] (Figure 2).

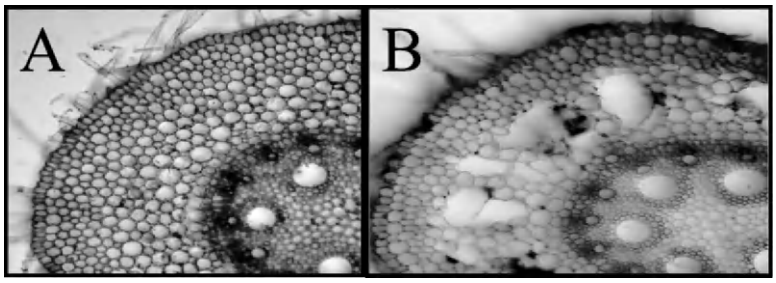

Figure 2. Transverse section of maize primary root about 15 mm from the base of the root. A. Root before waterlogging (aerobic conditions). B. Root 96 hours after the initiation of waterlogging, in which aerenchyma formation was observed in the mid cortex. Photographs provided by Ryosuke Watanabe (University of Tokyo).

Ethylene induces lysigenous aerenchyma formation in the roots of maize [30,31]. Hypoxia stimulates ethylene biosynthesis by enhancing of the activities of ACC

synthase and ACC oxidase [32], which indicates that the cell death in the root cortex of maize under low oxygen conditions occurs downstream of ethylene signal transduction. In addition, experiments with some specific inhibitors suggest that heterotrimeric G protein-related signaling, Ca^{2+} signaling, and protein phosphorylation are involved in the formation of lysigenous aerenchyma [29,33].

3.3. Formation of a barrier to radial oxygen loss (ROL)

Roots of rice and wetland species also contain a barrier to ROL in the basal zones [30,34,35]. This barrier, which is composed of a suberized exodermis and endodermis and a layer of lignified sclerenchymatous cells [36,37], enhances the amount of oxygen diffusing into the root apex and promotes the development of an aerobic rhizosphere around the root tip [38]. Thus, the effectiveness of the aerenchyma can be increased by the formation of a barrier to prevent ROL from roots.

4. ALLEVIATION OF POST-ANOXIC INJURY

In addition to being damaged under anaerobic conditions, plants may be damaged during re-aeration following anaerobic conditions. The latter type of damage is known as post-anoxic injury (also referred to as post-hypoxic injury by some authors) [39,40,41]. Post-anoxic injury appears to be caused by acetaldehyde and reactive oxygen species (ROS). Examples of the latter are superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). The production of ROS is immediately induced upon exposure of anaerobic plant tissues to normal oxygen tension and, as a result, proteins, nucleic acids and membranes can undergo severe peroxidation [42,43]. Plants have some defense mechanisms for scavenging ROS after re-aeration. Superoxide dismutase, ascorbate peroxidase and catalase (CAT) are mainly engaged in the detoxification of ROS in plants [1,44]. Re-aeration also induces the production of acetaldehyde, as a result of oxidation of ethanol, which is produced and accumulated by ethanolic fermentation under anaerobic conditions [45,46] (Figure 3). Ethanol is assumed to be rapidly oxidized to acetaldehyde by alcohol dehydrogenase (ADH) and/or CAT [39,47,48]. CAT probably oxidizes ethanol through its reduction of H_2O_2 that is produced during re-aeration [39,47]. Acetaldehyde is harmful to cells because of its tendency to form acetaldehyde-protein and acetaldehyde-DNA adducts [49,50]. Cells possess mechanisms to metabolize acetaldehyde. Aldehyde dehydrogenase (ALDH) catalyzes the conversion of aldehydes to the corresponding acids [51] (Figure 3). Rice has two mitochondrial ALDHs, ALDH2a and ALDH2b [52,53]. The amount of *ALDH2a* transcripts increased under submerged conditions, whereas the amount of *ALDH2b* transcripts decreased. Interestingly, re-aerated rice plants showed an intense ALDH2a induction, despite a decline of *ALDH2a* mRNA. Along with the increase of ALDH2a protein, acetaldehyde-oxidizing ALDH activity increased, thereby causing the acetaldehyde content to decrease in rice during re-aeration [54]. These findings suggest that rice *ALDH2a* mRNA is accumulated in order to quickly metabolize

acetaldehyde that is produced upon re-aeration, and that mitochondrial ALDH is involved in the alleviation of post-anoxic injury induced by acetaldehyde.

In conclusion, plants possess complex mechanisms to avoid and alleviate anoxic and post-anoxic injuries and to tolerate low oxygen conditions in combination with metabolic and morphological changes. Much more research is needed to fully understand the mechanisms for adapting to low oxygen stress in plants because many factors are intricately involved. In addition, it is not known how plants sense low oxygen levels. Further studies involving forward and reverse genetics and high-throughput techniques such as microarray analyses should elucidate the intricate mechanisms involved in adapting to and recovering from low oxygen conditions, as well as provide clues on how to produce crops that can tolerate such conditions.

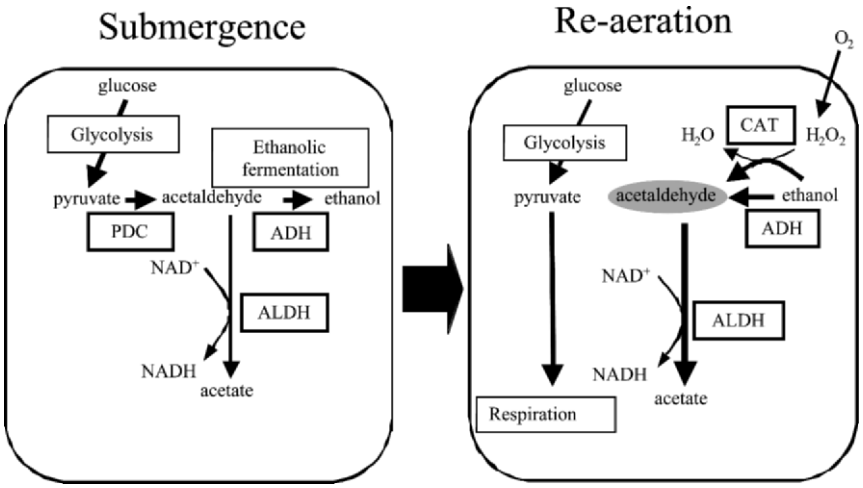

Figure 3. Proposed metabolic pathways of rice under submerged conditions and following re-aeration. Under submerged conditions (left), pyruvate, which is produced by pathways such as glycolysis, is converted to acetaldehyde by PDC. At the same time, acetaldehyde is converted to ethanol by ADH and to acetate by aldehyde dehydrogenase (ALDH). When the submerged plants are transferred to aerobic conditions (right), the anaerobically accumulated ethanol is rapidly oxidized to acetaldehyde by the reverse reaction of ADH and/or the peroxidation of ethanol by catalase (CAT) during the conversion of H_2O_2 to H_2O. Evidence suggests that ALDH efficiently detoxifies the phytotoxic acetaldehyde by the rapid increase of mitochondrial ALDH under re-aeration.

REFERENCES

1. Blokhina, O., Virolainen, E. and Fagerstedt, K.V. (2003) Antioxidants, oxidative damage and oxygen deprivation stress. *Ann. Bot.* **91,**179-194.
2. Dennis, E.S., Dolferus, R., Ellis, M., Rahman, M., Wu, Y., Hoeren, F.U., Grover, A., Ismond, K.P., Good,A.G. and Peacock, W.J. (2000) Molecular strategies for improving waterlogging tolerance in plants. *J. Exp. Bot*. **51,** 89-97.

3. Drew, M.C. (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 223-250.
4. Sachs M.M., Subbaiah C.C., Saab I.N. (1996) Anaerobic gene expression and flooding tolerance in maize. *J. Exp. Bot*. **47**, 1-15.
5. Davies, D.D., Grego, S. and Kenworthy, P. (1974) The control of the production of lactate and ethanol by higher plants. *Planta* **118,** 297-310.
6. Menegus, F., Cattaruzza, L., Chersi, A. and Fronza, G. (1989) Differences in the anaerobic lactate-succinate production and in the changes of cell sap pH for plants with high and low resistance to anoxia. *Plant Physiol.* **90,** 29-32.
7. Menegus, F., Cattaruzza, L., Mattana, M., Beffagna, N. and Ragg, E. (1991) Response to anoxia in rice and wheat seedlings. *Plant Physiol*. **95,** 760-767.
8. Ratcliffe, R.G. (1997) *In vivo* NMR studies of the metabolic response of plant tissues to anoxia. *Ann. Bot. Suppl. A* **79,** 39-48.
9. Gout, E., Boisson, A., Aubert, S., Douce, R. and Bligny, R. (2001) Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells: carbon-13 and phosphorous-31 nuclear magnetic resonance studies. *Plant Physiol*. **125,** 912-925.
10. Roberts, J.K.M., Callis, J., Wemmer, D., Walbot, V. and Jardetzky, O. (1984) Mechanisms of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. *Proc. Natl. Acad. Sci. USA* **81,** 3379-3383.
11. Fox, G.G., McCallan, N.R. and Ratcliffe, R.G. (1995) Manipulating cytoplasmic pH under anoxia: a critical test of the role of pH in the switch from aerobic to anaerobic metabolism. *Planta* **195,** 324-330.
12. Setter, T.L., Ella, E.S. and Valdez, A.P. (1994) Relationship between coleoptile elongation and alcoholic fermentation in rice exposed to anoxia. II. cultivar differences. *Ann. Bot*. **74,** 273-279.
13. Gibbs, J., Morrell, S., Valdez, A., Setter, T.L. and Greenway, H. (2000) Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. *J. Exp. Bot*. **51,** 785-796.
14. Quimio, C.A., Torrizo, L.B., Setter, T.L., Ellis, M., Grover, A., Abrigo, E.M., Oliva, N.P., Ella, E.S., Carpena, A.L., Ito, O., Peacock, W.J., Dennis, E.S. and Datta, S.K. (2000) Enhancement of submergence tolerance in transgenic rice overproducing pyruvate decarboxylase. *J. Plant Physiol*. **156,** 516-521.
15. Matsumura, H., Takano, T., Takeda, G. and Uchimiya, H. (1998) *Adh1* is transcriptionally active but its translational product is reduced in a *rad* mutant of rice (*Orizaya sativa* L.) , which is vulnerable to submergence stress. *Theor. Appl. Genet*. **97,** 1197-1203.
16. Vriezen, W.H., Zhou, Z. and Van Der Straeten, D. (2003) Regulation of submergence-induced enhanced shoot elongation in *Oryza sativa* L., *Ann. Bot*. **91,** 263-270.
17. Voesenek, L.A.C.J., Benschop, J.J., Bou, J., Cox, M.C.H., Groeneveld, H.W., Millenaar, F.F., Vreeburg, R.A.M. and Peeters, A.J.M. (2003) Interactions between plant hormones regulates submergence-induced shoot elongation in the flooding-tolerant dicot *Rumex palustris*. *Ann. Bot*. **91,** 205-211.
18. Jackson, M.B. (1985) Ethylene and responses of plants to soil waterlogging and submergence. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **36,** 145-174.
19. Stünzi, J.T. and Kende, H. (1989) Gas composition in the internal air spaces of deepwater rice in relation to growth induced by submergence. *Plant Cell Physiol*. **30,** 49-56.
20. Bleecker, A.B. and Kende, H.(2000) Ethylene: a gaseous signal molecule in plants. *Annu. Rev. Cell Dev. Biol.* **16,** 1-18.
21. Vriezen, W.H., Hulzink, R., Mariani, C. and Voesenek, L.A.C.J. (1999) 1-aminocyclopropane-1-carboxylate oxidase activity limits ethylene biosynthesis in *Rumex palustris* during submergence. *Plant Physiol*. **121,** 189-195.
22. Zarembinski, T.I. and Theologis, A. (1997) Expression characteristics of OS-ACS1 and OS-ACS2, two members of the 1-aminocyclopropane-1-carboxylate synthase gene family in rice (*Oryza sativa* L. cv. *Habiganj Aman* II) during partial submergence. *Plant Mol. Biol*. **33,** 71-77.
23. Van Der Straeten, D., Zhou, Z., Prinsen, E., Van Onckelen, H.A. and Van Montagu, M.C.(2001) A comparative molecular-physiological study of submergence response in lowland and deepwater rice. *Plant Physiol*. **125,** 955-968.
24. Mekhedov, S.L. and Kende, H. (1996) Submergence enhances expression of a gene encoding 1-aminocyclopropane-1-carboxylate oxidase in deepwater rice. *Plant Cell Physiol*. **37,** 531-537.

25. Hoffmann-Benning, S. and Kende, H. (1992) On the role of abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol*. **99,** 1156-1161.
26. Raskin, I. and Kende, H. (1984) The role of gibberellin in the growth response of submerged deepwater rice. *Plant Physiol*. **76,** 947-950.
27. Smirnoff, N. and Crawford, R.M.M. (1983) Variation in the structure and response to flooding of root aerenchyma in some wetland plants. *Ann. Bot*. **51,** 237-249.
28. Evans, D.E. (2003) Aerenchyma formation. *New Phytol*. **161,** 35-49.
29. Drew, M.C., He, C.-J. and Morgan, P.W. (2000) Programmed cell death and aerenchyma formation in roots. *Trends Plant Sci*. **5,** 123-127.
30. Jackson, M. and Armstrong. W. (1999) Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biol*. **1,** 274-287.
31. Gunawardena, A.H.L.A.N., Pearce, D.M., Jackson, M.B., Hawes, C.R. and Evans, D.E. (2001) Characterization of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.) *Planta* **212,** 205-214.
32. He, C.-J., Drew, M.C. and Morgan, P.W. (1994) Induction of enzymes associated with lysigenous aerenchyma formation in roots of *Zea mays* during hypoxia or nitrogen starvation. *Plant Physiol.* **105,** 861-865.
33. He, C.-J., Morgan, P.W. and Drew, M.C. (1996) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. *Plant Physiol.* **112,** 463-472.
34. Visser, E.J.W., Colmer, T.D., Blom, C.W.P.M. and Voesenek, L.A.C.J. (2000) Changes in growth, porosity, and radial oxygen loss from adventitious roots of selected mono- and dicotyledonous wetland species with contrasting types of aerenchyma. *Plant Cell Environ*. **23,** 1237-1245.
35. McDonald, M.P., Galwey, N.W. and Colmer, T.D. (2002) Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wetland and dryland grass species. *Plant Cell Environ.* **25,** 441-451.
36. Soukup, A., Votrubová, O. and Cízková, H. (2002) Development of anatomical structure of roots of *Phragmites australis. New Phytol*. **153,** 277-287.
37. De Simone. O., Haase, K., Müller, E., Junk, W.J., Hartmann, K., Schreiber, L. and Schmidt, W. (2003) Apoplasmic barriers and oxygen transport properties of hypodermal cell walls in roots from four amazonian tree species. *Plant Physiol*. **132,** 206-217.
38. Colmer, T.D. (2003) Aerenchyma and an inducible barrier to radial oxygen loss facilitate root aeration in upland, paddy and deep-water rice (*Oryza sativa* L.) *Ann. Bot*. **91,** 301-309.
39. Monk, L.S., Brandle, R. and Crawford, R.M.M. (1987) Catalase activity and post-anoxic injury in monocotyledonous species. *J. Exp. Bot*. **38,** 233-246.
40. VanToai, T.T. and Bolles, C.C. (1991) Postanoxic injury in soybean (*Glycine max*) seedlings. *Plant Physiol*. **97,** 588-592.
41. Crawford, R.M.M. and Walton, J.C., Wollenweber-Ratzer, B. (1994) Similarities between post-ischaemic injury to animal tissues and post-anoxic injury in plants. *Proc. Roy. Soc.* **102B,** 325-332.
42. Wollenweber-Ratzer, B and Crawford, R.M.M. (1994) Enzymatic defence against post-anoxic injury in higher plants. *Proc. Roy. Soc.* **102B,** 381-390.
43. Blokhina, O.B., Fagerstedt, K.V. and Chirkova, T.V. (1999) Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiol. Plant*. **105,** 625-632.
44. Mittler, R. (2002) Oxidative stress, antioxidants, and stress tolerance. *Trends Plant Sci*. **7,** 405-410.
45. Pfister-Sieber, M. and Brändle, R. (1995) Response of potato tubers to hypoxia followed by re-aeration. *Potato Res*. **38,** 231-239.
46. Zuckermann, H., Harren, F.J.M., Reuss, J. and Parker, D.H. (1997) Dynamics of acetaldehyde production during anoxia and post-anoxia in red bell pepper studied by photoacoustic techniques. *Plant Physiol*. **113,** 925-932.
47. Pavelic, D., Arpagaus, S., Rawyler, A. and Brändle, R. (2000) Impact of post-anoxia stress on membrane lipids of anoxia-pretreated potato cells. a re-appraisal. *Plant Physiol*. **124,** 1285-1292.
48. Kreuzwieser, J., Harren, F.J.M., Laarhoven, L.J.J., Boamfa, I., te Lintel-Hekkert, S., Scheerer, U., Hüglin, C. and Rennenberg, H. (2001) Acetaldehyde emission by the leaves of trees - correlation with physiological and environmental parameters. *Physiol. Plant*. **113,** 41-49.
49. Perata, P., Vernieri, P., Armellini, D., Bugnoli, M., Tognoni, F. and Alpi, A. (1992) Immunological detection of acetaldehyde-protein adducts in ethanol-treated carrot cells. *Plant Physiol*. **98,** 913-918.

50. Zhang, M., Nagata, S., Miyazawa, K., Kikuchi, H. and Esashi, Y. (1997) A competitive enzyme-linked immunosorbent assay to quantify acetaldehyde-protein adducts that accumulate in dry seeds during aging. *Plant Physiol*. **113,** 397-402.
51. Kirch, H.-H., Bartels, D., Wei, Y., Schnable, P.S. and Wood, A.J. (2004) The ALDH gene superfamily of *Arabidopsis*. *Trends Plant Sci*. **9,** 371-377.
52. Nakazono, M., Tsuji, H., Li, Y., Saisho, D., Arimura, S., Tsutsumi, N. and Hirai, A. (2000) Expression of a gene encoding mitochondrial aldehyde dehydrogenase in rice increases under submerged conditions. *Plant Physiol*. **124,** 587-598.
53. Tsuji, H., Tsutsumi, N., Sasaki, T., Hirai, A. and Nakazono, M.(2003) Organ-specific expressions and chromosomal locations of two mitochondrial aldehyde dehydrogenase genes from rice (*Oryza sativa* L.), ALDH2a and ALDH2b. *Gene* **305,** 195-204.
54. Tsuji, H., Meguro, N., Suzuki, Y., Tsutsumi, N., Hirai, A. and Nakazono, M. (2003) Induction of mitochondrial aldehyde dehydrogenase by submergence facilitates oxidation of acetaldehyde during re-aeration in rice. *FEBS Lett*. **546,** 369-373.

SECTION IV

PHYTOREMEDIATION

8. GENETIC ENGINEERING STRESS TOLERANT PLANTS FOR PHYTOREMEDIATION

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Abstract. Phytoremediation uses plants and associated microbes to remove, sequester, and detoxify contaminants, particularly trace elements. The great potential of this low-cost, low-management approach has spurred researchers to increase the efficiency of this natural process through the use of genetic engineering. Plants used for phytoremediation face a primary stress emanating from the high local concentrations of contaminants as well as possible secondary stresses such as extreme temperature, salinity, desiccation, flooding, and/or high light. Since the total amount of a contaminant removed is a product of the total biomass of the harvestable tissue and the concentration of the contaminant in those tissues, it is critical that phytoremediating plants tolerate contaminant stress through detoxification mechanisms rather than avoidance or exclusion mechanisms. At the same time, they should survive the sub-optimal growth conditions often associated with contaminated sites. For this reason, the introduced genes often play a role in increasing the stress tolerance of the engineered plants. Successful genetic engineering approaches range from the transfer of genes with specific detoxification function to overexpression of genes involved in ameliorating oxidative stress in general. Advances in related fields, including rapid genome sequencing, microarrays, and more sophisticated gene expression systems, should eventually lead to an increase in the number of engineered plants suitable for field-use.

1. INTRODUCTION

Phytoremediation is the use of plants and their associated microbes to remove, sequester, and detoxify contaminants from soil and water [1]. This process occurs naturally in plants. Frequently, contaminants, especially trace elements, are transported into plant roots because of their essentiality or chemical similarity to an essential element. In phytoextraction, the contaminants are further transported from root to shoot where they can be harvested in the above-ground biomass [1]. A subset of contaminants, particularly selenium and mercury, can be metabolized in the plant to volatile (gaseous) forms [1]. This process, referred to as phytovolatilization, is of special interest because it can potentially remove all the contaminant from the local ecosystem. Phytostabilization, the use of plants to contain soil pollutants by preventing erosion, will not be discussed in this review.

Phytoremediation is an attractive remediation approach for dealing with wastewater, soils, and sediments contaminated with heavy metals, metalloids,

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radionuclides, and organics, such as polyaromatic hydrocarbons and polychlorinated biphenyls [2]. Of particular importance are its low cost and low management requirements [3]. Currently used physicochemical methods are prohibitively expensive, forcing governments to make difficult choices of which sites to remediate [4]. Biopiles of microbes are a low-cost but difficult to manage approach that requires a constant carbon source and seeding to maintain the growth of "useful" microbes compared to that of other microbes that constantly invade the site [5]. In phytoremediation, sunlight provides the energy for plants (through photosynthesis) to generate carbon products to support microbial populations associated with the root system. Thus, phytoremediation offers an attractive alternative because it lessens the need for constant management and for energy inputs [6].

Phytoremediation has been achieved in a number of small-scale field experiments with a variety of native species [e.g. 7-9]. The adoption of phytoremediation for contaminant cleanup has been limited primarily by the inefficiency of natural plant processes. Most plants do not accumulate contaminants, either because they exclude their uptake or because they suffer reduced biomass and growth in the presence of the contaminant. Some plants on the other hand, known as hyperaccumulators, are tolerant of the contaminant (especially toxic trace elements) and may accumulate several thousands of parts per million in their aboveground tissues [10]. However, this high level of accumulation is generally offset by low biomass and slow growth rate, so that the overall efficiency of phytoremediation by hyperaccumulators is not high [11]. Other plants, known as secondary accumulators, are more successful at phytoremediation because they accumulate moderate concentrations of trace element contaminants while maintaining a relatively fast growth rate and high biomass [12,13]. Several attempts have been made to increase the phytoremediation efficiency of secondary accumulators through genetic engineering, an approach that has met with a number of successes [e.g. 14-26]. In our own laboratory for example, we have been able to genetically engineer Indian mustards that were able to outperform wildtype plants in removing selenium from toxic sediments under field conditions [27].

2. ENHANCING PHYTOREMEDIATION THROUGH GENETIC ENGINEERING

In phytoextraction, the total amount of contaminant removed (during one growth cycle) is equal to the concentration of contaminant in the shoot tissue multiplied by shoot biomass [28]. The ultimate goal of phytoextraction is clean up the contaminated soil and water in the shortest possible time, i.e., in as few growth cycles as possible. This may be achieved through genetic engineering. One approach, for example, is to overexpress metal transporters so as to increase the efficiency of uptake of the contaminant into root or shoot tissues [22, 29, 30]. This approach is complicated by the fact that the transgenic plant may not have the ability to detoxify the increased levels of contaminant it has accumulated [30]. Nevertheless, transgenic plants of *Arabidopsis thaliana* overexpressing YCF1 exhibited increased tolerance and accumulation of lead and cadmium because of increased

transport of these metals to the vacuole [31]. Another approach is to engineer plants to exude organic acids that alter rhizosphere pH so as to increase the solubility and bioavailability and therefore uptake of contaminants [32-34].

The ability of plants for phytoremediation can be greatly improved by increasing tolerance to environmental stresses. This allows the plant to maintain a high biomass and fast growth rate in environments potentially unfavorable for growth. Although the primary stress encountered by the plants is likely to be the toxicant to be remediated, the plant may also have to contend with other stresses, such as moderate to high levels of other contaminants, poor soil quality (pH, mineral deficiencies, etc…), aridity or flooding, and temperature variations [27].

Plants have evolved three general strategies for tolerating high concentrations of trace metal contaminants in soil and water. The first is to avoid the metals all together. This can be accomplished through such means as exuding organic acid chelates through the roots or by changing the pH in the rhizosphere so that trace metals are converted to a less bioavailable form. In several species of plants, for instance, tolerance to high soil concentrations of aluminum is correlated with root exudation of organic acids [35,36]. A second strategy is to trap toxicants in the roots through the over-production of chelating molecules [37]. This strategy is particularly effective for metals that exert their toxicity in the shoots, since they are prevented from being transported to the shoots. Finally, some plants tolerate metals by detoxifying them, either through chelation, sequestration, or metabolism to less toxic forms [38-41]. Only this latter strategy is useful for phytoextraction since the metals are accumulated in the easily harvestable plant shoots.

3. LESSONS FROM NATURE: DESIGNING STRESS TOLERANT PLANTS FOR PHYTOREMEDIATION

A key step in any genetic engineering project is to identify the appropriate genes to introduce into target plants suitable for phytoremediation. Such a target plant ideally should have a fast-growth rate, high biomass, and moderate tolerance to trace element contaminants and environmental stresses [42]. Examples of plants that have been used for this purpose include tobacco (*Nicotiana tabacum*), Indian mustard (*Brassica juncea*), and trees such as poplar (*Populus* sp.). The selection of genes to be introduced into target plant species may be identified through a variety of techniques. Early hybridization work provided some insight into the genetic basis of tolerance in a variety of species [43,44]. The production and screening of random mutants, particularly with the model plant species, *Arabidopsis thaliana*, has led to the discovery of a number of genes involved in heavy metal tolerance [45,46]. Under the premise that genes whose expression is modified by the presence of heavy metals are important in the plant's heavy metal stress response, modern microarrays will likely become more important in identifying such genes. That is because with current genetic engineering technology, we are generally altering the expression of only one or two genes, so we are merely tweaking the plant's natural abilities.

One special advantage of genetic engineering is the ability to transform plants with genes from other species rather than upregulating an already existing plant

stress response. The genomes of hyperaccumulating plant species are of interest to researchers seeking genes for genetic engineering. Hyperaccumulators are able to accumulate trace elements (e.g., Se, As, Pb, Ni, and Zn) to thousands of parts per million in their shoot tissues [40]. Generally, this adaptation is specific to only one element so that its genetic basis is often very specific rather than a general stress response. The problem with hyperaccumulators is that their phytoremediation efficiency is often limited by their slow growth rate and low biomass [11]. In genetic engineering, it is technically easier to transfer genes responsible for hyperaccumulation to fast-growing, high biomass plants than to increase the growth rate of hyper-accumulators themselves. For this reason, hyperaccumulators are currently viewed as gene sources rather than hosts for transformation.

Another source of potential genes for genetic engineering is the genomes of extremophile microbes, which have been discovered in a wide range of extreme environments that do not support the life of plants or higher organisms. Their rapid evolution has resulted in microbes that can tolerate such diverse stresses as extreme cold, heat, pH, salinity, and high metal concentrations [47]. In fact, there are microbes that conduct anaerobic respiration of selenium and arsenic [48]. There are, however, several complications with using microbial genes including different codon usage, difficulty in culturing, and the fact that gene regulation in a multicellular organism might be different.

4. GENETIC ENGINEERING STRESS-TOLERANT PLANTS FOR PHYTOREMEDIATION: SUCCESSES

Success in genetic engineering stress-tolerant plants has been the culmination of extensive work in identifying critical genes, which, on overexpression increase either tolerance to a specific metal contaminant or general stress tolerance that results in increased plant biomass.

An obvious strategy for increasing the tolerance of a plant to a toxic trace element is to increase its ability to convert the trace element to a less toxic form. Typically, such a plant would then be able to accumulate higher levels of this detoxified form. An example of this approach is the overexpression of the gene encoding selenocysteine methyltransferase (SMT) in Indian mustard [25]. The gene encoding this enzyme was first identified in the Se hyperaccumulator, *Astragalus bisulcatus*, and was determined to be a key component of the hyperaccumulation mechanism [49]. The toxicity of selenium is due in part to its chemical similarity to sulfur. The misincorporation of selenoamino acids analogs of the sulfur-amino acids, cysteine and methionine, into proteins can alter enzyme structure and activity. The role of SMT in hyperaccumulation is to methylate selenocysteine to the non-protein selenoamino acid, methylselenocysteine. Thus, selenium is detoxified by diverting selenium flow away from the selenoamino acids, and therefore, from proteins [50]. Transgenic Indian mustard overexpressing SMT exhibited increased selenium tolerance and accumulation [25].

Another specific detoxification pathway that has been transformed into plants for phytoremediation is the bacterial mer operon [51]. The merA and merB genes

encode mercuric reductase and methylmercury lyase, respectively. Expressed together these enzymes function to convert organic or inorganic mercury to elemental mercury that escapes from the plant in volatile form. A variety of plant species overexpressing merA and merB have increased tolerance and accumulation of both inorganic and organic mercury forms [52]. MerA and merB genes have been introduced into plants using chloroplast transformation (e.g. in tobacco) as well as the more common nuclear transformation approach [53]. Chloroplast transformation, although slightly more complicated, results in greater transgene expression, maternal transfer of transgenes, which restricts gene flow through pollen spread, and may afford a greater protective effect against metals with specific toxicity against the chloroplasts [54].

Specific detoxification mechanisms may be introduced at the same time as increasing expression of genes involved in plant stress response. A striking example of this is seen in the development of Arabidopsis overexpressing both arsC, encoding arsenate reductase, and gshII, the gene encoding γ-glutamylcysteine synthetase (ECS) for increased arsenic resistance [19]. Arsenate is the predominant form of bioavailable arsenic. The first-step in one proposed arsenic detoxification model is the reduction of arsenate to arsenite. Since a plant arsenate reductase gene was not available, the authors used a bacterial gene. Although arsenite is more toxic than arsenate, it has the advantage that it can be detoxified through chelation with thiol-containing compounds, such as glutathione (GSH) and phytochelatins (PCs). By overexpressing an arsenate reductase in Arabidopsis, the proportion of As present as toxic arsenite increased [19]. Because Arabidopsis would not be able to effectively detoxify this greater pool of arsenite without a concomitant increase in thiol-containing compounds, the authors overexpressed ECS in the same plants. ECS catalyzes a rate-limiting step in the production of PCs, short Cys-containing peptides known to chelate a variety of metals/metalloids [55].

Heavy metals may express their toxicities through individual symptoms such as stunted growth, root damage, necrosis, etc. However, the mechanism of damage may involve oxidative stress through the production of reactive oxygen species [56]. As such, genes involved in ameliorating oxidative stress may afford protection against a wide variety of trace element contaminants, which is an especially important asset for sites contaminated with multiple metals.

In order to genetically engineer Indian mustard for enhanced tolerance and accumulation of heavy metals, transgenic plants overexpressing glutathione synthetase (GS) and γ-glutamylcysteine synthetase (ECS) were developed. GS and ECS are enzymes that catalyze rate-limiting steps in the production of glutathione (GS) and phytochelatins (PC) [16,17]. The role of PCs in cadmium chelation and sequestration has been well established, and, as expected, these plants had enhanced cadmium tolerance and accumulation [16,17,57]. However, these plants have since been shown to tolerate and accumulate a wide variety of heavy metals [21]. Similarly, Indian mustard overexpressing the Arabidopsis gene encoding ATP sulfurylase (APS) was originally developed for Se phytoremediation [15]. APS catalyzes a rate-limiting step in the sulfur/selenium assimilation pathway in plants, and its overexpression results in increased selenate uptake and eventual reduction to selenite [58]. At the same time, though, APS overexpression also increases sulfate

uptake and reduction. Since reduced sulfur forms (such as Cys) are limiting substrates for the synthesis of GSH and PCs, the greater pool of reduced sulfur may allow for protection against heavy metals as well. Indeed, APS Indian mustard plants were more effective than wild type in tolerating and extracting a wide variety of heavy metals from contaminated soil [26].

Recently, Indian mustard lines overexpressing APS, ECS and GS, respectively, were studied in the first successful phytoremediation field trial of transgenic plants in the United States [27]. The plants, along with wild type, were grown on a mix of clean topsoil and high Se-sediment dredged from drainage water canals in central California. The growth conditions on this site were sub-optimal since the levels of soil salinity and extractable B are considered to be detrimental for normal plant growth, especially under the hot and arid climatic conditions commonly present in this part of California [59]. All three lines accumulated significantly higher concentrations of Se in their shoots, up to 430% more in the case of APS, even though the ECS and GS were not specifically thought to have increased Se [57]. The transgenics were at least equally tolerant to these soil and field conditions compared to wild type, despite their higher Se accumulation.

Despite their greater accumulation of selenium, the transgenic plants were as, or more, tolerant of the soil and field conditions as the wild type. The GS transgenic line was particularly tolerant of the contaminated soil [57]. GS plants grown on the sediment-soil mixture attained 80% of the biomass of plants of GS plants grown on control soil, indicating that these plants were more tolerant of the high levels of soil Se and sodium, as well as greater tolerance to other adverse growth conditions, such as heat or drought. Since the production of GSH is known to be part of the plant's protective response to a variety of stresses including salinity stress, chilling, heat shock, pathogen attack, active oxygen species, air pollution, and heavy metals, the better growth of the GS line on contaminated soil could well have been due to an elevated glutathione concentration [60-63]. Besides glutathione, increased levels of metallothioneins (translated heavy-metal binding peptides), superoxide dismutases, other reducing agents, such as ascorbate, and organic acids, such as malate and citrate, have been observed in response to multiple and seemingly diverse stresses such as wounding, pathogen attack, drought, heat, and metal stress [64-67]. This suggests that the overexpression of certain oxidative stress response genes could indeed afford a wide range of protection against the primary (metal contaminant) and secondary (environmental conditions) stresses faced by plants used in phytoremediation.

5. GENETIC ENGINEERING STRESS-TOLERANT PLANTS FOR PHYTOREMEDIATION: COMPLICATIONS

There have been a number of successes in the past ten years with respect to using genetic engineering to increase plant tolerance and accumulation of trace element contaminants. However, there is much to learn before genetically engineered plants can be used for large-scale phytoremediation processes. The biggest obstacle currently is public fear of genetically modified organisms. Although the use of

genetically engineered plants for phytoremediation is more acceptable than genetically modified food products, there are legitimate concerns to be addressed. One is that the super-accumulating plants will transport more contaminants from soils and water into the aboveground plant tissues so that contaminants may become more bioavailable to wildlife in the surrounding ecosystem [68]. Of particular concern is the risk to endangered wildlife populations. A whole ecosystem approach, accounting for total concentrations and chemical forms at different trophic levels, may be required to accurately evaluate the overall risks and benefits of phytoremediation in different environments.

Certain elements, such as selenium and mercury, can be metabolized to volatile forms, which are released as gases into the atmosphere and, therefore, removed from the local ecosystem. As such, genetic engineering to increase the flux through these metabolic pathways could be an effective means of alleviating risk to the local ecosystem [69,70].

Another concern is the possibility of transgene flow (either the transgene itself or selectable markers) to the ecosystem. Fortunately, researchers have been developing methods to address this concern [70]. Approaches such as chloroplastic engineering and induced sterility will reduce gene flow through pollen [54,71]. At the same time, methods for removing selectable marker genes following homozygous line selection, which typically encode antibiotic resistance, following homozygous line selection have been developed [72].

Researchers involved in genetic engineering plants for phytoremediation also face a number of other technical problems. Perhaps the most critical challenge for the field is to develop plants with truly superior levels of contaminant accumulation that still maintain near-normal biomass. Typically, the transgenic plants produced thus far have a several-fold (i.e., somewhat less than 10-fold) greater accumulation of contaminants than wild type. It is commonly believed that phytoremediation technology will be adopted only when the accumulation approaches 100- or 1000-fold more than wild type. If such concentrations are even possible has yet to be determined. It is clear that we have a great deal to learn if this barrier is to be overcome. In particular, we need to improve our ability to predict the effects of gene over-expression, since there are now several examples of overexpression of oxidative response genes that did not result in plants with increased phytoremediation potential [73]. Overexpression of the same gene in different species can even result in different effects, increasing tolerance in one and sensitivity in another [74]. The gathering and analysis of genome, transcriptome, proteome, and metabolome data along with more sophisticated modeling techniques should enhance our ability to predict the effect of transgenes on contaminant uptake and tolerance.

Similarly, genetic engineering can have undesired consequences. For instance, peptides and metabolites involved in heavy metal binding frequently play a role in essential-metal homeostasis and transport, and, consequently, plant development [75]. Overexpressing such proteins could lead to chelation and sequestration of essential heavy metals and/or a disruption of normal plant development. More generally, constitutive overexpression of a transgene may tax the plant's energy and substrate resources. Indeed, transgenic plants are frequently smaller and slower in developing than wild type plants when grown under control conditions. Here again,

more sophisticated expression systems may be needed to overcome these barriers. Examples of these are the use of stress-inducible or tissue-specific promoters that limit the expression of the transgene to the times and tissues when they are needed, allowing the plant to grow and develop normally [76].

6. FUTURE PROSPECTS

Even though the use of genetic engineering for phytoremediation is a very young field of research, it has had a number of important successes. A solid foundation has been laid through the demonstration that specific detoxification mechanisms can be successfully transferred from hyperaccumulators or microbes into plants. At the same time, the ability of general oxidative stress response genes to afford protection against multiple metals and environmental stresses suggests that high-biomass accumulating plants can be developed through genetic engineering. Advances in bioinformatics and the development of more sophisticated expression systems may be used to create efficient, publicly acceptable plants that can be used for wide-scale phytoremediation projects.

REFERENCES

1. Terry, N., Zayed, A.M., de Souza, M.P. and Tarun, A.S. (2000) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 401-432
2. Suresh, B. and Ravishankar, G.A. (2004) Phytoremediation. a novel and promising approach for environmental clean-up. *Crit. Rev. Biotechnol*. **24,** 97-124.
3. Glass, D.J. (2000) Economic potential of phytoremediation: In *Phytoremediation of Toxic Metals – Using Plants to Clean up the Environment* (Raskin, I. and Ensley, B.D., eds). New York.
4. Cunningham, S.D. and Berti, W.R. (2000) Phytoextraction and phytostabilization: technical, economic and regulatory considerations of the soil-lead issue: In *Phytoremediation of Contaminated Soil and Water* (Terry, N. and G. Bañuelos, G., eds). FL : CRC Press LLC, Boca Raton.
5. Cookson, J.T. (1995) *Bioremediation Engineering, Design and Application*. New York, McGraw-Hill, Inc.
6. Chaudhry, Q., Blom-Zandstra, M., Gupta, S. and Joner, E.J. (2005) Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. *Environ. Sci. Pollut. Res. Int*. **12,** 34-48.
7. Blaylock, M. (2000) Field demonstrations of phytoremediation of lead-contaminated soils. In *Phytoremediation of Contaminated Soil and Water* (Terry, N. and G. Bañuelos, G., eds). FL: CRC Press LLC, Boca Raton.
8. Horne, A.J. (2000) Phytoremediation by constructed wetlands: In *Phytoremediation of Contaminated Soil and Water* (Terry, N. and G. Bañuelos, G., eds). FL : CRC Press LLC, Boca Raton.
9. Bañuelos, G.S. (2000) Factors influencing field phytoremediation of selenium-laden soil. In *Phytoremediation of Contaminated Soil and Water* (Terry, N. and G. Bañuelos, G., eds). FL : CRC Press LLC, Boca Raton.
10. Brooks, R.R. (1994) Plants that hyperaccumulate heavy metals. In *Plants and the Chemical Elements: Biochemistry, Uptake, Tolerance and Ttoxicity* (Garago, M.E., ed). Weinheim, Germany: VCH Verlagsgesellsschaft, pp. 88-105.
11. Cunningham, S., Shann, J., Crowley, D. and Anderson, T. (1997) Phytoremediation of contaminated water and soil: In *Phytoremediation of Soil and Water Contaminants* (Kruger, E., Anderson, T. and Coats, J., eds) Washington, D.C. : ACS Symposium Series 664, pp. 2-17.
12. Bañuelos, G. and Schrale, G. (1989) Plants that remove selenium from soils. *Calif. Agric*. **43,** 19-20.
13. Bañuelos, G.S., Ajwa, H.A., Mackey, M., Wu, L., Cook, C., Akohoue, S. and Zambruzuski, S. (1997) Accumulation of selenium by different plant species grown under increasing sodium and calcium chloride salinity. *J. Environ. Qual*. **26,** 639-646.

14. Hasegawa, E. Terada, M. Sunairi, H. Wakita, F. Shinmachi, A. Noguchi, M. Nakajima and J. Yazaki, J. (1997) Genetic improvement of heavy metal tolerance in plants by transfer of the yeast metallothionein gene (CUP1). *Plant Soil.* **196,** 277-281.
15. Pilon-Smits, E.A.H., Hwang S., Mel Lytle C., Zhu Y., Tai J.C., Bravo R.C., Chen Y., Leustek, T. and Terry, N. (1999) Overexpression of ATP sulfurylase in indian mustard leads to increased selenate uptake, reduction, and tolerance. *Plant Physiol*. **119,** 123-132.
16. Zhu, Y., Pilon-Smits, E.A.H., Jouanin, L. and Terry, N. (1999) Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. *Plant Physiol*. **119,** 73-80.
17. Zhu, Y., Pilon-Smits, E.A.H., Tarun, A., Weber, S.U., Jouanin, L. and N. Terry, N. (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. *Plant Physiol*. **121,** 1169-1178.
18. Kramer, U. and Chardonnens, A.N. (2001) The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Appl. Microbiol. Biotechnol*. **55,** 661-672.
19. Dhanker, O.P., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A. and Meagher, R.B. (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. *Nat. Biotechnol*. **20,** 1140-1145.
20. Pilon-Smits, E.A.H. and Pilon, M. (2002) Phytoremediation of metals using. transgenic plants. *Crit. Rev. Plant Sci*. **21,** 439-456.
21. Bennett, L.E., Burkhead, J.L., Hale, K.L., Terry, N., Pilon, M. and Pilon-Smits, E.A.H. (2003) Analysis of transgenic indian Mustard plants for phytoremediation of metal-contaminated mine tailings. *J. Environ. Qual*. **32,** 432-440.
22. Pilon, M., Owen, J.D., Garifullina, G.F., Kurihara, T., Mihara, H., Esaki, N. and Pilon-Smits, EA.H. (2003) Enhanced selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. *Plant Physiol*. **131,** 1250-1257.
23. Song, W.Y., Sohn, E.J., Martinoia, E., Lee, Y.J., Yang, Y.Y., Jasinski, M., Forestier, C., Hwang, I. and Lee, Y. (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. *Nat. Biotechnol*. **21,** 914-919.
24. Van Huysen, T., Abdel-Ghany, S., Hale, K.L., LeDuc, D., Terry, N., Pilon-Smits, E.A.H. (2003) Overexpression of cystathionine-gamma-synthase enhances selenium volatilization in *Brassica juncea*. *Planta* **218,** 71-78.
25. LeDuc, D.L., Tarun, A.S., Montes-Bayon, M., Meija, J., Malit, M.F., Wu, C.P., AbdelSamie, M., Chiang, C.Y., Tagmount, A., deSouza, M., Neuhierl, B., Bock, A., Caruso, J. and Terry, N. (2004) Overexpression of selenocysteine methyltransferase in Arabidopsis and Indian mustard increases selenium tolerance and accumulation. *Plant Physiol*. **135,** 377-383.
26. Van Huysen, T., Terry, N. and Pilon-Smits, E.A.H. (2004) Exploring the selenium phytoremediation potential of transgenic Indian musturd overexpressing ATP sulfurylase or cystathionine- gamma - synthetase. *Int. J. Phytorem.* **6,** 111-118.
27. Bañuelos, G., Terry, N., LeDuc, D.L., Pilon-Smits, E.A.H. and Mackey, B. (2005) Field trial of transgenic Indian mustard plants shows enhanced phytoremediation of selenium-contaminated sediment. *Environ. Sci. Technol*. **39,** 1771-1777.
28. McGrath, S.P., Zhao, F.J. and Lombi E. (2002) Phytoremediation of metals, metalloids, and radionuclides. *Adv. Agron*. **75,** 1-56.
29. Curie, C., Alonso, J.M., Le Jean, M., Ecker, J.R. and Briat, J.F. (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J*. **347,** 749-755.
30. Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L. and Wagner, G.J. (2000) Expression of arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. *Plant Physiol.* **124,** 125-133.
31. Thomine, S., Wang, R., Ward , J.M,, Crawford, N.M. and Schroeder, J.I. (2000) Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. *Proc. Natl. Acad. Sci. USA*. **97,** 4991-4996.
32. Goto, F., Yoshihara, T. and Saiki, H. (1998) Iron accumulation in tobacco. plants expressing soyabean ferritin gene. *Transgenic Res*. **7,** 173-180.
33. Goto, F., Yoshihara, T., Shigemoto, N., Toki, S. and Takaiwa, F. (1999) Iron fortification of rice seed by the soybean ferritin gene. *Nat. Biotechnol*. **17,** 282-286.

34. Takahashi, M., Nakanishi, H., Kawasaki, S., Nishizawa, N.K. and Mori S. (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. *Nat. Biotechnol.* **19,** 466-469.
35. Delhaize, E. and Ryan, R. (1995) Aluminum toxicity and tolerance in plants *Plant* Physiol. **107,** 315.-321.
36. Larsen, P.B., Degenhardt, J., Stenzler, L.M., Howell, S.H. and Kochian, L.V. (1998) Aluminum-resistant Arabidopsis mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiol*. **117,** 9-17.
37. Medappa, K.C. and Dana, M.N. (1970) *J. Am. Soc. Hortic. Sci*. **95,** 107.
38. Zhou, J. and Goldsbrough, P. B. (1995). Structure, organization and expression of the metallothionein gene family in Arabidopsis. *Mol. Gen. Genet*. **248,** 318-328.
39. Cobbett, C.S. (2000) Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol*. **125,** 825-832.
40. Reeves, R.D. and Baker, A.J.H. (2000) Metal hyperaccumulating hlants: Phytoremediation of toxic metals: using plants to clean up the environment. Eds. I. Raskin and B.D. Ensley, Wiley, New York.
41. Lasat, M.M. (2002) Phytoextraction of toxic metals. J. Environ. Qual., **31,** 109-120.
42. Eapen, S. and D'Souza, S.F. (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnol. Adv*. **23,** 97-114.
43. Slootmaker, L.A.J. (1974) Tolerance to high soil acidity. in wheat related species, rye and triticale. *Euphytica* **23,** 505-513.
44. Eenink, A.H. and Garretsen, G. (1977) Inheritance of insensitivity of lettuce to a surplus of exchangeable manganese in steam sterilized soils. *Euphytica* **26,** 47-53.
45. Howden, R., Goldsbrough, P.B., Andersen, C.R. and Cobbett, C.S. (1995) Cadmium-sensitive, cad1 mutants of Arabidopsis thaliana are phytochelatin deficient. *Plant Physiol*. **107,** 1059-1066
46. Larsen, P.B., Degenhardt, J., Tai, C.Y., Stenzler, L.M., Howell, S.H. and Kochian, L.V. (1998) Aluminum-resistant Arabidopsis mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiol*. **117,** 9-17.
47. van den Burg, B. (2003) Extremophiles as a source for novel enzymes. *Curr. Opin. Microbiol*. **6,** 213-218.
48. Croal, L.R., Gralnick, J.A., Malasarn, D. and Newman, D.K. (2004) The Genetics of Geochemistry. *Annu. Rev. Genet*. **38,** 175-202.
49. Neuhierl, B. and Böck, A. (1996) On the mechanism of selenium tolerance in selenium-accumulating plants. Purification and characterization of a specific selenocysteine methyltransferase from cultured cells of Astragalus bisculatus. *Eur. J. Biochem*. **239,** 235-238.
50. Brown, T. and Shrift A (1981) Exclusion of selenium from proteins in selenium-tolerant *Astragalus* species. *Plant Physiol* **67,** 1951-953.
51. Summers, A.O. (1986). Organization, expression and evolution of genes for mercury resistance. *Annu. Rev. Microbiol*. **40,** 607-634.
52. Rugh, C.L., Bizily, S.P. and Meagher, R.B. (2000) Phytoremediation of environmental mercury pollution: In *Phytoremediation of Toxic Metals – Using Plants to Clean up the Environment* (Raskin, I. and Ensley, B.D., eds). New York.
53. Ruiz, O.N., Hussein, H.S., Terry, N. and Daniell, H. (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. *Plant Physiol*. **132,** 1344-1352.
54. Daniell, H., Khan, K.S. and Allison, L. (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci*. **7,** 84-89.
55. Grill, E., Loeffler, S., Winnacker, E.L. and Zenk. M.H.1989. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase) . *Proc. Natl. Acad. Sci. USA* **86,** 6838-6842.
56. Gallego, S.M., Benavídes, M.P. and Tomaro, M.L. (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Sci*. **121,** 151-159.
57. Zenk, M.H. (1996) Heavy metal detoxification in higher plants. *Gene*, **179,** 21-30.
58. de Souza, M. P., Pilon-Smits, E.A.H., Lytle, C.M., Hwang, S., Tai, J., Honma, T.S.U., Yeh, L. and Terry, N. (1998) Rate limiting steps in Se assimilation and volatilization by *Brassica juncea*. *Plant Physiol*. **117,**1487-1494.
59. Bañuelos, G.S., Ajwa, H.A., Wu, L., Guo, X., Akohoue, S. and Zambrzuski, S. (1997) Selenium-Induced Growth Reduction in*Brassica*Land Races Considered for Phytoremediation. *Ecotox. Environ. Safe.* **36,** 282-287.

