14 Citrus Fruits

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14.1 Introduction

14.1.1 Background

Citrus, belonging to the family Rutaceae, is one of the world's most important fruit crops with a total world production of 105 million metric tons. It is commercially grown in the tropical and subtropical regions around the world, primarily between the latitudes of 40°N to 40°S. The literature record of citrus domestication and cultivation history dates back to 2100 BC (Webber 1967; Scora 1988). It is considered to have originated from the Malay Archipelago and Southeast Asia, occurring from Northern India to China and in the South through Malaysia, the East Indies and the Philippines. Movement of citrus worldwide was achieved by distant explorers, traders, and church missionaries. More recent evidence suggests that Yunnan Province in the Southwest China may be the center of origin due to the diversity of species found there (Gmitter and Hu 1990). The network of rivers in this area could have provided an on-route dispersal to the south (Sauer 1993). Much has been written on the evolution of modern citrus cultivars and its broad diversity (Swingle and Reece 1967). Studies on the relationships between genera and species were carried out based mainly on morphological characteristics leading to the formulation of numerous classification systems. As there were differences of opinion among the taxonomists, the numbers of species of citrus classified by them were very controversial (Swingle 1943; Swingle and Reece 1967; Tanaka 1976), with Tanaka favoring the naming of many more species than Swingle. Barrett and Rhodes (1976) suggested that there were three true ancestral citrus species, namely, *C. maxima* (pummelos), *C. reticulata* (mandarins), and *C. medica* (citrons); all other species are viewed as introgressions of these ancestral forms. More recently, molecular marker evidence q(Nicolosi et al. 2000) has supported this hypothesis, though there may be other species that might also be considered as ancestral.

Citrus plants are small, spreading, evergreen trees with thorny shoots, growing to about 2–15 m tall. Leaves are unifoliate and alternate with more or less broadly winged petioles. Flowers are fragrant, usually white but sometimes pink or purple pigmented, perfect with 5 petals and 5 sepals, and are borne solitary or in short cymes. Citrus industries in many production areas generate substantial regional revenue. Brazil, the United States, and China (Fig. 1) are the three largest citrus producers in the world (FAO 2003). Citrus is primarily valued for the fruit which can be eaten as a fresh fruit, processed into juice, or added to dishes and beverages. The major types of edible citrus including the ancestral *Citrus* species and their introgressions along with their possible place of origin are listed in Table 1. Citrus is rich in vitamin C, flavonoids, acids, volatile oils, carotenoids, and other microelements.

In a wide genetic perspective, the general term citrus also includes species from two other closely related genera, *Poncirus* (trifoliate orange) and *Fortunella* (kumquat), which are sexually compatible with *Citrus* species. *Poncirus* is the most valuable genetic resource for genetic improvement of *Citrus*. Though its fruits are not edible, *Poncirus* is often used in the production of rootstocks as it possesses many resistance genes that are not found in *Citrus*. Resistance or tolerance to citrus tristeza virus (CTV), *Phytophthora* root rot, citrus nematode, cold accumulation, and other environmental stresses have been explored for use in scion and rootstock genetic improvement via conventional or molecular approaches (Cai et al. 1994; Gmitter et al. 1996; Tozlu et al. 1999a, b; Ling et al. 2000). In addition, the genetically dominant trifoliate leaf of *Poncirus* is a big advantage to develop mapping populations, as it allows the direct identifi-

Genome Mapping and Molecular Breeding in Plants, Volume 4
Fruits and Nuts
C. Kole (Ed.)

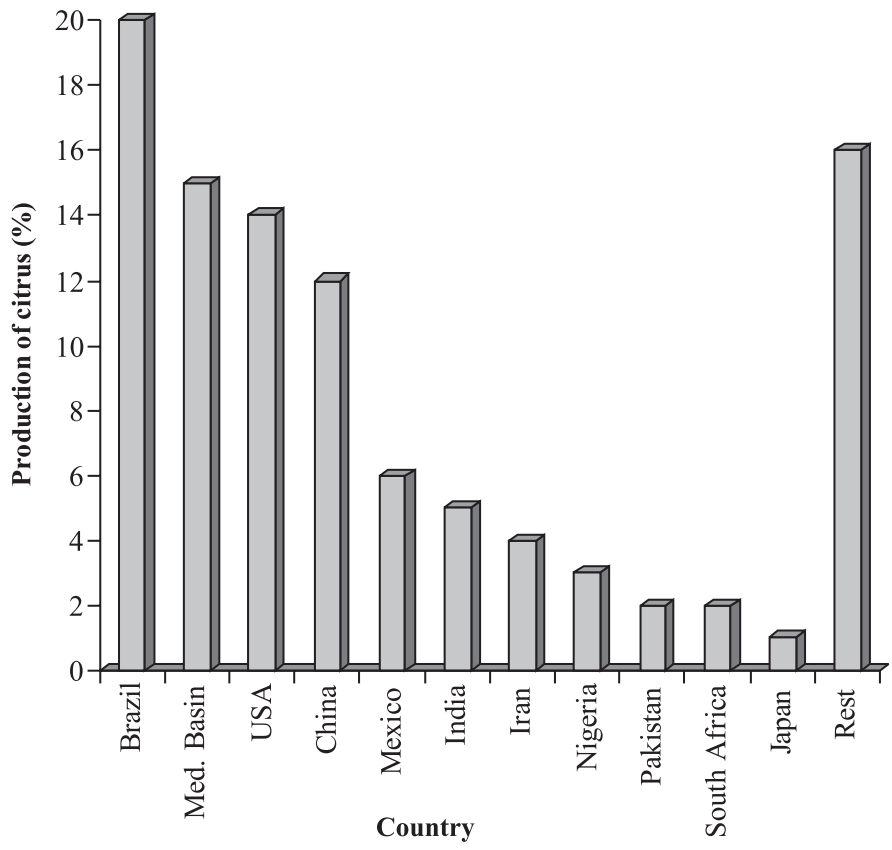

Fig. 1. World production of citrus (FAO, 2003)