60. May, M.J.,Vernoux, T., Leaver, C., Van Montagu, M. and Inzé, D. (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J. Exp. Bot*. **49,** 649-667.
61. Noctor, G., Arisi, A.C.M., Jouanin, L., Kunert, K.J., Rennenberg, H. and Foyer, C.H. (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants *J. Exp. Bot*. **49,** 623-647.
62. Kocsy, G., Galiba, G. and Brunold, C. (2001) Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants. *Physiol. Plant* **113,** 158-164.
63. Ruiz, J.M. and Blumwald, E. (2002) Salinity-induced glutathione synthesis in *Brassica napus*. *Planta* **214,** 965-969.
64. Farmer, E.E. and Ryan, C.A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* **4,** 129-134.
65. Buchanan-Wollaston, V. (1994) Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus*. Identification of a gene encoding a senescence-specific metallothionein-like protein. *Plant Physiol*. **105,** 839-846.
66. Choi, D., Kim, H.M., Yun, H.K., Park, J.A., Kim, W.T. and Bok, S.H. (1996) Molecular cloning of a metallthionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. *Plant Physiol*. **112,** 353-359.
67. Kramer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M. and Smith, J.A.C. (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379,** 635-638.
68. Moffat, A.S. (1995) Plants proving their worth in toxic metal cleanup. Science. **269,** 302-303.
69. Lin, Z.Q., Schemenauer, R.S., Cervinka, V., Zayed, A.M., Lee, A. and Terry, N. (2000) Selenium volatilization from a soil-plant system for the remediation of contaminated water and soil in the San Joaquin Valley. *J. Environ. Qual*. **29,** 1048-1056.
70. Meagher, R. B. (2000) Phytoremediation of toxic elemental and organic pollutants. *Curr. Opin. Plant Biol*. **3,** 153-162.
71. Greenland, A., Bell, P., Hart, C., Jepson, I., Nevshemal, T. and Wright, S. (1998) *71Symp. Soc. Exp. Biol.* **51,** 141.
72. Daniell, H., Muthukumar, B. and Lee, S.B. (2001) Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet*. **39,** 109-116.
73. Tepperman, J.M. and Dunsmuir, P. (1990) Transformed plants with elevated level of chloroplastic SOD are not more resistant to superoxide toxicity. *Plant Mol. Biol*. **14,** 501-511
74. Thomas, J.C., Davies, E.C., Malick, F.K., Endreszl, C., Williams, C.R., Abbas, M., Petrella, S., Swisher, K., Perron, M., Edwards, R., Osenkowski, P., Urbanczyk, N., Wiesend, W.N. and Murray, K.S. (2003) Yeast metallothionein in transgenic tobacco promotes copper uptake from contaminated soils. *Biotechnol. Prog*. **19,** 273-280.
75. Buchanan-Wollaston, V. (1994) Isolation of cDNA clones for genes that are expressed during leaf senescence in Brassica napus. Identification of a gene encoding a senescence-specific metallothionein-like protein. *Plant Physiol*. **105,** 839-846.
76. Pellegineschi, A., Reynolds, M., Pacheco, M., Brito, R.M., Almeraya, R., Yamaguchi-Shinozaki, K. and Hoisington, D. (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* **47,** 493-500.

SECTION V

OSMOTIC STRESSES

9. METABOLIC ENGINEERING OF GLYCINEBETAINE

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Abstract. Drought and salinity are among the worst scourges of agriculture. One effective mechanism to reduce damage from these stresses is the accumulation of high intracellular levels of osmoprotectant compounds. Glycinebetaine is a typical osmoprotectant. Recent studies demonstrated that the introduction of betaine accumulation pathways improves the stress tolerance of plants. Metabolic engineering is a useful technique to improve stress tolerance of important crops. The levels of betaine accumulation is determined by the rates of betaine synthesis, betaine uptake and efflux, and metabolisms. Although betaine is synthesized from choline in plants, some halotolerant cyanobacterium synthesizes betaine from glycine by three step methylation. Introduction of betaine synthesis pathways into plants have been examined. It is largely unknown how betaine is transported among plants. Only few betaine transporter genes have been isolated. In addition to the stress tolerance of plants, betaine uptake from foods would play an important role in human nutrition. As a methyl donor, betaine participates in the methionine cycle. In this review, recent progress on metabolic engineering of betaine will be described.

1. INTRODUCTION

Cells of many organisms experience a variety of environmental stresses such as high external osmolality, desiccation, and drought that reduce the amount of intracellular water [1-3]. In response to these water stresses, both prokaryotic and eukaryotic cells transport or synthesize highly soluble, low molecular weight compounds called osmolytes or osmoprotectants that allow the organism to take up and retain cellular water [4-6] and resume or sustain normal cellular processes. Osmolytes include *N*-methylated amino acids and amines (glycine betaine, sarcosine, and trimethylamine-*N*-oxide), amino acids (glycine, proline, and glutamate), and polyols (manitol and trehalose). Structures of some osmoprotectants are shown in Fig. 1.

Glycine betaine (*N*,*N*,*N*-trimethylglycine, GB) is one of the most widespread osmolytes, found in bacteria, halophilic archaebacteria, marine invertebrates, plants, and mammalians [5,6]. Recent research on several aspects of betaine attracts considerable attentions. These are on biosynthetic pathway of betaine [7-9], new concept "osmophobic effects" [10], transport of betaine [11,12] genetic engineering

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of betaine [13-16], and role of betaine on human health [17]. In this review, we discuss on these subjects.

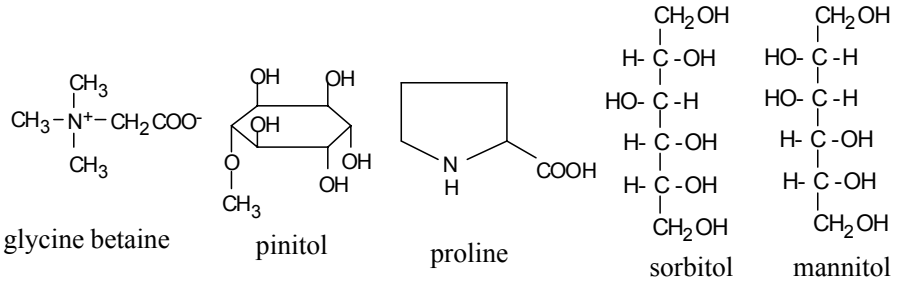


Figure 1. Structures of some osmoprotectants.

2. PHYSIOLOGICAL ROLES OF GLYCINE BETAINE

Betaine is found in microorganisms, plants, and animals [4-7] and is a significant component of many foods [17], including wheat, shellfish, spinach, and sugar beets. Betaine is a zwitterionic quaternary ammonium compound that is also known as trimethylglycine, glycine betaine, lycine, and oxyneurine. It is a methyl derivative of the amino acid glycine with a molecular weight of 117.2 and has been characterized as a methylamine because of its 3 chemically reactive methyl groups. Betaine was first discovered in the juice of sugar beets (*Beta vulgaris*) in the 19th century and was subsequently found in several other organisms. The physiologic function of betaine is either as an organic osmolyte to protect cells under stress or as a catabolic source of methyl groups via transmethylation for use in many biochemical pathways [17]. The principle role for betaine in plants and microorganisms is to protect cells against osmotic inactivation [4-6,18,19] Exposure to drought, high salinity, or temperature stress triggers betaine synthesis in chloroplast in plants [7,8], mitochondria in animal [20,21] or cytosol in bacteria [22], which results in its accumulation in the cells. Betaine is a compatible osmolyte that increases the water retention of cells, replaces inorganic salts, and protects intracellular enzymes against osmotically induced or temperature induced inactivation [23]. For example, spinach is grown in saline soil, and betaine can accumulate in amounts of up to 3% of fresh weight. This enables the chloroplasts to photosynthesize in the presence of high salinity [24].

3. OSMOPHOBIC EFFECTS

Protein stability plays an important role not only in its biological function but also in medical science and protein engineering. Osmolytes can protect proteins from the unfolding and aggregation induced by extreme environmental stress [10]. Significant progress on molecular mechanisms of osmolytes has been made recently [23,25]. It was found that the protective effects of osmolytes are a result of enhancing the

structural stability of native protein. The effects of osmolytes on preventing proteins against aggregation are due to the preferential increase of free energy of the activated complex (unfolded protein) which shifts the equilibrium between the native state and the activated complex to favor the native state (Fig. 2). The effect of osmolytes on the free energy of the native protein is small.

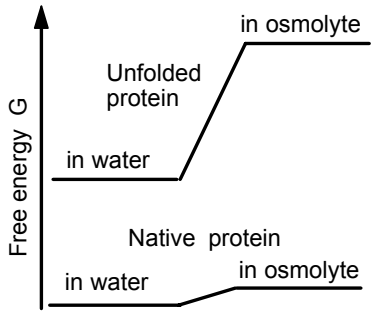


Figure 2. A schematic diagram of free energy in water and osmolyte.

The protection of enzyme activity by osmolytes is due to the enhancement of the structural stability of the whole protein rather than the active site.

In natural selection of organic osmolytes as protein stabilizers, it appears that the osmolyte was selected as molecules which exhibit the unfavorable interaction with the peptide backbone (osmophobic effect) [10,23,25]. Because the peptide backbone is highly exposed to osmolyte in the denatured state, the osmophobic effect preferentially raises the free energy of the denatured state, shifting the equilibrium in favor of the native state. Since the osmophobic effect is the interaction on the denatured state, the native state is relatively unfettered by the presence of osmolyte. The osmophobic effect is a new thermodynamic force in nature that complements the well-recognized hydrophobic interactions, hydrogen bonding, electrostatic and dispersion forces that drive protein folding.

Osmolytes are preferentially excluded from folded protein surface which is equivalent to preferential interaction with water rather than with the solute. As a result, the local concentration of the solute at the protein surface is less than its bulk concentration. Under high concentrations of osmolytes, the efficient osmophobic effects are anticipated which has been proposed to be the basis of their evolutionary selection [10,23,25]

4. BIOSYNTHETIC PATHWAYS OF GLYCINEBETAINE

Most known biosynthetic pathways of betaine include a two-step oxidation of choline: choline → betaine aldehyde → betaine (Fig. 3). The first step is catalyzed by choline monooxygenase (CMO) in plants [8], choline dehydrogenase (CDH) in animals [20] and bacteria [18,22], and choline oxidase (COD) in some bacteria [26]. The second step is catalyzed by NAD^+-dependent betaine aldehyde dehydrogenase (BADH) [7,18,21] in all organisms although in some bacteria, choline

dehydrogenase and choline oxidase also catalyze the second step. CDH is the membrane bound enzyme linked to respirately electron transport systems in *E. coli* and animals [18,20]. In mamalian, CDH [20] and BADH [21] are localized in mitochondria . COD is a soluble enzyme known in *Arthrobacter globiformis*.

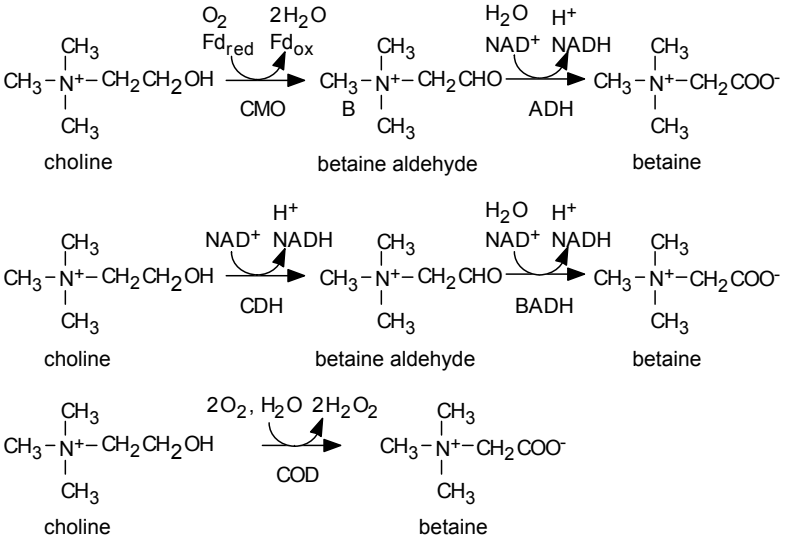


Figure 3. Betaine synthetic pathways from choline.

In plants, the first step is catalyzed by a novel Rieske-type iron-sulfur enzyme choline monooxygenase (CMO) which is not found in animals and bacteria [8]. CMO [8] and BADH [7] are localized in chloroplasts. CMO is not well known, having so far been found only in Chenopodiaceae (spinach and sugar beet) and Amaranthaceae [6-8], and not detected even in some betaine accumulating plants such as mangrove [27]. Betaine accumulating mangrove, *Avicennia marina,* contains two BADH genes, but CMO gene could not be detected [27]. CMO enzyme is soluble and insensitive to carbon monooxide, contains an Rieske-type iron-sulfur center, and consists of homo-dimer or -trimer of subunit *Mr* 42,000 [8]. These

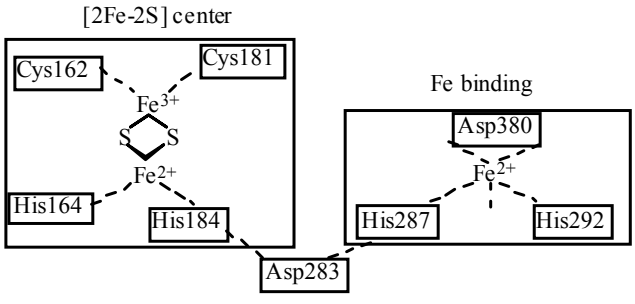


Figure 4. A schematic structure of active site of CMO.

properties are completely unrelated to CDH, COD, and cytochrome P-450 type monooxygenases. The importance of Cys181 and His287 for binding of Fe-S cluster and Fe was demonstrated (Fig. 4) [28]. Spinach BADH has very short or no transit peptide, suggesting the targetting signal in mature coding region. Barley has a salt inducible BADH localized in peroxisome [29]. It has a tri-peptide SKL at the C-terminus [29]. Betaine accumulating mangrove (*Avicennia marina*) and barley have at least two BADH genes [27,30]. One chloroplast and the other is peroxisome localized, BADHs, [27,30]. Since CMO gene could not be detected in both plants, physiological function of multiple BADHs and biosynthetic pathway of betaine are unknown. Although spinach BADH is dimers and *E. coli* BADH is tetramer, both BADHs catalyzed the oxidation of not only betaine aldehyde but also omega aldehyde [31]. The affinities for betaine aldehyde were similar in the spinach and *E. coli* BADHs, whereas those for omega-aminoaldehydes were higher in spinach BADH than in E. coli BADH [32]. In contrast, the mangrove (*Avicennia marina*) BADH efficiently catalyzed the oxidation of betainealdehyde, but not the oxidation of omega-aminoaldehydes and were more stable at high temperature than the spinach BADH [27].

Recently, it was shown that a halotolerant cyanobacterium *Aphanothece halophytica* isolated from Dead Sea, has a novel biosynthetic pathway of betaine from glycine [33]. Two N-methyltransferase genes were involved for it (Fig. 5). One of gene products (GSMT) catalyzed the methylation reactions of glycine and sarcosine with S-adenosylmethionine (AdoMet, SAM) acting as the methyl donor. The other one (DMT) specifically catalyzed the methylation of dimethylglycine to betaine. Both enzymes are active as monomers. Betaine, a final product, did not show the feed back inhibition for the methyltransferases even in the presence of 2 M. A reaction product, S-adenosyl homocysteine (AdoHcy), inhibited the methylation reactions with relatively low affinities. The co-expressing of two enzymes in *E. coli* increased the betaine level and enhanced the growth rates. Immunoblot analysis revealed the increased accumulation of these enzymes in *Aphanothece halophytica* cells under high salinity.

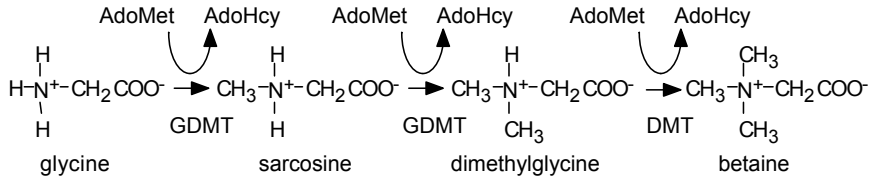


Figure 5. Betaine synthesis pathway by glycine methylation.

5. GENETIC ENGINEERING OF GLYCINE BETAINE

In the natural environment, plants often grow under unfavorable conditions, such as drought, salinity, chilling, high temperature, wounding, or strong light. These

conditions are known collectively as abiotic stresses which can delay growth and development, reduce productivity and, in extreme cases, cause the plant to die [1-3]. Glycine betaine is synthesized in some plant species at elevated rates in response to various types of environmental stress [5-8]. Whereas in many plants, such as *Arabidopsis*, rice (*Oryza sativa*), and tobacco (*Nicotiana tabacum*), betaine can not be detected or negligible level if any. Glycine betaine appears to be a critical determinant of stress tolerance. The concentrations of betaine is correlated with the level of tolerance. Moreover, exogenous application of glycine betaine improves the growth and survival of a wide variety of plants under various stresses.

Transgenic plants of various species have been produced that express CMO, CDH, or COD [13-16,28,33]. For the expression of CMO and CDH, BADH gene was also expressed although betaine synthesis occurs via the use of BADH gene from host plants. These plants accumulate glycine betaine at various levels and exhibit enhanced tolerance of several types of stress. The metabolic engineering of glycine betaine thus appear to be an effective method for improving stress tolerance. However, the accumulation levels of glycine betaine in transgenic plants are generally low (<5 μmol/g fresh weight (gFW)) compared with those in betaine accumulating plants that accumulate betaine, 4-40 μmol/gFW, under stress conditions. These facts suggest the importance of other factor(s) than CMO for the accumulation of glycine betaine. The importance of two factors, the availability of endogenous choline and the transport of choline across the chloroplast envelope have been demonstrated [13,14].

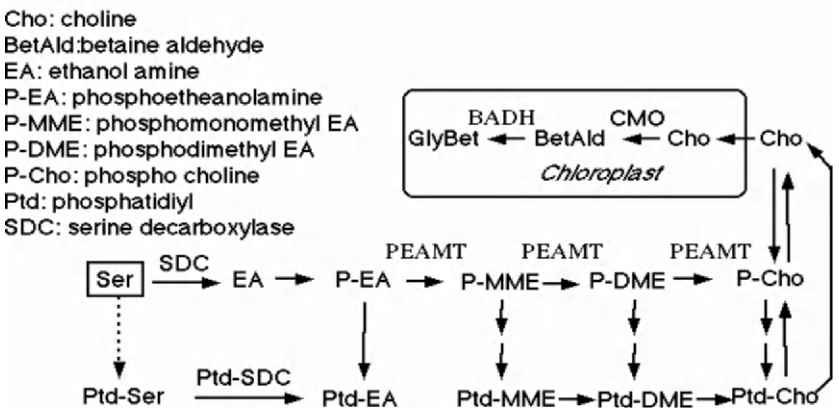


Figure 6. A schematic diagram of betaine synthesis from serine.

The key enzyme in the choline-biosynthetic pathway is phosphoethanolamine N-methyltransferase (PEAMT), which catalyzes each of the three methylation reactions that are required to convert phosphoethanolamine to phosphocholine (Fig.6) [34]. A gene for PEAMT has been isolated from spinach and used to transform tobacco plants together with CMO and BADH [34]. The transgenic plants that expressed PEAMT, CMO, and BADH contained up to 50-fold more free

choline and accumulated 30-fold more glycine betaine than plants transformed with the vector alone. Despite the simultaneous expression of CMO, BADH and PEAMT, the level of glycine betaine was still lower than the transgenic rice plants that expressed a *codA* gene in their cytosol [15,16,33]. Since it has been shown that plants synthesize ethanolamine by direct decarboxylation of serine, it is interesting to construct the transgenic plants overexpressing CMO, BADH, PEAMT, serine decarboxylase. Durring methylation reactions by PEAMT, S-AdoMet is converted to S-AdoHcy which is a potent inhibitor of methylation of S-AdoMet. Adenosine is produced by hydrolysis oh S-AdoHcy. Importance of adenosine kinase, which catalyzes the salvage synthesis of adenine monophosphate from adenosine and ATP, for transmethylation was demonstrated [35] .

Compared to CMO gene, the transgenic plants that express the COD gene seem to be more effective to confer the tolerance for abiotic stresses [15,16]. Endogenous glycine betaine contents as low as 0.1 μmol/gFW are apparently sufficient to confer high levels of tolerance in tomato plants, as achieved via transformation with the COD gene [36]. Exogenous application of H_2O_2 improved both chilling and oxidative tolerance concomitant with enhanced catalase activity [37]. During the oxidation of choline to glycine betaine catalyzed by COD, H_2O_2 is produced. Recent results suggest that this increase of H_2O_2 in COD transgenic plants might activate the H_2O_2-inducible protective mechanism, resulting in improved chilling and oxidative tolerances in glycine betaine-accumulating COD transgenic plants[36].

The potential role of N-methyltransferase genes for betaine synthesis was examined in a fresh water cyanobacterium *Synechococcus* sp. PCC 7942 [38]. The plasmids for control and for expressions of *E. coli bet*-genes (CDH/BADH), Na^+/H^+ antiporter from *Aphanothece halophytica* (ApNhaP1), and methyltransferase genes (ApGSMT/ApDMT), were introduced into *Synechococcus* cells. Under normal conditions, the *Synechococcus* cells transformed with these vectors could grow at almost the same rate. When the growth medium contained 0.4 M NaCl, the cells expressing vector alone and *bet*-cluster genes could not grow whereas the cells expressing ApGSMT/ApDMT could grow. Thus the betaine-synthesis by three-step methylation of glycine conferred salt tolerance more than that by choline oxidation. Neither control nor CDH/BADH expressing cells could grow in sea water from Mikawa bay of Aichi-prefecture in Japan, with psu 30.4, whereas the cells expressing ApGSMT/ApDMT and ApNhaP1 could grow in sea water (Fig. 7A). The betaine levels in the *Synechococcus* cells expressing ApGSMT/ApDMT were always higher than those in the cells expressing CDH/BADH genes (Fig. 7B). Especially, at 0.5 M NaCl, the betaine level was significantly high in the cells expressing ApGSMT/ApDMT whereas the betaine could not be measured in the cells expressing CDH/BADH due to no growth at this salinity. The betaine level of cells expressing ApGSMT/ApDMT at 0.5 M NaCl was about 5-fold higher than that in the cells expressing *bet*-cluster genes at 0.3 M NaCl, and was estimated as about 200 mM. In plants transformed with choline-oxidizing enzymes, it was shown that exogenous supply of choline enhanced the accumulation levels of betaine [13,14]. However, it was found that the exogenous addition of glycine, sarcosine, dimethylglycine, and SAM (AdoMet) to the cells expressing ApGSMT/ApDMT did not enhance the accumulation levels of betaine. Thus the levels of substrates were

sufficient and did not limit the rates of betaine synthesis in the transformed *Synechococcus* cells.

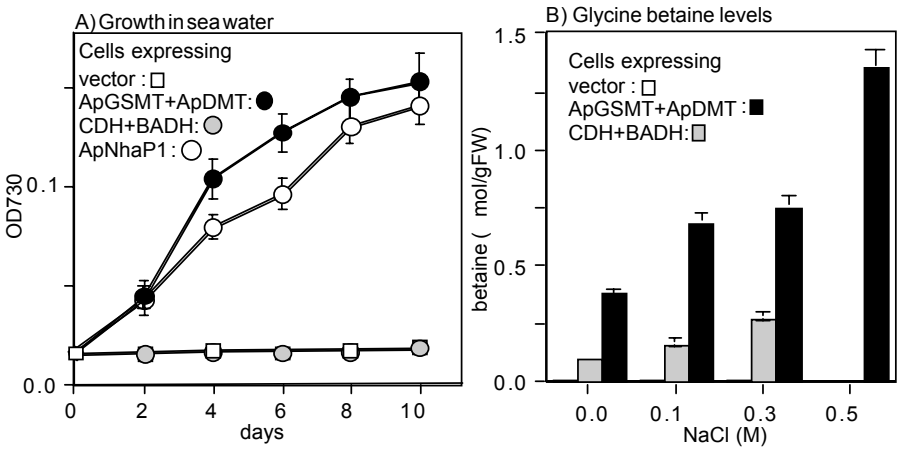


Figure 7. Growth and betaine accumulation of transformed freshwater cyanobacteria.

Genetic engineering of higher plants with betaine via choline oxidase (COD), choline dehydrogenase (CDH), and choline monooxygenase (CMO) conferred abiotic-stress tolerance. However, the accumulation levels of betaine were small even after supplying exogenous choline. To examine the potential role of N-methyltransferases for the accumulation of betaine in plants, the genes encoding ApGSMT and ApDMT with the 35S promoter were transferred into *Arabidopsis* plants [38]. The proteins and activities of ApGSMT and ApDMT were only detected in transgenic plants. As a reference, CMO transformant was also tested. Under normal growth conditions, no phenotype difference was observed among the wild-type and transgenic plants. Upon salt stress, the germination of wild-type seeds was more severely inhibited than that in the transformants. Among the transformants, the CMO transformant was more severely inhibited than the ApGSMT/ApDMT transformant. *Arabidopsis* plants expressing ApGSMT/ApDMT increased the tolerances for various abiotic stresses during the developmental stages more than wild-type and CMO transformants did. The stress tolerance of reproductive stage was also tested. The plants that are just before appearance of the primary inflorescence, were irrigated with 1/10 MS medium containing 0.2 M NaCl until harvesting of seeds. Although numbers of siliques and seed weight in wild-type and transgenic plants were similar under normal conditions, the transgenic plants expressing ApGSMT/ApDMT produced siliques about 15-fold higher than those in wild-type plants at high salinity. The ApGSMT/ApDMT expressing plants produced about 3- to 4-fold more siliques than those in CMO expressing plants. Thus,

ApGSMT/ApDMT expressing *Arabidopsis* plants increased the tolerance for salt stress during the reproductive stage and produced higher seed yields.

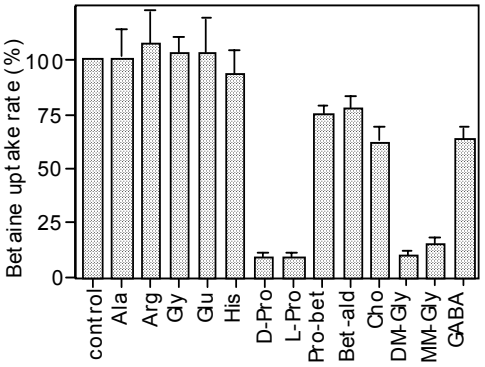
The levels of betaine in wild-type *Arabidopsis* plants were very low or negligible. By contrast, transgenic plants expressing ApGSMT/ApDMT accumulated betaine, and the amounts rose as the NaCl level was increased. The betaine levels at 150 mM NaCl were about 3-fold higher than those without addition of NaCl. Because the expression levels of ApGSMT and ApDMT did not change so much upon salt stress in transgenic plants, the increase of betaine would be caused by the activation of enzymes or by an increased supply of the precursors, glycine and SAM (AdoMet), upon salt stress. Large amounts of betaine were detected in leaves, stems, roots, and also flowers. By contrast, the CMO plants accumulated betaine preferencially in roots. The exogenous supply of glycine or dimethylglycine to the control growth medium enhanced the accumulation levels of betaine by ApGSMT/ApDMT plants more than 2-fold whereas sarcosine did not enhance the levels. When NaCl and glycine were included in the growth medium, the levels of betaine in leaves, stems, and roots of *Arabidopsis* plants expressing ApGSMT/ApDMT were about 1 to 2 μmol/gFW. In contrast, *Arabidopsis* plants expressing CMO accumulated betaine about 0.8 μmol/gFW in roots, but very low levels in leaves and stems. The accumulation of betaine at high levels in various organs of *Arabidopsis* plants expressing ApGSMT/ ApDMT would be the reason for the significant improvement against various abiotic stresses.

Addition of the methyl donor SAM (AdoMet) to *Arabidopsis* plants expressing ApGSMT/ApDMT that were grown in the MS medium with or without NaCl, enhanced the betaine levels slightly (~1.2-fold). On the other hand, additions of glycine and dimethylglycine, but not sarcosine, brought about much higher levels of betaine. Thus, SAM (AdoMet) is not a limiting factor for the accumulation of betaine in transgenic plants, but glycine levels are somewhat limiting. After 6 days salt stress (0.2 M NaCl), wild-type plants were almost all bleached and/or dead, whereas the CMO expressing plants supplied with choline survived. Almost all ApGSMT/ApDMT expressing plants without supply of glycine, still retained green leaves, but, if glycine was supplied to the ApGSMT/ApDMT expressing plants, the plant size was greater, and especially root length was longer than those plants to which glycine was not supplied. Thus, glycine was limiting for maximal salt stress tolerance, in the plants expressing ApGSMT/ApDMT. Consistent with the above conclusion, the levels of serine and glycine in the plants expressing ApGSMT/ ApDMT were significantly lower than those in the wild-type plants under salt-stress conditions, whereas the proline levels were similar.

6. TRANSPORT OF BETAINE

The increase of osmoprotectants is achieved either by altering metabolism (increasing biosynthesis and/or decreasing degradation) or by transport (increased uptake and/or decreased export) [11,12]. In plants, glycine betaine is synthesized in chloroplasts from choline via two step oxidations and must be transported for long distance upon the environmental stresses. However, very little is known about the

betaine transport in plants and the betaine transport activity was only directly demonstrated using a proline transporter from tomato (LeProT1) [39]. Recently, two full length and one partial betaine transporter genes were isolated from betaine accumulating mangrove *Avicennia marina* [40]. Their homologies to betaine transporters from bacteria and betaine/GABA transporters from mammalian cells were low, but high to proline transporters from *Arabidopsis* and tomato. Two full length transporters could complement the Na^+-sensitive phenotype of the *E. coli* mutant deficient in *betT, putPA*, *proP*, and *proU*. Both transporters could efficiently take up betaine and proline with similar affinities (Km, 0.32-0.43 mM) and maximum velocities (1.9-3.6 nmol/min mg protein) (Fig. 8). The uptakes of betaine and proline were significantly inhibited by mono- and di-methylglycine, but only partially inhibited by betaine aldehyde, choline, and GABA. Sodium- and potassium-chloride markedly enhanced betaine uptake rates with optimum concentrations at 0.5 M whereas sucrose showed only modest activation. Site-directed mutagenesis approach suggested the involvement of a periplasmic loop for the deregulation of betaine transport. Three proline transporters (AtProT1-3) have been reported in *Arabidopsis* plants, all of which are localized at the plasma membrane. The affinity of all three AtProTs was higher for glycine betaine (K_m = 0.1-0.3 mM) thant those for Pro (K_m = 0.4-1 mM). AtProT1 expressed in the phloem or phloem parenchyma cells throughout the whole plant. AtProT2 expressed in the epidermis and cortex cells in roots, whereas AtProT3 expressed at the above-ground parts of the plant, suggesting different roles in planta by different expression pattern. However, several amino acid permeases transport proline at higher affinity than AtProTs. Thus the role of ProT for abiotic stress tolerance is not clear. Genetic engineeringof glycine betaine has not been reported.



B) Kinetic parameters for AmT1 and AmT2

		Km (mM)	Vm
betaine uptake	AmT1	0.34	3.3
	AmT2	0.36	1.9
proline uptake	AmT1	0.43	3.6
	AmT2	0.32	2.7

Vm : nmol/min/mg protein

C) Km values of Arabidopsis amino acid permeases for plorine uptake

	Km (mM)		Km (mM)
AtLH2	0.013	AtAAP5	0.500
AtAAP1	0.060	AtAAP6	0.067
AtAAP2	0.140	AtProT1	0.360
AtAAP3	0.250	AtProT2	0.360
AtAAP4	0.134	AtProT3	0.380

Figure 8. Properties of mangrove betaine transporter.

7. BETAINE IN HUMAN NUTRITION

Since the above transgenic plants accumulates betaine in high amounts, we discuss on the effects of betaine accumulation on human health. The principal physiologic role of betaine for human is as an osmolyte and methyl donor. As an osmolyte, betaine protects cells, proteins, and enzymes from environmental stress as discussed in above. As a methyl donor, betaine participates in the methionine cycle primarily in the human liver and kidneys. Inadequate dietary intake of methyl groups leads to hypomethylation in many important pathways, including 1) disturbed hepatic methionine metabolism with elevated plasma homocysteine concentrations and decreased S-AdoHcy concentrations, and 2) inadequate hepatic fat metabolism leading to fatty accumulation. This alteration in liver metabolism would contribute to various diseases, including coronary, cerebral, hepatic, and vascular diseases. Betaine has been shown to protect internal organs, improve vascular risk factors, and enhance performance.

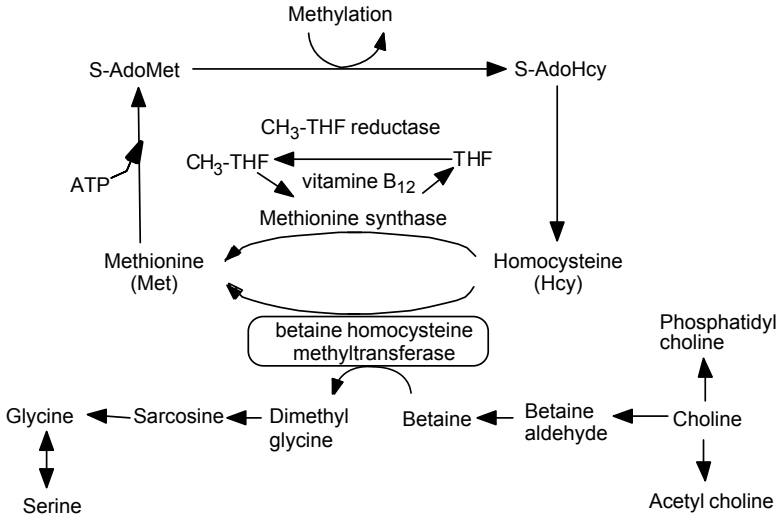


Figure 9. Metabolic pathways of betaine in mammalian cells.

Betaine has been used as a dietary feed supplement in animal nutrition for 50 years. Betaine is added to farmed fish feed as an osmolyte to protect fish from the stress upon the move from low to high salinity [17]. Betaine protects chick intestinal cells from coccidia infection, alleviates symptoms, and improves performance [41]. As a methyl donor, betaine provides the one-carbon units that can spare the amount of dietary methionine and choline required for optimal nutrition. For example, betaine improves growth and the efficiency of food utilization and reduces body fat in pigs and chicks [42]. Humans obtain betaine from foods that contain either betaine or choline-containing compounds. Betaine is present in foods in variable

amounts that depend on food items and their growth and stress conditions. Dietary intake of betaine can be estimated as 0.5~2.5 g per day [17,43,44]. A maijor metabolic fate of choline is the oxidation to betaine in the liver and kidney via a two-step process (Fig. 4). Other metabolic fate of choline is used for the synthesis of acetylcholine and phospholipids (Fig. 9). A diet of normal foods is estimated as 1 g choline per day [17,44].

In mammalian, betaine is transported mainly by betaine/gamma-aminobutyric acid transport system. Betaine showed rapid uptake and distribution, with a peak at 1~2 h in serum and 20-70 μmol betaine per liter serum at the resting concentrations [45]. Betaine is mainly eliminated by metabolism, not excretion, even at relatively high doses (100 mg/kg body wt) betaine [45] although urinary excretion of betaine is elevated in subjects with renal disease or diabetes [46]. Subacute and subchronic rat studies determined that betaine is nontoxic at all doses studied (0-5 % of the diet) [47]. Betaine is catabolized by transmethylation reactions [43] which involves the transfer of methyl groups via the methionine cycle (Fig. 9). Betaine homocysteine methyl transferase (BHMT) transfers a methyl group of betaine and produces dimethylglycine. The conversion of homocysteine to methionine is important to conserve methionine, detoxify homocysteine, and produce SAM (AdoMet). Elevated total homocysteine concentrations and low SAM (AdoMet) concentrations have been associated with chronic disease. The formation of methionine from homocysteine can also occur by 5-methyltetrahydrofolate (CH_3-THF). The enzyme methionine synthase transfers a methyl group of CH_3-THF to the cofactor cobalamin, vitamin B-12, which converts to methylcobalamin. Then methylcobalamin transfers the methyl to homocysteine to form methionine. Methylenetetrahydrofolate reductase (MTHFR) is involved in reforming CH_3-THF from CH_3-THF. BHMT is a zinc metalloenzyme with an optimum pH of 7.8 [48] that occurs in the human liver, kidney, and optic lens. Concentrations of liver BHMT increase when rats are fed diets containing betaine or choline [49]. Betaine increases serum methionine, trans-methylation rate, homocysteine remethylation, and methionine oxidation in healthy adults. Animals injected with betaine show a dose dependent increase in red blood cell SAM (AdoMet). Thus, transmethylation metabolic pathways closely interconnect choline, betaine, methionine, CH_3-THF, and B-12. The growing body of evidence shows that betaine is an important nutrient for the prevention of chronic disease. Success of transgenic plants which accumulate a large amount of betaine might have two merits, increase of crop yield under abiotic stress and improve of human health.

REFERENCES

1. Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **51,** 463-499.
2. Ramanjulu, S. and Bartels, D. (2002) Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environ*. **25,** 141-151.
3. Bohnert, H.J. and Cushman, J.C. (2002) Plants and environmental stress adaptation strategies. In: *Plant Biotechnology and Transgenic Plants* (Barz, M. and Oksman-Kaldentey, D., eds). New York : Marcel Dekker, pp. 635-664.

4. Lucht, J.M. and Bremer, E. (1994) Adaptation of Escherichia coli to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol. Rev*. **14,** 3-20.
5. Hanson, A.D., Rathinasabapathi, B., Rivoal, J., Burnet, M., Dillon, M.O. and Gage, D.A. (1994) Osmoprotective compounds in the *Plumbaginacea*e: a natural experiment in metabolic engineering of stress tolerance. *Proc. Natl. Acad. Sci. USA* **91,** 306-310.
6. McNeil, S.D., Nuccio, M.L. and Hanson, A.D. (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. *Plant Physiol*. **120,** 945-950.
7. Weretilnyk, E.A. and Hanson, A.D. (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. *Proc. Natl. Acad. Sci. USA* **87,** 2745-2749.
8. Rathinasabapathi, B., Burnet, M., Russell, B.L., Gage, D.A., Liao, P.C., Nye, G.J., Scott, P., Golbeck, J.H. and Hanson, A.D. (1997) Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycine betaine synthesis in plants: prosthetic group characterization and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **94,** 3454-3458.
9. Waditee, R., Tanaka, Y., Aoki, K., Hibino, T., Jikuya, H., Takano, J., Takabe, T. and Takabe, T. (2003) Isolation and functional characterization of N-methyltransferases that catalyze betaine synthesis from glycine in a halotolerant photosynthetic organism *Aphanothece halophytica*. *J. Biol. Chem*. **278,** 4932-4942.
10. Bolen, D.W. and Baskakov, I.V. (2001) The osmophobic effect: natural selection of a thermodynamic force in protein folding. *J. Mol. Biol*. **310,** 955-963.
11. Poolman, B., Spitzer, J.J. and Wood, J.M. (2004) Bacterial osmosensing: roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions. *Biochim. Biophys. Acta* **1666,** 88-104.
12. Kramer, R. and Morbach, S. (2004) BetP of *Corynebacterium glutamicum*, a transporter with three different functions: betaine transport, osmosensing, and osmoregulation. *Biochim. Biophys. Acta* **1658,** 31-36.
13. Nuccio, M.L., Rhodes, D., McNeil, S.D. and Hanson, A.D. (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol. **2,**128-134.
14. Rontein, D., Basset, G., and Hanson, A.D. (2002) Metabolic engineering of osmoprotectant accumulation in plants. *Metab. Eng*. **4,** 49-56.
15. Chen, T.H. and Murata, N.(2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol*. **5,** 250-257.
16. Sakamoto, A. and Murata, N. (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ*. **25,** 163-171.
17. Craig, S.A. (2004) Betaine in human nutrition. *Am. J. Clin. Nutr*. **80,** 539-549.
18. Kempf, B. and Bremer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol*. **170,** 319-330.
19. Ishitani, M., Nakamura, T., Han, S.Y. and Takabe T. (1995) Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol. Biol*. **27,** 307-315.
20. Perrino, L.A. and Pierce, S.K. (2000) Choline dehydrogenase kinetics contribute to glycine betaine regulation differences in chesapeake bay and atlantic oysters. *J. Exp. Zool*. **286,** 250-261.
21. Perrino, L.A. and Pierce, S.K. (2000) Betaine aldehyde dehydrogenase kinetics partially account for oyster population differences in glycine betaine synthesis. *J. Exp. Zool*. **286,** 238-249.
22. Lamark, T., Rokenes, T.P., McDougall, J. and Strom, A.R. (1996) The complex bet promoters of *Escherichia coli*: regulation by oxygen (ArcA), choline (BetI), and osmotic stress. *J. Bacteriol.* **178,**1655-1662.
23. Liu, Y. and Bolen, D.W. (1995) The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. *Biochemistry* **34,** 12884-12891.
24. Murata, N., Mohanty, P.S., Hayashi, H. and Papageorgiou, G.C. (1992) Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. *FEBS Lett*. **296,** 187-189.
25. Qu, Y., Bolen, C.L. and Bolen, D.W.(1998) Osmolyte-driven contraction of a random coil protein. *Proc. Natl. Acad. Sci. USA* **95,** 9268-9273.
26. Ikuta, S., Imamura, S., Misaki, H. and Horiuti, Y. (1977) Purification and characterization of choline oxidase from *Arthrobacter globiformis*, *J. Biochem. (Tokyo)* **82,** 1741-1749.

27. Hibino, T., Meng, Y.L., Kawamitsu, Y., Uehara, N., Matsuda, N., Tanaka, Y., Ishikawa, H., Baba, S., Takabe, T., Wada, K., Ishii, T. and Takabe, T. (2001) Molecular cloning and functional characterization of two kinds of betaine-aldehyde dehydrogenase in betaine-accumulating mangrove *Avicennia marina* (Forsk.) Vierh. *Plant Mol. Biol*. **45,** 353-363.
28. Hibino, T., Waditee, R., Araki, E., Ishikawa, H., Aoki, K., Tanaka, Y. and Takabe T. (2002) Functional characterization of choline monooxygenase, an enzyme for betaine synthesis in plants. *J Biol Chem*. **277,** 41352-41360.
29. Nakamura, T., Yokota, S., Muramoto, Y., Tsutsui, K., Oguri, Y., Fukui, K. and Takabe, T. (1997) Expression of a betaine aldehyde dehydrogenase gene in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. *Plant J.* **11,** 1115-1120.
30. Nakamura, T., Nomura, M., Mori, H., Jagendorf, A.T., Ueda, A. and Takabe, T. (2001) An isozyme of betaine aldehyde dehydrogenase in barley. *Plant Cell Physiol*. **42,** 1088-1092.
31. Trossat, C., Rathinasabapathi, B. and Hanson, A.D. (1997) Transgenically expressed betaine aldehyde dehydrogenase efficiently catalyzes oxidation of dimethylsulfoniopropionaldehyde and omega-aminoaldehydes. *Plant Physiol*. **113,** 1457-1461.
32. Incharoensakdi, A., Matsuda, N., Hibino, T., Meng, Y.L., Ishikawa, H., Hara, A., Funaguma, T., Takabe, T. and Takabe, T. (2000) Overproduction of spinach betaine aldehyde dehydrogenase in *Escherichia coli*. Structural and functional properties of wild-type, mutants and *E. coli* enzymes. *Eur. J. Biochem.* **267,** 7015-7023.
33. Hayashi, H., Alia., Mustardy, L., Deshnium, P., Ida, M. and Murata, N. (1997) Transformation of *Arabidopsis thaliana* with the *cod*A gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. *Plant J.* **12,** 133-42.
34. McNeil, S.D., Nuccio, M.L., Ziemak, M.J. and Hanson, A.D. (2001) Enhanced synthesis of choline and glycine betaine in transgenic tobacco plants that overexpress phosphoethanolamine N-methyltransferase. *Proc. Natl. Acad. Sci. USA* **98,** 10001-10005.
35. Moffatt, B.A., Stevens, Y.Y., Allen, M.S., Snider, J.D., Pereira, L.A., Todorova, M.I., Summers, P.S., Weretilnyk, E.A., Martin-McCaffrey, L. and Wagner, C. (2002) Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. *Plant Physiol*. **128,** 812-821.
36. Park, E.J., Jeknic, Z., Sakamoto, A., DeNoma, J., Yuwansiri, R., Murata, N. and Chen, T.H. (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *Plant J.* **40,** 474-487.
37. Jagendorf, A.T. and Takabe, T. (2001) Inducers of glycinebetaine synthesis in barley. *Plant Physiol*. **127,** 1827-1835.
38. Waditee, R., Bhuiyan, M.N., Rai, V., Aoki, K., Tanaka, Y., Hibino, T., Suzuki, S., Takano, J., Jagendorf, A.T., Takabe, T. and Takabe T. (2005) Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **102,** 1318-1323.
39. Schwacke, R., Grallath, S., Breitkreuz, K.E., Stransky, E., Stransky, H., Frommer, W.B. and Rentsch, D. (1999) LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. *Plant Cell* **11,** 377-392.
40. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., Hayakawa, S., Suzuki, S., Futsuhara, Y., Kawamitsu, Y., Takabe, T. and Takabe, T. (2001) Functional characterization of betaine/proline transporters in betaine-accumulating mangrove. *J. Biol. Chem.* **277,** 18373-18382.
41. Fetterer, R.H., Augustine, P.C., Allen, P.C. and Barfield, R.C. (2003) The effect of dietary betaine on intestinal and plasma levels of betaine in uninfected and coccidia-infected broiler chicks. *Parasitol Res*. **90,** 343-348.
42. Fernandez-Figares, I., Wray-Cahen, D., Steele, N.C., Campbell, R.G., Hall, D.D., Virtanen, E. and Caperna, T.J. (2002) Effect of dietary betaine on nutrient utilization and partitioning in the young growing feed-restricted pig. *J. Anim. Sci*. **80,** 421-428.
43. Olthof, M.R. and Verhoef, P. (2005) Effects of betaine intake on plasma homocysteine concentrations and consequences for health. *Curr. Drug Metab*. **6,** 15-22.
44. Zeisel, S.H., Mar, M.H., Howe, J.C. and Holden, J.M. (2003) Concentrations of choline-containing compounds and betaine in common foods. *J. Nutr*. **133,** 1302-1307.
45. Schwahn, B.C., Hafner, D., Hohlfeld, T., Balkenhol, N., Laryea, M.D. and Wendel, U. (2003) Pharmacokinetics of oral betaine in healthy subjects and patients with homocystinuria. *Br. J. Clin. Pharmacol*. **55,** 6-13.