cation of zygotic hybrids from true nucellar seedlings. *Fortunella* is an important edible fruit and also a resource for resistance to Asian citrus canker (ACC). In order to have a clear usage of the term "citrus" in this chapter, citrus, in regular font, will be a general term referring to any related species regardless of genus, and *Citrus*, in italic font with initial letter capitalized, will be a genus name representing only the *Citrus* species.

Citrus is vegetatively propagated. Selection of new citrus and related cultivars has been occurring for thousands of years and superior phenotypes have been selected from the wild for cultivation. Citrus has mainly two different breeding targets, viz., scion and rootstock. Yield and fruit quality (both for domestic as well as international market demand) are two of the most important considerations for scion improvement. A superior rootstock is desired to possess broad and durable resistance to pests, diseases, and other environmental stresses (particularly from soil and water). A rootstock should also grow vigorously, be compatible with the scion, and produce maximal numbers of seeds containing true nucellar embryos. Finally, rootstocks substantially influence both yield and fruit quality of the grafted scion cultivars, so these factors also are considered in the evaluation of new candidates. Though the objectives of breeding scion and rootstocks are different, overall breeding objectives may sometime address the aspects of both programs by improving traits such as disease resistance, cold tolerance, etc.

Conventional breeding is a very slow and difficult process due to long juvenility, large tree size, polyembryony, high heterozygosity, and self-incompatibility to some extent. There is a significant lack of knowledge regarding genetic mechanisms controlling the inheritance of agriculturally important traits (most of them may be quantitatively inherited), and only a few of them thus far have demonstrated a single gene inheritance pattern (Davies and Albrigo 1994). Through conventional hybridization, few new *Citrus* cultivars have been produced, although a few rootstocks were developed (Soost and Cameron 1975; Cameron and Soost 1984; Gmitter et al. 1992). Most of the commercially grown cultivars were derived from well-adapted native seedlings/varieties, spontaneous bud mutations, or artificial irradiations. For example, most Satsuma mandarin varieties were from field

Table 1. Four original wild and four hybrid *Citrus* edible species

Name	Scientific name	Possible place of origin
Citron	*C. medica*	India and China
Pummelo	*C. grandis*	Malaysia and India
Mandarin	*C. reticulata*	Southeast Asia
Lime	*C. aurantifolia*	East India
Sour orange	*C. aurantium*	Pummelo × Mandarin. China
Sweet orange	*C. sinensis*	Pummelo × Mandarin. China
Lemon	*C. limon*	Citron × Lime. Unknown, likely in China
Grapefruit	*C. paradisii*	Pummelo × Sweet orange. Barbados island

selection or bud mutation in Japan and China, as were grapefruits and oranges in the US (Hodgson 1967). Chromosomal rearrangements have also been involved in selection for seedlessness and other traits within cultivated citrus (Gmitter et al. 1992). A consequence of the mutational origin and diversification of many of the most important cultivar groups (including oranges, grapefruit, lemons, and certain categories of mandarins such as the Satsuma and Clementine cultivar groups) is that sexual hybridization is excluded as a strategy for genetic improvement. Inbreeding depression and the lack of phenotypic similarity to market expectations and definitions, when hybrids within groups are created, results in plants that are unacceptable to citriculture. A further consequence is that the ability to move useful genes within or among citrus cultivars and germplasm resources, for disease resistance or fruit quality for example, is reliant entirely upon alternatives such as genetic transformation.

Few studies have been conducted to understand the genetics of citrus. Knowledge and understanding of the genetic mechanisms that control important traits such as juvenility/maturity, disease resistance, cold tolerance and aspects of fruit ripening process are clearly lacking (Gmitter et al. 1992). The rapid development of molecular marker technologies has made it possible to investigate gene expression and has helped in construction and integration of genetic and physical maps of the economically important traits. The knowledge and establishment of genomics and bioinformatics have also provided efficient tools for tagging and cloning the genes, and have made the sequencing of the citrus genome plausible. This chapter will summarize the achievements of citrus genome research in the past thirty years, as well as ongoing efforts and planned genomic goals.