46. Lever, M., Sizeland, P.C., Bason, L.M., Hayman, C.M., Robson, R.A. and Chambers, S.T. (1994) Abnormal glycine betaine content of the blood and urine of diabetic and renal patients. *Clin. Chim. Acta* **230,** 69-79.
47. Hayes, K.C., Pronczuk, A., Cook, M.W., Robbins, M.C. (2003) Betaine in sub-acute and sub-chronic rat studies. *Food Chem. Toxicol*. 41, 1685-1700.
48. Evans, J.C., Huddler, D.P., Jiracek, J., Castro, C., Millian, N.S., Garrow, T.A. and Ludwig, M.L. (2002) Betaine-homocysteine methyltransferase: zinc in a distorted barrel. *Structure (Camb)* **10,** 1159-1171.
49. Park, E.I., Renduchintala, M.S., Garrow, T.A. (1997) Diet-induced changes in hepatic betaine-homocysteine methyltransferase activity are mediated by changes in steady-state levels of its mRNA. *J. Nutr. Biochem*. **8,** 541-545.

10. INDUCTION OF BIOSYNTHESIS OF OSMOPROTECTANTS IN HIGHER PLANTS BY HYDROGEN PEROXIDE AND ITS APPLICATION TO AGRICULTURE

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Abstract. We have found that hydrogen peroxide at low levels can induce synthesis of osmoprotectants, e.g. glycinebetaine in barley, and proline and sucrose in rice. Then we have succeeded in enhancing salt-stress tolerance by hydrogen peroxide treatment in rice, which is known to be one of the most salt-sensitive crops. Furthermore we have found that hydrogen peroxide treatment induces a higher sugar content in tomato and melon fruits. Hydrogen peroxide was effective in accelerating germination of a turf grass (Kentucky bluegrass) under cool conditions. Thus we conclude that application of hydrogen peroxide can be a useful technique for agriculture.

1. INTRODUCTION

Hydrogen peroxide is a familiar compound for use as an antiseptic. On the other hand, hydrogen peroxide is known to be one of the reactive oxygen species (ROS) produced in plants under various abiotic stresses, and leads to ultimate death. Recently another aspect of hydrogen peroxide has been found. Namely it acts as one of the signal molecules for stress tolerance in plants [1, 2]. For instance we found that hydrogen peroxide increased in leaves of barley and rice, when the plants were exposed to various stresses [3, 4]. Furthermore exogenously applied low levels (~10 mM) of hydrogen peroxide induced accumulation of an osmoprotectant, glycinebetaine, in barley leaves [5].

Rice is known to be a glycinebetaine non-accumulator [6]. Messenger RNA levels of P5CS and sucrose-phosphate synthase were increased in rice leaves by hydrogen peroxide treatment [3]. Both P5CS and sucrose-phosphate synthase are key enzymes for proline and sucrose synthesis, respectively, and they are thought to

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be major osmoprotectants in stressed rice leaves. Those observations spurred us to test the use of hydrogen peroxide for agriculture under abiotic stress conditions, since decrease of crop productivity by abiotic stresses (salt, drought, cold and heat) may threaten humanity with food shortages in the near future.

In this chapter, we would like to propose using hydrogen peroxide to improve growth and productivity of crops from abiotic stresses. While genetic modification of one gene or another has been shown to be helpful, there remains considerable public apprehension about GMO crops. Many consumers ask for 100% safety for genetically modified crops, just as for atomic energy power station. We find that hydrogen peroxide treatment can cause an even more significant increase in stress tolerance than current genetic changes, and has the advantage of disappearing rapidly after the application, with no residues to worry about.

2. HYDROGEN PEROXIDE TREATMENT INDUCES GLYCINEBETAINE ACCUMULATION IN BARLEY

For barley (*Hordeum vulgare*) plants, an important response to stress conditions is the induction of synthesis of the osmoprotectant glycinebetaine [7]. Its synthesis is induced by high salt [8, 9], drought and water deficits [10], cold stress (11) and heat stress [4].

Glycinebetaine is made from choline in higher plants in two steps, with glycinebetaine aldehyde as intermediate. In spinach the first step is performed by an interesting, chloroplast-localized enzyme, choline monooxygenase (CMO), which uses electrons from photosystem I via ferredoxin to help do the oxidation [12, 13]. The second step is catalyzed by betaine aldehyde dehydrogenase (BADH). In barley, we have found two BADH genes. One of them encodes the protein targeted to peroxisomes [6] and the other one encodes a cytosolic BADH protein [14]. The cytosolic BADH is the major one in leaves. On the other hand, peroxisomal BADH is the major one in roots [6, 14]. We have not found any CMO gene in Gramineae yet. It is very interesting that the intracellular localization of glycinebetaine synthesis is different between chenopods and Gramineae. We believe that Gramineae must have a unique CMO-like protein to convert choline to glycinebetaine aldehyde. We have been searching for the so far mysterious gene.We found that hydrogen peroxide applied to barley roots induced glycinebetaine synthesis in the leaves [5]. An organic peroxide, cumene hydroperoxide, also induced glycinebetaine accumulation. Hydrogen peroxide has an observable effect at 5 mM and brings on almost maximal glycinebetaine accumulation at 25 mM. We obtained indirect evidence for the participation of protein kinase C and phospholipase D in the signal transduction pathway, although the whole signal transduction sequence for glycinebetaine synthesis is not fully understood as yet in higher plants.

3. HYDROGEN PEROXIDE TREATMENT INCREASES ABIOTIC STRESS TOLERANCE IN RICE SEEDLINGS

Rice is a crop known to be very sensitive to salt stress. Therefore, many reports have appeared on enhancement of salt tolerance in rice by genetic engineering of only one gene in most cases. If hydrogen peroxide is a signal molecule in the induction of genes related to abiotic stress response or tolerance in rice, it might be possible to increase salt-stress tolerance by simply treating the plants with hydrogen peroxide. Internal hydrogen peroxide levels of 8-d-old seedlings went up transiently around 6 h, then decreased to the original level and increased again gradually during 12~48 h after treatment with 10 mM NaCl [3]. Then it was actually found that hydrogen peroxide treatment (10 μM) enhanced salt tolerance in rice seedlings. The treatment induced not only active oxygen scavenging enzyme activities, but also expression of transcripts for stress-related genes encoding sucrose-phosphate synthase, pyrroline-5-carboxylate synthase and small heat shock protein 26. Many other genes or proteins were increased by hydrogen peroxide, as well [15]. These results suggest that externally applied hydrogen peroxide can indeed increase salt tolerance in rice seedlings, by acting as a signal molecule for the abiotic stress response. Furthermore we have found that the abiotic nitric oxide donor sodium nitroprusside also increased salt tolerance in rice seedlings. Both hydrogen peroxide and nitric oxide increased heat tolerance in rice plants. However these effects occurred at an optimum concentration; excess amounts of either hydrogen peroxide or sodium nitroprusside decreased salt- or heat-stress tolerance in rice seedlings compared to untreated controls.

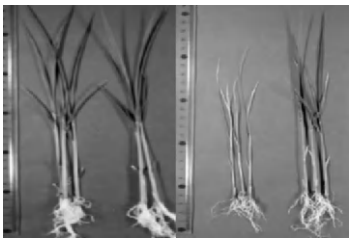

Figure 1. Effects of hydrogen peroxide on salt stress of rice.

4. HYDROGEN PEROXIDE TREATMENT PRODUCES SWEET FRUITS WITHOUT DECREASE IN THEIR PRODUCTIVITY

Since we noted that hydrogen peroxide treatment increased the expression of the sucrose-phosphate synthase gene in rice seedlings, we wondered if it also might increase the sugar content of fruits. We tested it with tomato and melon plants [16]. Hydrogen peroxide was applied to the soil from the beginning of flowering time, for

two months. The sucrose-phosphate synthase activity was increased about 2~3 fold in tomato and melon leaves, compared to those without the treatment. The sugar content was increased in leaves of the two plants. Since leaves supply the fruits with carbohydrate, the hope was that the treatment would make the fruits sweeter. Indeed, the sugar content increased by about 1.5 fold in the two fruits. It was a nice surprise that the hydrogen peroxide treatment did not decrease plant growth (Tables. 1 and 2) or their fruit productivity. Usually water shortage (drought stress) is given to make sweet fruits such as tomato with some reduction of fruit productivity [17]. We are now carefully determining if we can decrease the concentration of hydrogen peroxide in the treatment, and/or the frequency of application, which would save the farmer's labor. The best method will have to be established from the viewpoints of both safety and saving labor.

Table 1. Comparison of biomass increase between genetic engineering and hydrogen peroxide treatment. Relative value (100%) was defined as that of control condition.

Plant	Gene	Organism	Modification	Condition	Biomass	Reference
Genetic engineering						
Poplar	4-coumarate: coenzyme A ligase (antisense)	Poplar	Repressing lignin biosynthesis	Normal	150%	(20)
Poplar	GA 20-oxidase	*Arabidopsis*	Gibberellin	Normal	125%	(21)
Tomato	Sucrose phosphate synthase	Tomato	Sucrose metabolism	Saturated light and CO_2	120%	(22)
Poplar	Xyloglucanse	*Aspergllus aculeatus*	Cell wall	Sucrose	150%	(23)
Arabidopsis	Auxin-inducible gene ARGOS	*Arabidopsis*	Auxin signal downstream	Normal	150%	(24)
Tobacco	Fructose-1, 6-bisphoshatase and sedoptulose-1,7-bisphoshatase	Cyanobacterium (PCC 7942)	Calvin cycle	Normal	150%	(25)
Hydrogen peroxide treatment (20 mM H_2O_2)						
Tomato			Sugar metabolism	Normal	120%	Present study
Melon			Sugar metabolism	Normal	150%	Present study

Table 2. Comparison of plant growth, fruit yield and sugar content in tomato plant with various treatments. Relative value (100%) was defined as that of control condition.

Treatment	Condition	Plant growth	Fruit yield	Sugar content	Reference
Hydrogen peroxide	50 mM H_2O_2	95%	100%	150%	Present study
Drought stress	pF 2.5	86%	88%	140%	(17)
Salt stress	60 mM NaCl	75%	70%	130%	(26)

5. HYDROGEN PEROXIDE TREATMENT ENHANCES GERMINATION AND EARLY GROWTH OF KENTUCKY BLUEGRASS

A third application study was suggested by company (Kureha chemical industry) in Japan. A cool season turf grass, Kentucky bluegrass, takes more than 3 weeks to germinate at a temperature lower than 23°C. The grass is used frequently for golf courses, tennis courts, soccer grounds and airports. Furthermore turf grasses are used widely to protect soil from landslide and erosion. Soil erosion has been well known to be a serious problem in land desertification. To accelerate germination and early growth, the seeds were treated with growth regulators (gibberellin and benzyladenine), a detergent (silwet) and hydrogen peroxide [18]. It appeared that hydrogen peroxide accelerated significantly early growth, after germination of the seeds at 20°C. Interestingly hydrogen peroxide of very low concentrations (1~10 μM) was effective. This concentration is low enough to be accepted as an agrochemical.

Figure 2. Effects of hydrogen peroxide on the growth of melon.

6. CONCLUSIONS

We have shown three application studies on the use of hydrogen peroxide for agriculture under abiotic stresses. They are good examples indicating that basic research contributes to application for agriculture. Hydrogen peroxide is an inexpensive reagent and is easily decomposed in the soil by bacteria. Since in a number of these applications, it is effective at very low concentrations, there is no problem in using it as an agrochemical. With rapidly increasing knowledge of entire plant genomes, and understanding of gene functions and signal pathways [19], there should be further opportunities to find additional external chemicals that interact with the complex biochemical machinery of plants, and might thereby be of significant use in agriculture. We have indicated that inducers of glycinebetaine synthesis include inorganic salts (NaCl, KCl, LiCl and Na_2SO_4), oxidants (H_2O_2 and cumene hydroperoxide), and organic compounds (abscisic acid, polymixin B, *n*-butanol, salicylic acid and aspirin) [5]. Are there any other compounds having ability of a signal molecule for abiotic stress tolerance in plants? We have been searching for such compounds in our laboratory.

REFERENCES

1. Prasad, T.K., Anderson, M.D. and Stewart, C.R. (1994) Evidence for chilling induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *Plant Cell* **6,** 65-74.
2. Srivalli, B., Sharma, G. and Khanna-Chopra, R. (2003) Antioxidative defense system in an upland rice cultivar subjected to increasing intensity of water stress followed by recovery. *Physiol. Plant*. **119,** 503-512.
3. Uchida, A., Jagendorf, A.T., Hibino, T., Takabe, T. and Takabe, T. (2002) Effects of hydrogen peroxide and nitric oxide on both salt and heat tolerance in rice. *Plant Sci*. **163,** 515-523.
4. Uchida, A., Shi, W., Ozaki, K., Okamoto, M., Koshiba, T., Hirai, T., Hibino, T., Ichihara, S., Takabe, T. and Takabe, T. Induction of glycinebetaine accumulation is possibly triggered by H_2O_2 and ABA in a winter-type barley under heat stress. *Plant Sci*. submitted.
5. Jagendorf, A.T. and Takabe, T. (2001) Inducers of glycinebetaine synthesis in barley. *Plant Physiol.* **127,** 1827-1835.
6. Nakamura, T., Yokota, S., Muramoto, Y., Tsutsui, K., Oguri, Y., Fukui, K. and Takabe, T. (1997) Expression of a betaine aldehyde dehydrogenase gene in rice, a glycinebetaine non-accumulator, and possible localization of its protein in peroxisomes. *Plant J.* **11,** 1115-1120.
7. Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. **44,** 357-384.
8. Arakawa, K., Katayama, M. and Takabe, T. (1990) Levels of glycinebetaine and glycinebetaine aldehyde dehydrogenase activity in the green leaves, and etiolated leaves and roots of barley. *Plant Cell Physiol*. **33,** 833-840.
9. Arakawa, K., Mizuno, K., Kishitani, S. and Takabe, T. (1992) Immunological studies of betaine aldehyde dehydrogenase of barley. *Plant Cell Physiol*. **33,** 833-840.
10. Hitz, W.D, Ladyman, J.A.R and Hanson, A.D (1982) Betaine synthesis and accumulation in barley during field water stress. *Crop. Sci*. **22,** 47-54.
11. Kishitani, S., Watanabe, K., Yasuda, S,. Arakawa, K. and Takabe, T. (1994) Accumulation of glycinebetaine during cold acclimation and freezing tolerance in leaves of winter and spring barley plants. *Plant Cell Environ*. **17,** 89-95.
12. Brouquisse, R., Weigel, P., Rhodes, D., Yocum, C.F. and Hanson, A.D. (1989) Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. *Plant Physiol*. **90,** 322-329.
13. Rathinasabapathi, B., Burnet, M., Russel, B.L., Gate, D.A., Liao, P.C., Nye, G.J., Scott, P., Goldbeck, J.M. and Hanson, A.D. (1997) Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycinebetaine synthesis in plants: Prothetic group characterization and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **94,** 3454-3458.
14. Nakamura, T., Nomura, M., Mori, H., Jagendorf, A.T,, Ueda, A. and Takabe, T. (2001) An isozyme of betaine aldehyde dehydrogenase in barley. *Plant Cell Physiol*. **42,** 1088-1092.
15. Narita, Y., Taguchi, H., Nakamura, T., Ueda, A., Shi, W.M. and Takabe, T. (2004) Characterization of the salt-inducible methionine synthase from barley leaves. *Plant Sci*. **167,** 1009-1016.
16. Uchida, A., Takabe, T., Hayashi, T. and Takabe, T. (2004) Sugar content increases in tomato and melon fruits by hydrogen peroxide treatment. *Plant Growth Reg*. Submitted.
17. Hayata, Y., Tabe, T., Kondo, S. and Inoue, K. (1998) The effects of water stress on the growth, sugar and nitrogen content of cherry tomato fruit. *J. Japan Soc. Hort. Sci*. **67,** 759-766.
18. Takabe, T., Uchida, A. and Takabe, T. (2004) Effects of hydrogen peroxide on germination of Kentucky bluegrass. *Plant Biology 2004* 161.
19. Ueda, A., Kathiresan, A., Inada, M., Narita, Y., Nakamura, T., Shi, W.M., Takabe, T. and Bennett, J. (2004) Osmotic stress in barley regulates expression of different set of genes than salt stress does. *J. Exp. Bot.* **55,** 2213-2218.
20. Hu, W.J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.J. and Chiang, V.L. (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nat. Biotechnol*. **17,** 808-812.
21. Eriksson, M.E., Israelsson, M., Olsson, O. and Moritz, T. (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol*. **18,** 784-788.

22. Galtier N., Foyer C.H., Huber J., Voelker T.A. and Huber S.C. (1993) Effects of elevated sucrose-phosphate synthase activity on photosynthesis, assimilate partitioning, and growth in tomato (*Lycopersicon esculentum* var UC82B). *Plant Physiol*. **101,** 535-543.
23. Park, Y.W., Baba, K., Furuta, Y., Iida, I., Sameshima, K., Arai, M. and Hayashi, T. (2004) Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase in poplar. *FEBS Lett*. **564,** 183-187.
24. Hu, Y., Xie, Q. and Chua N.H. (2003) The Arabidopsis auxin-inducible gene ARGOS controls lateral organ size. *Plant Cell* **15,** 1951-1961.
25. Miyagawa, Y., Tamoi, M. and Shigeoka, S. (2001) Overexpression of a cyanobacterial fructose-1, 6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nat. Biotechnol*. **19,** 965-969.
26. Carvajal, M., Cerdá, A. and Martínez, V. (2000) Modification of the response of saline stressed tomato plants by the correction of cation disorders. *Plant Growth Reg*. **30,** 37-47.

SECTION VI

ION HOMEOSTASIS

11. NA^+/H^+ ANTIPORTERS IN PLANTS AND CYANOBACTERIA

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Abstract. Under salt stress, plants protect the deleterious effects of Na^+ by maintenance a high cytosolic K^+ to Na^+ ratio. Intracellular K^+ and Na^+ homeostasis is important for the activities of many cytosolic enzymes, maintaining membrane potential, and for the synthesis of osmoticum. Although salt-stress sensors remain elusive, some of components important for salt stress have been identified. Understanding how plants cope with excessive Na^+ in the environment is of great agricultural importance as soil salinity accounts for large yield losses in crops worldwide. This review focuses on the recent progresses in understanding of sodium ion homeostasis in plants and cyanobacteria and their applications to construct the salt-tolerant crop.

1. INTRODUCTION

Today, ~20% of the world's cultivated land and nearly half of all irrigated lands are affected by high salinity. Salinity is one of the most important factors to decrease the crop yield. Proper regulation of ion flux is necessary for cells to keep the concentrations of toxic ions low and to accumulate essential ions. Intracellular K^+ and Na^+ homeostasis is important for the activities of many cytosolic enzymes, maintaining membrane potential, and for the synthesis of osmoticum. To maintain high K^+/Na^+ ratio in cytosol, plant cells employ the primary active transport mediated by H^+-ATPases and the secondary transport mediated by channels and co-transporters. Under salt stress, Na^+ enters to plant cells through several pathways and Na^+ stress disrupts K^+ uptake by root cells. These processes decrease the K^+/Na^+ ratio in cytosol. When Na^+ accumulates to high levels in cytosol, it becomes toxic to enzymes. Excess Na^+ in cytosol could be removed by the active mechanisms to apoplastic space via plasma membrane or to vacuole via tonoplast membrane [1-3].

Unlike animal cells, Na^+-ATPases or Na^+/K^+-ATPases could not be found in most plant cells. Plant cells have to rely on Na^+/H^+ antiporters to extrude Na^+. Therefore, Na^+/H^+ antiporters would play much more important roles for salt tolerance in plants than in any other organisms. Although salt-stress sensors remain elusive, some of components important for salt stress have been identified. This review

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focuses on the recent progresses and prospects in understanding of sodium ion homeostasis in plants and cyanobacteria and their applications to construct the salt-tolerant crop.

2. NA^+/H^+ ANTIPORTER

Na^+/H^+ antiporters are ubiquitous membrane proteins that catalyze the exchange of Na^+ for H^+ across membranes. Na^+/H^+ antiporters are involved in diverse physiological processes, e.g., for salt tolerance [4-10], pH regulation [11, 12], cell volume [13], morphogenesis [14], and extruding the H^+ generated during metabolism. To date, many Na^+/H^+ antiporters have been reported as shown in Table1. These are 1) NhaA, 2) NhaB, 3) NhaC, 4) ChaA, 5) NhaP (NHE, NHX), 6) NapA (CHX), and 7) NhaD (NHD). Three antiporters, NhaA, NhaB and ChaA, are known in *E.coli*, and their functional properties have been extensively studied [15, 16]. In human, nine NHEs (NHE1-9) which have a similar structure, but different subcellular localization, have been reported [17]. NHE1 plays a key role in the regulation of internal pH, but not for Na^+ extrusion.

Genome sequencing of *Arabidopsis* suggests the presence of more than 38 Na^+/H^+ antiporter homologs [18]. These antiporters fall into three families: monovalent cation/proton antiporter-1 (CPA1) family (8 members, NHX1-8), monovalent cation/ proton antiporter-2 (CPA2) family (28 members, CHX1-28), and NhaD family (two members, NHD1-2). Most of these genes are not functionally characterized. Among 8 members of CPA1 family, the vacuolar AtNHX1 and the plasma membrane AtNHX7 (SOS1) are two extensively studied ones [19, 20, 21]. Whereas only limited information is available for CPA2 family. Thus far, two CPA2 exchangers, AtCHX15 [22] and AtCHX17 [23] are functionally characterized in *Arabidopsis*.

Table 1. Na^+/H^+ antiporters found in prokaryotic and eukaryotic cells.

Prokaryote	Eukaryote
NhaA, Bacteria	
NhaB, Bacteria	
ChaA, Bacteria	CAX yeast, plant (Ca^{2+}/H^+ antiporter but not Na^+/H^+ antiporter)
NhaP, Bacteria	NHX plant, yeast; NHE animal
NapA, Bacteria	CHX plant, Nhap1 yeast
NhaC, Bacteria	
NhaD, Bacteria	NhaD plant

Compared with plants, the Na^+/H^+ antiporter system in cyanobacteria seems to be simple. Since cyanobacteria conduct plant-like oxygenic photosynthesis, the Na^+/H^+ antiporter system in cyanobacteria could be used as a model system for the understanding of molecular mechanisms of salt-tolerance in plants. The entire genome sequences of several cyanobacteria, namely *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, *Thermosynechococcus elongatus* sp. BP-1, *Gloeobacter*

violaceus PCC 7421, *Prochlorococcus marinus* SS120, *Prochlorococcus marinus* MED4, *Prochlorococcus marinus* MIT9313, *Synechococcus* sp. WH 8102, are available (http://www.kazusa.or.jp/cyano/cyano.html). Among them, *Synechocystis* sp. PCC6803 is the most well studied organism. The genome sequence of *Synechocystis* sp. PCC 6803 revealed the presence of five putative Na^+/H^+ antiporter genes [24] (Table 2). Two of them (Syn-NhaP1 and Syn-NhaP2) belong to CPA1 family and exhibit some homology to NhaP from *Pseudomonas aeruginosa*, vacuole antiporter from *Arabidopsis* (AtNHX1), plasma membrane antiporter (SOS1), and mammalian NHE. Other three antiporters (Syn-NapA1, Syn-NapA2, and Syn-NapA3) belong to CPA2 family and are homologous to NapA from *Enterococcus hirae* [10, 25]. Functional properties of NhaP1 [25-27] and NapA [28] type Na^+/H^+ antiporters from cyanobacteria have been characterized.

Table 2. Comparison Na^+/H^+ antiporters in cyanobacterium, Synechocystis sp. PCC6803, and higher plant, Arabidopsis thaliana.

Synechocystis sp. PCC6803	*Arabidopsis thaliana*
NhaP1 (NhaS1, slr1727) NhaP2 (NhaS2, sll0273)	AtNHX1-8
NapA1 (NhaS3, slr0415) NapA2 (NhaS4, slr1595) NapA3 (NhaS5, slr0689)	AtCHX1-28
	AtNhaD1-2

3. NHAP TYPE NA^+/H^+ ANTIPORTERS FROM CYANOBACTERIA

Functional properties of all five Na^+/H^+ antiporter genes from *Synechocystis* sp. PCC6803 were examined using the salt-sensitive *E.coli* mutant in which three antiporters (*nhaA*, *nhaB*, and *chaA*) were disrupted [29]. Among them, only the NhaS1 (Syn-NhaP1) and NhaS3 (Syn-NapA1) complemented the salt-sensitive *E.coli* mutant. NhaS1 (Syn-NhaP1) and NhaS3 (Syn-NapA1) exhibited the exchange activity between $Na^+$and H^+ as well as Li^+ and H^+. NhaS1 (Syn-NhaP1) exhibited the exchange activities over a wide range of pH from 5 to 9. The activities were insensitive to amiloride [25]. The affinity of NhaS1 (Syn-NhaP1) for Na^+ is low, ~5 mM [10]. A homologous gene, Ap-NhaP1, was isolated from a halotolerant cyanobacterium, *Aphanothece halophytica*. Ap-NhaP1 exhibited the Na^+/H^+ antiporter activity over a wide range of pH between 5 and 9, but had virtually no exchange activity between Li^+ and H^+. Interestingly, Ap-NhaP1 exhibited the Ca^{2+}/H^+exchange activity at alkaline pH. Topological models of NhaS1(Syn-NhaP1) and Ap-NhaP1 suggests the presence of 11 transmembrane segments with a long hydrophilic C-terminal tail (Fig. 1). Importance of acidic amino acid (Asp139) in transmembrane segment for the exchange activity was demonstrated. The C-terminal tail of NhaS1(Syn-NhaP1) and Ap-NhaP1 have similar size, 123 amino acid residues in Syn-NhaP1 and 124 amino acid residues in Ap-NhaP1. But, their charges are different. Ap-NhaP1 has positive net charges (22+ 14-) while Syn-NhaP1 has

negative net charges (15+ 24-). A chimera ASNhaP, in which the C-terminal tail of Ap-NhaP1 was replaced with that of Syn-NhaP1, exhibited the exchange activity between Li^+ and H^+ which was not observed in the wild-type Ap-NhaP1. In contrast, the chimera SANhaP, in which the C-terminal tail of Syn-NhaP1 was replaced with that of Ap-NhaP1, exhibited the altered

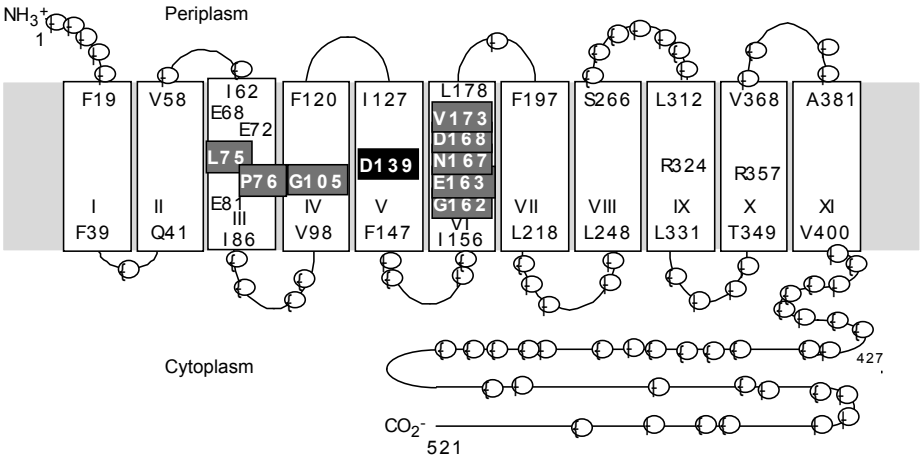

Figure 1. Hypothetical secondary structure model of the Ap-NhaP1 protein. The possible TMs of the Ap-NhaP1 sequence were deduced by a computer program TopPredII. Putative transmembrane helical segments are boxed and the first and the last amino acid of each segment are indicated. The number of positive and negative charges on the C-terminal hydrophilic tail is indicated.

the ion specificity from that of Syn-NhaP1. These facts demonstrate that the C-terminal tail of NhaP is involved in ion specificity.

SOS1 [5], AtNHX1 [30], mammalian NHE [31], and yeast Nha1p [32] have also a long C-terminal tail although their homology is low. In NHEs, the long C-terminal tail exposes to cytoplasmic space, mediates the interactions with other proteins, and involves in the regulation of the activity [33-35]. The C-terminal tail of yeast Nha1p involves in the osmotic adaptation [32] and localization of transporter [36]. In SOS1, the long C-terminal portion is necessary for salt tolerance. The single amino acid substitution mutants in the hydrophilic tail of SOS1, i.e. *sos1-8* (Gly777Glu) and *sos1-9* (Gly784Asp) were inactive [2]. In AtNHX1, deletion of the hydrophilic C-terminal tail caused a dramatic increase of Na^+/H^+ antiport activity [30]. The C-terminal of *AtNHX1* appears to be involved in the determination of the ion selectivity of the transporter. Thus, the functions of C-terminal tail in eukayotic Na^+/H^+ antiporters have various functions.

4. NAPA TYPE NA^+/H^+ ANTIPORTERS FROM CYANOBACTERIA

Synechocystis sp. PCC 6803 has three NapA type antiporters (NhaS3, NhaS4 and NhaS5). NhaS3 belongs to CPA2 family and shares the homology with *NapA* from *Enterococcus hirae* [37] *GerN* from *Bacillus cereus* [38]. Topological model of NhaS3 predicted 11 segments of transmembrane and a long C-terminal tail is absent. NhaS3 displayed the exchange activity of Na^+ or Li^+ for H^+ and had high affinity for Na^+ and Li^+ [10]. The homologous gene of NhaS3 was isolated from a halotolerant cyanobacterium, *Aphanothece halophytica*. It contains at least two homologous genes, namely Ap-NapA1-1 and Ap-NapA1-2. The importance of amino acids, Glu129, Asp225 and Asp226 in the transmembrane region and Glu142 in the loop region for the exchange activities was identified in ApNapA1-1 by mutagenesis experiment [28]. Another homologous gene, *ApNapA1-2* is probably an Na^+/H^+-K^+ antiporter. Ap-NapA1-2 could complemented the K^+-uptake deficient mutant as well as the Na^+-sensitive mutant [28].

5. KNOCKOUT OF CYANOBACTERIA NA^+/H^+ ANTIPORTERS

To clarify the physiological roles of antiporters in cyanobacteria, the analysis of gene knockout mutants was carried out in *Synechocystis* sp. PCC 6803. Four genes (*NhaS1, NhaS2, NhaS4, and NhaS5*) could be disrupted. But, *NhaS3* gene could not completely be disrupted [10, 39, 40]. The *Synechocystis* sp. PCC 6803 cells disrupted of *NhaS1, NhaS4, or NhaS5* exhibited normal growth or only weak salt-sensitive phenotype (Table3). The *NhaS3* gene seems to be most crucial for NaCl tolerance [39]. The cells partially disrupted of *NhaS3* exhibited severe salt-sensitive phenotype.

Table 3. Effects of gene knock-out of five putative Na^+/H^+ antiporters in Synechocystis sp. PCC 6803.

Putative Na^+/H^+ antiporters	Phenotypes	References
slr1727 (NhaS1, Syn-NhaP1)	No NaCl sensitivity	39
sll0273 (NhaS2, Syn-NhaP2)	weak salt sensitivity, shift the pH preference to acidic region	39, 41
sll0683 (NhaS3, Syn-NapA1)	significant salt sensitivity (only partially segregated)	10, 39
slr1595 (NhaS4, Syn-NapA2)	weak salt sensitivity	10, 39
slr0415 (NhaS5, Syn-NapA3)	No NaCl sensitivity	10
NhaS1/NhaS4	No NaCl sensitivity	40
NhaS1/NhaS5	No NaCl sensitivity	40
NhaS1/NhaS4/NhaS5	No NaCl sensitivity	40

It should be mentioned that the disruption of *NhaS1* in *Synechocystis* did not cause salt-sensitive phenotype. This fact suggests that NhaS1 antiporter does not play a crucial for salt tolerance in *Synechocystis*. However, the importance of NhaS1 (NhaP1) for salt tolerance was demonstrated in the case of Ap-NhaP1 from a

halotolerant cyanobacterium, *Aphanothece halophytica*[27]. The overexpressing of Ap-NhaP1 in a fresh water cyanobacterium *Synechococcus* sp. PCC 7942, significantly increased the salt tolerance of *Synechococcus* cells so that the transformed cells could grow in seawater [27].

The disruption of *NhaS2* caused the mutant cells sensitive to low sodium concentration and exhibited the increased sensitivity towards potassium ion, even at low concentrations. It has been proposed that the *NhaS2* is required for Na^+ influx [41]. Moreover, the disruption of *NhaS2* shifts the pH optimum of growth of *Synechocystis* cells to the acidic pH, suggesting that this antiporter is involved in pH regulation.

The disruption of *NhaS4* exhibited similar phenotype to that of *NhaS2*, but it was sensitive to acidic pH. The expressing of *NhaS4* in an *E.coli* mutant is tolerant to K^+ depleted medium, suggesting that *NhaS4* facilitates K^+ uptake [10].

The gene knockout analysis suggested that NhaS3 (NapA1) is the most important antiporter in cyanobacteria for salt tolerance. However, the physiological function of each antiporter in cyanobacteria is still unclear. Further studies are required to understand the role of Na^+/H^+ antiporters for salt tolerance in cyanobacteria.

6. PRIMARY PUMP IN PLANTS

Unlike animal cells, most plant cells do not have Na^+-ATPases or Na^+/K^+-ATPases. Therefore, plant cells have to rely on Na^+/H^+ antiporters to extrude Na^+ into non-toxic space. Since the energy of Na^+ transport is driven by proton-motive force that is created by H^+-ATPases and H^+-pyrophosphatases, they would be important for salt tolerance in plants [42]. The plasma membrane proton pump P-type ATPase (H^+-ATPase) is a single polypeptide with a molecular mass of about 100 kDa and plays a central role in transport across the plasma membrane. It mediates ATP-dependent H^+ extrusion to the extracellular space, thus creating the differences of pH and membrane potential across the plasma membrane (inside negative). The improvement of abiotic-stress tolerance in plants has not been reported by over- or suppress-expressions of plasma membrane H^+-ATPase. This might be due to the facts that the plasma membrane H^+-ATPase is involved in many physiological functions and consumes ATP. From the analysis of suppress expression of plasma membrane H^+-ATPase, its involvement for the pro-anthocyanidin formation, endomembrane function, has been demonstrated [43].

In plant cells, the vacuole is the largest intracellular H^+ bank. The acidification of the *Arabidopsis* vacuole is carried out by two systems: the vacuolar H^+-ATPase and the vacuolar H^+-pyrophosphatase. The vacuolar H^+-ATPase is a multisubunit complex whose subunits are encoded by at least 26 genes [44]. In contrast, the *Arabidopsis* H^+-pyrophosphatase is a single subunit protein although the *Arabidopsis* genome contains three homologs (AVP1-AVP3). The increase of proton pumping by overexpressing the plant V-type ATPases has not been reported. However, heterologous expression of plant vacuolar H^+-pyrophosphatase in yeast restored the salt tolerance to a salt-sensitive yeast mutant [45]. A potential advantage of the AVP1 overexpression is that this H^+ pump uses inorganic pyrophosphate, allowing

ATP to be conserved and used to improve the plant cell performance under a more demanding environment [46]. The transgenic *Arabidopsis* plants ectopically expressing AVP1 exhibited the increased tolerance to salt and drought stresses [46]. The size of transgenic plant increased due to the increase of cell number. Under normal growth conditions, the transgenic plants accumulated more solutes than the control plants. These data indicate that the overexpression of AVP1 also enhances the activity of various plant secondary transporters such as Na^+/H^+-antiporters [47].

Recently, the existence of Na^+-ATPase in moss *Physcomitrella patens* and a marine alga have been reported [48, 49]. In addition to Na^+-ATPase, SOS1-type Na^+/H^+-antiporter genes were also found in moss [48]. These results suggest that evolutionally early land plants had Na^+-ATPase, but they lost during the evolution to flowering plants.

7. SENSING SALT STRESS AND ENTRY OF SODIUM IN PLANTS

Figure 2 shows the simplistic model of salt-stress sensor, perception, and extrusion systems in plant cell. Little is known about Na^+ sensing in any cellular system. Theoretically, Na^+ can be sensed either before or after entering the cell, or both. Extracellular Na^+ may be sensed by a membrane receptor, whereas intracellular Na^+ may be sensed either by membrane proteins or by any of the many Na^+-sensitive enzymes in the cytoplasm. Zhu J.K. [23] proposed that the plasma-membrane Na^+/H^+-antiporter SOS1 is a possible Na^+-sensor. Several transporters such as sugar permease BglF in *Escherichia coli* [50] and betaine transporter in *Corynebacterium* [51] have a dual role in sensing and transporting. This hypothesis is worthwhile to be tested.

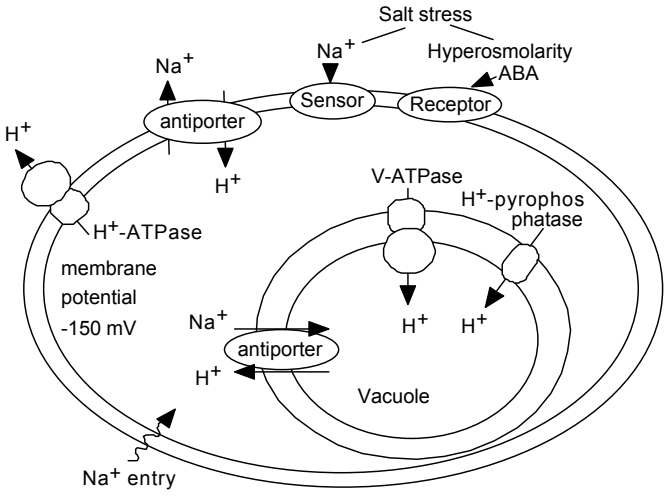

Figure 2. Schematic model of salt-stress sensor, perception, and extrusion systems.

Although ion specific signals are probably more important than hyperosmolarity in the regulation of Na^+ transport, salt stress also causes osmotic stress which activates the synthesis of abscisic acid (ABA). Osmotic stress activates the synthesis of abscisic acid (ABA), which can upregulate the transcription of vacuolar Na^+/H^+ antiporter gene *AtNHX1*. Osmotic stress may be sensed in part by stretch-activated channels and by transmembrane protein kinases such as two component histidine kinases [52] and wall-associated kinases [53].

The negative membrane potential across the plasma membrane of plant cells favors the passive transport of Na^+ into cells. Na^+ enters into plant cells through the high-affinity K^+ transporter HKT1 [55, 56] and also through non-selective cation channels [57]. In some plant species such as rice, Na^+ leakage into the transpiration stream via the apoplast can account for a major part of Na^+ entry into plants [58].

Although Na^+ influx led to deleterious effects to plant cells, but it is a part of the mechanism of salt tolerance. Functional analysis of AtHKT1 which transport Na^+ in *Arabidopsis* plants indicated the importance of Na^+ recirculation from shoots to roots via phloem sap. The mutants of this gene increased Na^+ accumulation in aerial organs, but decreased in roots [56].

8. NA^+ EFFLUX FROM PLASMA MEMBRANES IN PLANTS

The role of cellular efflux of Na^+ is not intuitive in multicellular plants, as Na^+ transported out of one cell would present a problem for neighboring cells. So the role of Na^+ efflux has to be considered in specific tissues and in the context of whole plants. In *Arabidopsis*, Na^+ efflux is catalyzed by the plasma-membrane Na^+/H^+ antiporter encoded by the SOS1 (AtNHX7) gene [2, 4, 6, 59]. SOS1 catalyzes the Na^+/H^+ exchange and cannot transport Li^+ or K^+ [6]. A physiological function of SOS1 is the Na^+ efflux into the root medium. SOS1 is also involved in the control of long-distance of Na^+ transport between roots and leaves. The expression of SOS1 is partially upregulated by salt stress at the posttranscriptional level. Increased SOS1 protein contributes to improve the salt tolerance in transgenic *Arabidopsis* [20]. Activity of the SOS1 antiporter is regulated by the SOS2 Ser/Thr protein kinase [6, 59], which is in turn regulated by the SOS3 Ca^{2+}-binding protein [60, 61]. SOS3 is a myristoylated calcium-binding protein that is capable of sensing the cytosolic calcium signal elicited by salt stress [62]. Loss-of-function *sos2* alleles show a salt-sensitive phenotype [21], as do *sos3* mutants when faced with the extra demand of Ca^{2+} deficiency [60].

9. VACUOLAR SEQUESTRATION OF NA^+ IN PALNTS

Vacuolar sequestration of Na^+ could contribute to decrease the concentration of Na^+ concentration in the cytoplasm. But it also contributes to maintain the osmotic pressure inside cells that can protect the water release into saline solutions. Other organelles, such as plastids and mitochondria, may also accumulate some Na^+ and thus contribute to the overall subcellular compartmentation of Na^+. In *Arabidopsis*, the AtNHX1-6 probably function for the Na^+ compartmentation [63]. AtNHX1 and

AtNHX2 are localized in the tonoplast membrane. Their transcript levels are upregulated by ABA or osmotic stress [64]. The transcript levels of vacuolar H^+-ATPase components also increased in response to salt stress [65]. Overexpression of AtNHX1 in *Brassica* and tomato [66] enhanced the salt tolerance of plants substantially.

10. CPA2 TYPE NA^+/H^+ ANTIPORTERS IN PLANTS

Arabidopsis genome has 28 putative CPA2 antiporters. But, limited information is available on these proteins. The probable functions of the *AtCHX* genes in CPA2 family have been reported very recently by the expression pattern in transgenic plants [67] and the functional analysis of suppress mutant [23]. It reveals that several *AtCHX* genes specifically expressed in the male gametophyte and sporophytic tissues and developmentally regulated during gametophyte [67]. The *AtCHX17*, which expressed in the epidermal and cortical cells of mature root zone, is believed to be involved in K^+ transport since the knock-out mutant plants accumulate less K^+ in the roots when the plants were subjected with salt stress or K^+ starvation [22]. Recently, it was shown that the AtCHX23 is a $Na^+(K^+)/H^+$ antiporter localized on the chloroplast envelope, although the exchange activity was not shown. AtCHX23 might be involved in the pH homeostasis and chloroplast development [23]. It should be mentioned that plants are the only unique organisms having so many CPA2 type antiporters. CPA2 antiporter is unknown in animals.

11. POTENTIAL ROLES OF NA^+/H^+ ANTIPORTERS FOR SALT TOLERANCE IN HIGHER PLANTS

The progress in genome sequence of higher plants increasesd the information on Na^+/H^+ antiporters. In addition to model plant *Arabidopsis*, the Na^+/H^+ antiporters from barley (*Hordeum vulgare*) [68], cotton (*Gossypium hirsutum*) [69], maize (*Zea mays*) [70], and rice (*Oryza sativa*) [71] have been characterized. Overexpression of the rice Na^+/H^+ antiporter, OsNHX1, enhanced the salt tolerance of transgenic rice cells and plants [71]. Numerous investigations have led to the conclusion that these genes play important roles to extrude Na^+ across plasma membrane, sequestrate Na^+ into tonoplast, and re-circulate Na^+ from shoot to root. However, there are many Na^+/H^+ antiporters of which functions are unknown. Little is known for the regulation of expression of various Na^+/H^+ antiporters.

For salt tolerance, K^+ homeostasis is an another important aspect. The transcript levels of several K^+ transporter genes are either down- or up-regulated by salt stress, probably reflecting the different capacities of plants to maintain K^+ uptake under salt stress. Unraveling additional stress-associated gene resources, from both crop plants and highly salt- and drought-tolerant model plants, will enable future molecular dissection of salt-tolerance mechanisms in important crop plants.

REFERENCES

1. Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. *Science.* **285,** 1256-1258.
2. Shi, H., Quintero, F.J., Pardo, J.M. and Zhu, J.K. (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *Plant Cell* **14,** 465-477.
3. Zhang, H.X. and Blumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotechnol*. **19,** 765-768.
4. Zhu, J.K. (2001) Plant salt tolerance. *Trends Plant Sci*. **6,** 66-71.
5. Shi, H., Ishitani, M., Kim, C. and Zhu, J.K. (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. *Proc. Natl. Acad. Sci. USA* **97,** 6896-6901.
6. Qiu, Q.S., Guo, Y., Dietrich, M.A., Schumaker, K.S. and Zhu, J.K. (2002) Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. USA* **99,** 8436-8441.
7. Apse, M.P., Sottosanto, J.B. and Blumwald, E. (2003) Vacuolar cation/H^+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1, the Arabidopsis vacuolar Na^+/H^+ antiporter. *Plant J*. **36,** 229-239.
8. Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA* **98,** 12832-12836.
9. Dover, N. and Padan, E. (2001) Transcription of *nhaA*, the main Na^+/H^+ antiporter of *Escherichia coli*, is regulated by $Na^+$and growth phase. *J. Bacteriol.* **183,** 644-653.
10. Inaba, M., Sakamoto, A. and Murata, N.. (2001) Functional expression in Escherichia coli of low-affinity and high-affinity $Na^+(Li^+)/H^+$ antiporters of *Synechocystis*. *J. Bacteriol*. **183,** 1376-1384.
11. Karpel, R., Olami, Y., Taglicht, D., Schuldiner, S. and Padan, E. (1988) Sequencing of the gene ant which affects the Na^+/H^+ antiporter activity in *Escherichia coli*. *J. Biol. Chem*. **263,** 10408-10814.
12. Krulwich, T.A., Ito, M. and Guffanti, A.A. (2001) The Na(+)-dependence of alkaliphily in *Bacillus*. *Biochim. Biophys. Acta* **1505,** 158-168.
13. Blumwald, E. (2000) Sodium transport and salt tolerance in plants. *Curr. Opin. Cell Biol*.**12,** 431-434
14. Soong, T.W., Yong, T.F., Ramanan, N. and Wang, Y. (2000) The *Candida albicans* antiporter gene CNH1 has a role in Na^+ and H^+ transport, salt tolerance, and morphogenesis. *Microbiology* **146,** 1035-1044.
15. Padan, E. and Schuldiner, S. (1996) Bacterail Na^+/H^+ antiporters: molecular biology, biochemistry, and physiology. In *Handbook of Biological Physics* (Konings, W.N., Kaback, H.R., and Lolkema, J.S., eds) . Amsterdam: Elsevier Science, vol.2, pp.501-531..
16. Rosen, B.P. (1986) Ion extrusion systems in *Escherichia coli*. *Methods Enzymol*. **125,** 328-336.
17. Brett, C.L., Donowitz, M. and Rao, R. (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *Am. J. Physiol. Cell Physiol*. **288,** C223-239.
18. Mäser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J., Sanders, D., Harper, J.F., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D.E., Kim, S.A. and Guerinot, M.L. (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiol*. **126,** 1646-1667.
19. Apse, M.P. and Blumwald, E. (2002) Engineering salt tolerance in plants. *Curr. Opin. Biotechnol*. **13,** 146-150.
20. Shi, H.., Lee, B.H., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol*. **21,** 81-85.
21. Zhu, J.K. (2003) Regulation of ion homeostasis under salt stress.*Curr. Opin. Plant Biol*. **6,** 441-445.
22. Cellier, F., Conejero, G., Ricaud, L., Luu, D.T., Lepetit, M., Gosti, F. and Casse, F. (2004) Characterization of AtCHX17, a member of the cation/H^+ exchangers, CHX family, from *Arabidopsis thaliana* suggests a role in K^+ homeostasis. *Plant J*. **39,** 834-846.
23. Song, C.P., Guo, Y., Qiu, Q., Lambert, G., Galbraith, D.W., Jagendorf, A. and Zhu, J.K. (2004) A probable $Na^+(K^+)/H^+$ exchanger on the chloroplast envelope functions in pH homeostasis and chloroplast development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **101,** 10211-10216.
24. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda,

M., Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). *DNA Res*. **30,** 185-209.

25. Hamada, A., Hibino, T., Nakamura, T. and Takabe, T. (2001) Na^+/H^+ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity. *Plant Physiol*. **125,** 437-446.
26. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A. and Takabe, T. (2001) Halotolerant cyanobacterium Aphanothece halophytica contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. *J. Biol. Chem*. **276,** 36931-36938.
27. Waditee, R., Hibino, T., Nakamura, T., Incharoensakdi, A. and Takabe, T. (2002) Overexpression of a Na^+/H^+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. *Proc. Natl. Acad. Sci. USA* **99,** 4109-4114.
28. Wutipraditkul, N., Waditee, R., Incharoensakdi, A., Hibino, T., Tanaka, Y., Nakamura, T., Shikata, M., Takabe, T. and Takabe, T. (2005) The Halotolerant Cyanobacterium *Aphanothece halophytica*, Containe NapA type Na^+/H^+ Antiporters involved in Salt Tolerance at Alkaline pH with Novel Ion Specificity. *Appl. Environ. Microbiol*. **71,** 4176-4184.
29. Ohyama, T., Igarashi, K. and Kobayashi, H. (1995) Physiological role of the chaA gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J. Bacteriol.* **176,** 4311-4315.
30. Yamaguchi, T., Apse, M.P., Shi, H. and Blumwald, E. (2003) Topological analysis of a plant vacuolar Na^+/H^+ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proc. Natl. Acad. Sci. USA* **100,** 12510-12515.
31. Wakabayashi, S., Pang, T., Su, X. and Shigekawa, M. (2000) A novel topology model of the human Na^+/H^+ exchanger isoform1. *J. Biol. Chem*. **275,** 7942-7949.
32. Kinclova, O., Potier, S. and Sychrova, H.(2002) Difference in substrate specificity divides the yeast alkali-metal-cation/H^+ antiporters into two subfamilies. *Microbiology* **148,** 1225-1232.
33. Lehoux, S., Abe, Ji., Florian, J.A. and Berk, B.C. (2001) 14-3-3 Binding to Na^+/H^+ exchanger isoform-1 is associated with serum-dependent activation of Na^+/H^+ exchange. *J. Biol. Chem*. **276,** 15794-15800.
34. Denker, S.P., Huang, D.C., Orlowski, J. Furthmayr, H. and Barber, D.L. (2000) Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskelton and cell shape independently of H^+ translocation. *Mol.Cel*. **6,** 1425-1436.
35. Pang, T., Su, X., Wakabayashi, S. and Shigekawa, M. (2001) Calcineurin homologous protein as an essential cofactor for Na^+/H^+ exchangers. *J. Biol. Chem*. **276,** 17367-17372.
36. Mitsui, K., Kamauchi, S., Nakamura, N., Inoue, H. and Kanazawa, H. (2004) A conserved domain in the tail region of the Saccharomyces cerevisiae Na^+/H^+ antiporter (Nha1p) plays important roles in localization and salinity-resistant cell-growth. *J. Biochem*. **135,** 139-148.
37. Strausak, D., Waser, M. and Solioz, M.. (1993) Functional expression of the *Enterococcus hirae* NaH-antiporter in *Escherichia coli*. *J. Biol. Chem*. **268,** 26334-26337.
38. Southworth, T.W., Guffanti, A.A., Moir, A. and Krulwich, T.A. (2001) GerN, an endospore germination protein of Bacillus cereus, is an Na^+/H^+-K^+ antiporter. *J. Bacteriol*. **183,** 5896-5903.
39. Wang, H.L., Postier, B.L. and Burnap, R.L. (2002) Polymerase chain reaction-based mutageneses identify key transporters belonging to multigene families involved in Na^+ and pH homeostasis of *Synechocystis* sp. PCC 6803. *Mol. Microbiol*. **44,** 1493-1506.
40. Elanskaya, I.V., Karandashova, I.V., Bogachev, A.V. and Hagemann, M. (2002) Functional analysis of the Na^+/H^+ antiporter encoding genes of the cyanobacterium *Synechocystis* PCC 6803.Biochemistry (Mosc). **67,** 432-440.
41. Mikkat, S. Milkowski, C., Hagemann, M. (2000) The gene sll0273 of the cyanobacterium *Synechocystis* sp. PCC6803 encode a protein essential for growth at low Na^+/K^+ ratios. *Plant Cell Environ*. **23,** 549-559.
42. Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiol. Mol. Biol. Rev.* **63,** 334-348.
43. Baxter, I.R., Young, J.C., Armstrong, G., Foster, N., Bogenschutz, N., Cordova, T., Peer, W.A., Hazen, S.P., Murphy, A.S. and Harper, J.F. (2005) A plasma membrane H^+-ATPase is required for the formation of proanthocyanidins in the seed coat endothelium of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **102,** 2649-2654.

44. Sze, H., Schumacher, K., Muller, M.L., Padmanaban, S. and Taiz, L. (2002) A simple nomenclature for a complex proton pump: VHA genes encode the vacuolar H^+-ATPase. *Trends Plant Sci*. **7,** 157-1561.
45. Kim, E.J., Zhen, R.G. and Rea, P.A. (1994) Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. *Proc. Natl. Acad. Sci USA*. **91,** 6128-6132.
46. Geigenberger, P., Hajirezaei, M., Geiger, M., Deiting, U., Sonnewald, U. and Stitt, M. (1998) Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversions, and increased levels of nucleotides in growing potato tubers. *Planta* **205,** 428-437.
47. Gaxiola, R.A., Li, J., Undurraga, S., Dang, L.M., Allen, G.J., Alper, S.L. and Fink, G.R. (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. *Proc. Natl. Acad. Sci. USA* **98,**11444-11449.
48. Benito, B. and Rodriguez-Navarro, A. (2003) Molecular cloning and characterization of a sodium-pump ATPase of the moss Physcomitrella patens. *Plant J*. **36,** 382-389.
49. Shono, M., Wada, M., Hara, Y. and Fujii, T. (2001) Molecular cloning of Na^+-ATPase cDNA from a marine alga, Heterosigma akashiwo. *Biochim. Biophys. Acta* **1511,** 193-199.
50. Chen, Q. Arents, J.C., Bader, R., Postma, P.W and Amster-Choder, O. (1997) BglF, the sensor of the *E. coli* bgl system, uses the same site to phosphorylate both a sugar and a regulatory protein. *EMBO J* **16,** 4617–4627.
51. Rubenhagen R, Morbach S, Kramer R. (2001) The osmoreactive betaine carrier BetP from *Corynebacterium glutamicum* is a sensor for cytoplasmic K^+. *EMBO J.* **20**, 5412-20.
52. Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. *Plant Cell* **11,** 1743-1754.
53. Kohorn, B.D. (2001) WAKs: cell wall associated kinases. *Curr. Opin. Cell Biol*. **13,** 529-533.
54. Qiu, Q.S., Barkla, B.J., Vera-Estrella, R., Zhu, J.K. and Schumaker, K.S. (2003) Na^+/H^+ exchange activity in the plasma membrane of *Arabidopsis thaliana*. *Plant Physiol.* **132,** 1041-1052.
55. Maser, P., Eckelman, B., Vaidyanathan, R., Horie, T., Fairbairn, D.J., Kubo, M., Yamagami, M., Yamaguchi, K., Nishimura, M., Uozumi, N. Robertson, W., Sussman, M.R. and Schroeder, J.I. (2002) Altered shoot/root NaR distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the NaR transporter AtHKT1. *FEBS Lett.* **531,** 157-161.
56. Berthomieu, P., Conejero, G., Nublat, A., Brackenbury, W.J., Lambert, C., Savio, C., Uozumi, N., Oiki, S., Yamada, K., Cellier, F., Gosti, F., Simonneau, T., Essah, P.A., Tester, M., Very, A.A., Sentenac, H. and Casse, F. (2003) Functional analysis of AtHKT1 in Arabidopsis shows that Na^+ recirculation by the phloem is crucial for salt tolerance. *EMBO J*. **22,** 2004-2014.
57. Amtmann, A. and Sanders, D. (1999) Mechanisms of NaR uptake by plant cells. *Adv. Bot. Res*. **29,** 75-112.
58. Yeo, A.R., Flowers, S.A., Rao, G., Welfare, K., Senanayake, N. and Flowers, T.J: (1999) Silicon reduces sodium uptake in rice (Oryza sativa L.) in saline conditions and this is accounted for by a reduction in the transpirational bypass flow. *Plant Cell Environ*. **22,** 559-565.
59. Quintero, F.J., Ohta, M., Shi, H., Zhu, J.K. and Pardo, J.M. (2002)) Reconstitution in yeast of the Arabidopsis SOS signaling pathway for NaR homeostasis. *Proc. Natl. Acad. Sci. USA* **99,** 9061-9066.
60. Liu, J. and Zhu, J.K. (1997) An arabidopsis mutant that requires increased calcium for potassium nutrition and salt tolerance. *Proc. Natl. Acad. Sci. USA* **94,** 14960-14964.
61. Liu, J., Ishitani, M., Halfter, U., Kim, C.S. and Zhu. J.K. (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acad. Sci. USA*. **97,** 3730-3734.
62. Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Shi, W., Zhu, J.K. (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. *Plant Cell* **12,** 1667-1677.
63. Blumwald, E., Aharon, G.S, and Apse, M.P. (2000) Sodium transport in plant cells. *Biochim. Biophys. Acta*. **1465,** 140-151.
64. Yokoi, S., Quintero, F.J., Cubero, B., Ruiz, M.T., Bressan, R.A., Hasegawa, P.M. and Pardo, J.M: (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. *Plant J.* **30,** 529-539.
65. Dietz, K.J., Tavakoli, N., Kluge, C., Mimura,T., Sharma, S.S., Harris, G.C., Chardonnens, A.N. and Golldack, D. (2001) Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level. *J. Exp. Bot* . **52,** 1969-1980.

66. Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA* **98,** 12832-12836.
67. Sze, H., Padmanaban, S., Cellier, F., Honys, D., Cheng, N.H., Bock, K.W., Conejero, G., Li, X., Twell, D., Ward, J.M. and Hirschi, K.D. (2004) Expression patterns of a novel AtCHX gene family highlight potential roles in osmotic adjustment and K^+ homeostasis in pollen development. *Plant Physiol*. **136,** 2532-2547.
68. Vasekina, A.V., Yershov, P.V., Reshetova, O.S., Tikhonova, T.V., Lunin, V.G., Trofimova, M.S. and Babakov, A.V. (2005) Vacuolar Na^+/H^+ antiporter from barley: identification and response to salt stress. *Biochemistry (Mosc)* **70,** 100-107.
69. Wu, C.A., Yang, G.D., Meng, Q.W. and Zheng, C.C. (2004) The cotton GhNHX1 gene encoding a novel putative tonoplast Na^+/H^+ antiporter plays an important role in salt stress. *Plant Cell Physiol*. **45,** 600-607.
70. Zorb, C., Noll, A., Karl, S., Leib, K., Yan, F. and Schubert, S.(2005) Molecular characterization of Na^+/H^+ antiporters (ZmNHX) of maize (*Zea mays* L.) and their expression under salt stress. *J. Plant Physiol.* **162,** 55-66.
71. Fukuda, A., Nakamura, A., Tagiri, A., Tanaka, H., Miyao, A., Hirochika, H. and Tanaka, Y. (2004) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. *Plant Cell Physiol*. **45,** 146-159.

12. STRUCTURAL AND FUNCTIONAL RELATIONSHIP BETWEEN CATION TRANSPORTERS AND CHANNELS

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Abstract. The K^+ channels as well as transporters for K^+ and Na^+ are essential to maintain high K^+ to Na^+ ratio in cytosol of plant cells under abiotic stresses. Recent progress on the structures of channels and ion transporters (such as symporters, antiporters, and pumps) together with several results from a variety of approaches indicated that the discriminate between K^+ channel and K^+ pump seems to be unclear at least molecular structural levels. In this review, we will discuss on the structural and functional relationship between cation transporters and channels.

1. INTRODUCTION

Under salt stress, plants maintain a high concentration of K^+ and a low concentration of Na^+ in the cytosol. They do this by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps. In many cases, H^+ pumps generate the driving force for transport of K^+ and Na^+. Over the past few years, molecular approaches associated with electrophysiological and structural analyses have greatly advanced our understanding of K^+ transporters. A large number of genes encoding K^+ transport systems have been identified, revealing a high level of complexity. Characterization of some transport systems is providing the exciting information at the molecular level on functions such as root K^+ uptake and secretion into the xylem sap, K^+ transport in guard cells, or K^+ influx into growing pollen tubes. It seems to become more unclear to discriminate between K^+ channel and K^+ pump at least molecular structural levels. In this review, we will discuss on the structural and functional relationship between cation transporters and channels.

2. ION TRANSPORTERS

Ion transporters in wide meaning, can be classified into transporters and channels in narrow meaning [1]. Transporters in narrow meaning are classified into 1) symporters or antiporters and 2) pumps such as H^+-ATPase and Na^+/K^+-ATPase. In the following, ion transporters are used in narow meaning if not mentioned. Ion transporters and channels perform their activities using H^+ (in some case, Na^+ or K^+) motive force (membrane potential and H^+ (Na^+ or K^+) chemical gradient) and ATP.

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Structural and functional aspects of cation transporters and channels are similar between prokaryotic and eukaryotic cells. In Figure 1, a schematic diagram of bacterial ion transporters in wide meaning is shown [2]. Electron transport systems generate H^+ (in some case, Na^+) electrochemical gradient (H^+ or Na^+ motive force). *Escherichia coli* uses H^+ motive force for the synthesis of ATP, uptake of nutrients, and extrusion of Na^+. In plants, ATP synthesis occurs in chloroplast via H^+-ATP synthetase using H^+ motive force driven by light energy. The H^+ motive forces are also produced by P-type H^+-ATPase localized in plasma membranes and vacuole membranes which are used for uptake of nutrients, and transport of Na^+.

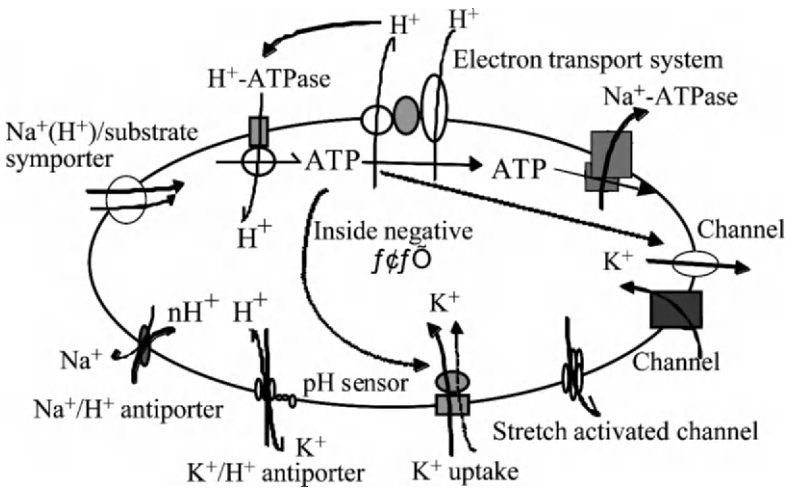

Figure 1. A schematic diagram of bacterial ion transporters (modified from Fig. 1 of ref. 2).

It is generally believed that ion transporters (such as symporters, antiporters and pumps) have substrates binding sites [3-7], and they change the conformation during ion transport [8]. The substrate (ion) binding sites are probably faced on both outside and inside of biological membranes. The structure of ion binding sites will change alternately during ion transport that is called as two faces Janus model. Historically, KcsA gene was first cloned from bacteria. KcsA protein was purified and reconstituted into proteoliposomes. It was shown that KcsA protein is K^+ channel [9]. Three dimensional X-ray structure of K^+ channel revealed that it has remarkable K^+ selective pore [10]. The pore was consisted from four conserved glycine. The X-ray structures of two prokaryotic Cl^--channels from *Salmonella* and *Escherichia coli* were solved [11]. Both structures reveal two identical pores, each pore being formed by a separate subunit within a homodimeric membrane protein. Individual subunits are composed of two roughly repeated halves that span the membrane with opposite orientations. This antiparallel architecture defines a selectivity filter in which a Cl^- ion is stabilized by electrostatic interactions with alpha-helix dipoles and by chemical coordination with nitrogen atoms and hydroxyl groups. The pore of water

channel has similar structural selectivity as seen in K^+ and Cl^- channels [12]. Human red cell aquaporin-1(AQP1) is the first functionally defined member of the aquaporin family. The aqueous pathway is lined with conserved hydrophobic residues that permit rapid water transport, whereas the water selectivity is due to a constriction of the pore diameter to about 3 A over a span of one residue. The atomic model provides a possible molecular explanation to a longstanding puzzle in physiology, how membranes can be freely permeable to water but impermeable to protons. Other channels with multi-substrate selectivity are also reported [13-16]. Water channels are considered to have always open conformation, whereas many other channels usually have the closed conformation. The pore structure of K^+ channel may not be altered when the K^+ channel is functionally opened by some signal. However, the gate-way to the pore will be opened. Dynamic changes of pore size structures have been reported in some channels [16, 17]. Mechanical structures of ion transporters and channels have been considered to be different. Ion transporters change their conformations during each steps of transport whereas channels do not change their conformations. However, the differences of basic structures between ion transporters and channels seem to become unclear, because transporters contain channel structure [18]

3. NA^+/H^+ ANTIPORTERS FROM BACTERIA, PLANT AND ANIMAL

Na^+/H^+ antiporters extrude Na^+ using H^+ motive force. At least, 8 families of Na^+/H^+ antiporters are reported, i.e. 1) NhaA, 2) NhaB, 3) ChaA (CAX), 4) NhaP (NHE, NHX), 5) NapA (CHX, Nha1, Sod2), 6) NhaC, 7) NhaD, and 8) Pha like system. NhaA, NhaB, and NhaC are Na^+/H^+ antiporters only found in bacteria. NhaA has 12 transmembrane segments [19,20]. The activity of *E. coli* NhaA strongly depends on pH which is a good example to study the molecular mechanisms of pH sensing, conformational changes, and control of internal pH. Recently, three dimensionally NhaA structure has been determined [21]. The amino acid sequence homology between NhaA and other antiporters were low, but some of the basic structure of them, NhaC for example, might have similarity to NhaA. NhaB has different structure from that of NhaA [22, 23], namely NhaB has 9 transmembrane segments with C terminus faced to periplasmic spase (outside). ChaA, NapA and NhaD are found not only in bacteria, but also in plants. ChaA (CAX) is functionally unique because it has Ca^{2+} extrusion activity [24, 25]. They might have unique structure. NapA [26] has homology to K^+ extrusion channel Kef C [27, 28] and K^+ efflux system of Yeast [29]. The structural information on NapA is desired because it works as a Na^+/H^+ antiporter whereas a homologous protein KefC works as a K^+ channel. NhaP (NHX/NHE) family is the most widely distributed Na^+/H^+ antiporters from bacteria to animal. Structural and functional studies of NhaP (NHX/NHE) from various species are interesting. Bacteria and plant NhaP (NHX) work for salt tolerance [30, 31] and it's C-terminal tail is involved for ion selectivity [32]. Animal NHE (NhaP) works as internal pH alkalization [33]. Na^+ movement is opposite between bacteria/plant NhaP (outward) and animal NHE (inward). Pha systems are

consisted of seven membrane proteins and reported that they work as K^+ or Na^+ transporters [34, 35].

4. K^+ UPTAKE SYSTEMS FROM BACTERIA, PLANT AND ANIMAL

One of the most widely distributed K^+ uptake pumps in animal is the Na^+/K^+-ATPase which extrudes Na^+ and uptakes K^+ during the hydrolysis of ATP to ADP and Pi. Its structure has some homology to Ca^{2+}-ATPase [3]. Bacteria have Kdp pump (P-type K^+-ATPase) that uptakes K^+ consuming ATP. The Kdp system requires at least three membrane proteins, KdpA, KdpB, and KdpC. KdpB is the menbrane bound ATPase protein and KdpA is considered to have K^+ ion passage route [36].

At least, 5 families of K^+ transporters (Trk, Ktr, HKT1, Kup, and Fku) which are powered by H^+ motive force, are reported. Trk system from bacteria requires three proteins, TrkA, TrkH and SapD [37]. TrkA and SapD are water soluble proteins that attach to the membrane protein TrkH. TrkH is considered to have K^+ ion passage route. Ktr system from bacteria requires at least two proteins, KtrA and KtrB [18]. KtrA is water soluble protein that attaches to the membrane protein KtrB. HKT1 is a Na^+ or K^+ transporter from plant [38]. As described below, the homology of TrkH, KtrB, and HKT1 is low and the evolutional distance among them is each other far, but they have common structure [39]. Kup is found from bacteria and plants [40,41]. So far, only one protein is required for Kup. Fku system is bacterial K^+ uptake system, its activity is low and K_m is high, and is considered as the fundamental K^+ uptake system [42]. It requires at least two proteins, FkuA and FkuB. FkuA is predicted as a water soluble protein that attaches to the membrane protein FkuB.

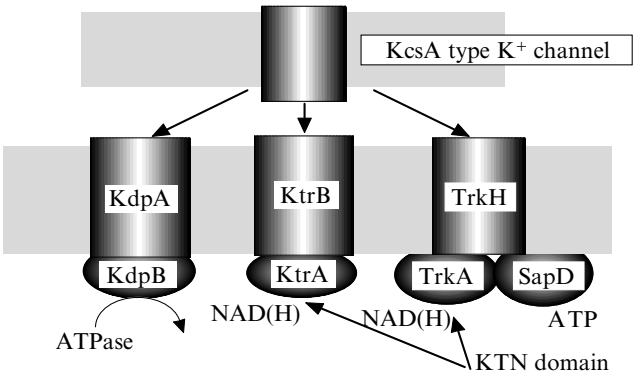

Figure 2. Similarity of transmembrane subunit (KdpA, KtrB and TrkH) in some K^+ uptake transporters with KcsA type K^+ channel.

Plant has two inward rectified K^+ channel families, KAT1/AKT1 and TWIK [19,43]. Homologous genes to KAT1/AKT1 and TWIK are found from animal.

Plant and animal have bacterial type K^+ channels, KcsA, although their physiological functions are not clear.

5. TRK AND KTR K^+ UPTAKE SYSTEMS

For the accumulation of K^+ by Trk system, the H^+ motive force is required [37]. SapD is a member of Trk system and contains the ATP binding site. ATP requirement in Trk system was demonstrated using Δ*atp*-strain [37]. It was shown that ATP regulates the activity of Trk system and a high cytoplasmic ATP concentration alone is not sufficient to drive K^+ transport. The H^+ motive force is the driving force for K^+ uptake.

Ktr system from *Vibrio alginolyticus* requires Na^+ for K^+ uptake [44]. In *Vibrio* Ktr, it is not clear whether Na^+ is transported as K^+/Na^+ symporter or Na^+ is involved in the activation of Ktr system.

6. K^+ TRANSPORTERS MAY DEVELOP FROM K^+ CHANNELS

Existence of KcsA type selective pore structures [9] are reported in transmembrane subunit (KdpA, KtrB and TrkH) of some K^+ uptake transporters (Kdp, Ktr and Trk) (Fig. 2) [39, 44, 45]. Similar pore structures are also found in HKT1 from plant [39]. These facts suggest that some of K^+ transporters may be developed from K^+ channels. If it is the case, an interesting question araises. Do inwardly rectified K^+ channels work as a K^+ transporter ? The answer is yes [43]. KAT1 and AKT1 are plant's inward rectified K^+ channels. These channels complimented the K^+ uptake deficient *E. coli* mutant strains.

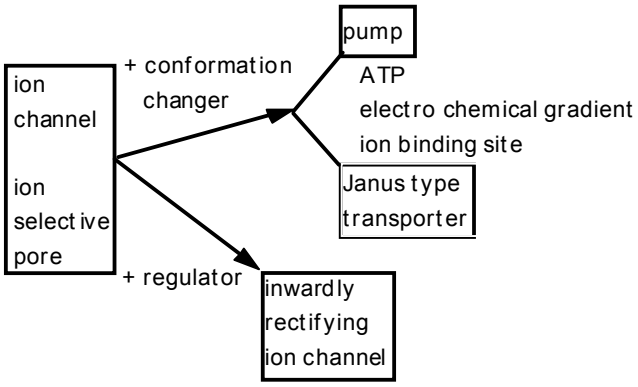

Figure 3. A hypothesison the evolutional relationship between channels and transporters.

KtrA that is attached to KtrB, is required for the transport activity of Ktr system [18]. Ktr system from plant requires one more protein, KtrE [46]. KtrA contains

KTN (K^+ transport nucleotide binding) domain that belongs to RCK domain which regulates the conductance of K^+. RCK domain is found in many prokaryotic and eucaryotic channels [47]. TrkA and SapD contain KTN domain or ATP binding site, respectively. KdpB is required for the Kdp system. KdpB works as ATPase. These facts suggest that K^+ transporters may be developed from K^+ channels with regulator (Fig. 3).

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REFERENCES

1. Stumpe, S., Schlosser, A., Schleyer, M. and Bakker, E. P. (1996) *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R. and Lolkema, J. S., eds). Amsterdam: Elsevier, Vol. **2,** pp. 473-499.
2. Nakamura, T. and Uozumi, N. (1999) Current topics of cation transporters in animal, plant and bacteria. *Tanpakushitsu Kakusan Koso* **44,** 1988-1995.
3. Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature* **405,** 647-655.
4. Murakami, S., Nakashima, R., Yamashita, E. and Yamaguchi, A. (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. *Natur* **419,** 587-593.
5. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H.R. and Iwata, S. (2003) Structure and mechanism of the lactose permease of *Escherichia coli*. *Science* **301,** 610-615.
6. Huang, Y., Lemieux, M.J., Song, J., Auer, M. and Wang, D.N. (2003) Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science* **301,** 616-620.
7. Ma, C. and Chang, G. (2004) Structure of the multidrug resistance efflux transporter EmrE from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **101,** 2852-2857.
8. Locher, K.P., Bass, R.B. and Rees, D.C. (2003) Structural biology. Breaching the barrier. *Science* **301,** 603-604.
9. Schrempf, H., Schmidt, O., Kummerlen, R., Hinnah, S., Muller, D., Betzler, M., Steinkamp, T. and Wagner, R. (1995) A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*. *EMBO J.* **14,** 5170-5178.
10. Doyle, D.A., Cabral, J.M., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* **280,** 69-77
11. Dutzler, R., Campbell, E.B., Cadene, M., Chait, B.T. and MacKinnon, R. (2002) X-ray structure of a ClC chloride channel at 3.0 A reveals the molecular basis of anion selectivity. *Nature* **415,** 287-294.
12. Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A. and Fujiyoshi, Y. (2000) Structural determinants of water permeation through aquaporin-1. *Nature* **407,** 599-605.
13. Chang, G., Spencer, R.H., Lee, A.T., Barclay, M.T. and Rees, D.C. (1998) Structure of the MscL homolog from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel. *Science* **282,** 2220-2226.
14. Bass, R.B., Strop, P., Barclay, M. and Rees, D.C. (2002) Crystal structure of *Escherichia coli* MscS, a voltage-modulated and mechanosensitive channel. *Science* **298,** 1582-1587.
15. Van den Berg, B., Clemons, W.M. Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S.C. and Rapoport, T.A. (2004) X-ray structure of a protein-conducting channel. *Nature* **427,** 36-44.
16. Miyazawa, A., Fujiyoshi, Y. and Unwin, N. (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **424,** 949-955.
17. Sukharev, S., Betanzos, M., Chiang, C.S. and Guy, H.R. (2001) The gating mechanism of the large mechanosensitive channel MscL. *Nature* **409,** 720-724.
18. Nakamura, T., Yuda, R., Unemoto, T. and Bakker, E.P. (1998) KtrAB, a new type of bacterial K^+-uptake system from *Vibrio alginolyticus*. *J. Bacteriol*. **180,** 3491-3494.
19. Williams, K.A. (2000) Three-dimensional structure of the ion-coupled transport protein NhaA. *Nature* **403,** 112-115.

20. Nakamura, T., Komano, Y. and Unemoto, T. (1995) Three aspartic residues in membrane-spanning regions of Na^+/H^+ antiporter from *Vibrio alginolyticus* play a role in the activity of the carrier. *Biochim. Biophys. Acta* **1230,**170-176.
21. Hunte, C., Screpanti E., Venturi, M., Rimon, A., Padan, E. and Michel H. (2005) Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature* **435**, 1197-1202.
22. Enomoto, H., Unemoto, T., Nishibuchi, M., Padan, E. and Nakamura, T. (1998) Topological study of *Vibrio alginolyticus* NhaB Na^+/H^+ antiporter using gene fusions in *Escherichia coli* cells. *Biochim. Biophys. Acta* **1370,** 77-86.
23. Nakamura, T., Fujisaki, Y., Enomoto, H., Nakayama, Y., Takabe, T., Yamaguchi, N. and Uozumi, N.. (2001) Residue aspartate-147 from the third transmembrane region of Na^+/H^+ antiporter NhaB of *Vibrio alginolyticus* plays a role in its activity. *J. Bacteriol*. **183,** 5762-5767.
24. Ivey, D.M., Guffanti, A.A., Zemsky, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S. and Krulwich, T.A. (1993) Cloning and characterization of a putative Ca^{2+}/H^+ antiporter gene from *Escherichia coli* upon functional complementation of Na^+/H^+ antiporter-deficient strains by the overexpressed gene. *J. Biol. Chem.* **268,** 11296-11303.
25. Waditee, R., Hossain, G.S., Tanaka, Y., Nakamura, T., Shikata, M., Takano, J., Takabe, T. and Takabe, T. (2004) Isolation and functional characterization of Ca^{2+}/H^+ antiporters from cyanobacteria. *J. Biol. Chem.* **279,** 4330-4338.
26. Waser, M., Hess-Bienz, D., Davies, K. and Solioz, M. (1992) Cloning and disruption of a putative NaH-antiporter gene of *Enterococcus hirae*. *J. Biol. Chem.* **267,** 5396-5400.
27. Reizer, J., Reizer, A. and Saier, M.H. Jr. (1992) The putative Na^+/H^+ antiporter (NapA) of *Enterococcus hirae* is homologous to the putative K^+/H^+ antiporter (KefC) of *Escherichia coli*. *FEMS Microbiol. Lett.* **73,** 161-163.
28. Ferguson, G.P., McLaggan, D. and Booth, I.R. (1995) Potassium channel activation by glutathione-S-conjugates in *Escherichia coli*: protection against methylglyoxal is mediated by cytoplasmic acidification. *Mol Microbiol* **17,** 1025-1033.
29. Ramirez, J. Ramirez, O. Saldana, C. Coria, R. and Pena, A. (1998) A *Saccharomyces cerevisiae* mutant lacking a K^+/H^+ exchanger. *J. Bacteriol.* **180,** 5860-5865.
30. Hamada, A., Hibino, T., Nakamura, T. and Takabe, T. (2001) Na^+/H^+ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity. *Plant Physiol*. **125,** 437-446.
31. Waditee, R., Hibino, T., Nakamura, T., Incharoensakdi, A. and Takabe, T. (12002) Overexpression of a Na^+/H^+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. *Proc. Natl. Acad. Sci. USA* **99,** 4109-4114.
32. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A. and Takabe, T. (2001) Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. *J. Biol. Chem*. **276,** 36931-36938.
33. Fafournoux, P. and Pouyssegur, J. (1996) *Handbook of Biological Physics,* eds. Konings, W. N., Kaback, H. R. and Lolkema, J. S. Amsterdam: Elsevier, Vol. **2,** pp. 369-380.
34. Putnoky, P., Kereszt, A., Nakamura, T., Endre, G., Grosskopf, E., Kiss, P. and Kondorosi, A. (1998) The *pha* gene cluster of Rhizobium meliloti involved in pH adaptation and symbiosis encodes a novel type of K^+ efflux system. *Mol. Microbiol*. **28**, 1091-1101.
35. Kosono, S., Morotomi, S., Kitada, M. and Kudo, T. (1999) Analyses of a Bacillus subtilis homologue of the Na^+/H^+ antiporter gene which is important for pH homeostasis of alkaliphilic *Bacillus* sp. C-125. *Biochim. Biophys. Acta* **1409,** 171-175.
36. Buurman, E.T., Kim, K.T. and Epstein, W. (1995) Genetic evidence for two sequentially occupied K^+ binding sites in the Kdp transport ATPase. *J. Biol. Chem.* **270,** 6678-6685.
37. Harms, C., Domoto, Y., Celik, C., Rahe, E., Stumpe, S., Schmid, R., Nakamura, T. and Bakker, E.P. (2001) Identification of the ABC protein SapD as the subunit that confers ATP dependence to the K^+-uptake systems Trk(H) and Trk(G) from *Escherichia coli* K-12. *Microbiology* **147,** 2991-3003.
38. Maser, P., Hosoo, Y., Goshima, S., Horie, T., Eckelman, B., Yamada, K., Yoshida, K., Bakker, E.P., Shinmyo, A., Oiki, S., Schroeder, J.I. and Uozumi, N. (2002) Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. *Proc. Natl. Acad. Sci. USA.* **99,** 6428-6433.

39. Durell, S.R., Hao, Y., Nakamura, T., Bakker, E.P. and Guy, H.R. (1999) Evolutionary relationship between K^+ channels and symporters. *Biophys. J.* **77,** 775-788.
40. Schleyer, M. and Bakker, E.P. (1993) Nucleotide sequence and 3'-end deletion studies indicate that the K^+-uptake protein Kup from *Escherichia coli* is composed of a hydrophobic core linked to a large and partially essential hydrophilic C terminus. *J. Bacteriol.* **175,** 6925-6931.
41. Maser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J., Sanders, D., Harper, J.F., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D.E., Kim, S.A. and Guerinot, M.L. (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiol*. **126,** 1646-1267.
42. Nakamura, T., Katoh, Y., Shimizu, Y., Matsuba, Y. and Unemoto, T. (1996) Cloning and sequencing of novel genes from *Vibrio alginolyticus* that support the growth of K^+ uptake-deficient mutant of *Escherichia coli*. *Biochim. Biophys. Acta* **1277,** 201-208
43. Uozumi, N., Nakamura, T., Schroeder, J.I. and Muto, S. (1998) Determination of transmembrane topology of an inward-rectifying potassium channel from *Arabidopsis thaliana* based on functional expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **95,** 9773-9778.
44. Tholema, N., Bakker, E.P., Suzuki, A. and Nakamura, T, (1999) Change to alanine of one out of four selectivity filter glycines in KtrB causes a two orders of magnitude decrease in the affinities for both K^+ and Na^+ of the Na^+ dependent K^+ uptake system KtrAB from *Vibrio alginolyticus*. *FEBS Lett*. **450,** 217-220.
45. Durell, S.R., Bakker, E.P. and Guy, H.R. (2000) Does the KdpA subunit from the high affinity K^+-translocating P-type KDP-ATPase have a structure similar to that of K^+ channels? *Biophys J.* **78,** 188-199.
46. Matsuda, N., Kobayashi, H., Katoh, H., Ogawa, T., Futatsugi, L., Nakamura, T., Bakker, E. P. and Uozumi, N. (2004) Na^+-dependent K^+ uptake Ktr system from the Cyanobacterium *Synechocystis* sp. PCC 6803 and its role in the early phase of cell adaptation to hyperosmotic shock. *J. Biol. Chem.* **279,** 54942-54962.
47. Jiang, Y., Pico, A., Cadene, M., Chait, B.T., and MacKinnon, R. (2001) Structure of the RCK domain from the *E. coli* K^+ channel and demonstration of its presence in the human BK channel. *Neuron* **29,** 593-601.

SECTION VII

NUTRITION

13. IS CELLULOSE SYNTHESIS ENHANCED BY EXPRESSION OF SUCROSE SYNTHASE IN POPLAR?

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Abstract. Sucrose synthase is believed to function in channeling UDP-glucosec from sucrose to various ß-glucan synthases. Expression of sucrose synthase in popla enhanced the incorporation of radioactive sucrose into cellulose, together with the metabolic recycling of fructose. The overexpression also enhanced the direct incorporation of the glucosyl moiety of sucrose into the glucan backbone of xyloglucan and increased recycling of fructose. These findings suggest that some of the sucrose loaded into the phloem of a poplar leaf is used directly by sucrose synthase associated with xyloglucan and cellulose synthases in the stem which may be a key function of sucrose synthase.

1. INTRODUCTION

A strategy on forest-tree biotechnology is to produce woody plants to grow faster and to increase cellulose deposition. It is not easy to apply traditional breeding methods but possible to use molecular breeding methods for their improvement, because woody plants have a long generation time. The overexpression of xyloglucanase in poplar enhanced growth, increased cellulose deposition, and modified the structure of the primary wall, as indicated by changes in Young's modulus [1]. The overexpression of cellulase also enhanced the growth by the trimming of cellulose microfibrils, which caused the loosening of xyloglucan intercalation and the enlargement of cells [2, 3]. Such genetic modification of cell wall in trees would be expected to produce fast-growing forest trees.

In the process of cellulose synthesis the glucose residue is transferred from UDP-glucose to 1,4-ß-glucan, which is used to elongate the glucan chain while releasing UDP:

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$$(1,4\text{-ß-glucan})_n + \text{UDP-glucose} = (1,4\text{-ß-glucan})_{n+1} + \text{UDP}$$

Therefore, UDP-glucose is required for cellulose synthesis. In higher plants, UDP-glucose is synthesized either by the action of UDP-glucose pyrophosphorylase or sucrose synthase. The sucrose synthase catalyzes the following freely reversible reaction:

$$\text{Sucrose} + \text{UDP} = \text{UDP-glucose} + \text{Fructose}$$

The production of UDP-glucose by sucrose synthase is an energy-conservative reaction with respect to ATP, but that by UDP-glucose pyrophosphorylase requires 2 molars of ATP for the synthesis of one molar UDP-glucose (Figure 1). If the two reactions by sucrose synthase and cellulose synthase mentioned above are combined, UDP is recycled between the two reactions, and the overall reaction becomes:

$$\text{Sucrose} + (1,4\text{-ß-glucan})_n = (1,4\text{-ß-glucan})_{n+1} + \text{Fructose}$$

The UDP released from UDP-glucose by ß-glucan synthase can be efficiently and rapidly recycled to produce UDP-glucose by sucrose synthase. Sucrose synthase prevents UDP build-up in cellulose biosynthesis. In fact, the expression of the S11E mutant mung bean sucrose synthase [4] in *Acetobacter xylinum* not only changed sucrose metabolism but also enhanced cellulose production by preventing the accumulation of UDP during cellulose biosynthesis [5], because UDP is the strongest endogenous inhibitor of cellulose synthase in *A. xylinum* [6]. It could be possible to produce transgenic poplars, in which cellulose deposition is enhanced by the overexpression of sucrose synthase. Certainly, sucrose synthase in trunk tissues is related to cambial wood production and heartwood formation [7]. The aim of the present review is to assess the effect of overexpression of sucrose synthase on the synthesis of cell wall polysaccharides in the poplar stem.

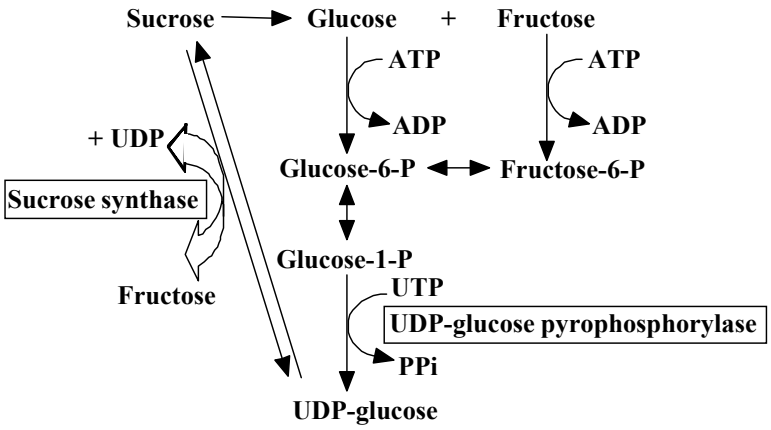

Figure 1. Pathways for UDP-glucose biosynthesis.

2. TRANSGENIC EXPRESSION OF SUPER SUCROSE SYNTHASE

Twenty five independent transgenic (trg) poplar lines expressed a S11E sucrose synthase [4] under the control of a 35S constitutive promoter. Seven typical lines which showed a similar growth pattern (about 30-cm stem length) were selected for further analysis. Sucrose synthase in the stem of the transgenic plants was detected at a position corresponding to the expected size of its polypeptide on a western blot and the activity of sucrose synthase was also higher in the transgenic plants than in the wild-type (wt) plants. About 60% of the carbohydrate in wt and trg stems was recovered in the 24% KOH-insoluble fraction (cellulose). The amounts of cellulose and non-cellulosic polysaccharides per dry weight were essentially the same in the seven transgenic lines and wild-type plants.

3. TRANSFER OF DUAL-LABELED SUCROSE FROM LEAF TO STEM

Sucrose is formed in leaf and loaded into the sieve tube by phloem-specific sucrose transporter. Then, the sugar is translocated via phloem to the stems, and incorporated into the sink tissues via companion cells either by a sucrose transporter or with sucrose cleavage. Here, we loaded [^{14}C-glucose][^{3}H-fructose]sucrose into the phloem of a poplar leaf and traced the incorporation of the glucose and fructose moieties through the petiole into hemicellulose and cellulose of the stem (Figure 2). The apical one-third of the leaf was excised and 4 µl of 1 mM [^{14}C-glucose][^{3}H-fructose]sucrose was directly fed into the central main vein on the cut surface. For loading with the reverse flat system [8], we loaded 4 µl of 1 mM [^{14}C-glucose][^{3}H-fructose]sucrose into a capillary tube pushed over the severed vein of a leaf. Then, the seedlings were cultured at 27 ^{o}C in 14-h light/10-h darkness. The radioactivity of each wall-polysaccharide fraction was determined with a liquid scintillation counter after feeding the dual-labeled sucrose.

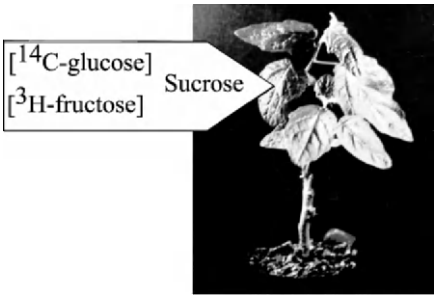

Figure 2. Loading [^{14}C-glucose][^{3}H-fructose]sucrose to the phloem of poplar leaf.

The efficiency of radioactivity transport of the total dual-labeled sucrose from the leaf to the stem was 10 to 11% and that from the leaf to root was 11 to 12% at the root, but less than 2% of the sucrose was converted to [^{3}H-glucose][^{14}C-fructose]sucrose in the phloem of basal petiole. When the reverse flat system was

used for loading the dual-labeled sucrose into the leaf phloem [8], the efficiency of the transport to the stem was increased up to 40% of the total radioactivity. In the phloem of basal petiole, however, the asymmetry between ^{14}C and ^{3}H in sucrose was not preserved, and ^{14}C and ^{3}H were detected at the same level in both glucose and fructose moieties (Table 1).

Table 1. Comparison of the asymmetry level of dual-labeled sucrose loaded by the reverse flat method and the direct method.

Loading method from leaf to stem	Efficiency of sucrose transport [^{14}C-fructose]sucrose	Conversion to [^{3}H-glucose]-%
Direct method	10	> 2
Reverse flat method	40	50

Table 2. Incorporation of [^{14}C-glucose][^{3}H-fructose]sucrose into hemicellulose and cellulose fractions of poplar stems after 24 h. Numbers in the table show pmol sugar incorporated from 1 mM [^{14}C-glucose (250 dpm/pmol)][^{3}H-fructose (250 dpm/pmol)]sucrose per mg dry weight after 24 h of incorporation. Values are averages of six samples per line.

Plant	Hemicelluloses		Cellulose	
	^{14}C	^{3}H	^{14}C	^{3}H
	pmol sugar incorporated/ mg dry weight			
wt	45.1	11.1	39.2	40.4
trg6	36.6	20.7	38.5	39.7
trg7	89.9	47.5	87.8	84.7
trg10	61.9	37.7	77.7	74.3
trg11	43.2	27.2	54.3	58.4
trg14	55.6	19.8	54.8	73.6
trg24	82.3	48.1	82.3	81.1
trg25	31.7	30.1	41.3	51.2

4. INCORPORATION OF DUAL-LABELED SUCROSE INTO HEMICELLULOSES AND CELLULOSE IN THE STEM

Overexpression of sucrose synthase in trg7, trg10, trg11, trg14 and trg24 enhanced the incorporation of both ^{14}C and ^{3}H into hemicelluloses (24% KOH-soluble fraction) and cellulose (24% KOH-insoluble fraction) compared with the wild type (Table 2). Furthermore, the overexpression of sucrose synthase markedly increased the incorporation of ^{3}H into hemicelluloses. At 24 h, after the incorporation, this overexpression enhanced the incorporation of ^{14}C and ^{3}H into hemicelluloses by factors of 2 and 4, respectively, and that into cellulose by a factor of 2, compared

with the wild-type. This incorporation of radioactivity was not significantly increased by the overexpression of sucrose synthase in trg6 and trg25, although the incorporation of the sucrose-derived fructosyl moiety (^{3}H) into hemicelluloses was increased 2- to 3-fold. It is likely that overexpression of sucrose synthase enhances the subsequent recycling of fructose.

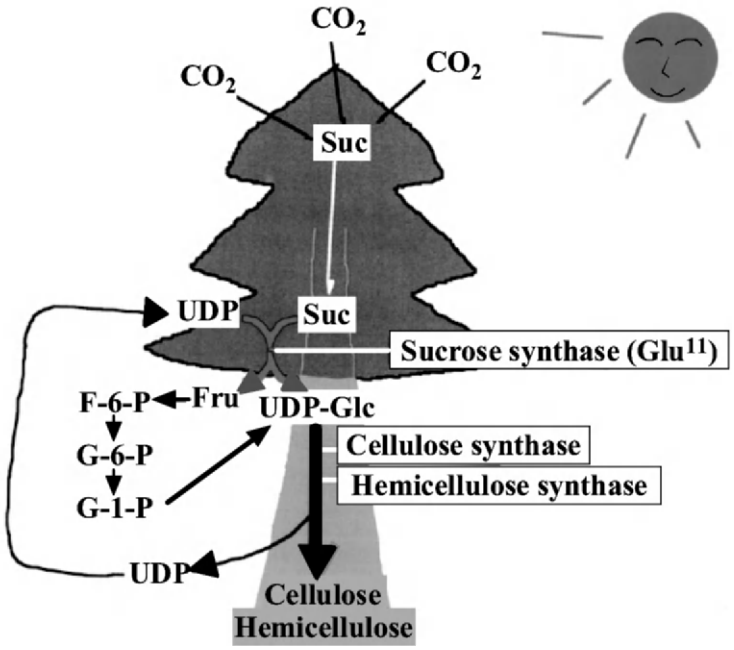

Figure 3. Metabolism of sucrose in poplar.