14.1.2 Early Knowledge of Citrus Genome and Genetics

Most citrus species, including those from other three distant relative genera, *Microcitrus*, *Eremocitrus* and *Clymenia*, are diploids with nine pairs of chromosomes ($2n = 2x = 18$), although polyploids have been reported. Many spontaneous and induced tetraploids have been used as breeding parents to produce seedless triploid varieties (Gmitter et al. 1992; Gmitter 1994), and numerous tetraploid somatic hybrids have been created as well, by protoplast fusion experiments (Grosser et al. 1996). Many citrus species are outcrossing (Roose et al. 1998). Cytogenetic studies revealed citrus has small but highly variable chromosomes (Naithani and Raghuvanshi 1958; Raghuvanshi 1962; Guerra 1984, 1993). Karyotypes based on Geimsa C-banding (Liang 1988) and staining with the intercalating fluorochromes chromomycin A3 (CMA) and 4′-6-diamidino-2-phenylindole (DAPI, Guerra 1993) show that many chromosome pairs must be heteromorphic. Staining citrus metaphase chromosomes with DAPI and CMA showed that several chromosomes contain large blocks of terminal heterochromatin (Miranda et al. 1997). Factors contributing to chromosomal heterozygosity in citrus include the origin of many accessions by interspecific hybridization and clonal propagation which allow accumulation of karyotypic rearrangements (Roose et al. 1998). The chromosomal identification of different genomes may be an additional and simple method of identifying citrus hybrids and could be of importance for future work on substitution lines. Citrus genome size is also relatively small, and the C value of *C. sinensis* was estimated to be 0.6 picogram per haploid DNA content (Guerra 1984), equivalent to approximately 367 Mb, which is nearly three times the size of *Arabidopsis* genome (125 Mb,

see the International Citrus Genome/Genomics Consortium home page, ICGC).

Most of the knowledge acquired on the inheritance of citrus traits was generally a by-product of the efforts of conventional breeding of rootstocks and scions. The dominance or recessiveness of these morphological traits was speculated according to the segregations of these phenotypes. Some characters such as cold hardiness, fruit acidity, leaf and rind oil, dwarfness, tolerance to chloride stress, resistance to *Phytophthora* and nematodes were roughly described as quantitative trait loci (QTLs). Characters such as polyembryony, trifoliate leaf and polyphenol oxidase-catalyzed browning appeared to be dominant over their allelic phenotypes, monoembryony (single gene), monofoliate leaf (two complimentary genes) and non-browning (single gene) (Soost and Cameron 1975). Rind texture was found to segregate as QTL with a dominant tendency (Yamamoto et al. 1990). According to early inoculation experiments and field survey, resistance to Asian citrus canker (ACC) was also thought to be a dominant trait (Lee 1918) and later, after genetic analysis of dozens of populations, it was assumed to be governed by a single dominant gene (Matsumoto and Okudai 1990). Using ELISA (enzyme-linked immunosorbent assay) on several *Poncirus*-derived populations, resistance to CTV was found to be dominant and was assumed to be controlled by a single gene (Yoshida et al. 1983; Yoshida 1985). By mapping the desired genes, it would be possible to improve the efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to genes influencing that trait. Efforts to develop localized linkage maps with associated molecular markers will be addressed in the following section.

14.2 Mapping of the Citrus Genome

Plant genome maps may include important linkage relationships among molecular markers and genes that breeders wish to manipulate for cultivar improvement, thereby increasing the efficiency of breeding programs. Long juvenile periods and large plant size combine to hinder conventional breeding of citrus by requiring large investments of time and land for characterization and evaluation of progeny. Mapping and sequencing of a citrus genome would help to elucidate gene function, gene regulation and expression. Genetic maps of citrus may provide the basis for early screening procedures, thus, permitting breeders to make initial selection among very young progeny based on the phenotype predicted by their genotype at molecular loci known to cosegregate with a particular phenotype (Durham et al. 1992).

The first linkage analysis of citrus genome began in the 1980s using isozyme markers. The two linkage groups, one with two markers and another with three markers, were found by a mapping program, Linkage-1 (Suiter et al. 1983) from 37 isozyme genes, using nine families of *C. grandis* Osbeck cv Acidless (pummelo) × *C. jambhiri* Lush. cv Florida and *C. grandis* Osbeck cv Chandler × *P. trifoliata* cv Webber-Fawcett (Torres et al. 1985). Codominant isozyme markers, though very limited, were contin-

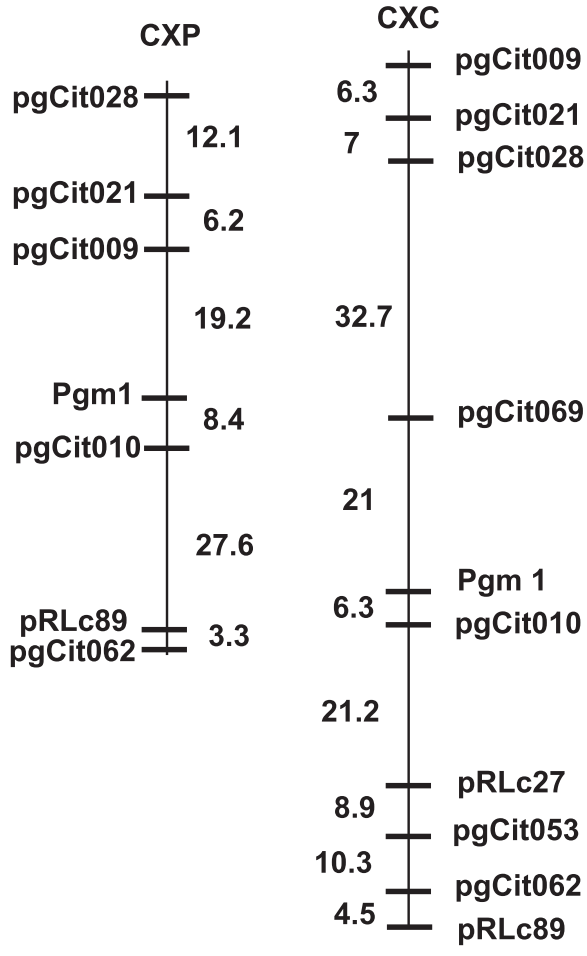

Fig. 2. Citrus linkage map (group 4) deduced from segregation data of two backcross populations of citrus using isozyme and RFLP markers (Durham et al. 1992)