5. CONCLUSION

Clear differences were not observed in the gross phenotype between the S11E transgenic and wild-type poplar lines, and the deposition of polysaccharides was not increased in the transgenic plants compared with that in the wild-type plants. This is probably because the level of expression was higher in the leaves than in the stem and the photoassimilated sucrose was arrested by the overexpressed sucrose synthase in the leaves. In addition, there might be some regulatory mechanism controlling the transport of free sugars and sugar derivatives from source to sink tissues in plants. Nevertheless, the overexpression of sucrose synthase might enhance hemicellulose and cellulose syntheses, together with fructose recycling (Figure 3). This overexpression might also enhance the direct incorporation of the glucosyl moiety of sucrose into the glucan backbone of xyloglucan and increase

recycling of fructose [9]. Xyloglucan synthase probably forms a putative synthase complex including sucrose synthase for utilizing this energy for the transfer of the glucosyl residue in Golgi (Figure 4). Thus, xyloglucan 4-ß-glucosyltransferase probably has a catalytic site and mechanism similar to those in the glucosyltransferases of cellulose [10], callose [11] and (1→3),(1→4)-ß-glucan [12]. The enhancement of the glucan synthesis was therefore derived not only from the direct transfer of the glucosyl moiety of sucrose via UDP-glucose, but also from the recycling of the fructose moiety by overexpression of sucrose synthase. The enhanced fructose recycling was probably caused by the production of more UDP-glucose and fructose by the higher activity of sucrose synthase.

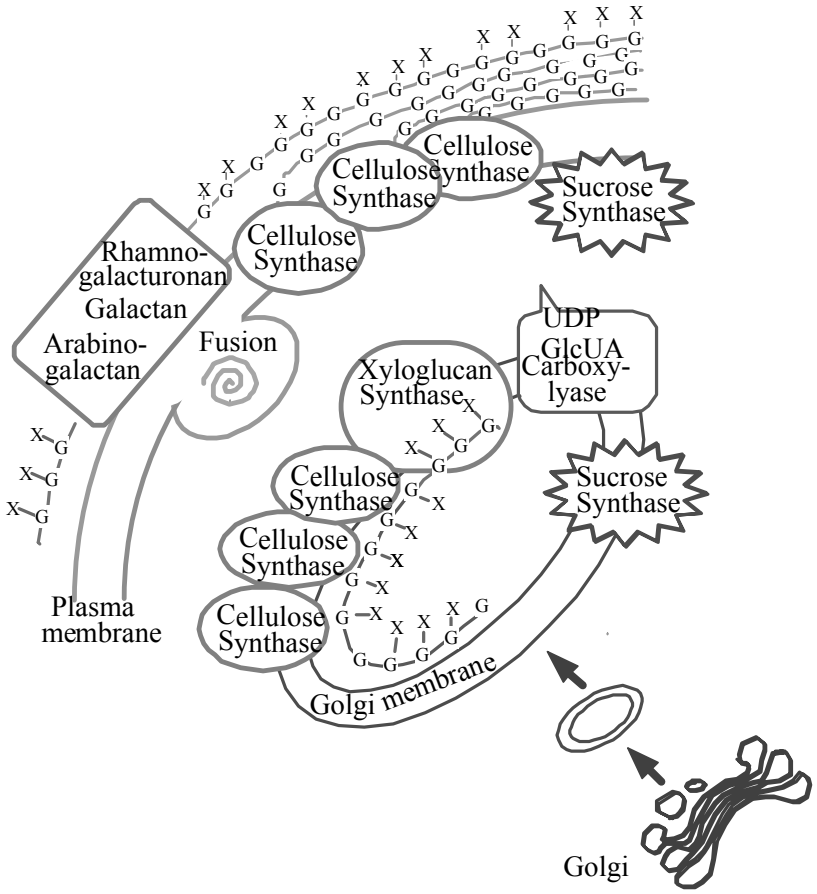

Figure 4. Sucrose synthases associated with cellulose synthase and xyloglucan synthase.

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REFERENCES

1. Park, Y.W., Baba, K., Furuta, Y., Iida, I., Sameshima, K., Arai, M.and Hayashi, T. (2004) Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase in poplar. *FEBS Lett.* **564,** 183-187.
2. Shani, Z., Dekel, M., Tsabary, G., Raphael, G. and Shoseyov, O. (2004) Growth enhancement of transgenic poplar plants by overexpression of *Arabidopsis thaliana* endo-1,4̄-ß-glucanase (*cel1*). *Mol. Breeding* **14 ,** 321-330.
3. Park, Y.W., Tominaga, R., Sugiyama, J., Furuta, Y., Tanimoto, E., Samejima, M., Sakai, F. and Hayashi, T. (2003) Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana*. *Plant J.* **33,** 1099-1106.
4. Nakai, T., Konishi, T., Zhang, X-Q., Chollet, R., Tonouchi, N., Tsuchida, T., Yoshinaga, F., Mori, H., Sakai, F., and Hayashi, T. (1998) An increase in apparent affinity for sucrose of mung bean sucrose synthase is caused by *in vitro* phosphorylation or directed mutagenesis of Ser^{11}. *Plant Cell Physiol*. **39,**
1337-1341.
5. Nakai, T., Tonouchi, N., Konishi, T., Kojima, Y., Tsuchida, T., Yoshinaga, F., Sakai, F., and Hayashi, T. (1999) Enhancement of cellulose production by expression of sucrose synthase in *Acetobacter xylinum*. *Proc. Nati. Acad. Sci. USA* **96,** 14-18.
6. Benziman, M., Delmer, D.P. and Aloni, Y. (1983) Unique regulatory properties of the UDP-glucose: 1,4̄-ß-D-glucan synthase of *Acetobacter xylinum*. *J. Appl. Polym. Sci*. **37,** 131-143.
7. Hauch, S. and Magel, E. (1998) Extractable activities and protein content of sucrose-phosphate synthase, sucrose synthase and neutral invertase in trunk tissues of *Robinia pseudoacacia* L. are related to cambial wood production and heartwood formation. *Planta* **207,** 266-274.
8. Sovonick, S.A., Geiger, D.R. and Fellows, R.J. (1974) Evidence for active phloem loading in the minor veins of sugar beet. *Plant Physiol*. **54,** 886-891.
9. Konishi, T., Ohmiya, Y. and Hayashi, T. (2004) Evidence that sucrose loaded into the phloem of a poplar leaf is used directly by sucrose synthase associated with various ß-glucan synthases in the stem. *Plant Physiol*. **134,** 1146-1152.
10. Carpita, N.C. and Vergara, C.E. (1998) A recipe for cellulose. *Science* **279,** 672-673.
11. Hong, Z., Zhang, Z., Olson, J.M. and Verma, D.P.S. (2001) A novel UDP-glucose transferase is part of the callose synthase complex and interacts with phragmoplastin at the forming cell plate. *Plant Cell* **13,** 769-780.
12. Buckeridge, M.S., Vergara, C.E. and Carpita, N.C. (1999) The mechanism of synthesis of a mixed-linkage (1→3),(1→4)-ß-D-glucan in maize. Evidence for multiple sites of glucosyl transfer in the synthase complex. *Plant Physiol***. 120,** 1105-1116.

14. NITROGEN METABOLISM IN CYANOBACTERIA UNDER OSMOTIC STRESS

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Abstract. Osmotic stress can interfere with the metabolism of inorganic and organic nitrogen compounds. Osmotic stress mostly in the form of high salinity affects several points of inorganic nitrogen assimilation pathway. The uptake of nitrate by cyanobacteria under salt stress can be increased or decreased depending on the species. One transport system for nitrate with either low or high affinity can be found in cyanobacteria, in contrast to more than one transport system in most bacteria and higher plants. Similar to nitrate uptake, the response of nitrate reduction to high salinity is favorable in some but unfavorable in other cyanobacteria. Nitrogen fixing activity is usually inhibited or depressed by salt stress in most cyanobacteria. In the halotolerant cyanobacterium *Aphanothece halophytica*, the efficient transport of glycine betaine requires the presence of Na^+, which is also the case for the transport of nitrate. The increase of polyamines in response to ionic and osmotic stress has recently been found in *Synechocystis* sp. PCC 6803. This increase is attributed to the increase in the level and the stability of the arginine decarboxylase transcript. For the reserved nitrogenous compound, cyanophycin, its content is increased in *Scytonema* sp. but decreased in Synechocystis sp. PCC 6803 as a response to salt stress.

1. INTRODUCTION

The growth of microorganisms in general depends on the availabilities of basic nutrients containing carbon, nitrogen and, to a lesser extent, phosphorus. Although carbon metabolism plays a vital role in growth and development of most microorganisms, it is also true that nitrogen metabolism is equally important due to the fact that nitrogen is a component of two essential macromolecules, namely, proteins and nucleic acids.

Cyanobacteria are prokaryotic organisms with an elaborate and highly organized system of internal membranes which function in photosynthesis. They can be found in almost every conceivable habitat, from seawater to freshwater to dry rock to soil. Owing to the phylogenetic relationship of cyanobacteria to chloroplasts, a lot of studies have been performed using cyanobacteria as a model for plant cells. The ubiquitous distribution of cyanobacteria is made possible as a result of the remarkable capacities of cyanobacteria to adapt to varying environmental conditions [1]. The studies on an adaptation to salt or osmotic stress have been done mostly in a few marine and euryhaline cyanobacteria. Particular attention is focused on the characterization of the osmoprotective response to salt or osmotic stress. The

Ashwani K. Rai and Teruhiro Takabe (eds.), Abiotic Stress Tolerance in Plants, 195-212.

osmolytes reported to be accumulated in cyanobacteria in response to osmotic stress are similar to those found in other prokaryotes and eukaryotes, i.e. sucrose, glucosylglycerol, trehalose and quaternary ammonium compounds like glycine betaine and glutamate betaine [2]. Information on nitrogen metabolism in relation to salt or osmotic stress that have been reported for cyanobacteria has been very limited. The present chapter attempts to reveal the changes in the metabolism of nitrogenous compounds both inorganic and organic, in some cyanobacteria under the influence of salt or osmotic stress. For the sake of simplicity, osmotic stress is meant to include the stress caused by the increased concentration of extracellular non-penetrating solutes as well as that of sodium chloride which can be transported across the membranes.

2. OSMOTIC STRESS AND NITRATE UPTAKE

Studies on nitrate uptake in cyanobacteria have been hampered by the fact that it is quite difficult to demonstrate a significant internal concentration of nitrate in cyanobacteria. Some investigators therefore studied nitrate uptake by demonstrating the disappearance of nitrate from the medium around the cells. The entry of nitrate into the cells can also be monitored by radiotracer techniques. However, the short half-life of $^{13}NO_3$, about 10 min, precludes its utilization for nitrate uptake studies. Another point to consider when studying the transport or the uptake of nitrate is that the transported nitrate should not be subject to further metabolism, i.e. its reduction to nitrite by nitrate reductase. Experimental systems suitable for this purpose include the assay of nitrate uptake in the dark where in vivo nitrate uptake is minimal [3], or the assay in the light but using the tungstate treated cells where nitrate reductase is inactive [4], or the assay in the light using mutant cells deficient in nitrate reductase [5]. For all three assays mentioned above, the time course of intracellular nitrate accumulation is similar. Nitrate uptake was shown in *Anacystis nidulans* R2 to exist as a sodium/nitrate cotransport [5]. This is in contrast to what observed in higher plants where the high affinity transport follows a mechanism of cotransport of nitrate and proton [6, 7]. It should be mentioned that recently a Na^+-dependent nitrate transport has been reported for a marine higher plant *Zostera marina* L. [8]. The uptake of nitrate coupled to that of Na^+ enabled the steep inwardly-directed electrochemical potential for Na^+ to drive net accumulation of nitrate within the cells. The sodium/nitrate cotransport has also been confirmed in a halotolerant cyanobacterium *Aphanothece halophytica* (*A. halophytica*) [9]. Both the unstressed and salt-stressed cells showed Na^+-dependent nitrate uptake. Unlike in higher plants which usually contain both low-and high-affinity nitrate uptake systems, many cyanobacteria contain only one nitrate uptake system of either low or high affinity. High affinity transport systems with K_s values of 1.6 and 31 μM were reported for *Anacystis nidulans* [5] and *Anabaena* sp. PCC 7120 [10], respectively. The low affinity transport system for nitrate occurred in a halotolerant cyanobacterium *A. halophytica* with a K_s value of 416 μM [9]. The affinity was not altered when *Aphanothece* cells were grown in salt-stress medium. However, these cells exhibited a reduction in V_{max} of nitrate uptake by about 50%. Experiments in a nitrogen-fixing

salt tolerant cyanobacterium *Anabaena torulosa* gave different results from those observed for *A. halophytica*. When *A. torulosa* was grown in salt-stress medium containing 170 mM NaCl, the increased rate of nitrate uptake was observed [11]. This increased nitrate uptake was proposed to be involved in salt tolerance mechanism of *A. torulosa* via the inhibition of Na^+ influx. This was based on the fact that the increase of nitrate uptake contributed to only marginal increase in the intracellular levels of nitrate during salt stress. It is therefore unlikely that the accumulated nitrate forming a very minor component of the internal osmoticum can act as an effective osmolyte. The protection of cyanobacterial cells from salt stress by nitrate was also reported for the freshwater cyanobacterium *Anabaena doliolum* [12]. The influx of Na^+ into the cells was minimum when nitrate was simultaneously available to the cells suggesting that the control of Na^+ influx is critical for salt tolerance.

In contrast, the fact that nitrate uptake was reduced in salt-stressed *A. halophytica* suggested the non-involvement of nitrate uptake in salt stress tolerance. The mechanism for salt stress adaptation in *A. halophytica* has been attributed to the accumulation of glycine betaine [13, 14] and the efflux of Na^+ catalyzed by the functional Na^+/H^+ antiporter [15]. The question remains as to whether *A. torulosa* also employs Na^+/H^+ antiporter for Na^+ extrusion in addition to inhibition of Na^+ influx as salt tolerance mechanism in the presence of nitrate.

Not only that the extrusion of Na^+ is important for the homeostasis of ions during the adaptation to osmotic stress, but the influx of K^+ is also crucial in this adaptation in cyanobacteria. A recent study in *Synechocystis* sp. PCC 6803 has shown that the uptake of K^+ mediated by Na^+ - dependent Ktr system plays a crucial role in the regulation of cell turgor after hyperosmotic stress [16]. A mutant deficient in KtrB was sensitive to long term hyperosmotic stress imposed by either NaCl or sorbitol. Furthermore, this mutant failed to reaccumulate K^+ to its original level after an osmotically – induced initial loss of K^+.

The effect of NaCl on nitrate uptake has also been reported for *Anabaena azollae* in association with a freshwater fern *Azolla pinnata*. The presence of 20 mM NaCl in the uptake medium caused an increased nitrate uptake in *Azolla pinnata-Anabaena azollae* association [17]. Furthermore, NaCl could also enhance the affinity of the *A. pinnata-A. azollae* association for nitrate. One interesting characteristic of this association was that the associated cyanobacterium was not able to transport nitrate. The cyanobiont freshly isolated from the association was demonstrated to show no uptake of nitrate. The ability to transport nitrate was developed after a lag of 60 min during which time the inhibitory substances of the cyanobiont might have been depleted, thus triggering the onset of nitrate uptake.

The effects of salt stress on nitrate uptake can also vary considerably in plants depending on species and experimental conditions. Nitrate uptake rates were decreased by 50% in wheat in the presence of 60 mM NaCl [18]. For barley, a relatively salt-tolerant plant, nitrate uptake was severely inhibited when salinity was applied at 200 mM NaCl [19]. Recently it has been reported that the inhibition of nitrate uptake by salt in barley was caused mainly by the osmotic effects of salts [20]. This conclusion was drawn from the demonstration that the same decrease of nitrate uptake was achieved by sorbitol or betaine at corresponding osmolalities. So

far there has been no such conclusion for the inhibition of nitrate uptake by salt in cyanobacteria.

The regulation of nitrate transport has been studied extensively in a unicellular non-nitrogen fixing cyanobacterium *Synechococcus* sp. PCC 7942. Nitrate uptake is completely and reversibly inhibited by the presence of ammonium in the medium [21]. Recently the inhibition of nitrate uptake by ammonium has also been shown in *A. halophytica* [9]. Both the unstressed and salt-stressed *Aphanothece* cells were sensitive to ammonium at concentration greater than 100 μM. Furthermore, the inhibition of nitrate uptake by ammonium appeared to be slightly more effective in salt-stressed cells than in unstressed cells. The inhibition of nitrate uptake could not be relieved by preincubation of the cells with methionine sulfoximine, an inhibitor of ammonium assimilation process. This observation is in contrast with some studies in higher plants including cyanobacteria [22-23], but similar to other reportes [26-28]. Bearing this in mind, it is thus likely that ammonium itself rather than an assimilation product is the agent responsible for feedback inhibition of nitrate uptake in *A. halophytica*.

Nitrite is able to inhibit the uptake of nitrate in *A. halophytica* [9]. The low K_i of nitrite suggests that the inhibition is of a competitive type, i.e. both nitrite and nitrate enter the cells by the same uptake system. A nitrite-nitrate bispecific transporter also exists in *Synechococcus* sp. PCC 7942 [29] and *Anacystis nidulans* [5]. Nevertheless, a recent study in a filamentous, heterocystous cyanobacterium, *Nostoc* ANTH has indicated the existence of separate nitrite and nitrate uptake systems [30]. The chlorate-resistant mutant of *Nostoc* ANTH lacking nitrate uptake activity still retained nitrite uptake capacity. The inhibition of nitrate uptake by nitrite was similarly observed for both unstressed and salt-stressed *Aphanothece* cells [9]. The uptake of nitrate in *A. halophytica* was insensitive to either chloride or phosphate suggesting different uptake system for these two anions.

There have been attempts to study the relationship between carbon and nitrogen assimilation in cyanobacteria. Experiments in *Synechococcus* sp. PCC 7942 indicated that the carbon control and the nitrogen control of nitrate transport were independent at both the physiological and the molecular level [20]. The mutant strain NC2 showed high nitrate transport activity insensitive to ammonium but sensitive to inhibitors of CO_2 fixation. Although the carbon control and the nitrogen control of nitrate transport are not necessarily linked, the transport of nitrate was still dependent upon active carbon assimilation since nitrate uptake was severely inhibited by pretreatment of *Synechococcus* sp. PCC 7942 cells with DL-glyceraldehyde, an inhibitor of CO_2 fixation. Similar observation was also reported for *A. halophytica*, under both unstress and salt-stress conditions.

An ATP-binding cassette (ABC) – type transporter which is the product of the *nrtA*, *nrtB*, *nrtC*, and *nrtD* genes is involved in nitrate-nitrite uptake by the freshwater cyanobacteria *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120 [31–34]. On the other hand, the nitrate-nitrite uptake is mediated by a carrier belonging to the major facilitator superfamily in the marine cyanobacteria *Synechococcus* sp. PCC 7002 and *Trichodesmium* sp. WH 9601 [35, 36]. The gene encoding this carrier has been named *nrtP* [35]. The energy source for ABC-type

transporter is likely from ATP [37] whereas it is still uncertain whether NrtP permease uses a gradient of H^+ or Na^+ [35]. Based on this information it has been suggested that utilization of nitrate by cyanobacteria under salt stress environments may be mediated by NrtP rather than NrtABCD transporter [36]. The two transporters have not been found to co-exist in cyanobacteria.

Recently the *nrtD* gene of *Synechocystis* sp. PCC 6803 has been shown to respond differently to salt stress and hyperosmotic stress. Treatment of cells with 0.5 M NaCl for 30 min resulted in about 3.5-fold repression of *nrtD* whereas treatment with 0.5 M sorbitol produced little or no repression of *nrtD* [38]. The authors suggested that *Synechocystis* sp. PCC 6803 might recognize salt stress and hyperosmotic stress as different stimuli. It is worth noting, however, that these experiments utilized NaCl with about two-fold higher osmolality than sorbitol.

3. OSMOTIC STRESS AND NITRATE REDUCTION

Following the uptake of nitrate into the cell, it is sequentially reduced first to nitrite and then to ammonium by ferredoxin-nitrate reductase and ferredoxin-nitrite reductase, respectively. The genes required for the assimilation of nitrate in the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 are organized in the so-called *nir* operon [39]. The gene encoding nitrite reductase, *nirA*, is the first gene of the operon, followed by the genes encoding the nitrate transporter (*nrt ABCD*) and finally the nitrate reductase gene (*narB*) (Fig. 1). A similar gene organization is also found in other freshwater cyanobacteria: *Synechocystis* sp. PCC 6803 [40], *Anabaena* sp. PCC 7120 [34, 41], *Plectonema boryanum* [42], and *Phormidium laminosum* [43]. The effects of various inorganic nitrogen compounds on the regulation of *nir* operon have been investigated. For example, this operon is expressed at high levels only when ammonium is absent from the growth medium [34, 44]. Either nitrate or nitrite can have positive effect on the mRNA levels of the operon.

In the marine cyanobacterium *Synechococcus* sp. PCC 7002, the *nrtP* gene is also clustered with *narB* but these two genes appear to be independently transcribed (Fig. 1). The expression of *narB* is regulated by the nitrogen sources in the medium. Highly expressed *narB* occurs in nitrate-containing medium whereas its expression is low in medium containing ammonium or urea [35]. In another marine cyanobacterium *Trichodesmium* sp. WH 9601, a gene cluster with the structure *nirA-nrtP-narB* has been reported but its expression has not yet been characterized [36] (Fig. 1). The genes encoding the nitrate/nitrite assimilation system of the marine cyanobacterium *Synechococcus* sp. WH 8103 have been cloned [45]. The *narB* and *nirA* are clustered on the chromosome but are organized in separate transcriptional units (Fig. 1). A homologue of *nrtP* is located upstream of *narB*.

Effects of salinity on nitrate reductase have been studied mostly in higher plants and bacteria. Salt stress suppressed nitrate reductase in the leaves of sugar beet with the decreased activity being observed with the increase of the salt level [46]. Similar effects of salt stress and osmotic stress on the decrease of nitrate reductase activity have also been reported in some other higher plants [47, 48]. The effect of salt stress

on nitrate reductase in higher plants was suggested to involve the expression rather than the post-translational modulation of the enzyme [48].

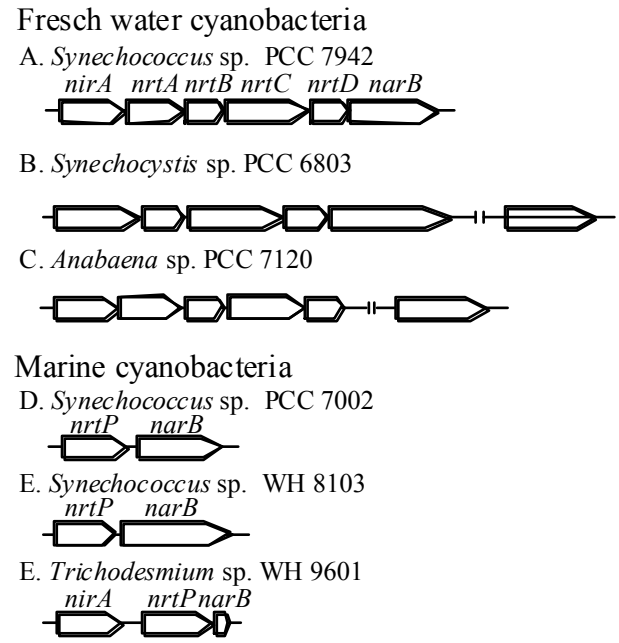

Figure 1. Comparative organization of the nitrate assimilation gene clusters between freshwater (A, B, C) and marine (D, E, F) cyanobacteria. The nirA gene in Synechococcus sp. PCC 7002 has not been identified whereas this gene is organized in a separate transcriptional unit in Synechococcus sp. WH 8103. Note the differences in the nitrate/nitrite transporters between the freshwater and marine cyanobacteria, i.e. nrt ABCD versus nrtP. Genes are drawn approximately to scale, and block arrows show the direction of transcription.

Nitrate reductase purified from the halophilic bacterium *Haloferax mediterranei* showed a strong dependence of its activity and stability on the presence of high concentration of NaCl. Maximum activity occurred at 80^{o}C at 3.1 and 2.2 M NaCl [49]. The enzyme was stable after 90 h in the presence of 4.3 M NaCl. In another bacterium *Rhodococcus* sp. RB 1 which is able to thrive in media with up to 0.9 M NaCl or KCl, nitrate reductase was still active in vivo in the presence of 3 M NaCl or KCl [50]. The maximum enzyme activity was detected at 0.5 M salt. Similarly the enzyme from a moderately halophilic bacterium *Ochrobactrum anthropi* could retain its activity up to 100 g/l NaCl [51]. For phototrophic bacterium like *Rhodobacter capsulatus* E1F1, the cells grown in 0.2 M NaCl showed a decrease in nitrate reductase activity [52]. This decreased activity was probably caused by repression of the enzyme. The enzyme activity could be restored when glycine betaine was present in the medium.

In cyanobacteria, very little study has been done with regard to the effect of salt stress on nitrate reductase. The filamentous cyanobacterium *Anabaena* sp. PCC 7120 showed an increase of nitrate reductase activity in saline medium with increasing concentration of NaCl [53]. It was also shown that the presence of NaCl in nitrate uptake medium also promoted nitrate uptake. The accumulated nitrate would then possibly act as a major signal affecting nitrate reductase expression and activity under salinity [48]. *Anabaena azollae* in association with a fast-growing fern *Azolla* has been shown to retain higher activity of nitrate reductase under salt-exposed than non-exposed association [17]. In addition, the affinity of the enzyme for nitrate was also increased under saline condition. With regard to the effect of salinity on the activity of nitrate reductase, there have been conflicting results. Salinity decreased the enzyme activity in potato [47], tomato [54], and cassava [55] but increased in soybean [56], rye grass [57], lettuce [58] and cyanobacterium *Anabaena* sp. PCC 7120 [53]. There is no evidence for a direct effect of salinity on nitrate reductase activity. It is likely, however, that salinity modulates nitrate reductase activity through nitrate uptake since the enzyme activity is mostly determined by nitrate flux into the metabolic pool [59].

4. OSMOTIC STRESS AND NITROGEN FIXATION

Nitrogen fixation in living organisms is the process by which dinitrogen is reduced to ammonia which is catalyzed by the nitrogenase enzyme. Due to the extreme sensitivity of this enzyme to oxygen, nitrogen fixation by microorganisms occurs when oxygen can be prevented from inactivating the nitrogenase. Heterotrophic bacteria require an oxygen-limited environment, which permits nitrogen fixation to occur when the respiration rate of the cells equals or exceeds the rate of diffusion of oxygen to the cell surface [60]. In this regard, the nitrogen-fixing cyanobacteria encounter a problem during oxygenic photosynthesis when the amount of oxygen evolved in the light exceeds non-inhibitory levels for the nitrogenase enzyme. Several filamentous cyanobacteria produce specialized cells, heterocysts, which protect the nitrogenase from oxygen [61]. However, aerobic nitrogen fixation is also possible in non-heterocystous, filamentous cyanobacteria such as *Oscillatoria* spp. and unicellular cyanobacteria such as *Gloeothece* spp. and *Synechococcus* sp. SF1.

Studies on the effect of salinity on the nitrogenase enzyme have been done mainly in rhizosphere bacteria such as *Azospirillum brasilense*. Nitrogenase activity was reported to be more sensitive to salt stress than cellular growth on combined nitrogen [62]. High salt concentrations were strongly inhibitory to the biosynthesis of nitrogenase in *A. brasilense* [63] and *Klebsiella pneumoniae* [64]. Recent experiments in *A. brasilense* have demonstrated that NaCl up to 300 mM could severely inhibit the biosynthesis of nitrogenase more than the nitrogenase activity itself [65]. In cyanobacteria, Fernandes et al. [66] showed that two nitrogen-fixing *Anabaena* strains exhibited different responses to salinity and osmotic stresses with regard to nitrogenase activity. *Anabaena* sp. strain L-31, a salt-sensitive strain, displayed significant osmotolerance whereas *Anabaena torulosa*, a salt-tolerant strain was relatively osmosensitive. Salinity and osmotic stresses affected nitrogenase

activity differently. Nitrogen fixation in both strains was severely inhibited by the ionic, i.e. exogenously added NaCl severely inhibited whereas sucrose remarkably enhanced acetylene reduction activity by 2.5 to 3-fold. Such differential sensitivity to salinity-osmotic stresses occurred irrespective of the inherent tolerance of the two strains to salt-osmotic stress. Supplementation of ammonium to the medium could confer significant protection against salinity stress but was ineffective against osmotic stress. Furthermore, unique salt stress- or osmotic stress-specific proteins were also induced in both *Anabaena* strains, indicating differential regulation of protein synthesis by the two stresses. While sucrose, a permeable osmolyte, has a positive effect on nitrogenase activity in *Anabaena* sp. strain L-31, it turns out that a non-permeable osmolyte (mannitol or polyethylene glycol) shows no influence on nitrogenase activity [67]. This suggests that the positive effect of sucrose is not caused by an osmotic stress. In addition, the effect of sucrose is mediated by the increased synthesis of dinitrogenase reductase (Fe-protein of nitrogenase complex). This conclusion is based on the evidence that the remarkable enhancement of Fe-protein can be clearly detected by immunoblot analysis of sucrose-grown cells [67]. The Fe-protein levels in cells stressed with non-permeable osmolytes or with NaCl are not significantly different from those in unstressed cells. This strengthens the viewpoint that the ionic and osmotic stresses differentially regulate cyanobacterial nitrogenase activity.

Another study in *Anabaena* sp. PCC 7120 also showed the inhibition of nitrogenase activity by increasing NaCl concentrations up to 200 mM [53]. Interestingly, complete removal of NaCl from the nutrient solution markedly reduced nitrogenase activity. An increase of almost 11-fold enzyme activity was observed in the presence of 0.5 mM NaCl, indicating a role for Na^+ as a nitrogenase activator. On the other hand treatment with 60 mM NaCl caused more than 50% reduction of nitrogenase activity in *Nostoc muscorum* [68]. The complete loss of enzyme activity occurred by treatment with 90 mM NaCl.

The nitrogenase activity of *Anabaena azollae* in association with a fast growing fern *Azolla* was depressed by salinity [17]. However, the severity of the inhibition of nitrogenase activity by NaCl was reduced when nitrate was present in the medium, indicating the protection of nitrogenase from salt toxicity by nitrate. The inhibition of nitrogenase activity by NaCl is reported to be due to the disturbance of electron transport, loss in ability of heterocysts to protect nitrogenase from oxygen due to disturbed plasma membrane permeability, inadequate supply of reductants and ATP [69 – 71]. Another likely explanation for nitrogenase inactivation by salt is that there may be a deviation of cellular energy to osmoregulatory processes, in particular, to the efflux of Na^+ [66].

The salinity effects on nitrogenase activity have also been conducted in estuarine planktonic cyanobacteria [72]. Nitrogenase activity was significantly reduced in *Cylindrospermopsis raciborskii* at higher than 2 g/l NaCl after 22 h, whereas significantly reduced activity was observed at higher than 15 g/l NaCl for *Anabaena aphanizomenoides*. High salinity produced no change or slight activation of nitrogenase activity in three other cyanobacteria, *Anabaenopsis* sp., *Nodularia* FL2f, and *Nodularia* UP16A. It is interesting to note that these studies showed that the maximum photosynthetic rates were observed under salt stress which were in

contrast to previous reports for the chlorophyte *Chlamydomonas reinhardtii* [73] and the filamentous cyanobacterium *Spirulina platensis* [74]. The increased photosynthesis could be explained as increased demand for carbon by these salinity acclimation mechanisms. For instance, initial stimulation of photosynthesis was reported for *Nostoc* in response to salinity, which was followed by reduced photosynthesis and accumulation of sucrose as an internal osmolyte [75].

5. OSMOTIC STRESS AND OSMOLYTES

The response of organisms exposed to osmotic stress is the accumulation of osmolytes to prevent plasmolysis of the cells. The osmolytes found in cyanobacteria are similar to those present in other prokaryotes or in eukaryotes, i.e. glycine betaine, sucrose, trehalose and glucosylglycerol. For most cyanobacteria, all the osmolytes can be synthesized. However, cyanobacteria are also able to transport the osmolytes, if available, from the environments.

Glycine betaine is a quaternary nitrogenous compound which plays an important role in osmoregulation in various organisms. Glycine betaine has been implicated as a major osmolyte in a number of halophilic or marine cyanobacteria including *Synechocystis* DUN 52 [76], *Spirulina subsalsa* [77] and *A. halophytica* [13, 14]. In the filamentous cyanobacterium *Spirulina subsalsa*, betaine aldehyde dehydrogenase was responsible for the oxidation of choline to form glycine betaine [78]. Recently two *N* – methyltransferase genes have been isolated from *A. halophytica* [79]. The two gene products (ORF 1 and ORF 2) could methylate glycine to give the final product glycine betaine, indicating a three-step methylation from glycine to synthesize glycine betaine. This mechanism of glycine betaine synthesis has been reported in extreme halophiles [80, 81]. Importantly, the increase in the salinity resulted in an increased accumulation of these two gene products. The function of *N*-methyl transferases for the synthesis of glycine betaine seems primarily responsible for the adaptation to salt stress in *A. halophytica*. This stems from the fact that salt stress led to an increased accumulation of glycine betaine without exogenous supply of choline [12]. Very recently the potential of *N*-methyltransferases has been demonstrated in freshwater cyanobacterium *Synechococcus* sp. PCC 7942 with ability to grow in seawater and in Arabidopsis plants with improved seed yield under salt stress [82]. A previous study in *Synechococcus* sp. PCC 7942 also showed no detectable intracellular choline when growing cells in the absence of choline, suggesting little or no physiological role of intracellular choline in cyanobacteria [83]. Initial studies on the uptake of choline in *A. halophytica* have also been done to investigate the role of choline as an osmoregulatory solute. Under salt-stress condition, the choline uptake rate was two-fold higher than that under unstress condition [84].

Freshwater cyanobacteria normally accumulate disaccharides and glucosylglycerol in response to osmotic stress [85]. However, freshwater cyanobacteria can acquire resistance to salt stress by synthesizing some other osmolytes apart from disaccharides and glucosylglycerol. Nomuraetal [83] have shown that *Synechococcus* sp.

PCC 7942 transformed with *E. coli bet* genes could produce glycine betaine and rendered the cells tolerant to salt stress.

The uptake system of glycine betaine in *A. halophytica* had very high affinity for glycine betaine, i.e. its K_m was 2 μM [86]. The uptake of glycine betaine was enhanced when cells were grown under high concentration of salt. High rates of glycine betaine uptake have been shown to be characteristic of cyanobacteria able to synthesize or accumulate glycine betaine as an endogenous osmolyte. For instance, *Synechocystis* sp. PCC 6714 which normally contains glucosylglycerol as a major osmolyte showed about 100-fold reduction of glycine betaine uptake rate compared to that observed for *A. halophytica* [86].

Another organic nitrogen compound, polyamine, has recently received some attention in terms of its role for abiotic stress adaptation [87]. Polyamines are positively charged nitrogenous compounds found in all prokaryotes and eukaryotes. Putrescine, spermidine and spermine are three main polyamines most commonly present in higher plants. Generally putrescine and spermidine are more abundant whereas spermine is present in lower or trace amounts. Studies of the response of polyamine to abiotic stress especially osmotic stress have been done mostly in plant systems. For example, a salt-induced increase in the endogenous polyamine content has been reported in a number of plants including tomato, maize, sorghum and wheat [88-90]. In bacteria low osmotic stress could enhance the production of putrescine in *Vibrio parahaemolyticus* in the presence of arginine [91]. This increase of putrescine was not due to the increase in the specific activity of arginine decarboxylase (ADC). The increase of putrescine occurred in parallel with the cation efflux from cells and in vitro experiments showed inhibition of arginine decarboxylase by NaCl and KCl. The authors suggested that the reduction in the concentrations of Na^+ and K^+ predominantly present in cells might cause the increase in activity of the preexisting ADC leading to the enhancement of putrescine production. In another bacterium *Rhizobium fredii* P220, an increase in external osmolality did not affect putrescine content but reduced the level of homospermidine, an analog of spermidine [92].

The effects of salt and osmotic stresses on polyamine contents have been reported recently in a cyanobacterium *Synechocystis* sp. PCC 6803. Salt stress by NaCl induced a slight increase in the total cellular polyamine contents whereas osmotic stress by sorbitol resulted in a three-fold increase in total polyamine contents [93]. The less profound response of polyamine content observed for salt-stressed cells than that for osmotic-stressed cells suggested that an osmotic effect rather than an ionic effect was the main signal triggering changes in polyamines. A detailed analysis indicated that spermine and spermidine were specifically induced by salt stress and osmotic stress, respectively. The underlying mechanism for the increase of polyamines appeared to be mediated by the increase of the expression of ADC genes (slr 1312 and slr 0662). The abundance of ADC mRNA was maximum by treatment with 550 mM NaCl followed by that of 700 mM sorbitol treatment. The increased abundance of ADC mRNA is partly attributed to its stability. The reverse transcription-PCR analysis of the salt-stressed and osmotic-stressed cells showed that a significant level of ADC mRNA was detected after 120 min incubation

with rifampicin, an inhibitor of transcriptional initiation. In contrast, no ADC mRNA was detected after 120 min incubation in unstressed cells. The stabilization of ADC transcript could arise as a result of the formation of polyamine-ADC mRNA complex. The high content of spermidine and spermine in the stressed cells of *Synechocystis* would facilitate the formation of such a complex. With related studies in *E. coli* 90% of the total spermidine was complexed with RNA [94]. On the other hand the stabilization of ADC transcript might be facilitated or mediated by glucosylglycerol, a main osmolyte in *Synechocystis*. The levels of polyamine accumulated in *Synechocystis* cells under both salt stress and osmotic stress were too low to account for any osmotic importance. The increase of different classes of polyamine in *Synechocystis* therefore represented the acclimatization of the cells toward the stress rather than the direct response mechanism. Hence the main role of polyamines may not be to balance ionic strength but rather to specifically protect sensitive proteins and membranes from the deleterious effects of toxic ions.

Another organic nitrogenous osmolyte, proline, has recently been reported to accumulate in *Nostoc muscorum* under 100 mM NaCl stress [68]. Under this condition an almost two-fold increase of intracellular proline level was observed. When salt-stressed cells were further provided with externally added proline, a much greater level of intracellular proline was detected, i.e. about nine-fold increase compared to the control. This indicated the existence of a salt-induced proline uptake system in *N. muscorum*. This was substantiated by the observation that treatment with 100 mM NaCl in the presence of 1 mM proline resulted in nearly 100% survival of *N. muscorum* whereas no survival occurred in the absence of proline.

6. OSMOTIC STRESS AND RESERVED NITROGENOUS COMPOUNDS

The main nitrogen reserves in cyanobacteria under conditions of unbalanced nutrition are cyanophycin and phycobilisomes. Cyanophycin, composed of polyaspartate and arginine, is found only as a high molecular weight nitrogen reserve in many cyanobacteria. The diazotrophic *Cyanothece* sp. ATCC 51142 grown under nitrogen fixing condition was reported to accumulate cyanophycin granules which occurred after nitrogenase activity commenced [95]. For the non-diazotrophic *Synechocystis* sp. PCC 6803, phycobilisomes appeared to be the main nitrogen reserve. The knock-out mutants (*cphA* and *cphB* genes) could grow under nitrogen-deficient growth conditions but needed to degrade phycobilisomes as a nitrogen reserve. Not only the nutrient stress, but also the salt stress affects cyanophycin accumulation. *Scytonema* sp. isolated from the central Australian desert showed the accumulation of cyanophycin in response to the salt stress [96]. The amount of accumulated cyanophycin depended on the concentration of NaCl to which the cells were exposed, a maximum concentration was detected at 150 mM NaCl. Interestingly, further enhancement of cyanophycin accumulation occurred with the supplementation of KNO_3. This indicated that the cells could readily incorporate nitrate from the environment into nitrogen reserves under conditions of growth limitation due to environmental stress. Nevertheless, the capacity of the cells to synthesize

cyanophycin from nitrate might be species-specific since a *Calothrix* species was unable to do so [97].

The accumulation of cyanophycin in cyanobacteria has also been demonstrated when its degradation was disrupted. The putative glycoprotease encoded by the *gcp* gene has been suggested to be responsible for the degradation of cyanophycin in *Synechocystis* sp. PCC 6803 [98]. The deletion of *gcp* gene led to reduced salt tolerance possibly mediated by nitrogen starvation of the cells. In the wild type *Synechocystis* cells, salt stress tolerance could be attained in conjunction with the decrease in cyanophycin insuring sufficient supply of nitrogen. In contrast, the *gcp* mutant showed a reduction in salt stress tolerance with accompanying increase in cyanophycin with a resultant nitrogen starvation. The nitrogen requirement for the *gcp* mutant under salt stress could be partially met by the degradation of phycobiliproteins as evidenced by a slight decrease of their absorption spectra at 620-630 nm. The freshwater cyanobacterium *Synechocystis* sp. PCC 7942 grown in seawater also showed a substantial reduction in the absorption spectra at 630 nm [99] suggesting that phycobiliproteins degradation might be a product of salt stress.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Although we have learned much about nitrogen metabolism under abiotic stresses in many living systems, it is still in its early stage for studies done in cyanobacteria. Nevertheless, some important findings with respect to the effect of osmotic or salt stress on nitrogen metabolic pathways can be summarized as shown in Figure 2. Osmotic stress can exert, depending on species, either stimulatory or inhibitory effect on most inorganic nitrogen pathways. In the case of organic nitrogen pathways it appears that the osmotic stress results in the activation of the pathways leading to the accumulation of nitrogenous compatible solutes.

A recent review on the nitrogen control in cyanobacteria [100] has emphasized the importance of the *ntcA* gene in the regulation of nitrogen assimilation. Much work has been devoted to the effects of nitrogen sources but little study has been done on the effects of abiotic stresses. For instance, it was suggested that in response to abiotic stress such as cold stress and osmotic stress, *Anabaena* 7120 enhanced its nitrogen assimilation by inducing the expression of *ntc A* gene [101]. The effects of osmotic stress imposed by penetrating and non-penetrating solutes on the regulation of this gene at both transcription and translation level might provide further insight into the mechanisms eliciting the signal for osmotic stress adaptation in cyanobacteria which might be amenable for application in higher plants. Comparison of NtcA proteins between freshwater and marine cyanobacteria shows very high pairwise identities of 76.9 to 88.4% [96], suggesting little difference in the protein itself. However, it is not known whether there exist different sets of genes that are activated by NtcA proteins between cyanobacteria living in freshwater and marine environments.

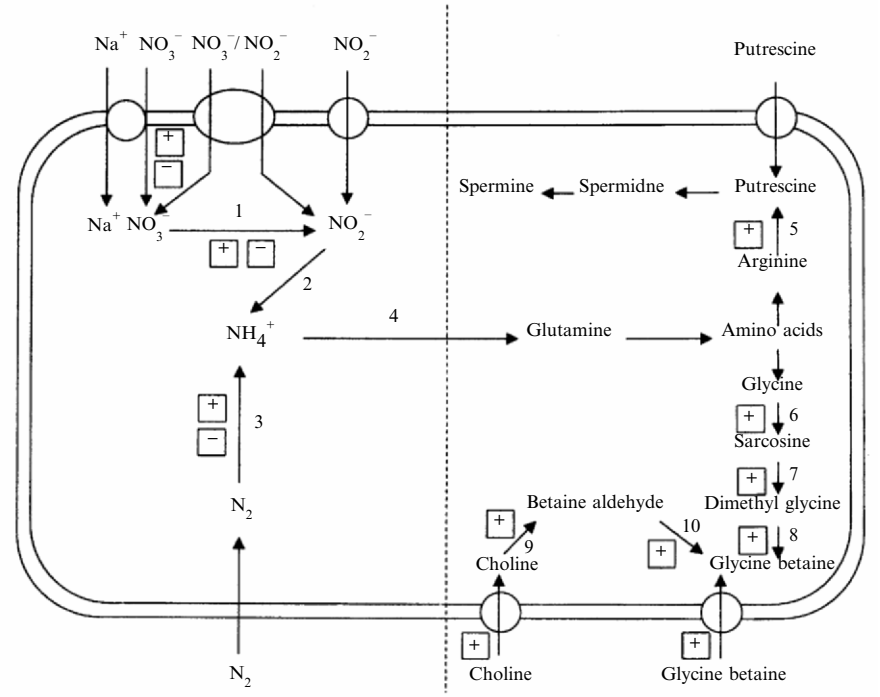

Figure 2. Inorganic (left half) and organic (right half) nitrogen metabolic pathways in cyanobacteria as regulated by osmotic stress. 1, nitrate reductase; 2, nitrite reductase; 3, nitrogenase; 4, glutamine synthetase; 5, arginine decarboxylase; 6, 7, 8, N-methyl-transferases.

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REFERENCES

1. Tandeau de Marsac, N. and Houmard, J. (1993) Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanism. *FEMS Microbiol. Rev*. **104,** 119-190.
2. Reed, R.H. and Stewart, W.D.P. (1988) Osmotic adjustment and organic solute accumulation in unicellular cyanobacteria from freshwater and marine habitats. *Marine Biol*. **88,** 1-9.
3. Sivak, M.N., Lara, C., Romero, J.M., Rodriguez, R. and Guerrero, M.G. (1989) Relationship between a 47 kDa cytoplasmic membrane polypeptides and nitrate transport in *Anacystis nidulans. Biochem. Biophys. Res. Commun*. **158,** 257-262.
4. Romero, J.M., Lara, C. and Guerrero, M.G. (1989) Determination of intracellular nitrate. *Biochem. J*. **259,** 545-548.

5. Rodriguez, R., Lara, C. and Guerrero, M.G. (1992) Nitrate transport in the cyanobacterium *Anacystis nidulans* R2: kinetic and energetic aspects. *Biochem. J*. **282,** 639-643.
6. Mistrik, I. and Ullrich, C.I. (1996) Mechanism of anion uptake in plant roots: quantitative evaluation of H^+ / NO_3^- and H^+ / $H_2PO_4^-$ stoichiometries. *Plant Physiol. Biochem*. **34,** 629-636.
7. Crawford, N.M. and Glass, A.D.M. (1998) Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci*. **3,** 389-395.
8. Garcia-Sanchez, M.J., Jaime, M.P., Ramos, A., Sanders, D. and Fernandez, J.A. (2000) Sodium-dependent nitrate transport at the plasma membrane of leaf cells of the marine higher plant *Zostera marina* L. *Plant Physiol*. **122,** 879-885.
9. Incharoensakdi, A. and Wangsupa, J. (2003) Nitrate uptake by the halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress and salt-stress conditions. *Curr. Microbiol*. **47,** 255-259.
10. Rai, A.K. and Tiwari, S.P. (1999) Mutants of the cyanobacterium *Anabaena* sp. PCC 7120 altered in nitrate transport and reduction. *Curr. Microbiol*. **39,** 237-243.
11. Reddy, B.R., Apte, S.K. and Thomas, J. (1989) Enhancement of cyanobacterial salt tolerance by combined nitrogen. *Plant Physiol*. **89,** 204-210.
12. Rai, A.K. and Abraham, G. (1995) Relationship of combind nitrogen-sources to salt tolerance in freshwater cyanobacterium *Anabaena doliolum*. *J. Appl. Bacteriol.* **78,** 501-506.
13. Ishitani, M., Takabe, T., Kojima, T. and Takabe, T. (1993) Regulation of glycine betaine accumulation in the halotolerant cyanobacterium *Aphanothece halophytica*. *Aust. J. Plant Physiol*. **20,** 693-703.
14. Incharoensakdi, A. and Wutipraditkul, N. (1999) Accumulation of glycine betaine and its synthesis from radioactive precursors under salt-stress in the cyanobacterium *Aphanothece halophytica*. *J. Appl. Phycol*. **11,** 515-523.
15. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A. and Takabe, T. (2001) Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+ / H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. *J. Biol. Chem*. **276,** 36931- 36938.
16. Matsuda, N., Kobayashi, H., Katoh, H., Ogata, T., Futatsugi, L., Nakamura, T., Bakker, E. P. and Uozumi, N. (2004) Na^+ - dependent K^+ uptake Ktr system from the cyanobacterium *Synechocystis* sp. PCC 6803 and its role in the early phase of cell adaptation to hyperosmotic shock. *J. Biol. Chem.* **279,** 54952-54962.
17. Rai, A.K. and Rai, V. (2003) Effect of NaCl on growth, nitrate uptake and reduction and nitrogenase activity of *Azolla pinnata-Anabaena azollae*. *Plant Sci*. **164,** 61-69.
18. Botella, M.A., Martinez, V., Nieves, M. and Cerda, A. (1997) Effect of salinity on the growth and nitrogen uptake by wheat seedlings. *J. Plant Nutr*. **20,** 793-804.
19. Aslam, M., Huffaker, R. and Raine, D. (1984) Early effects of salinity on nitrate assimilation in barley seedlings. *Plant Physiol*. **76,** 321-325.
20. Peuke, A.D. and Jesccke, W.D. (1999) The characterization of net nitrate uptake by salt in salt-tolerant barley (*Hordeum vulgare* L. cv. California Mariout). *J. Exp. Bot*. **50,** 1365-1372.
21. Rodriguez, R., Kobayashi, M., Omata, T. and Lara, C. (1998) Independence of carbon and nitrogen control in the post translational regulation of nitrate transport in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *FEBS Lett*. **432,** 207-212.
22. Lee, R.B. and Rudge, K.A. (1986) Effects of nitrogen deficiency on the absorption of nitrate and ammonium by barley plants. *Ann. Bot*. **57,** 471-486.
23. Lee, R.B., Purves, J.V., Rateliffe, R.G. and Sakes, L.R. (1992) Nitrogen assimilation and the control of ammonium and nitrate absorption by maize roots. *J. Exp. Bot*. **43,** 1385-1396.
24. Lara, C., Rodriguez, R. and Guerrero, M.G. (1993) Nitrate transport in the cyanobacterium *Anacystis nidulans*. *Physiol. Plant*. **89,** 582-587.
25. Sakamoto, T. and Bryant, D.A. (1999) Nitrate transport and not photoinhibition limits growth of the freshwater cyanobacterium *Synechococcus* species PCC 6301 at low temperature. *Plant Physiol*. **199,** 785-794.
26. Kronzucker, H.J., Glass, A.D.M. and Siddiqi, M.Y. (1999) Inhibition of nitrate uptake by ammonium in barley : analysis of component fluxes. *Plant Physiol*. **120,** 283-291.
27. De la Haba, P., Aguera, E. and Maldonado, J.M. (1990) Differential effects of ammonium and tungsten on nitrate and nitrite uptake and reduction by sunflower plants. *Plant Sci*. **70,** 21-26.

28. Aslam, M., Travis, R.L. and Huffaker, R.C. (1994) Stimulation on nitrate and nitrite afflux by ammonium in barley (*Hordeum vulgare* L.) seedlings. *Plant Physiol*. **106,** 1293-1301.
29. Maeda, S. and Omata, T. (1997) Substrate binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrite. *J. Biol. Chem*. **272,** 3036-3041.
30. Bhattacharya, J., Singh, A.K. and Rai, A.N. (2002) Isolation and characterization of a chlorate resistant mutant (Clo-R) of the symbiotic cyanobacterium *Nostoc* ANTH : heterocyst formation and N_2-fixation in the presence of nitrate, and evidence for separate nitrate and nitrite transport systems. *Curr. Microbiol*. **45,** 99-104.
31. Luque, I., Flores, E. and Herrero, A. (1994) Nitrate and nitrite transport in the cyanobacterium *Synechococcus* sp. PCC 7942 are mediated by the same permease. *Biochim. Biophys. Acta* **1184,** 296-298.
32. Maeda, S.-I. and Omata, T. (1997) Substrate binding lipoprotein of the cyanobacterium *Synechococcus* sp. PCC 7942 involved in the transport of nitrate and nitrite. *J. Biol. Chem*. **272,** 3036-3041.
33. Omata, T., Andriesse, X. and Hirano, A. (1993) Identification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* sp. PCC 7942. *Mol. Gen. Genet*. **236,** 193-202.
34. Frias, J.E., Flores, E. and Herrero, A. (1997) Nitrate assimilation gene cluster from the heterocyst forming cyanobacterium *Anabaena* sp. strain 7120. *J. Bacteriol*. **179,** 477-486.
35. Sakamoto, T., Inoue-Sakamoto, K. and Bryant, D.A. (1999) A novel nitrate / nitrite permease in the marine cyanobacterium *Synechococcus* sp. PCC 7002. *J. Bacteriol*. **182,** 7363-7372.
36. Wang, Q., Lee, H. and Post, A.F. (2000) Nitrate assimilation genes of the marine diazotrophic, filamentous cyanobacterium *Trichodesmium* sp. strain WH 9601. *J. Bacteriol*. **182,** 1764-1767.
37. Flores, E. and Herrero, A. (1994) Assimilatory nitrogen metabolism and its regulation. In *The Molecular Biology of Cyanobacteria* (Bryant, D.A., ed.). Kluwer Academic Publishers, pp. 487-517.
38. Kanesaki, Y., Suzuki, I., Allakhverdiev, S.I., Mikami, K. and Murata, N. (2002) Salt stress and hyperosmotic stress regulate the expression of different set of genes in *Synechocystis* sp. PCC 6803. *Biochem. Biophys. Res. Commun*. **290,** 339-348.
39. Omata, T. (1995) Structure, function and regulation of the nitrate transport system of the cyanobacterium *Synechococcus* sp. PCC 7942. *Plant Cell Physiol*. **36,** 207-213.
40. Kaneko, T. et al (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res*. **3,** 109-136.
41. Cai, Y. and Wolk, C.P. (1997) Nitrogen deprivation of *Anabaena* sp. strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate. *J. Bacteriol*. **179,** 258-266.
42. Suzuki, I., Kikuchi, H., Nakanishi, S., Fujita, Y., Sugiyama, T. and Omata, T. (1995) A novel nitrite reductase gene from the cyanobacterium *Plectonema boryanum. J. Bacteriol*. **177,** 6137-6143.
43. Merchand, F., Kindle, K.L., Llana, M.J., Serra, J.L. and Fernandez, E. (1995) Cloning and sequencing of the nitrate transport system from the thermophilic filamentous cyanobacterium *Phormidium laminosum* : comparative analysis with the homologous system from *Synechococcus* sp. PCC 7942. *Plant Mol. Biol*. **28,** 759-766.
44. Kikuchi, H., Aichi, M., Suzuki, I. and Omata, T. (1996) Positive regulation by nitrite of the nitrate assimilation operon in the cyanobacteria *Synechococcus* sp. strain PCC 7942 and *Plectonema boryanum*. *J. Bacteriol*. **178,** 5822-5825.
45. Bird, C. and Wyman, M. (2003) Nitrate / nitrite assimilation system of the marine picoplanktonic cyanobacterium *Synechococcus* sp. strain WH 8103: effect of nitrogen source and availability on gene expression. *Appl. Environ. Microbiol*. **69,** 7009-7018.
46. Ghoulam, C., Foursy, A. and Fares, K. (2002) Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environ. Exp. Bot*. **47,** 39-50.
47. Ghosh, S.C., Asanuma, K., Kusutani, A. and Toyota, M. (2001) Effect of salt stress on some chemical components and yield of potato. *Soil Sci. Plant Nutr*. **47,** 467-475.
48. Abd-El Baki, G.K., Siefritz, F., Man, H.M., Weiner, H., Kaldenhoff, R. and Kaiser, W.M. (2000) Nitrate reductase in Zea mays L. under salinity. *Plant Cell Environ*. **23,** 515-521.

49. Martinez-Espinosa, R.M., Marhuenda-Egea, F.C. and Bonete, M.J. (2001) Assimilatory nitrate reductase from the haloarchaeon *Haloferax mediterranei* : purification and characterization. *FEMS Microbiol. Lett*. **204,** 381-385.
50. Blasco, R., Martinez-Luque, M., Madrid, M.P., Castillo, F. and Moreno-Vivian, C. (2001) *Rhodococcus* sp. RB 1 grows in the presence of high nitrate and nitrite concentrations and assimilates nitrate in moderately saline environments. *Arch. Microbiol*. **175,** 435-440.
51. Kesseru, P., Kiss, I., Bihari, Z. and Polyak, B. (2002) The effects of NaCl and some heavy metals on the denitrification activity of *Ochrobactrum anthropi*. *J. Basic Microbiol*. **42,** 268-276.
52. Igeno, M.I., Moral, C.G.D., Castillo, F. and Caballero, F.J. (1995) Halotolerance of the phototrophic bacterium *Rhodobacter capsulatus* E1F1 is dependent on the nitrogen source. *Appl. Environ. Microbiol*. **61,** 2970-2975.
53. Rai, A.K. and Tiwari, S.P. (1999) Response to NaCl of nitrate assimilation and nitrogenase activity in the cyanobacterium *Anabaena* sp. PCC 7120 and its mutants. *J. Appl. Microbiol*. **87,** 877-883.
54. Cramer, M.D. and Lips, S.H. (1995) Enriched rhizosphere CO_2 concentrations can ameliorate the influence of salinity on hydroponically grown tomato plants. *Physiol. Plant*. **94,** 425-432.
55. Potluri, S.D.P. and Prasad, P.V.D. (2001) In-vitro studies on the effects of varying levels of sea-salt on two cassava cultivars. *Trop. Agri*. **78,** 62-65.
56. Bourgeais-Chaillou, P., Perez-Alfocea, F. and Guerrier, G. (1992) Comparative effect of N-sources on growth and physiological responses of soybean exposed to NaCl-stress. *J. Exp. Bot*. **254,** 1225-1233.
57. Sagi, M., Savidov, N.A., L'vov, N.P. and Lips, S.H. (1997) Nitrate reductase and molybdinum cofactor in annual ryegrass as affected by salinity and nitrogen source. *Physiol. Plant*. **99,** 546-553.
58. Blom-Zandstra, M. and Lampe, J.E.M. (1985) The role of nitrate in the osmoregulation of lettuce (*Latuca sativa* L.) grown at different light intensities. *J. Exp. Bot*. **36,** 1043-1052.
59. Ferrario, S., Valadier, M. and Foyer, C.H. (1998) Over-expression of nitrate reductase in tobacco delays drought-induced decrease in nitrate reductase activity and mRNA. *Plant Physiol*. **117,** 283-292.
60. Yates, M.G. (1977) Physiological aspects of nitrogen fixation. In *Recent Developments in Nitrogen Fixation* (Newton, W. et al, eds). Academic Press, pp. 219-270.
61. Wolk, C.P. (1982) Heterocysts. In *The Biology of Cyanobacteria* (Carr, N.G. and Whitton, B.A., eds.) pp. 359-386, University of California Press.
62. Rao, A.V. and Venkateshwarulu, B. (1985) Salt tolerance of *Azospirillum brasilense*. *Acta Microbiol. Hung*. **32,** 221-224.
63. Jofre, E., Rivarola, V., Balegno, H. and Mori, G. (1998) Differential gene expression in *Azospirillum brasilense* under saline stress. *Can. J. Microbiol*. **44,** 929-936.
64. Le Rudulier, D., Yang, S.S. and Csonka, L.N. (1982) Nitrogen fixation in *Klebsiella pneumoniae* during osmotic stress: effect of exogenous proline or proline-overproducing plasmid. *Biochim. Biophys. Acta* **719,** 273-283.
65. Tripathi, A.K., Nagarajan, T., Verma, S.C. and Le Rudulier, D. (2002) Inhibition of biosynthesis and activity of nitrogenase in *Azospirillum brasilense* Sp7 under salinity stress. *Curr. Microbiol*. **44,** 363-367.
66. Fernandes, T.A., Iyer, V. and Apte, S.K. (1993) Differential responses of nitrogen fixing cyanobacteria to salinity and osmotic stresses. *Appl. Environ. Microbiol*. **59,** 899-904.
67. Fernandes, T.A. and Apte, S.K. (2000) Differential regulation of nitrogenase activity by ionic and osmotic stresses and permeable sugars in the cyanobacterium *Anabaena* sp. strain L-31. *Plant Sci*. **150,** 181-189.
68. Bhargava, S., Saxena, R.K., Pandey, P.K. and Bisen, P.S. (2003) Mutational engineering of the cyanobacterium *Nostoc muscorum* for resistance to growth-inhibitory action of LiCl and NaCl. *Curr. Microbiol*. **47,** 5-11.
69. Tel-Or, E. and Melhamed-Harel, H. (1981) Adaptation to salt of photosynthetic apparatus in cyanobacteria. In *Photosynthesis* (Akoyunoglou, G., ed.), pp. 455-462, Bablan International Science Services.
70. Blumwald, E., Mehlhorn, R.J. and Packer, L. (1983) Ionic osmoregulation during salt adaptation of the cyanobacterium *Synechococcus* 6311. *Plant Physiol*. **73,** 377-380.
71. Apte, S.K. and Thomas, J. (1983) Impairment of photosynthesis by sodium deficiency and its relationship to nitrogen-fixation in cyanobacterium *Anabaena torulosa*. *FEMS Microbiol. Lett*. **16,** 153-157.

72. Moisander, P.H., McClinton III, E. and Paerl, H.W. (2002) Salinity effects on growth, photosynthetic parameters, and nitrogenase activity in estuarine planktonic cyanobacteria. *Microbiol Ecol*. **43,** 432-442.
73. Neale, P.J. and Melis, A. (1989) Salinity-stress enhances photoinhibition of photosynthesis in *Chlamydomonas reinhardtii*. *J. Plant Physiol*. **134,** 619-622.
74. Zeng, M-T. and Vonshak, A. (1998) Adaptation of *Spirulina platensis* to salinity-stress. *Comp. Biochem. Physiol*. Part A **120,** 113-118.
75. Blumwald, E. and Tel-Or, E. (1982) Osmoregulation and cell composition in salt-adaptation of *Nostoc muscorum*. *Arch. Microbiol*. **132,** 168-172.
76. Mohammad, F.A.A., Reed, R.H. and Stewart, W.D.P. (1983) The halophilic cyanobacterium *Synechocystis* DUN 52 and its osmotic responses. *FEMS Microbiol. Lett*. **16,** 287-290.
77. Gabbay-Azaria, R., Tel-Or, E. and Schonfeld, M. (1988) Glycine betaine as an osmoregulant and compatible solute in the marine cyanobacterium *Spirulina subsalsa*. *Arch. Biochem. Biophys*. **261,** 333-339.
78. Gabbay-Azaria, R. and Tel-Or, E. (1993) Mechanisms of salt tolerance in cyanobacteria. In *Plant Responses to The Environment* (Gresshoff, P.M., ed.), pp. 692-698, CRC Press.
79. Waditee, R., Tanaka, Y., Aoki, K., Hibino, T., Jikuya, H., Takano, J., Takabe, T. and Takabe, T. (2003) Isolation and functional characterization of N-methyltransferases that catalyze betaine synthesis from glycine in a halotolerant photosynthetic organism *Aphanothece halophytica*. *J. Biol. Chem*. **278,** 4932-4942.
80. Galinski, E.A. and Truper, H.G. (1994) Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol. Rev*. **15,** 95-108.
81. Nyyssola, A., Kerovuo, J., Kaukinen, P., von Weymarn, N. and Reinikainen, T. (2000) Extreme halophiles synthesize betaine from glycine by methylation. *J. Biol. Chem*. **275,** 22196-22201.
82. Waditee, R., Bhuiyan, N. H., Rai, V., Aoki, K., Tanaka, Y., Hibino, T., Suzuki, S., Takano, J., Jagendorf, A. T., Takabe, T. and Takabe, T. (2005) Genes for direct methylation of glycine provides high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 1318-1323 .
83. Nomura, M., Ishitani, M., Takabe, T., Rai, A.K. and Takabe, T. (1995) *Synechococcus* sp. PCC 7942 transformed with *Escherichia coli bet* genes produces glycine betaine from choline and acquires resistance to salt stress. *Plant Physiol*. **107,** 703-708.
84. Incharoensakdi, A. and Karnchanatat, A. (2003) Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*. *Biochim. Biophys. Acta* **1621,** 102-109.
85. Reed, R.H., Borowitzka, L.J., Mackay, M. A., Chudek, J.A., Foster, R., Warr, S.R.C., Moore, D.J. and Stewart W.D.P. (1986) Organic solute accumulation in osmotically stressed cyanobacteria. *FEMS Microbiol. Rev*. **39,** 51-56.
86. Moore, D.J., Reed, R.H. and Stewart, W.D.P. (1987) A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesising cyanobacteria. *Arch. Microbiol*. **147,** 399-405.
87. Bouchereau, A., Aziz, A., Larher, F. and Martin-Tanguy, J. (1999) Polyamines and environmental challenges: recent development. *Plant Sci*. **140,** 103-125.
88. Aziz, A., Martin-Tanguy, J. and Larher, J. (1999) Salt stress-induced proline accumulation and changes in tyramine and polyamine levels are linked to ionic adjustment in tomato leaf discs. *Plant Sci*. **145,** 83-91.
89. Erdei, L., Szegletes, Z., Barabas, K. and Pestenacz, M. (1996) Responses in polyamine titer under osmotic and salt stress in sorghum and maize seedlings. *J. Plant Physiol*. **147,** 599-603.
90. Erdei, L, Trivedi, S., Takeda, K. and Matsumoto, H. (1990) Effects of osmotic and salt stresses on the accumulation of polyamines in leaf segments from wheat varieties differing in salt and drought tolerance. *J. Plant Physiol*. **137,** 165-168.
91. Yamamoto, S., Yamasaki, K., Takashina, K., Katsu, T. and Shinoda, T. (1989) Characterization of putrescine production in non-growing *Vibrio parahaemolyticus* cells in response to external osmolality. *Microbiol. Immunol*. **33,** 11-21.
92. Fujihara, S. and Yoneyama, T. (1993) Effects of pH and osmotic stress on cellular polyamine contents in the soybean rhizobia *Rhizobium fredii* P220 and *Bradyrhizobium japonicum* A1017. *Appl. Environ. Microbiol*. **59,** 1104-1109.

93. Jantaro, S., Maenpaa, P., Mulo, P. and Incharoensakdi, A. (2003) Contents and biosynthesis of polyamines in salt and osmotically stressed cells of *Synechocystis* sp. PCC 6803. *FEMS Microbiol*. *Lett*. **228,** 129-135.
94. Igarashi, K. and Kashiwagi, K. (2000) Polyamines: mysterious modulators of cellular function. *Biochem*. *Biophys*. *Res*. *Commun*. **271,** 559-564.
95. Li, H., Sherman, D.M., Bao, S. and Sherman, L.A. (2001) Pattern of cyanophycin accumulation in nitrogen-fixing cyanobacteria. *Arch*. *Microbiol*. **176,** 9-18.
96. Page-Sharp, M., Behm, C.A. and Smith, G.D. (1998) cyanophycin and glycogen synthesis in a cyanobacterial *Scytonema* species in response to salt stress. *FEMS Microbiol*. *Lett*. **160,** 11-15.
97. Liotenberg, S., Campbell, D., Rippka, R., Houmard, J. and Tandeau de Marsac, N. (1996) Effect of the nitrogen source on phycobiliprotein synthesis and cell reserves in a chromatically adapting filamentous cyanobacterium. *Microbiology* **142,** 611-622.
98. Zuther, E., Schubert, H. and Hagemann, M. (1998) Mutation of a gene encoding a putative glycoprotease leads to reduced salt tolerance, altered pigmentation, and phycocyanin accumulation in the cyanobacterium *Synechoystis* sp. PCC 6803. *J*. *Bacteriol*. **180,** 1715-1722.
99. Waditee, R., Hibino, T., Nakamura, T., Incharoensakdi, A. and Takabe, T. (2002) Overexpression of a Na^+ / H^+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. *Proc*. *Natl*. *Acad*. *Sci*. *U.S.A*. **99,** 4109-4114.
100. Herrero, A., Muro-Pastor, A.M. and Flores, E. (2001) Nitrogen control in cyanobacteria. *J*. *Bacteriol*. **183,** 411-425.
101. Mori, S., Castoreno, A. and Lammers, P. J. (2002) Transcript levels of rbcR1, ntcA, and rbcL/S genes in cyanobacterium *Anabaena* sp. PCC 7120 are down regulated in response to cold and osmotic stress. *FEMS Microbiol. Lett.* **213,** 167 - 173.

SECTION VIII

STRUCTURAL RESPONSES

15. ULTRASTRUCTURAL EFFECTS OF SALINITY STRESS IN HIGHER PLANTS

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Abstract. Salinity stress induces various types of ultrastructural changes in higher plant cells. These structural changes provide useful information as to the underlying mechanism of salinity stress. In this report the ultrastructural effects of salinity (NaCl) stress in crop plants especially in rice are described based on the research work conducted in our laboratory. Relevant research results are also described and the possible mechanisms of ultrastructural changes are discussed.

1. EFFECTS OF SALINITY STRESS ON ROOT CELL ULTRASTRUCTURE

Salinity is one of the most serious problems that limit plant growth and crop yield. Salinity affects almost 10^9 ha of land, which corresponds to 7% of the global land surface [1]. In addition about 5% of the cultivated land is affected by salinity [2] and about 20% of irrigated land is suffering from secondary salinization due to inappropriate treatment of irrigation system [3]. Therefore, salinity stress is an important research subject to increase global agricultural production to cope with the increasing world population. There have been a number of studies on physiological and molecular biological responses of plants to salinity [4, 5], but information is also accumulating on the ultrastructure of plants under salinity. Salinity stress induces various types of ultrastructural changes in higher plant cells. These structural changes provide useful information as to the underlying mechanism of salinity stress. They would also provide some insight into practical methods to cope with the salinity stress in crop plants. In this report the ultrastructural effects of salinity (NaCl) stress in crop plants especially in rice are described based on the research work conducted in our laboratory. Relevant research results are also described and the possible mechanisms of ultrastructural changes are discussed.

Ashwani K. Rai and Teruhiro Takabe (eds.), Abiotic Stress Tolerance in Plants, 215-226.

2. EFFECTS OF SALINITY STRESS ON ROOT CELL ULTRASTRUCTURE

The roots are directly exposed to the soil and affected by salinity. Therefore, this organ must tolerate the salinity stress to keep the whole plant alive [6]. In fact growth inhibition by salinity is more prominent in roots than in shoots at least in the seedling stage of rice [7]. The root tip is also suggested to function as a sensor for different kinds of stress [8]. Therefore, investigation on salinity-induced ultrastructural changes in roots is important.

Rahman et al. [7] examined the effects of salinity stress on the seminal root tip ultrastructure in rice seedlings. Salinity treatment accelerated vacuolation in root cap cells and cortical cells (Fig. 1). However, the cells in the meristem and the central cylinder were less vacuolated than in control plants. An increase in endoplasmic

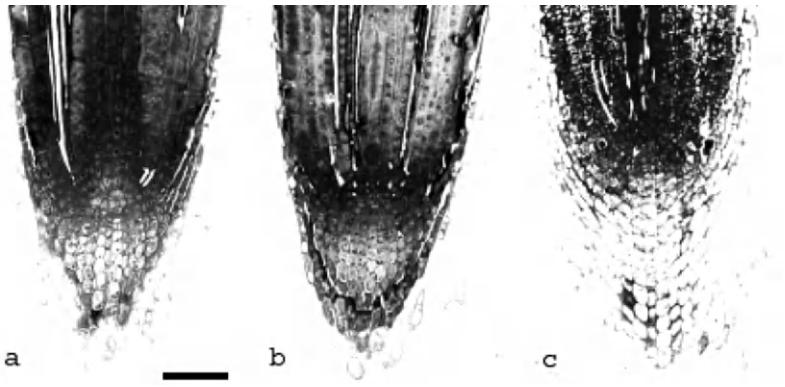

Figure 1. Light micrographs of longitudinal sections of root tip cells of rice grown on agar medium for 5 days (Bar=5 μm). a: control, b: 0.3% NaCl, c: 1% NaCl.

reticulum was observed in the vacuolated root cap cells and the cortical cells. Myelin figures were often observed in the vacuoles. Myelin figures were also observed in the endoplasmic reticulum. In fact some of the vacuoles appeared to be derived from partial inflation of the endoplasmic reticulum. After treatment with NaCl at a higher concentration (1% in agar medium) root cap cells were expanded and proliferated up to the upper (basal) region of the root (Fig. 1). Root cap cells in the peripheral region of normal root tips secrete mucilaginous polysaccharides, which cover and protect the root tip. This mucilage is known to be synthesized in Golgi bodies and secreted out of the plasma membrane via secretory vesicles. Golgi bodies with well-developed cisternae were abundant and many secretory vesicles derived from Golgi bodies were observed in the peripheral root cap cells. These secretory vesicles were filled with electron dense materials. These figures reflect a high activity in mucilage production in the peripheral root cap cells of control plants. However, the peripheral root cap cells of the plants treated with NaCl were highly vacuolated and not active in mucilage production. Golgi bodies in the remaining cytoplasm were sparse and their secretory vesicles were small and devoid of electron

dense materials (Fig. 2). The vacuolation of root tip cells may be an adaptive response to accumulate excess ions under salinity, which protects the cytoplasm from toxic levels of ions. The vacuolation of root cap cells and cortical cells may also function to separate ions from more vulnerable meristematic cells and procambial cells. However, the suppression of mucilage production in the peripheral root cap cells should have adverse effects on the root growth.

Vacuolation and vesiculation of root cells are also reported for salinity-stressed barley [9], *Sorghum* [10] and bean [11]. Vacuolation is prominent in the epidermis and cortical cells but is not apparent in the central cylinder. Some of the epidermal cells of salinity-stressed *Sorghum* plants showed a development of wall ingrowth, which is a characteristic feature of the transfer cell [12] and may function in the excretion of salt from symplast to apoplast [10]. In *Bacopa* plants numerous multicelluar root hairs are developed under moderate salinity stress (5 g/L NaCl) but are disappeared at higher NaCl treatments [13]. Increase of air cavity (aerenchyma)

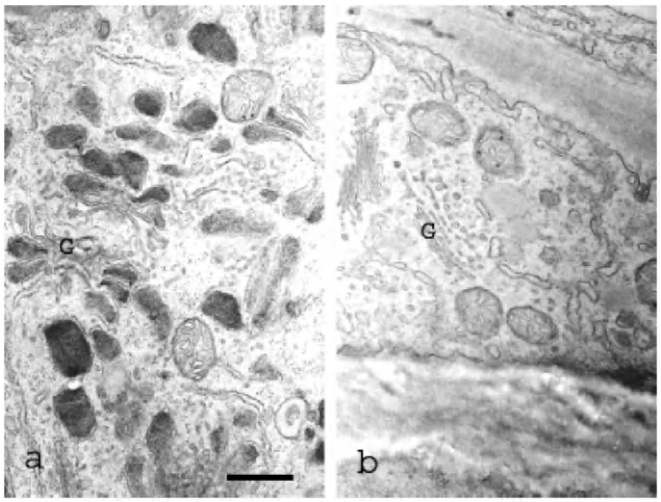

Figure 2. Electron micrographs of peripheral root cap cells of rice grown on agar medium for 5 days (Bar=0.5 μm). a: control, b: 1% NaCl. G, Golgi body.

in the cortex of stem and root is also reported for *Bacopa* plants treated with NaCl [13], however, the cortical cell death which leads to the formation of aerenchyma is suppressed in roots of rice under NaCl stress [14]. The suppression of aerenchyma formation should have additional adverse effects on the growth of rice under salinity. Damages to the structure of mitochondria are reported for maize roots under various stresses including salinity [15] and for salinity-stressed barley roots [9]. Increase in the number of mitochondria in the epidermis and the cortex is reported in *Sorghum* roots after salt treatment [10], which may be concerned with the energy supply for the selective transport of ions under salinity stress [16].

3. EFFECTS OF SALINITY STRESS ON LEAF ULTRASTRUCTURE

3.1. General obserbations

Salinity stress affects the ultrastructure of root cells and the root growth was inhibited severely compared with the shoot growth in the seedling stage. However, the inhibition of root growth itself is not lethal to the plant as far as the shoot is living. In fact a rice cultivar Nipponbare, which is relatively sensitive to salinity, grew better under salinity than salinity-tolerant Bangladesh cultivars, when they were compared in the seedling stage [17]. However, when rice plants are grown autotrophically and treated with NaCl, chlorosis develops in the leaves and finally the plants die. Salinity-tolerant cultivars perform better in this condition. Therefore, the effects of salinity stress on leaf cells are more important for the plant life and crop production.

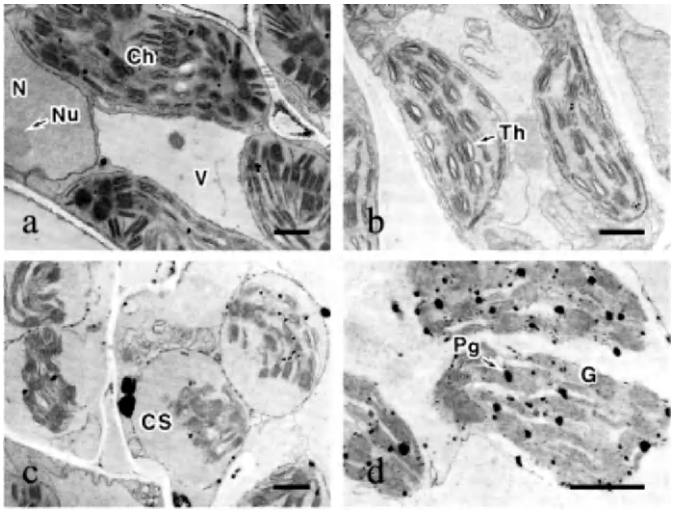

Figure 3. Chloroplasts of rice grown on media containing various concentrations of NaCl (Bars=1 μm̃a: control in soil culture, b: 0.3% NaCl in water culture for 8 days, c: 1% NaCl in soil culture for 14 days, d: 1% NaCl in soil culture for 14 days. Ch, chloroplast; CS, chloroplast stroma; G, granum; N, nucleus; Nu, nucleolus; Pg, plastoglobule; Th, thylakoid.

Rahman et al. [18] examined the salinity-induced ultrastructural changes in leaves of rice. The first indication of ultrastructural damage in leaf cells was swelling of thylakoids in chloroplasts (Fig. 3b). The swelling of thylakoids is discernible while no visible symptoms appear in the leaf. In later stages of salinity treatment, chloroplasts were swollen (Fig. 3c) and finally the chloroplast envelopes were disrupted. Numerous plastoglobuli were observed at this stage (Fig. 3d). Other

organelles were less sensitive to salinity compared with chloroplasts, but the inner structure of mitochondria was degraded in the later stages of salinity stress. Cellular structure was totally degenerated when the plasma membrane was disrupted. NaCl is mainly transported via vascular bundles [19], so the vascular tissues may be affected first by salinity. However, the vascular tissues were not so severely damaged as mesophyll cells. Destruction of vascular cells was occasionally observed in the later stages of salinity treatment (Fig. 4c).

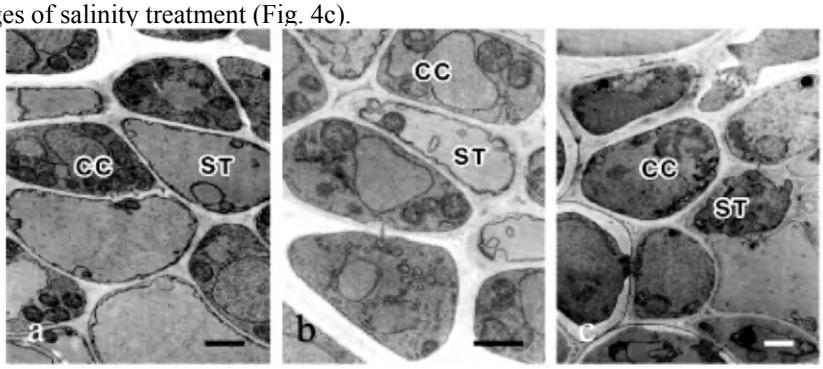

Figure 4. Vascular tissues of rice grown on soil treated with various concentrations of NaCl for 14 days (Bars=1 μm). a: control, b: 0.3% NaCl, c: 1% NaCl. CC, companion cell; ST, sieve tube.

Swelling of thylakoids induced by salinity stress is also reported for barley [9] but not in tomato [20, 21, 22] and *Argyranthemum* [23]. Increase in the size of starch grains together with the size of chloroplasts is reported in the latter cases. In some plants vascular parenchyma cells especially companion cells are differentiated into the transfer cells with well developed wall ingrowth. These cells may function in the exclusion of salt from leaves and the damage to the transfer cells is correlated with the appearance of symptoms in the leaves [24]. Transfer cells are known to be the most sensitive to salinity in the vascular cells [23, 24].

3.2. Comparison of the effects of salinity stress and drought stress on chloroplast ultrastructure

Swelling of thylakoids in chloroplasts is a typical symptom of salinity stress in rice plants. Salinity stress has both ionic and osmotic effects [19]. Yamane et al. [25] treated rice plants in hydroponic culture with NaCl and polyethylene glycol (PEG) both at a water potential of -1.0 MPa for 3 days. NaCl induced the swelling of thylakoids and a slight destruction of the chloroplast envelope. PEG caused severe destruction of the chloroplast envelope, but the thylakoids did not swell (Fig. 5). They suggested that the ionic effects of NaCl induced the swelling of thylakoids and the osmotic effects caused the destruction of chloroplast envelope.

Yamane et al. [26] further examined the effects of drought stress with different intensities and durations on the ultrastructure of chloroplasts of rice plants in soil.

Although a slight swelling of thylakoids was sometimes observed in the cells adjacent to the vascular bundle, the thylakoids were generally less affected by drought stress than the chloroplast envelope. Therefore, salinity stress and drought stress have different effects on the ultrastructure of the chloroplasts, and the swelling of thylakoid is characteristic of salinity stress.

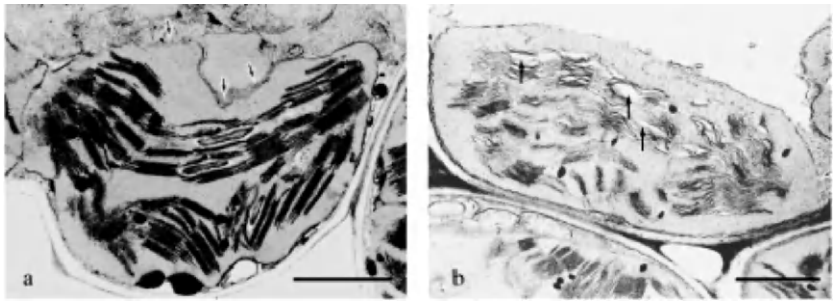

Figure 5. Ultrastructure of chloroplasts in rice plants treated with PEG (a) and NaCl (b) for 2 days (Bars=1 μm). Swelling of thylakoids is observed with NaCl treatment (b) while changes in envelopes are prominent with PEG treatment (a) (arrows).

3.3. Light dependency of salinity-induced damage in leaf cells

Salinity stress is often observed to affect the shoots more severely in light than in the dark. However, light dependency of salinity-induced damage is difficult to demonstrate. Since salt is mainly transported by the transpiration stream to the shoot, it might be natural that the salt concentration in the shoot is higher and the damage severer in light than in the dark when the transpiration is reduced due to stomatal closure. In addition a long-term experiment on salinity stress in the dark is difficult because plants can not live long in the dark.

Mitsuya et al. [27] developed an *in vitro* culture system of sweet potato, in which plantlets grew almost equally in both light and dark conditions. They examined foliar ultrastructure after 30 days of culture with NaCl. Salinity-induced ultrastructural damage was observed in the cytoplasm in both light and dark conditions. However, the inner membranes of etioplasts in the plants in the dark maintained their ultrastructural integrity while the thylakoids of chloroplasts in plants in the light were severely damaged. Therefore, the degradation of thylakoids by salinity seems dependent on light. However, etioplasts and chloroplasts were compared in this experiment. The possibility of low sensitivity of etioplasts to salinity is not excluded. Salt concentration in the plantlets in the light and dark conditions was not estimated either.

When rice plants were grown on hydroponic culture solutions containing various concentrations of NaCl, the sodium concentration in the shoot was always higher in light than in the dark at the same NaCl concentration (Fig. 6). A similar trend was

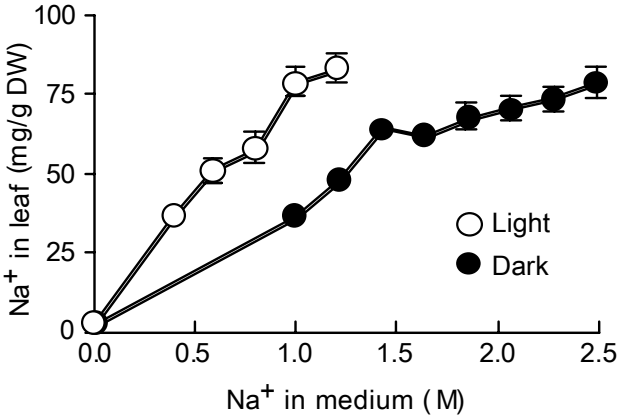

Figure 6. Na content in leaf blades of rice cultured in a solution containing various concentrations of NaCl for 24 h in a light or dark condition.

obtained for chloride concentration. Mitsuya et al. [28] examined the leaf tissues containing various concentrations of Na and Cl from rice plants treated with various concentrations of NaCl for 24 h in light and dark conditions. They found a decrease in the chlorophyll content and the damage to the chloroplasts only in leaf tissues of the plants kept in light. The chlorophyll content was unchanged and the thylakoids appeared intact in the plants grown in the dark even though the tissues contained higher concentrations of Na and Cl than those of the plants grown in light. This demonstrated the light dependency of salinity-induced chloroplast damage.

3.4. Involvement of reactive oxygen species in salinity-induced damage in chloroplast ultrastructure

Involvement of reactive oxygen species is known in a wide range of environmental stresses [29, 30]. The light dependency of salinity-induced chloroplast damage suggests involvement of reactive oxygen species (ROS). Swelling of thylakoids is also observed in the chloroplasts of plants treated with air pollutants and other stresses and has been suggested to be induced by ROS [31]. Hernandez et al. [32] also suggested the involvement of ROS in the damage of the thylakoidal structure by NaCl stress. ROS are produced during photosynthesis even in normal chloroplasts without stress. Figure 7 is a simplified scheme of the process of ROS production in chloroplasts as reviewed by Asada [33]. In the normal photosynthetic electron transport, electrons of H_2O are transported to NADPH via photosystem II (PSII) and photosystem I (PSI). Reducing power in the form of NADPH is used in the Calvin cycle to assimilate carbohydrates. However, under strong light or under some stress conditions or even under normal conditions, excess electrons or overproduction of reducing power produces ROS [33]. Singlet oxygen molecules ($^1O_2$) are produced by excited chlorophyll molecules near the PSII. Superoxide anions (O_2^-) are produced

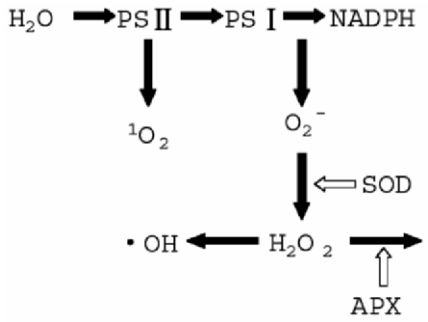

Figure 7. A simplified process of the production of reactive oxygen species. APX, ascorbate peroxidase; SOD, superoxide dismutase.

by electron transfer from the electron acceptors of the PSI to oxygen. Superoxide anions are converted to hydrogen peroxide (H_2O_2) by the reaction of superoxide dismutase (SOD). Hydrogen peroxide is detoxyfied by ascorbate peroxidase (APX) and converted to water by the ascorbate-glutathione cycle. However, the remaining hydrogen peroxide is converted to more toxic hydroxyl radicals ($\cdot OH$) by the Fenton reaction. Under normal conditions, however, ROS are mostly removed by antioxidants and scavenging enzymes.

To investigate the involvement of ROS Yamane et al. [34] examined the effects of antioxidants or scavengers of ROS. If the ROS are involved in salinity-induced chloroplast degradation, scavengers should protect the chloroplast from degradation. The following antioxidants were examined: Tiron (1,2-dihydroxybenzene-3,5-disulfonate), a scavenger of O_2^- ; ascorbate (AsA), a versatile antioxidant reacting with different antioxidants including H_2O_2 and $\cdot OH$; DABCO (1,4-diazabicyclo-[2,2,2]-octane), a scavenger of $^1O_2$; and benzoate, a scavenger of $\cdot OH$. Segments were cut from the middle portion of rice leaves and incubated in 200 mM NaCl solution for 24h in light after 12 h incubation with or without antioxidants. NaCl caused swelling of thylakoids in chloroplasts of segments without antioxidant pretreatment. AsA completely and benzoate partially suppressed the swelling of thylakoids. However, Tiron and DABCO did not suppress the effects of salinity on chloroplast ultrastructure. Therefore, $\cdot OH$ and H_2O_2 seem to be involved in the thylakoid swelling by salinity stress.

Yamane et al. [34] further examined enzyme activities relating to the metabolism of ROS in the leaf segments treated with NaCl. Total activity of superoxide dismutase (SOD) increased considerably by NaCl treatment. Particularly Fe-SOD, which is located in chloroplasts, increased nearly ten times. Ascorbate peroxidase (APX) activity was not different from the control. Catalase (CAT) activity was rather decreased compared with the control.

These results are interpreted as follows. Salinity stress blocks the normal electron flow. Overreduction of the electron transport system increases the production of O_2^-. An increased activity of SOD effectively converts O_2^- into H_2O_2.

However, APX activity is unfortunately not increased to cope with the increased production of H_2O_2. Therefore, the excessive H_2O_2 is converted to more toxic $\cdot OH$ radicals by the Fenton reaction. Thus the salinity-induced ultrastructural changes in chloroplasts seem to be caused by $\cdot OH$ and H_2O_2. At present the mechanism of increased production of ROS by salinity stress is not known. Some researchers suggest that stomatal closure upon salt stress may limit CO_2 supply, which then causes the overreduction of the photosynthetic electron transport system. However, stomatal closure is also a typical plant response to drought stress. Ultrastructural damages in chloroplasts caused by salinity stress are completely different from those caused by drought stress as described above. Although ROS has also been reported to be involved in drought stress [30], the mechanism of the increased production of ROS and the types of ROS involved in salinity stress seem to be different from those involved in drought stress.

3.5. Ameliorative effect of exogenous glycinebetaine on chloroplast ultrastructure

Glycinebetaine is known as a compatible solute produced in plant cells and functions as an osmoprotectant under salinity stress. In addition glycinebetaine has protective effects on various enzymes, membranous structures and organelles [35]. Therefore, glycinebetaine is useful under salinity stress to protect cellular structures and functions. However, rice plants cannot synthesize glycinebetaine [36], therefore they are more sensitive to salinity than those plants that can synthesize and accumulate glycinebetaine.

When rice seedlings were supplied with exogenous glycinebetaine, they accumulated glycinebetaine and showed enhanced salt tolerance [37]. Rahman et al. [38] further examined the effects of exogenous glycinebetaine on the ultrastructure of rice seedlings under salinity stress. Rice seedlings were pretreated with 0 - 10 mM glycinebetaine in hydroponic culture for 3 days and were then treated with 150 mM NaCl for 4 days. Glycinebetaine was not effective in alleviating the salinity-induced inhibition of root growth or rather enhanced the inhibition. On the contrary glycinebetaine at 5 mM effectively alleviated the inhibition of shoot growth and the ultrastructural damages in leaf cells such as swelling of thylakoids in chloroplasts and destruction of mitochondria. However, glycinebetaine could not prevent the vacuolation in root tip cells or rather increased vacuolation. The concentration of Na was significantly lower and the concentration of K was significantly higher in the shoots of the plants pretreated with glycinebetaine than in the plants without application of glycinebetaine under salinity. One of the ameliorative effects of glycinebetaine may be to function in the cytoplasm of roots as an osmoprotectant and to balance the osmotic pressure between the cytoplasm and the vacuole while Na is accumulating in the vacuole. The vacuolation of root tip cells seems to be an adaptive response to store Na and to reduce transportation of Na to the shoot. It is confirmed again that the reduction in root growth under salinity is not lethal to plants as compared with the damages in the shoot.

Makela et al. [21] reported that foliar application of glycinebetaine increased the size of starch grains in chloroplasts of tomato under salinity stress. According to

their interpretation the increase of starch grains seems to be a reflection of increased net photosynthesis and not a result of impaired translocation of photosynthates under salinity.

4. CONCLUSION

Electron microscopic observation is effective to detect the ultrastructural damage in plant cells under stress. It also helps consider the underlying mechanisms of stress effects. The vacuolation of root tip cells and the swelling of thylakoids in chloroplasts are major ultrastructural changes in rice plants under salinity. The vacuolation may be an adaptive response to accumulate Na in root tip cells and to protect the meristematic cells. It may also function to reduce transfer of Na to the shoot. The swelling of thylakoids seems to be a result of photochemical oxidation. Hydoxyl radical and hydrogen peroxide seem to be major ROS causing the thylakoid swelling. Based on these observations, genetic manipulation to reduce ROS, especially hydroxyl radical and hydrogen peroxide, seem to be promising to confer salinity tolerance to rice plants. Chemical treatments and development of cultivation practices to reduce ROS and to increase endogenous antioxidant levels may also be useful. For example the application of cytokinin [39] and the pretreatment with methyl viologen [40] was found effective to reduce the photooxidative damage caused by salinity.

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REFERENCES

1. I. Szabolcs, I. (1994) Soils and salinisation. In *Handbook of Plant and Crop Stress* (Pessarakali, M., ed). New York: Marcel Dekker, pp. 3-11.
2. Munns, R., Cramer, G.R. and Ball, M.C. (1999) *Carbon Dioxide and Environmental Stress* (Luo, L. and Mooney, H.A., eds). Academic Press, London.
3. Flowers, T.J., Garcia, A., Koyama, M. and Yeo, A.R. (1997) Breeding for salt tolerance in crop plants: the role of molecular biology. *Acta Physiol. Plant* **19**, 427-433.
4. Volkmar, K.M., Hu, Y. and Steppuhn, H. (1998) Physiological responses of plants to salinity. *Can. J. Plant Sci.* **78,** 19-27.
5. Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 463-499.
6. Hajibagheri, M.A., Yeo, A.R. and Flowers, T.J. (1985) Salt. tolerance in *Suaeda maritima* (L.) Dum.: Fine structure and ion concentrations in the apical region in roots. *New Phytol*. **99,** 331-343.
7. Rahman, M.S., Matsumuro, T., Miyake, H. and Takeoka, T. (2001) Effects of salinity stress on the seminal root tip ultrastructures of rice seedlings (*Oryza sativa* L.). *Plant Prod. Sci*. **4,** 103-111.
8. Colmer, T.D., Fan, T.W.M., Higashi, R.M. and Lauchli, A. (1994) Interactions of Ca^{2+} and NaCl stress on the ion relations and intracellular pH of *Sorghum bicolor* root tips: an in vivo 31P-NMR study. *J. Exp. Bot*. **45,** 1037-1044.
9. El-Banna, Y. and Attia, T. (1999) Root tip meristematic cell and leaf chloroplast structure in three barley (*H. vulgare* L.) genotypes exposed to salinity stress.*Cytologia*, **64,** 69-76.