uously used in the later DNA marker-based linkage maps due to their low cost and feasibility (Durham et al. 1992; Garcia et al. 1999). Isozymes may be influenced by the environment as well as by the stage of development of the plant and its organs, thus making the method less reliable; however many citrus isozyme locus linkage studies were conducted using selected systems that were found to be invariant when run on starch gels and using different stages or tissues sources. Throughput of such marker systems was extremely low.

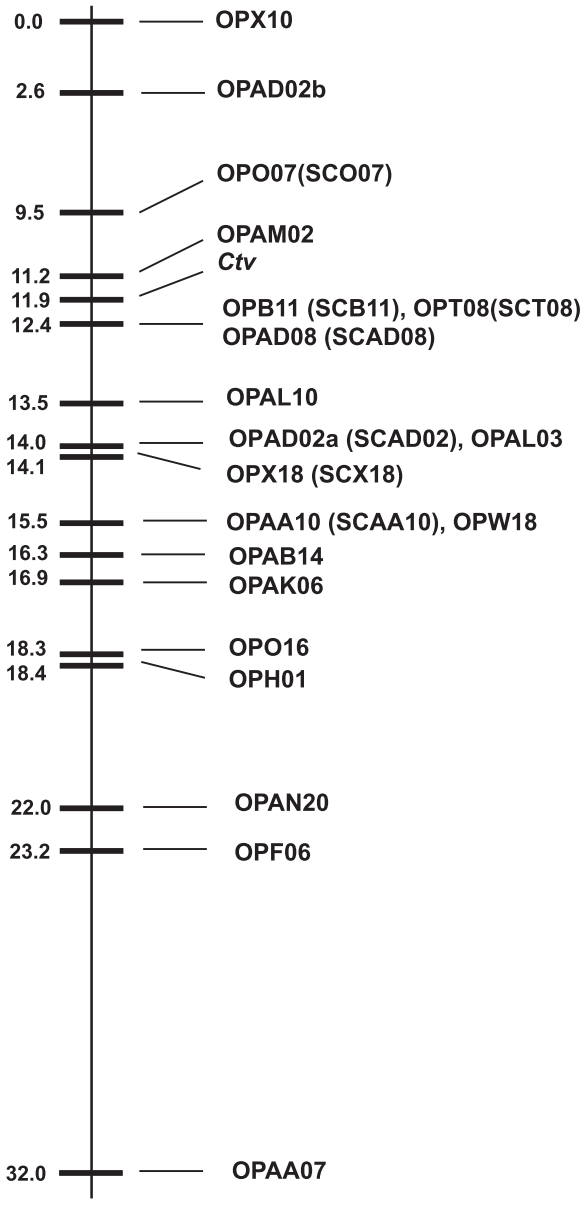

Fig. 3. A localized linkage map of *Ctv* region of *Poncirus trifoliata* constructed using RAPD and SCAR markers (Deng et al. 1997)

Several researchers have used restriction fragment length polymorphism (RFLP), a DNA based marker, for citrus mapping (Liou 1990; Durham et al. 1992; Jarrell et al. 1992; Liou et al. 1996). After cloning and characterizing RFLP markers in *Citrus*, Liou (1990) developed a citrus RFLP-based map, comprising of 29 RFLP and 8 isozyme loci in eight linkage groups. Three other RFLP-based linkage maps have been developed within citrus. Each was constructed from highly heterozygous intergeneric crosses which allowed a range of segregating characteristics to be genetically dissected. One map contained 46 markers (Jarrell et al. 1992) while the other two, constructed within the same cross, had a total of 62 markers (Durham et al. 1992) and each contained 11 linkage groups. Durham et al. (1992) were the first to demonstrate the potential of combining RFLP and isozyme analyses (Fig. 2) for developing a genetic map for citrus and reported that a total of 11 isozymes and 58 RFLPs segregated in a monogenic fashion.

Citrus maps, using other DNA based molecular markers such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP) were also constructed for obtaining much higher density of entire genome coverage (Cai et al. 1994; Luro et al. 1994, 1996; Weber and Moore 1996; Simone et al. 1998; Ling et al. 1999; Roose et al. 2000; Sankar and Moore 2001). Several traits of horticultural importance including CTV resistance (Gmitter et al. 1996), nematode resistance (Ling et al. 2000), fruit acidity (Fang et al. 1997) and dwarfing (Cheng and Roose 1995) have been tagged with RAPD markers. The second of the two maps developed by Durham et al. (1992) was extended to include 109 RAPD markers, which condensed to nine linkage groups with a total length of 1,192 cM estimated to cover 70–80% of the *Citrus* genome (Cai et al. 1994).