10. Koyro, H.W. (1997) Ultrastructural and physiological changes in root cells of *Sorghum* plants (*Sorghum bicolor x S. sudanensis* cv. Sweet Sioux) induced by NaCl. *J. Exp. Bot*. **48,** 693-706.
11. Cachorro, P., Olmos, E., Ortiz, A. and Cerda, A. (1995) Salinity-induced changes in the structure and ultrastructure of bean root cells. *Biol. Plant* **37,** 273-283.
12. Offler, C.E., McCurdy, D.W., Patrick, J.W. and Talbot, M.J. (2003) Transfer cells: cells specialized for a special purpose. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **54,** 431-454.
13. Ali, F., Ibrahim, A.A., Srivastava, P.S. and Iqbal, M. (1999) Structural changes in root and shoot of *Bacopa monniera* in response to salt stress. *J. Plant Biol.* **43,** 222-225.
14. Samarajeewa, P.K., Barrero, R.A., Umeda-Hara, C., Kawai, M. and Uchimiya, H. (1999*)* Cortical cell death, cell proliferation, macromolecular movements and r Tip 1 expression pattern in roots of rice (*Oryza sativa* L.) under NaCl stress *Planta* **207,** 354-361.
15. Ciamporova, M. and Mistrik, I. (1993). The ultrastructural response of root cells to stressful conditions. *Environ. Exp. Bot*. **33,** 11-26.
16. Koyro, H.W., Stelzer, R. and Huchzermeyer, B. (1993*).* ATPase activities and membrane fine structures of rhizodermal cells from *Sorghum* and *Spartina* roots grown under mild salt stress. *Bot. Acta* **106,** 110-119.
17. M.S. Rahman, H. Miyake and Takeoka, Y. (2001) Effect of sodium chloride salinity on seed germination and early seedling growth of rice (*Oryza sativa* L.). *Pakistan J. Biol. Sci*. **4,** 351-355.
18. Rahman, M.S., Matsumuro, T., Miyake, H. and Takeoka, Y. (2000) Salinity-induced ultrastructural alterations in leaf cells of rice (*Oryza sativa* L.). *Plant Prod. Sci*. **3,** 422-429.
19. Munns, R. and Termaat. A. (1986) Whole-plant response to salinity. *Aust. J. Plant Physiol*. **13**, 143-160.
20. Khavari-Nejad, R.A. and Y. Mostofi, Y. (1998) Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. *Photosynthetica,* **35,** 151-154.
21. Makela, P., Karkkainen, J. and Somersalo, S. (2000) Effect of glycine-betaine on chloroplast ultrastructure, chlorophyll and protein content, and RuBPCO activities in tomato grown under drought or salinity *Biol. Plant* **43,** 471-475
22. Sam, O., Ramirez, C., Coronado, M.J., Testillano, P.S. and Risueno, M.C. (2003) Changes in tomato leaves induced by NaCl stress: leaf organization and cell ultrastructure *Biol. Plant* **47,** 361-366.
23. Morales, M.A., Sanchez-Blanco, M.J., Olmos, E., Torrecillas, A. and Alarcon, J.J. (1998) Changes in the growth, leaf water relations and cell ultrastructure in *Argyranthemum coronopifolium* plants under saline conditions. J. *Plant Physiol*. **153,** 174-180.
24. Boughanmi, N., Michonneau, P., Verdus, M.C., Piton, F., Ferjani, E., Bizid, E. and Fleurat-Lessard, P. (2003) Structural changes induced by NaCl in companion and transfer cells of Medicago sativa blades. *Protoplasma* **220,** 179-187.
25. Yamane, K., Kawasaki, M., Taniguchi, M. and Miyake, H.(2003) Differential effect of NaCl and polyethylene glycol on the ultrastructure of chloroplasts in rice seedlings. *J. Plant Physiol*. **160,** 573-575.
26. Yamane, K., Hayakawa, K., Kawasaki, M., Taniguchi, M. and Miyake, H. (2003) Bundle sheath chloroplasts of rice are more sensitive to drought stress than mesophyll chloroplasts. *J. Plant Physiol*. **160,** 1319-1327.
27. Mitsuya, S., Takeoka, Y. and Miyake, H. (2000) Light dependency of salinity-induced chloroplast damages. *J. Plant Physiol*. **157,** 661-667.
28. Mitsuya, S., Kawasaki, M., Taniguchi, M. and Miyake, H. (2003) Light dependency of salinity-induced chloroplast degradation *Plant Prod. Sci.* **6,** 219-223.
29. Blokhina, O., Virolainen, E. and Fagerstedt, K.V. (2003) Antioxidants, oxidative damage and oxygen deprivation stress. *Ann. Bot*. **91,** 179-194.
30. Apel, K. and Hint, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **55,** 373-399.
31. Miyake, H., Furukawa, A., Totsuka, T. and Maeda, E. (1984) Differential effects of ozone and SO2 on the fine structure of spinach leaf cells. *New Phytol*. **96**, 215-228
32. Hernandez, J.A., Olmos, E., Corpas, F.J., Sevilla, F. and Del Rio, L.A. (1995) Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Sci*. **105,** 151-167.
33. Asada, K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 601-639.

34. Yamane, K., Rahman, M.S., Kawasaki, M., Taniguchi, M. and Miyake, H. (2004) Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.). *Plant Prod. Sci.* **7,** 292-300.
35. D. Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. 44 (1993) 357-384.
36. Rathinasabapathi, B., Gage, D.A, Mackill, D.J. and Hanson, A.D. (1993) Cultivated and wild rices do not accumulate glycinebetaine due to deficiencies in two biosynthetic steps. *Crop Sci.* **33,** 534-538.
37. Harinasut, P., Tsutsui, K., Takabe, T., Nakamura, M., Takabe, T. and Kishitani, S. (1996) Exogenous glycine betaine accumulation and increased salt-tolerance in rice seedlings. *Biosci. Biotech. Biochem*. **60,** 366-368.
38. Rahman, M.S., Miyake, H. and Takeoka, Y. (2002) Effects of exogenous glycinebetaine on growth and ultrastructure of salt-stressed rice seedlings (*Oryza sativa* L.). *Plant Prod. Sci*. **5,** 33-44.
39. Mitsuya, S., Kawasaki, M., Taniguchi, M. and Miyake, H. (2003) Relationship between salinity-induced damages and aging in rice leaf tissues. *Plant Prod. Sci*. **6,** 213-218.
40. Yamane, K., Rahman, M.S., Kawasaki, M., Taniguchi, M. and Miyake, H. (2004) Pretreatment with a low concentration of methyl viologen decreases the effects of salt stress on chloroplast ultrastructure in rice leaves (*Oryza sativa* L.). *Plant Prod. Sci*. **7,** 435-441.

SECTION IX

DEVELOPMENT OF BIOTECHNOLOGY

16. GENETIC DIVERSITY OF SALINE COASTAL RICE (*ORYZA SATIVA* L.) LANDRACES OF BANGLADESH

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Abstract. Traditional rice landraces from coastal Bangladesh are adapted not only to soil salinity but also to excess magnesium, calcium and sulphate as well as zinc deficiency. They can also tolerate water inundation to a certain extent, because they may be subjected to tidal saline intrusion as well as water stagnation during the monsoon season. These landraces are diverse with respect to their morphology, saline stress response as well as yield components. Characterization of these landraces can suggest how they survive in adverse soils and indicate suitable target genes for transfer to modern rice varieties. Genetic variation of 31 landraces (LRs) collected from the saline coastal belt of Bangladesh, salt tolerant Pokkali, Nona Bokra and sensitive IR29 and BRRIdhan 29 were analyzed with 60 evenly distributed rice microsatellite DNA markers. A total of 196 reproducible polymorphic alleles were identified from the band loci. Computation of genetic similarity with this data, using Jaccard's coefficient followed by UPGMA clustering, divided the landraces into 6 distinct groups. Three groups were composed of LRs only from the highly saline southwest. Two groups consisted of LRs from the mild to moderately saline mid-east and northeast coasts. The sixth group was heterogeneous, with LRs from the northeast, LRs from the southwest and Nona Bokra. Pokkali and Gunshi, a LR of the southwest, branched out individually. Morphological observations of plant type, such as tiller, leaf and flag leaf angle, height, as well as yield in non-saline soil indicated low variability among the different LRs. The measure of seedling Na and K concentration, Na/K ratios, affected leaf area as well as survival under salinity stress in hydroponics identified 6 LRs from the highly saline southwest as the most tolerant. UPGMA clustering using the Pearson product-moment correlation coefficient suitable for the quantitative physiological data on seedling saline stress, grouped all 6 LRs with Pokkali. When the landrace DNAs were analyzed with DNA markers linked to the major QTL of Pokkali within a 5cM region of chromosome 1, no similarity was detected between any of the traditional cultivars and Pokkali. Primers homologous to the coding region and part of the upstream as well as downstream region of the SOD, LEA and SalT genes when used for amplification show polymorphic DNA bands. These analyses thus revealed that some of the salt tolerant landraces of the coastal region have unique polymorphic loci, quite distinct from the popular salt tolerance donor Pokkali as well as Nona Bokra. The similarity and distinction between the cultivars chosen for the study can be used as a valuable tool for further study of specific landraces and also suggest a proper choice of parents for mapping or breeding purposes.

Ashwani K. Rai and Teruhiro Takabe (eds.), Abiotic Stress Tolerance in Plants, 229-244.

1. INTRODUCTION

Bangladesh needs to increase its rice production by more than 50% in the next 15 years to keep up with its population growth. With its high population density, there is no scope of new agricultural lands being used for the increased production of rice that is needed. One alternative is to use unfavorable land, which remains fallow, for most of the year except for the monsoon season, such as the salt-affected coastal areas in the south of Bangladesh. Salinity tolerance is complex both genetically as well as physiologically. A wide variation in physiological response to salinity stress has been observed in halophytes as well as tolerant glycophytes [1]. Evaluating tolerance is made more complex by variation in sensitivity to salt during the life cycle of the plant. The impact of NaCl varies with its concentration, its rate of accumulation, the presence of other ions, such as K^+, Mg^{2+} and Ca^{2+}, the duration of stress, its time of onset relative to the major stages of plant development, the genotype of the plant and environmental factors such as temperature, humidity and light intensity. A rapidly transpiring rice plant can change its leaf water ten times an hour in daylight [2], which is 1000% of its body weight. The rice plant has to replace this water from a soil solution over which it has no control. In case it encounters saline water, tolerance depends on the plant conserving a defined micro-environment in the cytoplasm, regulated in respect to both the quantity and quality of inorganic ions. Study of the popular rice landraces growing in the saline coast may identify target traits for incorporation into commercial varieties.

During progressive salt stress, three related problems are encountered, appearing one after the other: water deficit, ion toxicity and nutrient deficiency. Active water uptake into the xylem of plants is through the transcellular and symplastic pathways, driven by the difference in osmotic potential across the plasma membranes of root cells. Once in the xylem, the water flow is controlled by the leaf stomata aperture and the difference in vapor pressure between the intercellular spaces and air [3]. A third passive water uptake system is the apoplastic pathway, where water molecules move in the intercellular spaces until hydrophobic barriers such as Casparian strips block their progress. If the osmotic potential of saline water balances the osmotic potential of the cytosol, net water uptake by the transcellular and symplastic pathways cease [3]. Salt-treated roots produce ABA, which is transported to the leaves via the xylem and leads to stomatal closure. This response is crucial to the survival of the plant, because it can minimize the water deficit, caused by reduction in net water uptake. Plants may wilt at this time, which is countered by accumulation of osmotically active solutes such as glycine betaine and proline [4, 5] .

The ABA-induced closure of stomata prevents CO_2 uptake for photosynthesis. Since the leaf continues to absorb light, photons carry on driving photosynthetic electron transport. The lack of an ultimate electron acceptor causes the generation of active oxygen species when the products of the light reactions (electrons) and oxygen meet. The major product is superoxide O_2^- [6]. Superoxide dismutase converts superoxide to H_2O_2 which can be converted non-enzymatically into the hydroxyl radical $OH^{\cdot}$ which, can cause extensive damage to all cellular components [7]. H_2O_2 is detoxified by catalases and peroxidases and the reducing power of the electrons generated due to photosynthesis are used up for this purpose via several

intermediates [3]. Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) acts as an oxygenase when there is lack of CO_2 due to stomatal closure. In this case the photosynthetic reducing power is used for the production of ammonium ions. Increased production of glutamine synthetase, therefore takes care of the ammonium ions and enhances stress tolerance [8].

In order to survive the water deficit, water uptake by the symplastic and transcellular pathways has to resume in salt tolerant plants, even if partially. One of the main changes is the accumulation of Na^+ and Cl^- ions in the vacuoles of root cells, while K^+ ions accumulate in the cytosol. This differential compartmentation is done by ion selective transporters and channels, which remains incompletely understood. At low external concentrations, potassium may enter roots through K carriers, while at higher concentrations, non-selective cation channels are probably involved [9]. Channels that are activated when the transmembrane potential is hyperpolarized are highly selective for K^+. Other channels, which are activated when the membrane potential is depolarized are less selective and can allow Na^+ entry into cells [10]. Na^+ can also enter via the KUP/HAK/KT (K^+ uptake permease/high affinity K^+/K^+) transporters, cyclic-nucleotide-gated channels, LCT (low affinity cation) transporters and HKT (high affinity potassium) transporters but there is a big variation in the roles of each of these transporters in different species [10, 11]. HKT1 plays a role in net Na^+ accumulation in wheat [12] and into the distribution of ions between roots and shoots of Arabidopsis [13]. Rice homologues of the HKT transporter have shown differential shut down of expression in presence of Na^+ in the salt tolerant and sensitive rice varieties, Pokkali and IR29 respectively. Two HKT homologues have been found in Pokkali with a predicted amino acid identity of 91% to each other. One homologue OsHKT1 appears to be a Na^+ transporter, while OsHKT2, a Na^+-K^+ coupled transporter. Halophytes have a superior capacity to compartmentalize K^+ ions in the cytosol and Na^+ ions in the vacuole of older leaves [14]. Salt tolerant glycophytes such as rice have also been shown to efficiently partition Na^+ away from photosynthesizing leaves as well as the flag leaf [15]. Vacuolar Na^+/H^+ antiporters are responsible for compartmentalization of Na^+ into the vacuoles of older leaves and are thought to play an important role in salt tolerance [16].

One million hectares of the coastal area of Bangladesh is affected by mild to moderate salinity. The variation in soil salinity levels is dependent on the season as well as the location of rivers flowing into the basin from the north and into the Bay of Bengal (Fig. 1). In general, coastal salinity levels start gradually increasing from November at the beginning of the dry season and peaks in March until start of the annual rains, from April till October. The Sundarban forest area spans one-third of the southern portion of 3 districts in Bangladesh and is known to be highly saline. From the west to east, these are respectively, Satkhira, Khulna and Bagerhat. Salinity levels gradually decline from west to east; Satkhira being highly saline with

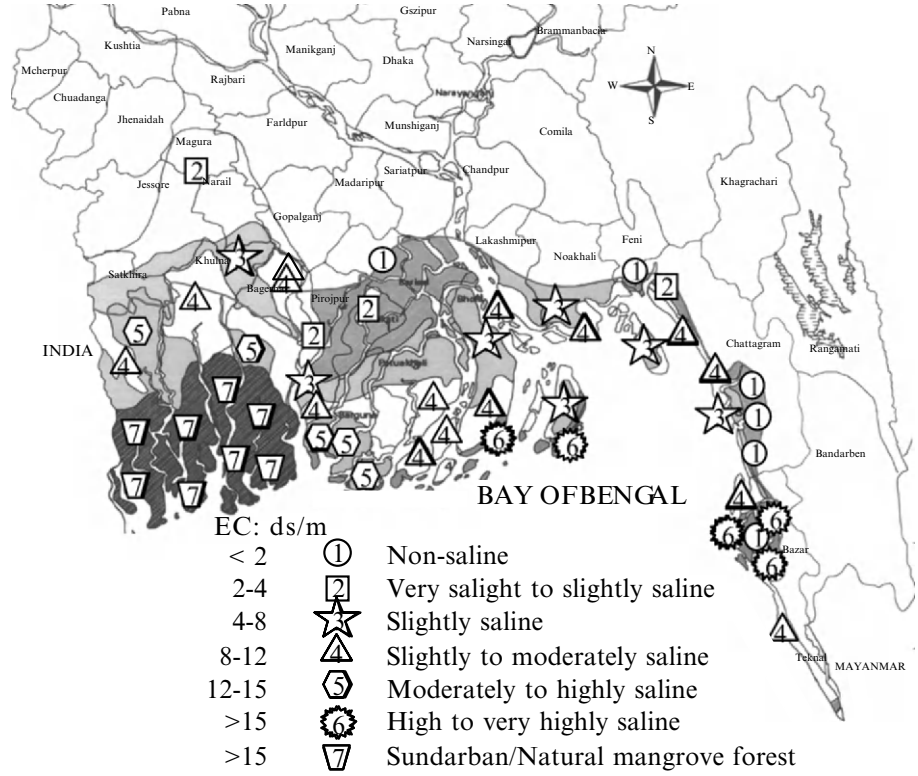

Figure 1. Map of coastal area of Bangladesh affected by salinity. Electrical conductivity of different districts is color-coded as well as numbered. Source: Soil Research Development Institute, SRDI, Bangladesh.

70% of the area having soil salinity levels of 4-16 dS/m [17]. Further eastwards, there are the coastal regions of Patuakhali, Barguna, Barisal and Pirojpur. These areas are slightly saline (2-4 dS/m), with some pockets being non-saline. The coastline extends north-eastwards, where there are the districts of Lakshmipur, Noakhali and Feni. The salinity in Noakhali is moderate (4-8 dS/m), and extends to larger areas compared to Lakshmipur and Feni. The coastal line moves southwards into Chittagong and Cox's Bazaar, where there are pockets of high salinity as well as mild levels [17]. In the moderate to highly saline southwest coastal areas, farmers can only grow a single rice crop during the monsoon season when the salinity levels are relatively low. In the rainfed lowlands of Satkhira and Khulna in the southwest, landraces still account for 80% of the top 20 rice varieties, despite introduction of modern varieties [18]. Soil salinity in this region can be moderate even during the monsoon season, if there is lower than average or early rainfall. The popular landraces of this region are therefore well adapted to the prevailing soil stresses, including soil salinity. In the slight to moderate saline northeastern coast, farmers

grow Aus rice, which is planted in the dry season in late March, when soil and canal water salinity levels are known to be moderate [19]. Landraces in the eastern coast of Noakhali and Feni form 60% of the top 20 varieties grown there in the dry season [18]. These varieties should therefore possess some resistance to salt stress, particularly at the seedling stage.

Bangladesh coastal areas are affected by both tidal saline intrusion as well as water stagnation because of lowlands in the monsoon season (July to October), upward or lateral movement of saline ground water during the dry season (November to May) and willful inundation with brackish water for shrimp cultivation [17]. Soil profiles in the coastal areas of Bangladesh have an excess of Magnesium, Calcium, and Sulphate and are generally deficient in Zinc [17, 19, 20]. Characterization of the landraces adapted to the variable nature of the saline coastal region of Bangladesh with respect to any of the different mechanisms used to combat saline stress will help identify useful donors for these traits. These can then be used to transfer to modern varieties. Study of the mechanism of stress tolerance in landraces can also indicate whether the trait can be separated from yield components in order to attempt improvement in their yields. Any improved rice variety will make major impacts in the livelihoods of the resource-poor farmers of the region, since it has been shown that introduction of modern rice varieties resulted in the production of staple food for 46% of the Bangladesh population between the years 1985-1997 [21].

This chapter reports on the morphological assessment of 31 rice (*O. sativa*) landraces collected from the Bangladesh coast. Traits noted were plant height, days to maturity, panicle length and type, tiller, leaf and flag leaf angle, and yield components under normal soil. Response to salinity stress at the seedling stage were evaluated by Na^+ and K^+ concentrations, ratio and leaf damage area [20]. The DNA from the landraces were further characterized by scoring polymorphism in simple sequence repeats or SSR markers evenly distributed over the 12 chromosomes [22, 23]. DNA markers homologous to proteins involved in detoxification under salt stress such as superoxide dismutase (AB026724) was also used. In addition DNA markers homologous to three genes important in abiotic stress tolerance were used, for example, SalT (Z25811), the Ca^{2+}-binding protein, calmodulin (calmod. Z12828) as well as LEA3 (AF046884) [24]. Calmodulin is part of the network of signal transduction pathways centered on calcium ions as second messengers in stress tolerance [25]. LEA3, a late embryogenesis abundant protein 3 accumulates in cells to protect them against ionic changes accompanying various stresses [26, 27]. SalT accumulates in rice leaf sheaths and roots in response to salt and drought but its role is not clear [28, 29, 30]. The DNA from the traditional landraces were also checked for the presence or absence of markers linked to the major salinity tolerance QTL, called Saltol of Pokkali within a 5cM region of chromosome 1 [31]. Only markers, which were polymorphic between IR29 and Pokkali were used.

2. RELATEDNESS OF TRADITIONAL LANDRACES BASED ON MORPHOLOGICAL CHARACTERS

Morphological traits of the 31 traditional landraces were recorded in normal soil conditions at the BRRI station fields. The yield components, panicles per plant, spikelets per panicle, percent fertile grains and 1000 grain-weight were published earlier [20]. Data on panicles with respect to type, secondary branching, exsertion as well as spikelet properties of the traditional landraces collected following IRRI recommendations [32] showed considerable variability (data not shown). Table 1 shows data on days to maturity, height, tiller number, leaf, tiller and flag leaf angle and yields of landraces in normal soil (average of 10 randomly chosen hills). These data were collected according to the description by Chang and Bardenas [33].

Table 1. Days to maturity, height, tiller number with panicles (eff.), without panicles (non eff.), leaf, tiller and flag leaf angle and yields of landraces in normal soil (average of 10 randomly chosen hills).

Sl. no.	Cultivars	Life cycle (days)	Plant height (cm)	No. of Tillers			Leaf angle	Tiller angle	Flag leaf angle	Yield gm/plant
				Eff.	Non eff.	Total				
1	Binnatoa	130	116.4	12.1	0.3	12.4	33.7	31.2		5.0
2	Boilam	120	113.0	15.6	0	15.6	28.9	40.5	43.2	-
3	Gheegoj	152	131.8	10.1	0.2	10.3	30.7	20.9	24.8	27.6
4	Kajalshail	150	127.1	12.3	0.4	12.7	19.1	21.0	63.5	20.9
5	Madhumalati	143	137.5	14.8	0.2	15.0	23.5	27.3	19.9	17.8
6	Nonashail	139	128.4	11.7	0.3	12.0	22.7	31.0	24.8	
7	Gunshi	154	148.9	8.3	0.3	8.6	30.3	40.0	87.5	-
8	Kaliboro	121	108.9	24.4	0.7	25.1	30.1	22.6	67.0	-
9	Soloi	126	115.1	13.4	0.1	13.5	28.0	32.6	30.3	-
10	Khaiyaboro	127	122.6	12.7	0.6	13.3	22.0	36.8	63.4	-
11	Hida	145	143.4	12.3	0.4	12.7	27.1	32.1	44.0	-
12	Razashail	128		10.8	0.3	11.1	24.0	20.5	25.3	6.8
13	Ashfal	162	143.3	8.3	0.3	8.6	27.7	19.6	38.2	16.0
14	Benapole	143	146.6	10.8	0.2	11.0	26.9	26.9	27.0	10.1
15	Dakshail	145	146.6	10.4	0.3	10.7	34.6	20.3	27.0	6.8
16	Hoglapata	153	135.7	12.7	0.3	13.0	33.6	17.5	33.3	22.0
17	Horkuch	158	145.7	9.2	0.2	9.4	41.7	22.5	107.5	19.5
18	Jamainaru	158	117.1	12.3	0.4	12.7	19.1	21.0	16.7	28.7
19	Kachra	156	144.3	11.6	0.1	11.7	37.5	20.3	62.0	24.3
20	Kalmilata	142	143.7	10.6	0.2	10.8	36.1	19.9	98.2	19.8
21	Lakshmikajal	156	136.9	10.9	0.1	11.0	30.1	11.0	86.9	22.5
22	Mohini	142	135.0	8.7	0.3	9.0	32.2	18.5	92.5	18.7
23	Morichshail	150	136.2	8.2	0.2	8.4	36.9	12.0	105.0	28.1
24	Patnai Balam	148	133.5	9.4	0.2	9.6	33.4	21.0	37.0	24.3
25	Raniselute	152	138.2	11.0	0.2	11.2	29.8	16.8	62.0	20.9
26	Capsule	140	142	12.1	0.1	12.2	36.1	20.6	107.4	7.6
27	Chinikanai	141	137.5	8.3	0.1	8.4	33.1	19.2	100.8	6.9
28	Jatai Balam	142	141.4	11.0	0.1	11.1	32.2	31.8	55.5	35.7
29	Moynamati	135	133.6	11.0	0.1	11.1	30.6	29.4	50.0	13.1
30	Pokkali	121	157.2	6.3	0.3	6.6	22.3	35.0	14.0	15.1
31	BRRIdhan40		127.9	11.0	0	11.0	30.8	32.2	24.4	31.7

*Yield was calculated from yield components obtained from our previous publication, Lisa et al. [20].

Table 2. Percent survival, area leaf affected, shoot and root reduction in height, shoot sodium and potassium concentration and ratio in hydroponically- grown 45 day-old seedlings of 31 landraces, 27 days after saline stress application at 12dS/m.

Sl. no.	Variety name	Origin	Percent survival	Percent leaf area affected	Percent shoot length reduction	Percent leaf Na at 12 dS/m	Percent leaf K at 12 dS/m	Shoot Na/K ratio at 12 dS/m
1*	Binnatoa	Noakhali, MNE2	60.0 c-i	50.0 e-j	9.5 abc	1.98 a	1.35 bc	1.47 a-d
2*	Boilam 3538	Noakhali, MNE2	20.0 kl	80.0 m-q	25.5 ijk	2.78 a-h	1.28 c	2.00 a-d
3	Gheegoj 2413	Noakhali, Het	33.3 jk	70.0 k-o	21.0 f-j	3.03 a-i	1.98 abc	2.06 a-d
4	Kajalshail 612	Noakhali, Het	66.7 b-g	40.0 b-g	9.5 abc	2.96 a-i	2.20 abc	1.4 a-d
5	Madhumalati 614	Noakhali, Het	15.0 kl	85.0 opq	21.8 g-j	3.24 c-i	1.94 abc	1.69 a-d
6	Nonashail	Noakhali, Het	8.3 l	86.7 opq	33.5 l	3.27 d-i	1.73 abc	2.52 de
7	Gunshi 3869	Barisal	63.3 b-h	40.0 b-g	16.5 c-h	3.06 a-i	2.28 abc	1.35 a-d
8*	Kaliboro 1281	Faridpur[1], MNE1	18.33 kl	81.7 n-q	24.7 ijk	3.56 ghi	1.63 abc	1.81 a-d
9*	Soloi 1713	Faridpur[1], MNE1	10.0 l	91.7 pq	31.2 kl	3.29 d-i	1.58 abc	2.16 bcd
10*	Khaiyaboro 4539	Habigonj[1], MNE2	40.0 ij	76.7 l-p	28.1 f-j	3.46 e-i	1.89 abc	1.79 a-d
11	Hida	Rajshahi, Het	-	-	-	-	-	-
12	Rajashail 1061	Narail[1], Het	20.0 kl	83.3 n-q	20.0 e-j	3.50 f-i	1.77 abc	2.28 cd
13	Ashfal	Khulna, SW2	80.0 abc	26.7 abc	6.9 ab	2.39 a-e	2.67 a	0.91 ab
14	Benapole	Khulna, SW1	76.7 a-d	30.0 a-d	4.7 a	2.49 a-g	2.47 abc	1.03 abc
15	Bajramuri	Khulna, SW3	-	-	-	-	-	-
16	Dakshail	Khulna, SW3	46.7 g-j	56.7 g-k	8.3 abc	3.11 b-i	1.82 abc	1.85 a-d
17	Hoglapata	Khulna, SW1	56.7 d-i	43.3 c-h	10.3 a-d	3.03 a-i	2.01 abc	1.66 a-d
18	Horkuch	Khulna, SW1	70.0 a-f	33.3 a-e	7.4 ab	2.59 a-h	2.61 ab	1.05 abc
19	Jamainaru2039	Khulna, Het	80.0 abc	23.3 ab	9.0 abc	2.17 abc	2.68 a	0.81 a
20	Kachra 973	Khulna, Het	60.0 c-i	46.7 d-i	11.4 a-d	3.08 b-i	2.16 abc	1.5 a-d
21	Kalmilata	Khulna, SW2	63.3 b-h	40.0 b-g	10.7 a-d	2.71 a-h	2.37 abc	1.17 abc
22	Lakshmikajol	Khulna, SW3	73.3 a-e	36.7 b-f	7.5 ab	2.81a-h	2.45 abc	1.18 abc
23	Mohini	Khulna, SW1	43.33 hij	63.3 i-m	13.02 a-f	3.41 e-i	1.63 abc	2.25 cd
24	Morichshail	Khulna, SW1	63.3 b-h	40.0 b-g	10.5 a-d	2.97 a-i	2.26 abc	1.4 a-d
25	Patnai Balam	Khulna, Het	70.0 a-f	30.0 a-d	8.3 abc	2.387 a-e	2.66 a	0.89 ab
26	Raniselute	Khulna, SW1	50.0 f-j	40.0 b-g	9.2 abc	3.32 e-i	2.04 abc	1.82 a-d
27	Capsule	Satkhira, SW2	83.3 ab	26.7 abc	13.3 b-f	2.20 a-d	2.56 abc	0.86 a
28	Chinikanai	Satkhira, SW1	16.7 kl	86.7 opq	24.3 h-k	2.83 a-i	1.83 abc	1.65 a-d
29	Dhulubeej	Satkhira, SW2	60.0 c-i	40.0 b-g	13.0 a-f	2.15 ab	1.81 abc	1.19 abc
30	Jatai Balam	Satkhira, SW2	53.3 e-j	50.0 e-j	12.5 a-e	2.47 a-f	2.51 abc	1.12 abc
31	Moynamoti	Satkhira, SW2	60.0 c-i	43.3 c-h	11.0 a-d	3.07 b-i	2.15 abc	1.48 a-d
32	Pokkali	Sri Lanka	90.0 a	16.0 a	7.8 ab	2.19 abc	2.2 abc	1.03 abc
33	BRRIdhan29	MV	5.7 l	96.0 q	14.4 b-g	3.90 i	1.48 abc	3.40 ef

Cultivars marked with an asterisk (1-8) are photoperiod insensitive. Means followed by a common letter are not significantly different at the 5% level by DMRT.

Gheegoj, Jamainaru and BRRIdhan 40 were found to be similar in agronomic characteristics, while the others were grouped separately, if clustered into a dendrogram (not shown). However the coefficient of dissimilarity was small. BRRIdhan 40 is a photoperiod sensitive salt tolerant modern variety released by

BRRI. However its plant type is intermediate, when compared to standard modern varieties. Therefore, Gheegoj and Jamainaru also have intermediate plant type. Salt tolerance of Jamainaru is close to Pokkali, whereas Gheegoj was found to be moderate [20]. See below and Table 2.

3. ASSESSMENT OF RESPONSE TO SALT STRESS APPLICATION AT THE SEEDLING STAGE

Evaluation of physiological response under seedling saline stress (12 dS/m) identified 6 landraces whose performance was not significantly different from Pokkali, the benchmark, or reference cultivar for assessment of tolerance [20]. These cultivars are Ashfal, Benapole, Jamainaru, Lakshmikajol, Patnai Balam and Horkuch, all farmer popular landraces of the Khulna region (Table 2). Data on percent survival, leaf area affected, reduction in shoot height, Na and K concentration as well as ratio, grouped these six cultivars with Pokkali in a dendrogram based on computation of similarity using the Pearson's product moment coefficient using the NTSYS-PC version 2.11f (Fig. 2) [34] as described earlier [20]. All the cultivars that grouped with Pokkali had lower Na/K scores (0.81-1.05) while that of Pokkali was 1.03. Low Na/K ratios at the seedling stage are associated with tolerance due to Na exclusion as well as partitioning of Na into older leaves [15, 35]. The capacity of plants to maintain low Na/K ratio has been correlated with salt tolerance scores as reviewed by Maathuis and Amtmann [36] and Tester and Davenport [11]. Under IRRI's standard system of evaluation [37], all 6 landraces scored between 3-5, which was marked according to the percent leaf area affected after about a 25-day period of salt stress and when the age of the seedlings was 45 days. Under these conditions, the tolerant check, Pokkali scored 3 and the sensitive check BRRIdhan 29 scored 9. We found the correlation between Na/K ratios and either percent leaf area affected ($r^2 = 0.69$) or tolerance score ($r^2 = 0.63$), to be highly significant for all the landraces. Sodium concentrations were expressed as percent of dry weight and these were found to be higher than reported values for Pokkali [38], so that our Na/K ratios were proportionately higher for all cultivars. The higher concentration of Na can be explained by the fact that the sensitive control BRRIdhan 29 needs 5-7 days longer to score 9 compared to IR29, the sensitive standard used by IRRI. Since BRRIdhan 29 is a farmer-accepted cultivar in the non-saline coastal region, we chose it as the sensitive check.

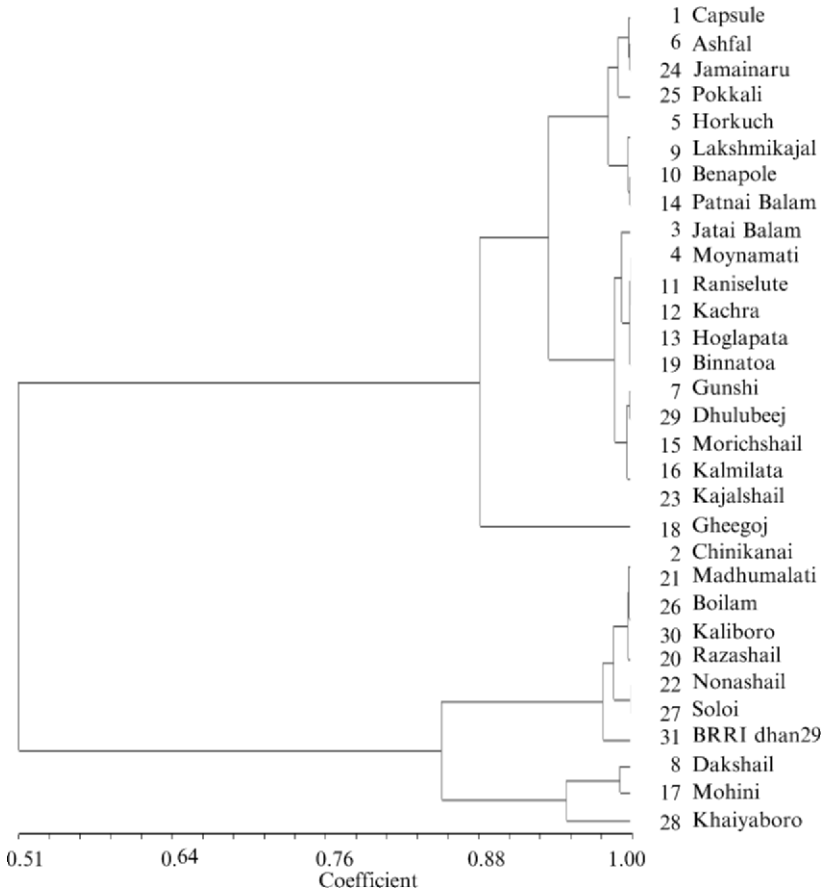

Figure 2. Grouping of LRs based on data obtained after salt stress to seedlings in hydroponics. The data included percent survival, leaf area affected, reduction in shoot height, Na and K concentration as well as ratio.

4. SHARED SSR ALLELES

A total of 196 reproducible polymorphic bands or alleles were identified using 60 microsatellite (SSR) primers [22, 23] after amplification of the DNA from the 35 rice genotypes. The 60 microsatellite primers were evenly distributed over the 12 rice chromosomes as described in detail previously [20]. Genetic relatedness between the 31 LRs as well as the traditional standards, Pokkali, Nona Bokra and sensitive IR29 and BRRIdhan 29 based on their shared SSR alleles was computed using Jaccard's coefficient followed by clustering into a dendrogram [39] (Fig. 3)

after analysis of the data using the NTSYS-PC software [20]. Six groups are apparent, at the coefficient value of 0.366. Three are named SW1, SW2 and SW3,

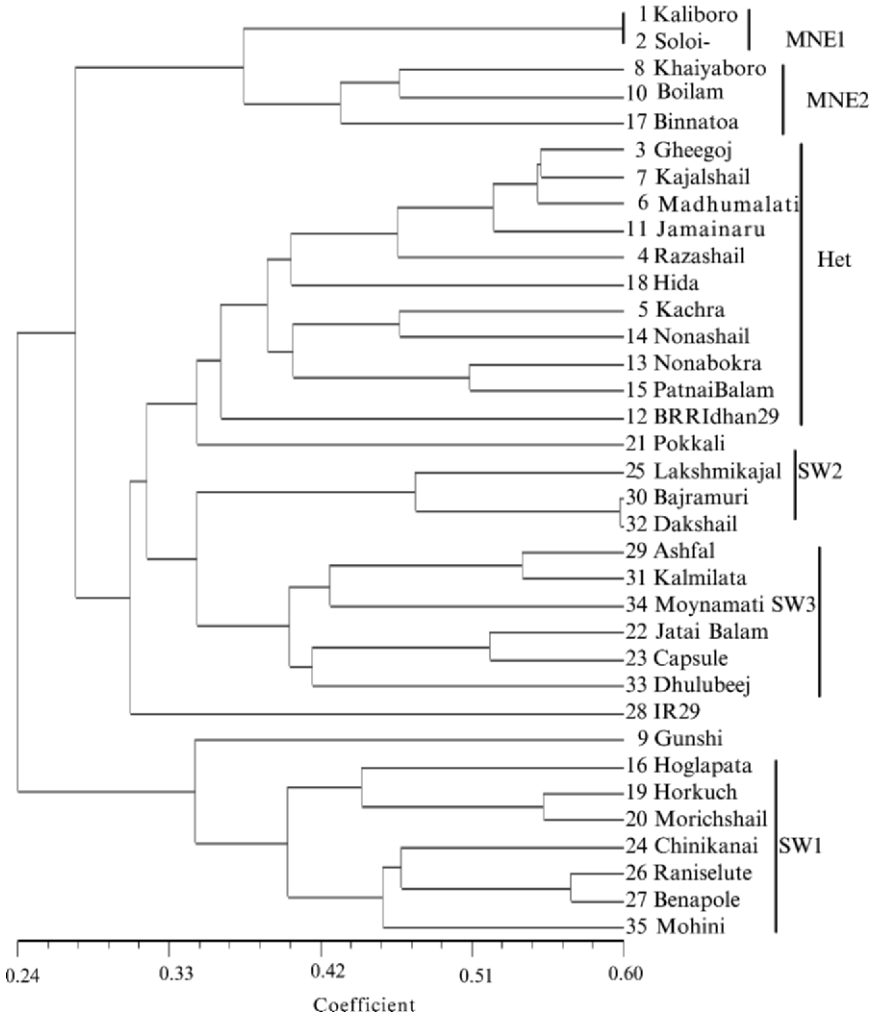

Figure 3. Grouping of LRs based on shared bands (alleles) after amplification of their DNA with microsatellite primers.

all of which contain photoperiod sensitive landraces from the highly saline southwest coastal regions of Satkhira and Khulna. Two more groups, called MNE1 and MNE2 have photoperiod insensitive landraces from the mildly saline coast of the mid northeast, Noakhali, as well as non-saline Faridpur and Habigonj, further

north from the above coastal region. The 6th group of the dendrogram consists of landraces from all the different coastal regions, which include 3 landraces from the southwest, 4 from the mid-northeast, 2 from non-coastal areas as well as Nona Bokra. The internationally well-known standard for saline tolerant rice, Pokkali as well as Gunshi, a landrace grown in the mildly saline Barisal (Table 2), did not group with any of the 30 other landraces. Salt sensitive IR29 and BRRIdhan 29 (BRRI-released modern variety) also show independent lineage. Comparison of the matrix of similarity generated with the cophenetic matrix of the dendrogram showed a good fit ($r = 0.79$), indicating the reliability of the groups within the tree diagram (Fig. 3).

Farmer's preference for these above landraces, despite introduction of modern varieties [18] suggests greater adaptability of these landraces in the variable coastal environment, which includes unpredictable rainfall, variable water stagnation in low lands, depending upon the season, sea-water intrusion due to upstream withdrawal of water, again depending upon the amount of rainfall in the upper riparian areas in our neighboring country. The landraces are also adapted to the ion toxicities as well as deficiencies of the coastal soils. Computation of the relatedness of the landraces based on their SSR alleles, separated cultivars growing in the southwest (SW1, SW2 and SW3) from those in the mid-northeast (MNE1 and MNE2). Due to lower levels of saline stress in the mid-northeast, farmers grow Aus or dry season photoperiod insensitive varieties, which are seeded in April. Some of these cultivars are also popular in regions north from the coast (Table 2). These are the ones forming the groups MNE1 and MNE2. Therefore genetic variation relates to adaptability of the cultivars in distinct coastal regions. The southwest coastal soil has a higher amount of Ca, K and Fe compared to the mid-northeast, while its sulfur toxicity less [20]. Its P and organic content is higher and drainage poor due to prevalence of clayey soil, in contrast to poor organic matter and clay loam soil in the mid-northeast.

5. POLYMORPHISM IN LANDRACES WITH RESPECT TO ABIOTIC STRESS PROTEINS

There was no difference between amplified products from the traditional landraces when primers homologous to the calmodulin gene were used. However amplified DNA from primers homologous to LEA3 and SalT showed distinct polymorphism between Pokkali, sensitive IR29 and BRRIdhan 29 as well as the other landraces (Table 3). Primers homologous to SOD gave amplification of polymorphic DNA between Pokkali, IR29 and most LRs. However BRRIdhan 29 and Pokkali were not polymorphic (Table 3). In the case of LEA3, the undigested product between Pokkali and BRRIdhan29 were identical but IR29 was different. However when the amplified DNA were digested either with *Taq* I or *Hae* III, all the three were clearly polymorphic. Among the traditional landraces, at least 4 different patterns of digested DNA polymorphic to Pokkali were obtained. Moons and coworkers have reported the existence of a small OsLEA3 gene family [40].

Table 3. List of primers homologous to genes/chromosomal location used to check polymorphism in rice landraces.

Chromosomal location/ gene type	Primer locus	Primer sequence	Tm and expected product size	Polymorphic status with Pokkali in specific gel type	Comments
Abiotic stress tolerance	SOD AB026724	F atgcaagccatcctcgc R ctacaacggggtcagcccaa	50.5°C, 636bp	+ PAGE**	10 LRs, Nona Bokra & sensitive BRRIdhan 29 same as Pokkali
	SalT 5'3'*	F ccacgaagactatgacgctggtg R ctttgaccactgggaatcaagg	55°C, 574bp	+ PAGE	3 LRs and Nona Bokra same as Pokkali
	LEA3 5'3'*	F gcttaggatcaatggcttcccacc R ccaaagggaaatcattcacggcgtc	58°C 941bp	+ PAGE	
	Calmod 5'3'*	F cgcgcgcgcctgcgtcgccaatgg R cgatgcttcaacttacttggcc	55°C, 1254bp	- agarose	
Chromosome 1 Markers 60.6-71.2cM	AP003206 60.6cM	F ttctcatcgcaccatctctg R ggaggaggaggaggaagaag	61°C, 375bp	- sequence	All LRs same as Pokkali sensitive BRRIdhan 29 different
	RM3412 62.5cM	F aaagcaggttttcctcctcc R cccatgtgcaatgtgtcttc	55°C, 211bp	+ PAGE	
	AP003722 63.9cM	F cggctcttcctccattgttc R ttgaccgaattacccccctct	60°C, 301bp	+ sequence	
	CP06224 64.4cM	F gtagcatgcaaccgtggtag R tactgctgctacccttgttc	55°C, 170bp	+ PAGE	
	RM140 64.9cM	F tgcctcttccctggctcccctg R ggcatgccgaatgaaatgcatg	55°C, 261bp	+ sequence	
	RM493 64.9	F tagctccaacaggatcgacc R gtacgtaaacgcggaaggtg	55°C, 211bp	+ PAGE	
	AP003211 65.4cM	F gtgtcatcgtgtccttgtgc R cgtcaaaacagaatgagtcca	55°C, 216bp	+ agarose	18 LRs, BRRIdhan 29 and Nona Bokra same as Pokkali
	AP004358 66.6cM	F gagggagggttgatgatagg R aataaaaccggcactcttga	57°C, 385bp	+ PAGE	
	AP002869 67.6cM	F gccttctctaccagcctcct R aagggagcagtggtgacttg	57°C, 233bp	+ sequence	
	CP10136 71.2cM	F gctctacaatggtttgtgag R gaggttatcaggtagaacgc	55°C, 170bp	+ PAGE	

* Primers were located in the 5'-and 3'- untranslated regions immediately outside the coding region of the genes and therefore amplify the entire coding region as well as any intron [24]. **PAGE: polyacrylamide gel electrophoresis.

6. POLYMORPHISM IN DNA MARKERS LINKED TO THE MAJOR SALT TOLERANT LOCUS IN POKKALI

Working with recombinant inbred lines between Pokkali and IR29 [41] we have linked the major QTLs for Na^+ and K^+ concentration as well as Na^+/K^+ to a 5cM region of chromosome 1 [31]. Primers within 1 cM distance of each other (Table 3) within this region were used to amplify DNA from the traditional landraces as well as Pokkali. All the traditional landraces were polymorphic with respect to the molecular weight of the bands obtained when compared to Pokkali. A dendrogram based on Jaccard's coefficient for qualitative data [39], clustered the traditional

landraces into 6 groups (Fig. 4). Pokkali was totally separated at a coefficient value of 0.88. Therefore it seems apparent that the major QTLs responsible for salt tolerance of Pokkali are not shared by any of the traditional landraces of coastal Bangladesh.

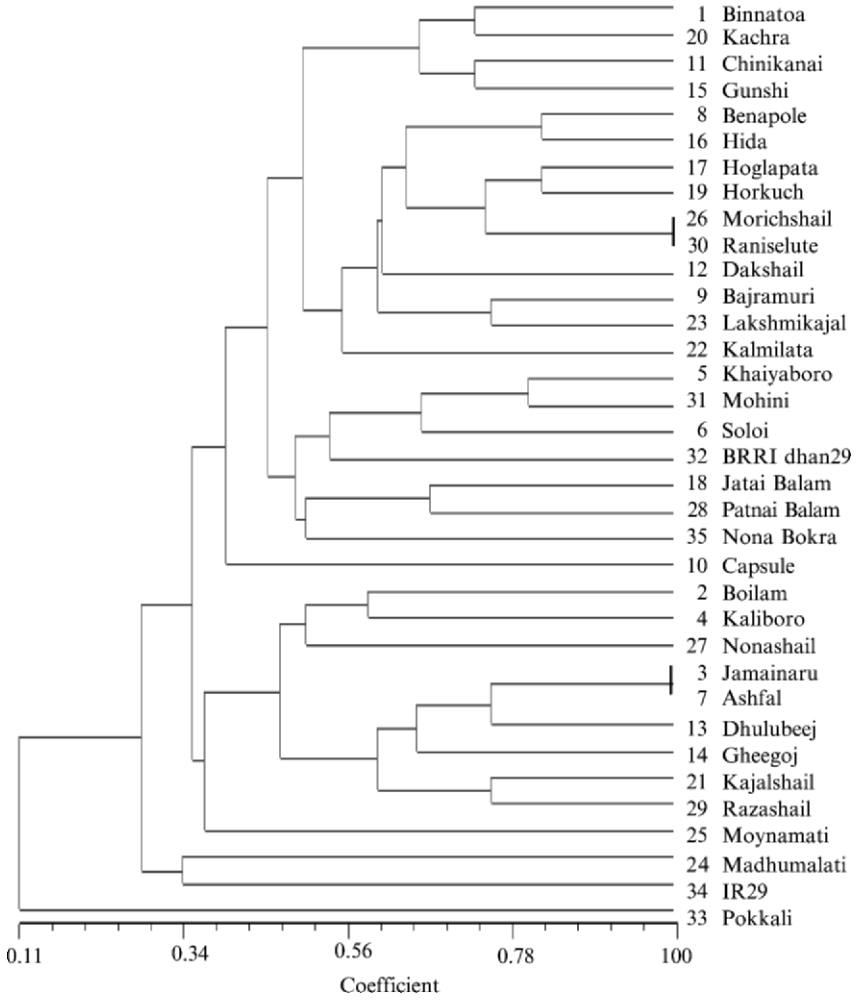

Figure 4. Grouping of LRs based on their polymorphism with Pokkali DNA using primers homologous to a 5 cM region of chromosome 1 which was shown to be linked to the major salinity tolerance QTL of Pokkali (see text for explanations).