ISSRs and AFLPs provide a relatively high-throughput polymorphism facilitating the development of dense maps that are effective for identifying markers linked to major genes (Roose 2000). Sanker and Moore (2001) evaluated the usefulness of ISSR analysis in generating markers to extend the genetic linkage map of citrus using a backcross population previously mapped (Durham et al. 1992) with RFLP, RAPD and isozyme markers (Cai et al. 1994). The new map has an improved distribution of markers along the linkage groups with fewer gaps; marker

Table 2. Crosses used for genetic maps of citrus genome

No	Crosses	Cross types	Progeny	Country
1a	*C. grandis* cv. Acidless × *C. jambhiri* cv. Florida	*Citrus* F1	35	USA
1b	*C. grandis* cv. Chandler × *P. trifoliata* cv. Webber-Fawcett	Intergeneric F1	360	USA
2	LB 1-21 (*C. reticulata* cv. Clementine × *C. paradisi* cv. Duncan) × *C. reticulata* cv. Clementine	*Citrus* BC1	65	USA
3	*C. grandis* cv. Thong Dee × USDA 17-40 (*C. grandis* cv. Thong Dee × *P. trifoliata* cv Pomeroy)	Intergeneric BC1	65	USA
4	Sacaton (*C. paradisi* × *P. trifoliata*) × Troyer (*C. sinensis* × *P. trifoliata*)	Intergeneric F1	60	USA
5a	*C. grandis* × *C. grandis*	*Citrus* self F1	52	France
5b	*C. reshni* × *P. trifoliata*	Intergeneric F1	52	France
6	*C. auratium* × *C. latipes*	*Citrus* F1	120	Italy
7a	*C. auratium* × *P. trifoliata* cv Flying Dragon	Intergeneric F1	66	Spain
7b	*C. volkameriana* × *P. trifoliata* cv Rubidoux	Intergeneric F1	80	Spain
7c	Self-pollation of *P. trifoliata* cv Flying Dragon	*Poncirus* self F1	57	Spain
8	*C. sunki* × *P. trifoliata*	Intergeneric F1	80	Brazil

order showed partial or complete conservation in the linkage groups, suggesting that ISSR markers are suitable for genetic mapping in citrus. Fang et al. (1998) identified RAPD and ISSR markers linked to the *Ctv* region in *P. trifoliata*. The genome map of an intergeneric backcross population of citrus, constructed using AFLP, gave 16 linkage groups covering 910.7 cM; when combined with the RAPD-based map, it generated 14 linkage groups covering 1,031.7 cM (Ling et al. 1999). Recupero et al. (2000) reported data on *C. aurantium* and *C. latipes* molecular maps based on a two-way pseudo-testcross mapping strategy using AFLP, RAPD, and RFLP markers.

Dominant markers like RAPDs, ISSRs and AFLPs are useful in the specific population in which they are identified, but are difficult to apply to other populations due to their biallelic nature, which reduces the probability of polymorphism (Roose 2000). To overcome the problems associated with RAPDs, they have also been converted into sequence characterized amplified region (SCAR) markers. This is done by cloning and sequencing RAPD products, designing longer specific primers based on the sequence and amplifying DNA under stringent conditions. SCARs have been developed linked to the *Ctv* resistance gene (Fig. 3) from *P. trifoliata* (Deng et al. 1997). SCAR markers have also been used in the studies on inheritance of citrus nematode resistance (Ling et al. 2000).

Table 2 lists the major crosses that were used in Table 3 for construction of different versions of citrus maps. As these maps (developed in different laboratories) share few common markers, there has generally been very little effort to inter-relate them. Since RAPD or AFLP markers are dominant, they are hardly used as chromosome-anchored markers for general reference and comparative mapping. Hence, recently, locus-specific DNA molecular markers such as simple sequence repeats (SSRs), expressed sequence tags (ESTs), or sequence tagged sites (STS) have also been integrated into these maps (Kijas et al. 1995; Kijas et al. 1997; Ruiz and Asins 2003). Roose et al. (2000) tested nine trinucleotide SSR markers developed by Kijas et al. (1997) in a population derived from *C. taiwanica* × *P. trifoliata* and observed that one of the primer pairs tested had the segregation type desired for combining maps. Cristofani et al. (2000) also used SSRs to construct linkage maps of *P. trifoliata* and *C. sunki*. They further reported a total of 78 RAPD, 3 SSR and 10 AFLP markers to fall into 11 linkage groups of *C. sunki* and 73 RAPD, 4 SSR and 9 AFLP to fall in 11 linkage groups of *P. trifoliata*. The integration of SSR markers into a linkage map of *Citrus* has demonstrated the utility of this marker type for genetic analysis within wide intergeneric crosses and the potential to act as "anchor loci" to align linkage maps from different crosses and laboratories (Kijas et al. 1997). However, their number was still too lim-