7. CONCLUSION

The LRs, Ashfal, Benapole, Jamainaru, Patnai Balam have good salinity tolerance scores and possess intermediate plant type. The LRs, Hoglapata, Kajalshail and Raniselute have moderate tolerance scores and intermediate plant type. Their genetic background based on SSR alleles is different from the traditional donor Pokkali and originates from 3 different groups (Fig. 3). There is polymorphism between these LRs and Pokkali with respect to proteins involved in abiotic stress such as LEA3, SalT and SOD. Although these LRs show low Na/K ratio as well as low Na concentrations and low leaf damage when subjected to salt stress, these do not share QTLs with Pokkali responsible for low Na/K and low Na concentrations (Fig. 4). It will be worthwhile to study the physiology of these LRs further under saline stress up to maturity along with using them as donors in breeding programs in order to widen the genetic basis of salt tolerance in existing modern rice varieties

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REFERENCES

1. Flowers, T.J. (2004) Improving crop salt tolerance. *J. Exp. Bot*. **55,** 307-319.
2. Yeo, A.R., Flowers, T.J., Ragab, R. and Pearce, G.R. (1997) The role of crop salt tolerance in the development of an integrated management plan for irrigation with saline water. In: *Water Management, Salinity and Pollution Control Towards Sustainable Irrigation in the Mediterranean Region.* (Hamdy, A., ed). Paris: Centre International de Haute Etudes Agromiquest Meditteraneennes, Volume IV, pp. 19-35.
3. Bennett, J. and Khush, G.S. (2003) Enhancing salt tolerance in crops through molecular breeding: a new strategy. *J. Crop Prod*. **7,** 11-65.
4. Jagendorf, A.T. and T. Takabe, T. (2001) Inducers of glycinebetaine synthesis in barley. *Plant Physiol*. **127,** 1827-1835.
5. Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C. and Szabados, L. (1997) Differential expression of two P5CS genes controlling proline accumulation during salt stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in *Arabidopsis*. *Plant J.* **12,** 557-569.
6. Badger, M.R., von Caemmerer, S., Ruuska, S. and Nakano, H. (2000) Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and rubisco oxygenase. *Phil. Trans. R. Soc. Lond. B Biol. Sci*. **355,** 1433-1446.
7. Ishida, H., Makino, A. and Mae, T. (1999) Fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase by reactive oxygen species occurs near gly-329. *J. Biol. Chem.* **274,** 5222.-5226.
8. Hoshida, H. Tanaka, Y. Hibino, T., Hayashi, Y., Tanaka, A., Takabe, T. and Takabe, T. (2000) Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Mol. Biol*. **43,** 103-111.
9. Demidchik, V., Davenport, R.J. and Tester, M. (2002) Non-selective cation channels in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **53,** 67-107.
10. Maser, P., Gierth, M. and Schroeder, J.I. (2002) Molecular mechanisms of potassium and sodium uptake in plants. *Plant Soil* **247,** 43-54.

11. Tester, M. and Davenport, R. (2003) Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot*. **91,** 503-527.
12. Laurie, S., Feeney, K.A., Maathuis, F.J.M., Heard, P.J., Brown, S.J. and Leigh, R.A. (2002) A role for HKT1 in sodium uptake by wheat roots. *Plant J.* **32,** 139-149.
13. Maser, P., Eckelman, B., Vaidyanathan, R., Horie, T., Fairbairn, D.J., Kubo, M., Yamagami, M., Yamaguchi, K., Nishimura, M., Uozumi, N. Robertson, W., Sussman, M.R. and Schroeder, J.I. (2002) Altered shoot/root NaR distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the NaR transporter AtHKT1. *FEBS Lett.* **531,** 157-161.
14. Yeo, A. (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. *J. Exp. Bot.* **49,** 915-928.
15. Moradi, F., Ismail, A.M., Gregorio, G.B. and Egdane, J.A. (2003) Salinity tolerance of rice during reproductive development and association with tolerance at the seedling level. *Ind. J. Plant Physiol*. **8,** 105.
16. Blumwald, E., Aharon, G.S. and Apse, M.P. (2000) Sodium transport in plant cells. Biochim Biophys Acta **1465,** 140-151.
17. Karim, Z. and Iqbal, A. (2001) Impact of land degradation in Bangladesh. Changing Scenario in Agricultural Land Use. Compilation of data from the geographical information system (GIS) project. Bangladesh Agricultural Research Council publication, pp. 106.
18. Bose, M.L., Isa, M.A., Bayes, A., Sen, B. and Hossain, M. (2001) Impact of modern rice varieties on food security and cultivar diversity. In: *Rice Research for Food Security and Poverty Alleviation*. (Peng, S. and Hardy, B., eds). IRRI publication, pp. 617.
19. Panaullah, G.M. (1993) Soil salinity and associated problems in connection with crop production in the coastal regions of Bangladesh. In: *Proceedings of the Workshop on Coastal Salinity and Crop Production in Bangladesh*. Bangladesh Rice Research Institute Publication, pp. 1-30.
20. Lisa, L.A., Seraj, Z.I., Elahi, C.M.F., Das, K.C., Biswas, K., Islam, M.R., Salam, M.A. and Gomosta, A.R. (2004) Genetic variation in microsatellite DNA, physiology and morphology of coastal saline rice (*Oryza sativa* L.) landraces of Bangladesh. *Plant Soil,* **263,** 213-228.
21. Hossain, M. (1998) Rice research, technological progress and the impact on the rural economy: the Bangladesh case. In *Impact of Rice Research. Manila, Philippines* (Pingali, P. and Hossain, M. eds). Thailand Development Research Institute and International Rice Research Institute, p.311.
22. McCouch, S.R., Chen, X., Panaud, O., Temnykh, X., Zu, Y, Cho, Y.G., Huang, N., Ishii, T. and Blair, M. (1997) Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Mol. Biol*. **35,** 89-99.
23. Temnykh, S., Park, W.D., Ayers, N., Cartinhour, S., Hauck, N., Lipovich, L., Cho, Y.G., Ishii, T. and McCouch, S.R. (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theor. Appl. Genet*. **100,** 697-712.
24. Rangan, L., Constantino, S., Khush, G.S., Swaminathan, M.S. and Bennett, J. (1999) The feasibility of PCR-based allele mining for stress tolerance genes in rice and related germplasm. *Rice Genet. Newslett* **16,** 43.
25. Epstein, E. (1998) How calcium enhances plant salt tolerance. *Science* **280,** 1906-1907.
26. Skriver, K. and Mundy, J. (1991) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* **2,** 503-512.
27. Wise, M.J. and Tunnacliffe, A. (2004) POPP the question:what do LEA proteins do. *Trends Plant Sci* **9,** 13-17.
28. Claes, B.R., Dekeyser, R., Villarvoel, R., Van den Bulke, M., Montagu, M.V. and Caplan, A. (1990) Characterization of a rice gene showing organ specific expression in response to salt and drought. *Plant Cell* **2,** 19-27.
29. Garcia, A.B., de Almeida, Engler. J., Claes, B., Villarroel, R., Van Montagu, M., Gerats, T. and Caplan, A. (1998) The expression of the salt responsive gene Salt from rice is regulated by hormonal and developmental cues. *Planta* **207,** 172-180.
30. Tseng, T.C., Tsai, T.H., Lue, M.Y. and Lee, H.T. (1995) Identification of sucrose-regulated genes in cultured rice cells using mRNA differential display. *Gene* **161,** 179-182.
31. Elahi, C.M.F., Seraj, Z.I., Rasul, N.M., Das, K.C., Biswas, K., Salam, M.A., Gomosta, A.R., Tumimbang, E., Adorada, D., Gregorio, G.B. and Bennett, J. (2004) Breeding rice for salinity tolerance using the Pokkali allele: finding a linked marker. In: *In Vitro Culture, Transformation and Molecular Markers for Crop Improvement.* (Islam, A.S., ed). New Hampshire, USA: Science Publishers Inc.

32. IRRI, Descriptors for rice *Oryza sativa* L. (1980) International Rice Research Institute and International Board for Plant Genetic Resources.
33. Chang, T.T. and Bardenas, E.A. (1965) The morphology and varietal characteristics of the rice plant. pp. 40.
34. Pearson K. (1896) Mathematical contributions to the theory of evolution: III Regression, heredity and panmixia. *Philos. Trans. Royal Soc*. **187,** 253-318.
35. Flowers, T.J. and Yeo, A.R. (1995) Breeding for salinity resistance in crop plants: where next? *Aust. J. Plant Physiol*. **22,** 875-884
36. Maathuis, F.J.M. and Amtmann, A. (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. *Ann. Bot*. **84,** 123-133.
37. IRRI, Standard evaluation system manual. (1996) International Rice Research Institute, Los Banos, Philippines.
38. Lee, K.S., Choi, W.Y., Ko, J.C., Kim, T.S. and Gregorio, G.B. (2003) Salinity tolerance of japonica and indica (*Oryza sativa* L.) at the seedling stage. *Planta* **216,** 1043-1046.
39. Jaccard, P. (1908) Nouvelles rescherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat*., **44,** 223-270.
40. Moons, A., DeKeyser, A. and Montagu, M.V. (1997) A group 3 LEA cDNA of rice, responsive to abscisic acid, but not to jasmonic acid, shows variety-specific differences in salt stress response. *Gene* **191,** 197-204.
41. Gregorio, G.B. (1997) Tagging salinity tolerance genes in rice using AFLP. Ph.D. thesis, University of the Philippines at Los Banos.

17. DEVELOPMENT OF MARKER-FREE AND GENE-EXCHANGE VECTORS, AND ITS APPLICATION

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Abstract. For the next decade, recombinant DNA technologies will make rapid progress and generate many kinds of transgenic crops and trees. It will become more crucial for commercialization of transgenic crops and trees to reduce their environmental impacts and to increase their reliability. In this review, I describe two approaches to address these problems.

1. INTRODUCTION

Dominant genes encoding either antibiotic or herbicide resistance are widely used as selectable markers in plant transformation [1]. Selectable marker genes remain in transgenic plants, and their gene products need to be assessed for safety and environmental impact [2]. Regardless of their scientific assurance, recent public concerns about their safety have caused considerable delays in the market performance of transgenic crops. Although transgenic trees do not need the assessment for their food safety, the use of antibiotic resistance genes as selectable markers also causes the particular concern that these genes may be transferred into pathogenic bacteria. The removal technology of marker genes is very useful to obtain both regulatory and public approval for transgenic trees due to reduce their environmental impacts.

It is difficult to introduce a second gene of interest into a transgenic plant that already contains a resistance gene as a selectable marker. There are a large number of desirable traits and genes worth incorporating into plants, but only a limited number of selectable marker genes are available for practical use. The problem becomes even more difficult if one wants to introduce a number of genes, and it is impossible to introduce them simultaneously [1]. It is desirable, therefore, to develop a system for the removal of selectable marker genes to produce environmentally safe transgenic plants and pyramid a number of transgenes by repeated transformation. We developed the MAT (multi-auto-transformation) vector system [3,4] which can remove marker genes without sexual crossings and stack valuable genes in sterile

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clones, is a promising technology to simplify obtaining both regulatory and public approval and to accelerate commercialization of transgenic trees (Figs. 1, 2).

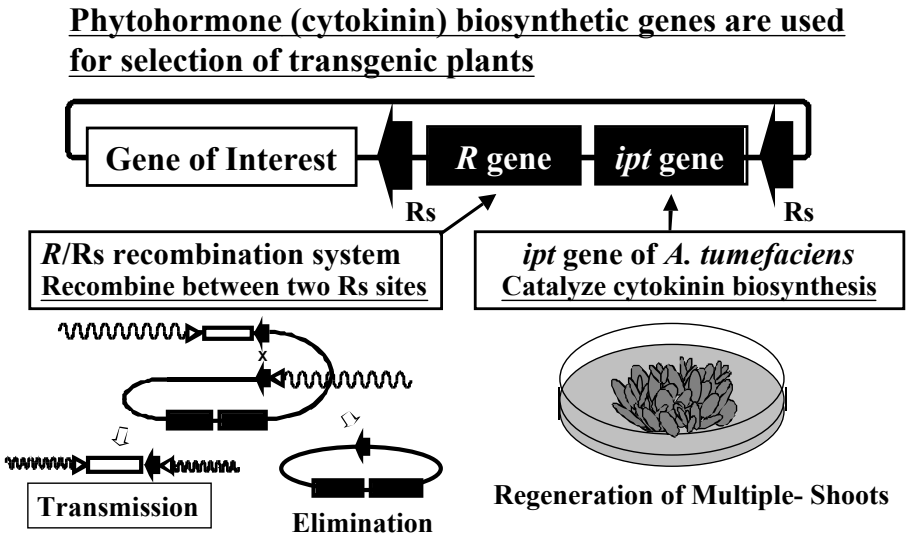

Figure 1. Schematic figure for ipt-type MAT vector

2. MAT VECTOR SYSTEM

MAT vector system uses the cytokinin genes for selection of transgenic plants. The *ipt* gene encodes the enzyme isopentenyl transferase which catalyzes the condensation of isopentenyl pyrophosphate with adenosine mono phosphate (AMP) to produce isopentenyl AMP, a precursor of several cytokinins [3]. Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants from cultured cells after transformation. When a chimeric *ipt* gene under the control of the (CaMV) 35S promoter was introduced into plant cells, transgenic cells proliferated and adventitious shoots differentiated in hormone-free medium. These transgenic plants exhibited an extremely shoot phenotype (ESP) and loss of apical dominance. Therefore, it is easy to detect visually transgenic plants that carry a functional *ipt* gene. Chimeric *ipt* genes are not commonly used as selectable markers because the resulting transgenic plants lose apical dominance and are unable to root due to overproduction of cytokinins.

The maize transposable element *Ac* has the ability to move to new locations within a genome. We used *Ac* to remove the chimeric *ipt* gene from transgenic cells of ESP shoots after transformation. In the transposition process, about 10% of the *Ac* elements that excise do not reinsert and therefore disappear, or reinsert into a sister chromatid that is subsequently lost by somatic segregation. When the chimeric *ipt* gene is inserted into *Ac* in the MAT vector, the chimeric *ipt* gene may transpose or

become lost along with *Ac* in transgenic cells. Consequently, we were able to obtain phenotypically normal transgenic plants that had lost the chimeric *ipt* gene.

"Kitakami Hakuyo" is elite clones of hybrid aspen (*Populus sieboldii* x *P. grandidentata*) used for paper production. The hybrids are vegetatively propagated to maintain the elite genome. The current co-transformation systems for eliminating selectable marker genes cannot be applied to the hybrids because they need sexual

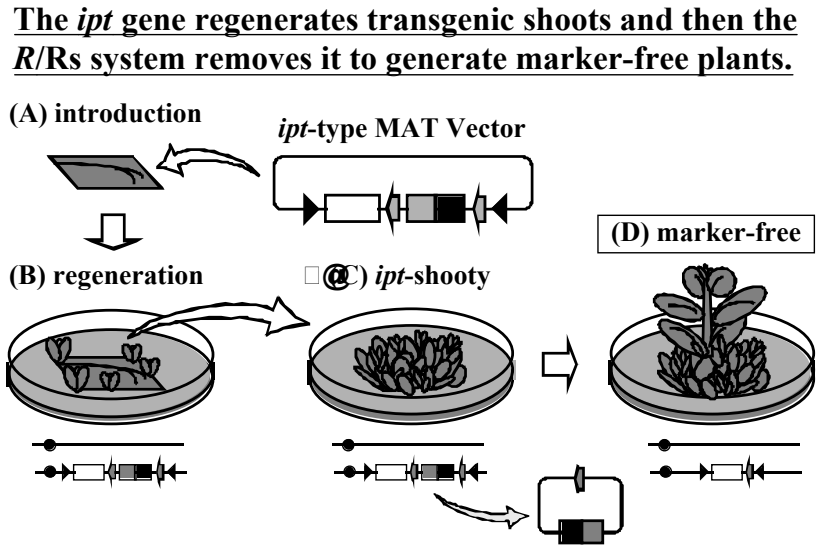

Figure 2. Schematic figure for two-step transformation

crossings to generate marker-free plants. We developed the GST-MAT vector (pRBI11) to generate marker-free transgenic aspen plants independent of sexual crossing at the stage of an *in-vitro* grown primary transformant (Fig. 3) [5]. In the MAT vector, the chimeric isopentenyltransferase (*ipt*) gene is combined with the site-specific recombination system (recombinase gene *R*/ recognition site Rs) to remove it from the transgenic cells after transformation (Fig. 1, 2). The GST-MAT vector pRBI11 has the *ipt* gene with the ribulose 1,5-bisphosphate carboxylase/ oxygenase small subunit (*rbc*S) 3B promoter and the *R* recombinase gene with the glutathione S-transferase (GST)-II-27 promoter (Fig. 3).

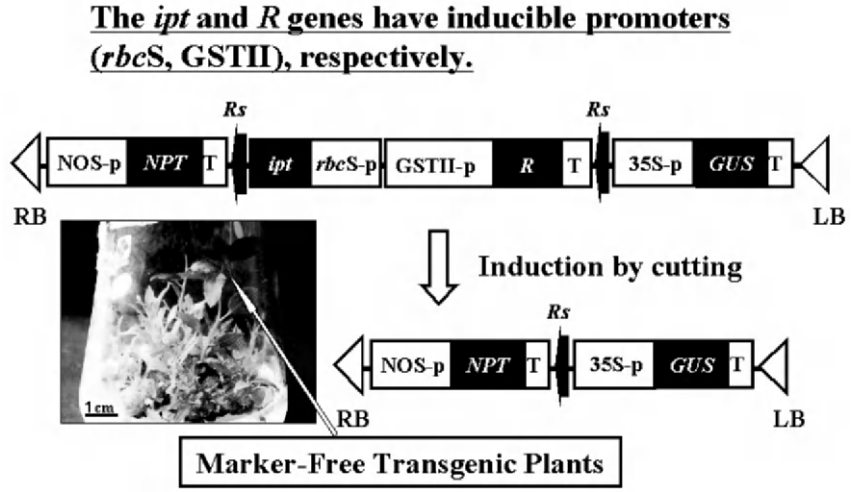

Figure 3. GST-MAT vector for hybrid aspen

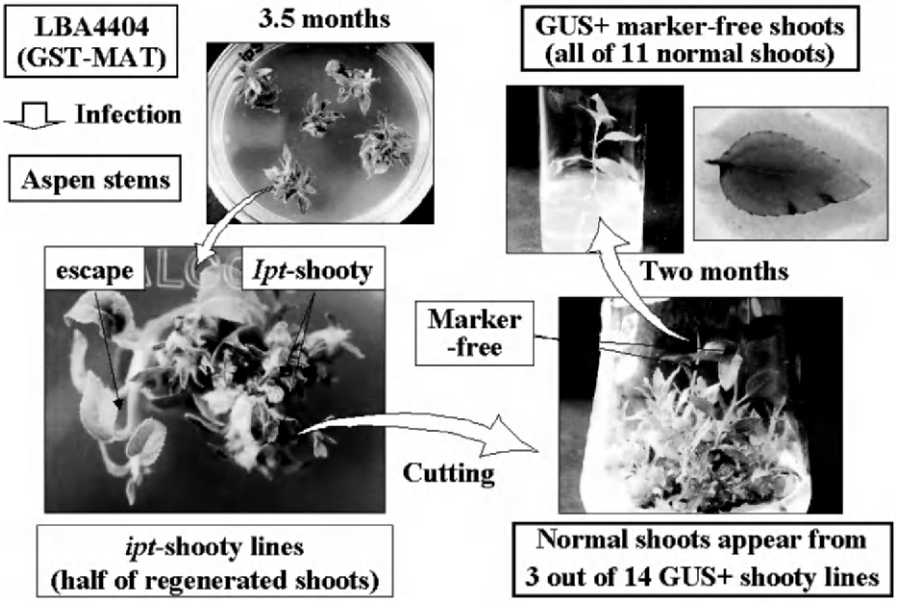

Figure 4. Transformation of hybrid aspen

The *rbc*S 3B promoter is induced by light and expressed actively only in green tissues. The GST-II-27 promoter is induced by wounding and expressed actively in the cutting sites of stem segments. We infected stem segments of hybrid aspen with *A. tumefaciens* containing the pRBI11 vector and cultivated on the hormone-free

modified MS agar medium (800 mg/l ammonium nitrate, 2 g/l potassium nitrate) containing 500 mg/l carbenicillin without kanamycin (nonselective medium) (Fig. 4). Within three months after infection, about half of regenerated shoots were clearly identified as *ipt*-shooty phenotypes. The *rbc*S 3B-*ipt* gene efficiently produced ß-glucuronidase (GUS)-positive *ipt*-shooty plants from 38.0 % of infected stem segments. We independently cut 14 GUS-positive *ipt*-shooty clones into small pieces and transferred then to a modified MS medium containing 0.5 mg/l zeatin and 500 mg/l carbenicillin (shoot-inducing medium). Eleven normal shoots appeared from three of 14 (21.4%) *ipt*-shooty clones within 2 months of induction by wounding. We subjected eight developed shoots to PCR analysis. All eight normal shoots were marker-free transgenic plants and no non-transgenic escapes appeared.

3. SITE-DIRECTED INTEGRATION (SDI) VECTOR SYSTEM

Agrobacterium and particle bombardment have been widely used to transform a wide variety of plant species. However, current transformation methods have two drawbacks for manipulation of the plant genome, a lack of control of the copy number, and a lack of specificity in the site of integration. To overcome these drawbacks, more precise and predictable technologies are highly desirable for increasing the quality of transgenic events and for manipulating the plant genome. The site-directed integration (SDI) system [6] may offer a solution to upgrade the reliability of transgenic technologies and their suitability for genome manipulation. Since precise integration of a desired gene into a well-characterized genomic locus would improve the stability of gene expression and the reliability of transgenic plants.

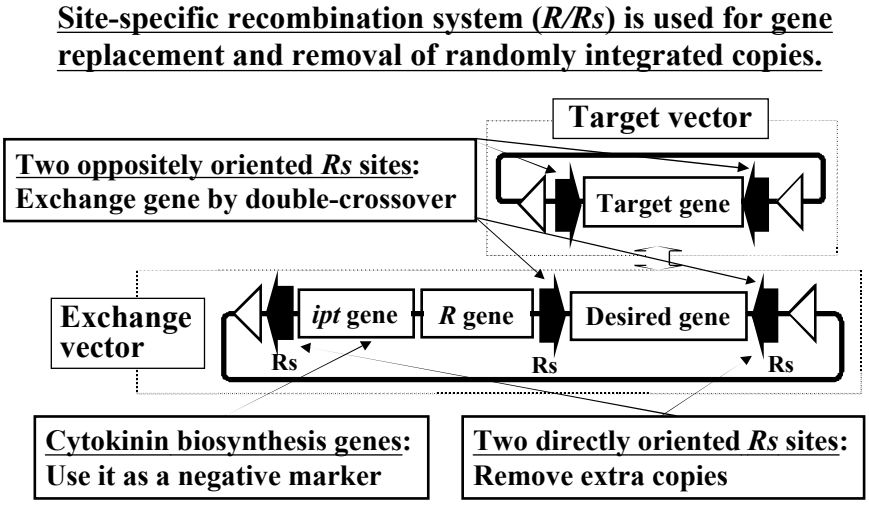

Figure 5. Construction of SDI vectors

Recombinase catalyzes gene replacement and removal.

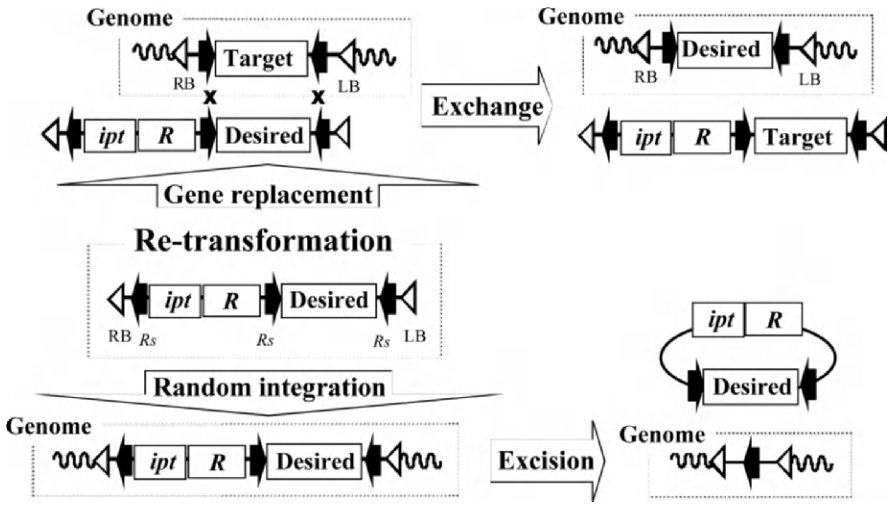

Figure 6. Principle of SDI system

We developed the SDI system to replace a target gene with a desired gene by using a site-specific recombination system (*R*/Rs) (Fig. 5, 6) [7]. This system consists a target vector to introduce the target gene and an exchange vector to re-introduce the desired gene (Fig. 7). We introduced a target vector into tobacco plants and

The SDI system consists of a target vector with a target gene and an exchange vector with a desired gene.

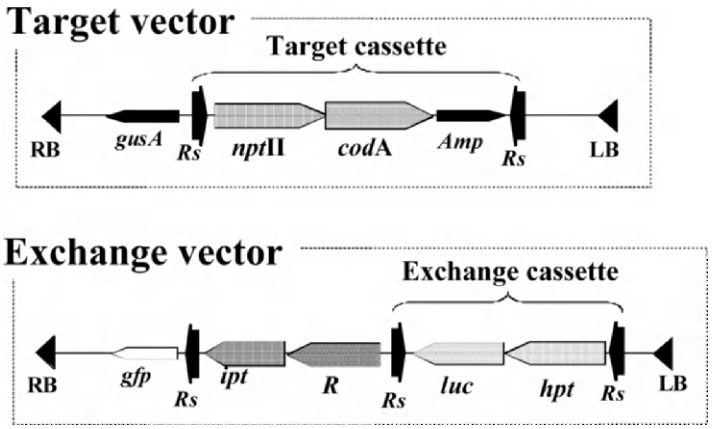

Figure 7. Diagram of SDI system

produced transgenic plants containing a single copy of target genes (neomycin phosphotransferase gene *npt*II, cytosine deaminase gene *cod*A) in a homozygous or heterozygous condition (Fig. 8). We re-introduced an exchange vector containing desired genes (hygromycin phosphotransferase gene *hpt*, firefly luciferase gene*luc*) into the transgenic plants by *Agrobacterium* (Fig. 6). Two oppositely oriented recognition site (Rs) sites flank both the target and desired genes, and recombinases catalyze double-crossover between the two Rs sites to replace the target genes with the desired genes. Transgenic plants with an exchanged copy were selected on shoot-inducing medium containing hygromycin.

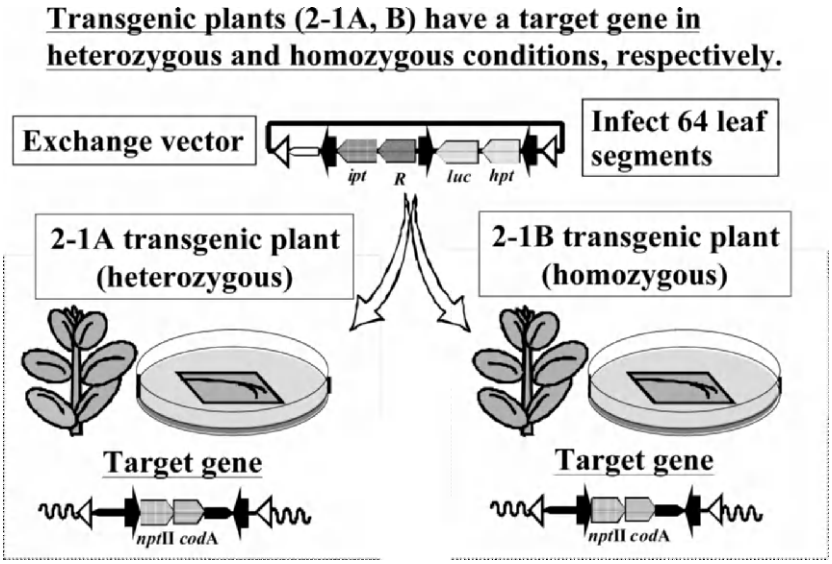

Figure 8. Targeted transgenic lines

Two directly oriented Rs sites flank the isopentenyl transferase gene (*ipt)*, recombinase gene gene (*R*) and desired genes, and recombinases remove randomly integrated copies of these genes. Incompletely integrated copies with a partial deletion remain on a chromosome. The *ipt* gene in these copies overproduces cytokinin and inhibits rooting. Transgenic plants with only an exchanged copy were selected as a normal phenotype on root-inducing hormone-free medium.

Our results indicate that 8% and 17% of independent normal transgenic plants from randomly selected calluses have a complete exchanged copy in homozygous and heterozygous conditions, respectively (Fig. 9, 10). Interestingly, the SDI system can be used to efficiently replace a target gene with a desired gene in a homozygous condition. Furthermore, the selection efficiency of transgenic plants with an exchanged copy can be improved by using the *cod*A gene as a negative marker. Thus, this system may be effective for transformation of cereal crops since seeds that have a target gene in a homozygous condition are available.

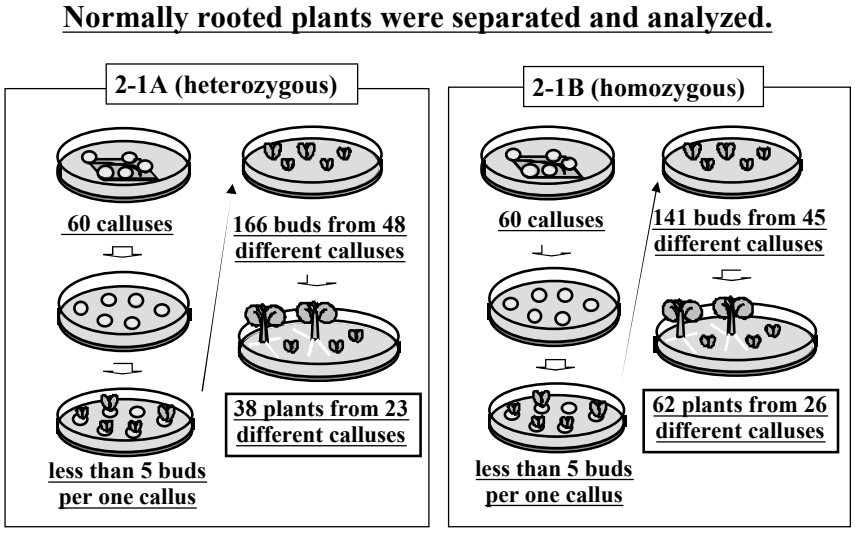

Figure 9. Transformation and selection

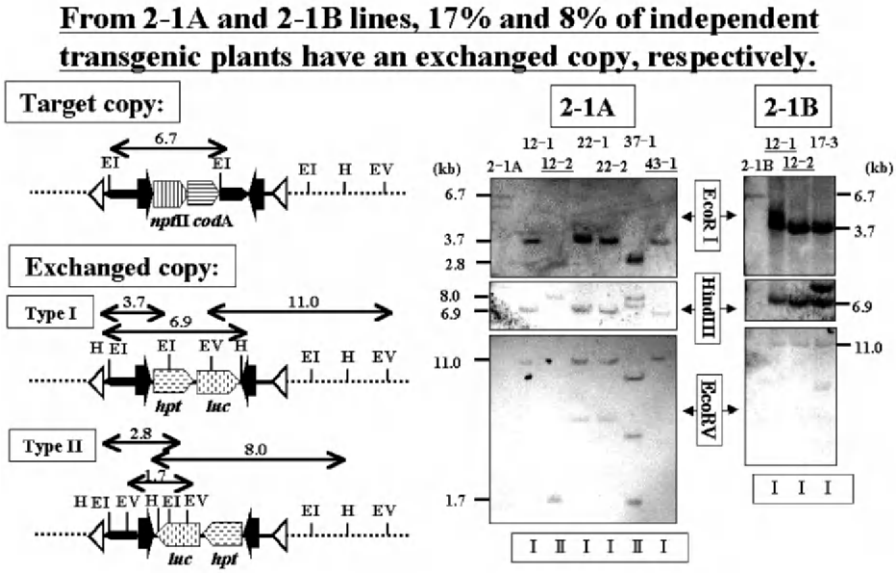

Figure 10. Southern analysis

REFERENCES

1. Yoder, J.I. and Goldsbrough, A.P. (1994) Transformation systems for generating marker-free transgenic plants. *Bio/Technology* **12**, 263-267.
2. Bryant, J. & Leather, S. (1992) Removal of selectable marker genes from transgenic plants needless sophistication or social necessity. *Trends Biotechnol.* **10**, 274-275.
3. Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M. (1997) Selection of marker-free transgenic plants using the isopentenyl transferase gene as a selectable marker. *Proc. Natl. Acad. Sci. USA* **94**, 2117-2121.
4. Ebinuma, H., Komanine, A. (2001) MAT (Multi-Auto-Transformation) vector system. The oncogenes of Agrobacterium as positive markers for regeneration and selection of marker-free transgenic plants. In Vitro Cell Dev Biol-Plant **37,**114-119.
5. Ebinuma, H., Sugita, K., Matsunaga, E., Endo, S. and Yamada, K. (2002) GST-MAT vector for the efficient and practical removal of marker genes from transgenic plants. In: Jackson, J.F., Linskens, H.F. and Inman, R.B. ed. Molecular methods of plant analysis. Vol22. "Testing for genetic manipulation." Springer-Verlag GmbH & Co. KG, Heidelberg, pp. 95-117.
6. Nanto, K., Yamada-Watanabe, K. and Ebinuma, H. (2005) Agrobacterium-mediated RMCE approach for gene replacement. *Plant Biotech. J*. **3,** 203-214.
7. Ebinuma, H., Sugita, K., Matsunaga, E., Endo, S., Yamada, K., Komamine, A. (2001) Systems for the removal of a selection marker and their combination with a positive marker. Plant Cell Rep 20: 383-392

18. TOWARD THE DEVELOPMENT OF BIOTECHNOLOGY IN ASIA

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Abstract. Biotechnology involves the harnessing of natural processes of microbes, plant and animal cells for the human life. In this chapter, we review the status of biotechnology at the commercial level, especially the development of genetically modified crops in Asia.

1. INTRODUCTION

Nature and scope of biotechnology have become broader and more complex from the simple classical fermentation to the modern genetic engineering. The biotechnology should fulfil the following conditions: increase the crop productivity; conserve the biodiversity; keep the sustainable agriculture; keep high productivity even under abiotic and biotic stresses; improve the economical and social benefits; and alleviate the poverty in the developing countries [1].

In the last decade, Asia has achieved the rapid growth including the biotechnologies but at a phase lagging behind developed countries. The development of biotechnology is affected by various factors such as a) funding, b) capabilities, c) public awareness, d) risk management, e) public sector investment in agriculture, f) private sector investment in biotechnology, g) Overseas Development Administration (ODA) for biotechnology, and h) intellectual property rights [2]. Development of biotechnology in Asia has been made possible through the various networking. Some of them are presented in Table 1 [3]. All networks cited are generally for the development of biotechnology for a specific commodity. The network can function through collaborative research, training, and exchange of information. A distinct feature of the Papaya Biotechnology Network of the Southeast Asia is the involvement of the private sector which forms a so-called "partnership arrangement". In this review, we review the status of biotechnology at the commercial level, especially the development of genetically modified crops in Asia.

Ashwani K. Rai and Teruhiro Takabe (eds.), Abiotic Stress Tolerance in Plants, 255-260.

Table 1. Summary of some networking for biotechnology in Asia.

Network	Lead Institution	Year Estab-lished	Countries Involved	Funding Source
Asian Rice Biotechnology Network	IRRI	1993	China, Indonesia, Pakistan, Philippines, Thailand (full),Vietnam, Bangladesh, and Sri Lanka (Associate)	Asian Development Bank (ADB), German Gov't. Bundesministerium fur Technische Zusam minarbeit (BNZ)
Asian Maize Biotechnology Network	CMMYT	1998	China, India, Indonesia, Philippines, Thailand	ADB
Papaya Biotechnology Network of Southeast Asia	ISAAA	1998	Malaysia, Indonesia, Philippines, Thailand, Vietnam	Private Sector "partnership arrangement" 1) delayed ripening gene of Zeneca 2) PRSV-R gene of Monsanto
Cassava Biotechnology Network (CBN)	Int'l Center for Tropical Agriculture	1992	Asian Countries	Netherlands Directorate General for International Cooperation, IDRC
Intermediary Biotechnology Service	Int'l Service for National Agricultural Research (ISNAR)	1993	China, Indonesia, Malaysia, Philippines, Thailand, Taiwan, Vietnam	Governments of Japan and UK, Netherlands, Directorate General for International Cooperation; Swiss Agency for Development and Cooperation
Asia Network for Small-scale Agricultural Biotechnology (ANSAB)	ISNAR	1992	Bangladesh, India, Indonesia, Nepal, Philippines, Sri Lanka, Thailand, Vietnam	Appropriate Technology International (ATI)
Seed Network for Asia and the Pacific (SNAP)	Asia and the Pacific Seed Association (APSA) and Thai Depart-ment of Agricultural Extension	1999	Asian Countries	FAO

2. STATUS OF BIOTECHNOLOGY IN VARIOUS COUNTRIES IN ASIA

China

Based on a report made in 2000 by Dr. Zhangliang Chen, Vice President of Peking University and Director of the National Laboratory of Protein Engineering and Plant genetic Engineering, China started the research on transgenic crops in 1983, initiated the field-test in 1989, and established the Regulatory Office of Genetic Engineering Safety Administration in 1996. In 1997, Monsanto and the Chinese Academy of Agricultural Sciences (CAAS) started a commercial production of Bt cotton. This was followed with commercial production of virus resistant and shelf life- altered tomato, virus resistant sweet pepper, and flower color-altered petunia. Field trials and biosafety test for other major crops have been continued. Since 1999, no other crops have been approved for commercialization due to external, mostly European pressures.

The amount of biotech products of China is fourth in the world, first the U.S., then Argentina, Canada. and China followed. But China has more professionals in the field than any other country in the world. With the full support by government, China developed the biotechnology on GM crops very rapidly [4]. While its industry is at its infancy, 65 GM plants have already been licensed for environmental release. In comparison, less than 50 GM plants are approved in U.S. A.

In 2002, China grew 2.1 million hectares of transgenic crop (4% of the total global transgenic crop area) and displayed the highest percentage year-on-year growth with a 40% increase in its GM crop area of Bt cotton between 2001 and 2002 [1].

Following to the U.S.A., China is second in the production of maize in the world. Approximately 130 million MT of maize are produced on 25 million hectares. An average yield loss of 10% by Asian corn borer prompted China to conduct Bt maize yield trials as early as 1997. Field trials with Bt maize show the yield gains in favor of Bt maize about 9-23% (1997~2003).

On rice biotechnology, China has three leading institutes, namely China National Rice Research Institute (CNRRI) in Zhejiang, the National Hybrid Rice Engineering Technical Centre (NHRETC) in Hunan and the Institute of Genetics and Developmental Biology (IGDB) in Beijing. The Chinese government has deeply involved in plant breeding programs to deliver high-yield rice varieties [5].

Indonesia

Indonesian government established the National Committee on Biotechnology in 1988 to prepare the national biotechnology policy and the development program. The economic crises in 1997 prompted them to make the changes in priorities of biotechnologies to include: 1) immediate application of existing biotechnology for food production, 2) strategic research, and 3) increased participation of private companies. In addition to Universities, various private companies also conducted biotechnology. Biosafety regulations were established in 1997 covering genetic manipulation of microbes, plants, fish and livestock, and amended in 1999 to include plantation and forestry plants and food products [6].

In 2001, Indonesia started the commercializing of GM cotton. The global transgenic crop area in 2001 and 2002, were 52.6 and 58.7 million hectares, respectively. Indonesia grew 1% of global transgenic crops that is ninth among the top twelve countries. About 2,700 farmers continue to grow Bt cotton in South Sulawesi, Indonesia [1].

In 2002, Indonesia was the ninth among the top ten countries in maize production. Among 602 million MT, 9.3 million MT (1.54%) are produced by BT maize. They were produced by 3.3 out of 140 million hectares (2.36%) and 6.2 out of 196 million maize farmers (3.15%).

Philippines

Development of transgenic crops such as rice with insect and pathogen resistance, papaya and mango with delayed ripening trait, papaya with resistance to ring spot virus, banana with resistance to bunchy top virus, and coconut with modified fatty acid is ongoing at several institutions [7].

In December, 2002, Monsanto announced the approval of Bt maize containing the cry1Ab gene that confers the resistance to Asian corn borer which is a major pest in most parts of Asia. Bt maize is the first GM crop to be approved in the Philippines. The approval was made after a series of field trials from 2001.

Singapore

As early as the 1980s, the Institute of Molecular and Cell Biology (IMCB) affiliated to the National University of Singapore was established. Drug companies such as Glaxo/Welcome offered the research funding for studies on the central nervous system. Other studies are on cell regulation, neurology, recombinant proteins, DNA/peptide synthesis and sequencing, and HIV. In 1990, Singapore BioInnovations (SBI) was established to take the charge of commercialization of local bio-technology and to attract foreign investors to Singapore.

Thailand

Aas early as 1983, the National Center for Genetic Engineering and Biotechnology (BIOTECH) was establishe. As of 2000, six limited field trials of Bt corn and Bt cotton and confined experiments of GM tomato, rice, corn, with various traits had been conducted in Thailand. Importation of 40 species of GMOs has been allowed only for experimentation and not commercialization. The government of Thailand has maintained no commercialization of GM crops as a national policy.

Vietnam

As a result of the economic reforms initiated by the Vietnam government over the past 10 years, the crop production, especially of rice, increased. The priority researches in Biotechnology in Vietnam are GM rice, maize, potato, and sweet potato resistant to pest and diseases, and abiotic stress. The main institutes for biotechnological research are the Institute of Biotechnology (IBT) at the National Center of Natural Science and Technology, Institute of Agricultural Genetics (IAG), and Institute of Agricultural Sciences (IAS) [8].

In addition to rice and maize, fruits and vegetables such as asparagus and leafy green vegetables are important targets that should be improved by biotechnology. Legumes and livestock are also importance. The development of transgenic crops which could reduce the viral and fungal disease is not well developed, but already tested at laboratory levels. Various interesting genes have been cloned or imported from other countries (Table 2) and have been applied to several plants.

The enetic modification is one of important crop biotechnologies which have been applied on several crop plants (Table 3). To promote the application of biotechnology in agriculture and health care, the Vietnam government has set up the fund in the amount of approximately US$ 130M to support the companies who commercialise the biotechnological products.

Table 2. Genes used in Vietnam for plants (Nguyen, 2000).

Gene	Expression	Origin
1. Cry IA (a,b,c,d)	Insect resistance	Ottawa Univ., Canada
2. GNA	Bacterial resistance	John Inne Institute, England
3. Xa 21	Bacterial resistance	UC, Davis, USA
4. Asp1	Increase store protein	Demegen, USA
5. Chitinase	Fungal resistance	UG, Belgium
6. P5CS	Drought tolerance	VUB, Belgium
7. OAT	Drought tolerance	VUB, Belgium
8. HAL	Drought tolerance	PUV, Spain
9. Nha	Salt tolerance	PUV, Spain
10. Bar	Herbicide tolerance	PMB, France
11. Dhpds	Drought tolerance	VUB, Belgium
12.CP	RSV resistance	IBT, Vietnam
13. ACC antisense	Increase shelf-life	IBT, Vietnam
14. Chil442	Chilling tolerance	IBT, Vietnam
15. Tps	Drought tolerance	PUV, Spain
16. myb family	Rice crop improvement	NIAR, Japan

Table 3. Crop biotechnology in Vietnam (Nguyen, 2000).

Crops	Biotechnology	Crops	Biotechnology
Sugarcane	Germplasm, borer resistance	Soybean	Abiotic stress tolerance, Rhizobia for Mekong Delta soil
Maize	Diagnosis	Cassava	Propagation
Potato	*In Vitro Tuberization*	Cotton	Transgenic BT plants
Sweet potato	BT transgenic plants	Fruits	PSV resistance (papaya)
Rice	Hybrid, gene transformation		

Malaysia

Historically, the development of agricultural biotechnology in Malaysia is divided into three phases. 1) Establishment Phase (prior to 1995). The basic infrastructures for molecular and cellular biology laboratories were establishment. These are Malaysian Research and Development Institute (MARDI), Palm Oil Research

Institute of Malaysia (PORIM), now Malaysian Palm Oil Board (MPOB), and Rubber Research Institute of Malaysia (RRIM), now Malaysian Rubber Board (MRB), and various universities such as Universiti Putra Malaysia (UPM), Universiti Kabangsaan, Malaysia (UKM), University of Malaysia (UM), and Universiti Sains Malaysia (USM). 2) Development Phase (1996-2000). The National Biotechnology Directorate (BIOTEK) was established. BIOTEK promotes the commercialization of biotechnology. BIOTEK covers the areas of plant-, animal-, food-, environmental/ industrial-, medical-biotechnology, molecular biology and bio-pharmacy. In this phase, some of pharmaceutical companies have moved to make the biotech-based pharmaceuticals. Companies involved in agricultural biotechnology are mainly on plantation (palm oil), herbal-based, and on aquaculture for sustainable fish and prawn breeding.

Overview of the Development of Biotechnology in Asia

India intends to have a biotechnology policy as quickly as possible to supply the pest-resistant and drought-resistant seeds with high nutritional values. South Africa planted 400,000 hectares of GM crops, and a large percentage was grown by smallholder emergent farmers. GM food had spread throughout the South African food chain during the past six years and been consumed by 40-million people. Commercial farmers producing GM maize on dry land realised higher yields on average of 400 kg/ha. GM cotton farmers producing under irrigation also increased yields by 400 kg/ha. Thus GMO food production is growing worldwide.

The Cartagena Protocol on Biosafety is an international agreement on biosafety. The protocol makes clear that products from new technologies must be based on the precautionary principle and allow developing nations to balance public health against economic benefits. It will for example let countries ban imports of a genetically modified product if they feel there is not enough scientific evidence the product is safe. Both development of biotechnology and regulatory frameworks for assuring food safety are important subjects for future.

REFERENCES

1. James, C. (2003) Global review of commercialized transgenic crops: 2002 Feature: Bt Maize. *ISAAA Briefs No. 29*. Ithaca, NY: ISAAA, pp. 1-182.
2. Matsumoto, T. (2000) Managing technology transfer in agricultural biotechnology. In *Proceedings of the Regional Conference on Agri-Biotech*. Bangkok. BIC-SEARCA, pp.1-167.
3. Sriwatanapongse, S. (2000) Regional development in agricultural biotechnology: Capacity building in the 21st century. In *Proceedings of the Regional Conference on Agri-Biotech*. Bangkok. 2000. BIC-SEARCA. pp. 1-167.
4. Macer, D.R.J., Azariah, J. and P. Srinives, P. (2000) Attitudes to biotechnology in Asia. *International Journal of Biotechnology* **2,** 313-332.
5. Brookes, G. and Barfoot, P. (2003) GM Rice: Will this lead the way for global acceptance of GM crop technology? *ISAAA Briefs No. 28*, Ithaca, NY: ISAAA, pp. 1-53.
6. Sukara, E. and Slamet-Loedin, L.H. (2000) Agricultural biotechnology in Indonesia. In *Proceedings of the Regional Conference on Agri-Biotech*. Bangkok. BIC-SEARCA. pp. 1-167.
7. Halos, S.C. (2000) Agricultural biotechnology in the Philippines. In *Proceedings of the Regional Conference on Agri-Biotech*. Bangkok. BIC-SEARCA, pp.1-167.
8. Nguyen, T.V. (2000) Agricultural biotechnology in Vietnam. In *Proceedings of the Regional Conference on Agri-Biotech*. Bangkok. BIC-SEARCA. pp. 1-167.

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