Table 3. Genetic maps developed for citrus genomes

References	Marker types	Markers	Linkages	cM	Crosses No[a]
Torres et al. 1985	isozymes	5	2	–	1a, 1b
Liou 1990	RFLP, isozymes	35	8	314	2
Durham et al. 1992	RFLP, isozymes	52	11	533	3
Jarrell et al. 1992	RFLP, isozymes	38	10	351	4
Cai et al. 1994	RAPD, RFLP, isozymes	189	9	1,192	3
Luro et al. 1996	RAPD	34 for *C. grandis*	7	600	5a, 5b
		95 for *Poncirus*	12	1,503	
Kijas et al. 1997	RFLP, ISSR	48	12	410	4
Simone et al. 1998	AFLP, RAPD, RFLP	247 for *C. aurantium*	20	1,000	6
		92 for *C. latipes*	12	600	
Ling et al. 1999	AFLP, RFLP, isozyme	337	11	1,026	3
Garcia et al. 1999	RAPD, RFLP, CAPS, isozyme	69	3	–	7b
Cristofani et al. 1999	RAPD	63 for *C. sunki*	10	732	8
		62 for *P. trifoliata*	8	866	
Roose et al. 2000	RAPD, RFLP, ISSR	156	16	701	4
Sankar and Moore 2001	ISSR, RAPD, RFLP, isozymes	310	9	874	3
Ruiz and Asins 2003	RAPD, SSR, IRAP	48 for *Poncirus*	10	–	7a, 7b, 7c
		120 for *C. auratium*	17		

[a] Cross number is cited from Table 2.

ited at that time to expand their comparative and integrative usage in subsequent citrus genomic exploration.

Recently, ESTs have proven to be powerful tools for gene discovery, gene mapping and for the analysis of quantitative traits. ESTs are generated by large-scale sequencing of randomly picked clones from cDNA libraries constructed from mRNA isolated at a particular development stage and/or tissue; these sequences are available from the public domain such as GenBank (Guo et al. 2004). From the total EST sequence database, a representative set of unigenes are derived and their functions are compared to genes of known function from other organisms. Arrays can be designed using the unigenes to observe the spatial and temporal expression profiles of the available citrus genes. Many ESTs also contain SSR sequences, and through data mining these can be identified and exploited, thus increasing greatly the number of SSRs available as anchoring loci. Development of EST-based genetic maps covering the entire citrus genome is under way and will lay the basis of integration with physical maps for future genome sequencing (Chen et al. 2006).

14.3 Molecular Tagging and Cloning of Specific Genes and QTLs

Molecular marker technologies have provided tools to tag the genes of known phenotypes by developing localized molecular linkage maps, which are essential for a map-based cloning (MBC) approach and marker-assisted selection (MAS) breeding programs. With QTL mapping, the roles of specific resistance loci can be described, race-specificity of partial resistance genes can be assessed, and interactions between resistance genes, plant development and the environment can be analyzed. Resistance to CTV, apparently, was the first phenotype in citrus for which a localized map was developed (Gmitter et al. 1996). The linkage map contained DNA markers associated with its designated conferring gene, *Ctv*. The *Ctv* gene was identified from *Poncirus* and was assumed to be a single dominant gene (Yoshida et al. 1983; Yoshida 1985, 1993). This map was developed using a bulked segregant analysis (BSA) approach (Michelmore et al. 1991) and RAPD markers. Since then, several labs have independently made efforts to

map and clone *Ctv*. Some of these maps shared several common markers (Gmitter et al. 1996; Fang et al. 1998), while others did not (Mestre et al. 1997a; Cristofani et al. 1999). Two BAC contigs (Fig. 4) with integrated fine genetic maps have been constructed (Deng et al. 1997, 2001; Yang et al. 2001), resulting in full length sequencing of the locus spanning several hundreds of kilobases and identification of the candidate genes (Yang et al. 2003; Gmitter et al. unpublished data). These putative *Ctv* gene(s) are now under further confirmation using genetic transformation and complementation tests. However, evidence after prolonged CTV challenge has suggested that there may be more than one CTV resistance genes involved (Mestre et al. 1997b). Under a similar prolonged CTV challenge on a population of citradias (derived from the cross between sour orange and *Poncirus*), one CTV resistance gene was later mapped in a different location within linkage group 4 of *Poncirus*. The change of mapping position was interpreted as a deviation from the single gene hypothesis, which could be QTLs (Bernet and Asins 2003). Further, by QTL analysis of CTV-citradia interactions, considering CTV accumulated titer as a quantitative trait, up to five minor QTLs were detected besides the previously located major *Ctv* gene (Asins et al. 2004). There were other pathogen resistant phenotypes mapped. For example, it was found that a major QTL, designated *Tyr1*, controls resistance to citrus nematode (Ling et al. 2000), and it was adjacent to the *Ctv* region (Ling 1999). Nineteen putative QTLs (8 in *C. volkameriana* and 11 in *P. trifoliata*) controlling the number of fruits per tree were detected in the *C. volkameriana* and *P. trifoliata* progeny (Garcia et al. 2000). Mapping of QTLs associated with freezing tolerance was accomplished using a *C. grandis* × *P. trifoliata* F_1 pseudotestcross population (Weber et al. 2003). Other QTLs controlling some fruit characters and tolerances to salt and cold stress were characterized and DNA linkage maps for these traits have also been constructed (Table 4).

Molecular maps, particularly of those with very closely flanking or co-segregating DNA markers with the gene(s) of interest, may be very useful for fur-

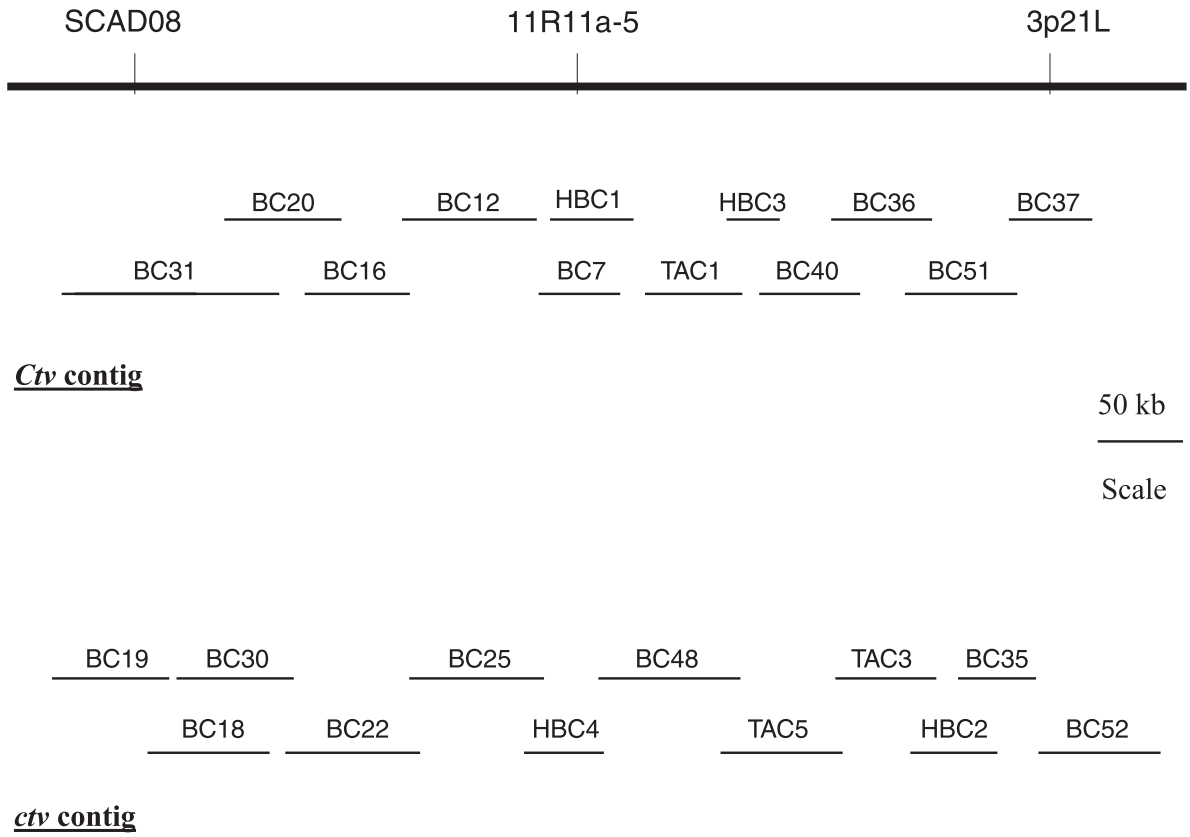

Fig. 4. BAC contigs of CTV resistance gene region (*Ctv* contig) and its allelic susceptibility gene region (*ctv* contig, Deng et al. 2001)

ther genomic manipulation, MBC and MAS breeding programs (Recupero et al. 2000; Asins 2002). MBC, also called positional cloning, is an approach using comprehensive genetics, genomics and bioinformatics tools to isolate gene(s) without prior knowledge of gene product. In theory, flanking or co-segregating DNA markers associated with the gene of interest are identified and a contig covering the target gene region with large insert genomic DNA clones (usually bacterial artificial chromosomes or BACs) are constructed. Sequencing of the spanning physical region and subsequent analysis of the sequence using various gene prediction programs will result in identification of the gene candidate sequences that can be confirmed by complementary test after genetic transformation into the host plant. This strategy has been employed to isolate *Ctv*, a single dominant gene from *P. trifoliata* that confers resistance to CTV (Gmitter et al. 1998; Deng et al. 2001). The resistance gene contig consists of 20 BACs and is approximately 550 kb in length. The susceptibility gene contig, derived from the susceptible citrus chromosome region consists of 16 BACs and extends to about 450 kb (Deng et al. 2001). Although it now takes less than 1 person-year to isolate a gene in *Arabidopsis* (Jander et al. 2002), application of similar tools in cloning citrus gene(s) would take a much longer time to reach the goal. For example *Ctv*, the only citrus gene upon which extensive MBC efforts have been made, has taken about ten years to reach the last step partly due to genetic restraints. However, MBC will make trait-specific improvement of citrus cultivars and rootstocks possible. Isolation and deployment of gene(s) of interest will greatly benefit the citrus industries and would enable a deeper understanding of the citrus genome.

Morphological traits such as dominant trifoliate leaf, considered to be the earliest MAS marker, were easily used to distinguish zygotic hybrids from nucellar seedlings. However, they cannot be used with varieties without such distinct characters. Therefore, common MAS is carried out with the aid of generic biochemical and DNA based markers. Isozymes, RAPDs and EST-SSRs have also been used in the identification of hybrids (Soost and William 1980; Nageswara Rao et al. 2006). The use of DNA based molecular markers to select rootstocks that

Table 4. Mapped citrus phenotypes

Phenotypes	Genotypes	Genetic map
Resistances to		
Citrus tristeza virus (CTV)	Single dominant[a]	Gmitter et al. 1996
Nematode	QTLs	Ling et al. 2000
Citrus variegated chlorosis (CVC)	QTLs	Oliveira et al. 2002
Alternaria	QTLs	Dalkilic et al. 2005
Asian citrus canker (ACC)	QTLs[a]	Choi et al. 2005
Citrus leaf miner (CLM)	QTLs	Bernet et al. 2005
Tolerance to		
Cold accumulation	QTLs	Cai et al. 1994
Na^+ stress	QTLs	Tozlu et al. 1999a
Cl^- stress	QTLs	Tozlu et al. 1999a
Salinity	QTLs	Tozlu et al. 1999b
Freezing	QTLs	Weber et al. 2003
Characters of		
Dwarfing	Single dominant	Cheng and Roose 1995
Acidity	QTLs	Fang et al. 1997
Apomixis	QTLs	Garcia et al. 1999
Nucellar embryony	QTLs	Kepiro and Roose 2000
Yield and seed number	QTLs	Garcia et al. 2000
Rooting	QTLs	Siviero et al. 2003

[a] Different genotypes, which may or may not be from the same locus, were found to have the same phenotype name.

may contain many of the desired resistances to CTV, nematode, *Phytophthora,* etc., will be of very high cost-efficiency as compared to traditional greenhouse or field screening approaches using inoculation. Blind tests of several rootstock selection populations of known phenotypes using DNA markers associated with CTV and nematode resistance genes indicated that MAS is a very promising and highly effective tool for breeding programs (Gmitter et al. unpublished data). In addition, molecular markers have also been widely applied on phylogenetic and taxonomic studies (Herrero et al. 1996; Fang and Roose 1997; Fang et al. 1997; Bret et al. 2001).

14.4 Citrus Genome Plan and Future Trends

The expanding capabilities of genomics and bioinformatics have the potential to revolutionize the entire field of citrus biology and genetics, and they offer promise of greatly improving the cultivars that are grown through precise and targeted manipulations of the genome. The foundations for the future are currently being laid by the international citrus genomics community. Many citrus genetic linkage maps have been developed in different laboratories in the past two decades (Table 3). Due to the use of very few common markers, it is difficult to interrelate the various linkage groups identified on these maps. Hence, it is essential to develop a reference map of the model genotype (Chen et al. 2006). The reference map should include a set of markers that are highly polymorphic and which can be mapped in various populations. This will allow various maps to be compared and combined. Also, more molecular markers are needed to saturate the genome. Long term studies of the nature and mode of inheritance of economically important traits must be pursued to link these difficult-to-evaluate traits more easily to the scored markers (Gmitter et al. 1992). Limited efforts have been made for single-case characterization of a citrus genome in a few areas such as resistance gene candidates (RGCs) (Deng et al. 2000; Deng and Gmitter 2003; Bernet et al. 2004), retrotransposons (Asins et al. 1999; Bernet and Asins 2003), microsatellites (Kijas et al. 1995; Ahmad et al. 2003), satellites (Fann et al. 2001), variations from fragment restriction (Liou et al. 1996), methylation (Cai et al. 1996), and individual gene expressions (Moriguchi et al. 1998; Shimada et al. 2005). Physical maps of citrus, integrated with genetic linkage maps, are also required for efficient localization and isolation of the genes, for studying the organization and evolution of the genome, and as an initial step for efficient whole genome sequencing by serving as the scaffold onto which genomic sequence will be assembled.

Large-scale citrus genome plans have been launched in several countries, and the International Citrus Genome Consortium (ICGC) has been initialized, to provide general guidance and overall goals on a citrus genome sequencing plan. The countries that are leading in planned citrus genome sequencing research are Brazil, US, Spain, Japan, France, Australia, China, Israel and Italy (Machado et al. 2005; Omura et al. 2005; Roose et al. 2005; Talon et al. 2005). Those activities, already done, being done, and to be done, include sequencing ESTs, developing microarray platforms for expression and genotyping studies, constructing genetic maps using ESTs or SNPs, fingerprinting large insert clones to develop physical maps, integrating genetic and physical maps, and eventually the complete sequencing of one or two citrus genome(s). A great number of citrus EST sequences, for example, over 200,000 in Brazil, and over 100,000 in the US, have been acquired (Fig. 5). A citrus GeneChip® from Affymetrix, containing 30,264 citrus unigene probe sets and other important contents, has been recently released (Close et al. 2006). Some are partially or completely released to the public database such as GenBank, and others are still kept for in-house use. Regardless of the availability, these contributors and other citrus genomic research communities have been utilizing comprehensive bioinformatics tools to categorize them, to design gene chips, to explore genetic information such as SSRs and SNPs, for large-scale gene expression and function studies, genetic and physical mapping, and comparison with other advanced genomes (Hisada et al. 1997; Fujii et al. 2003; Boscariol et al. 2005; Terol et al. 2005). Great progress on citrus genome research has been, and is being made, by these efforts eventually leading to the completion of an international citrus genome plan.

Citrus genomic technology is essential for the sustainability and future viability of the world's citrus industries. Most of the critical goals for scion improvement, such as resistance to devastating diseases or quantum changes in fruit quality attributes (color,

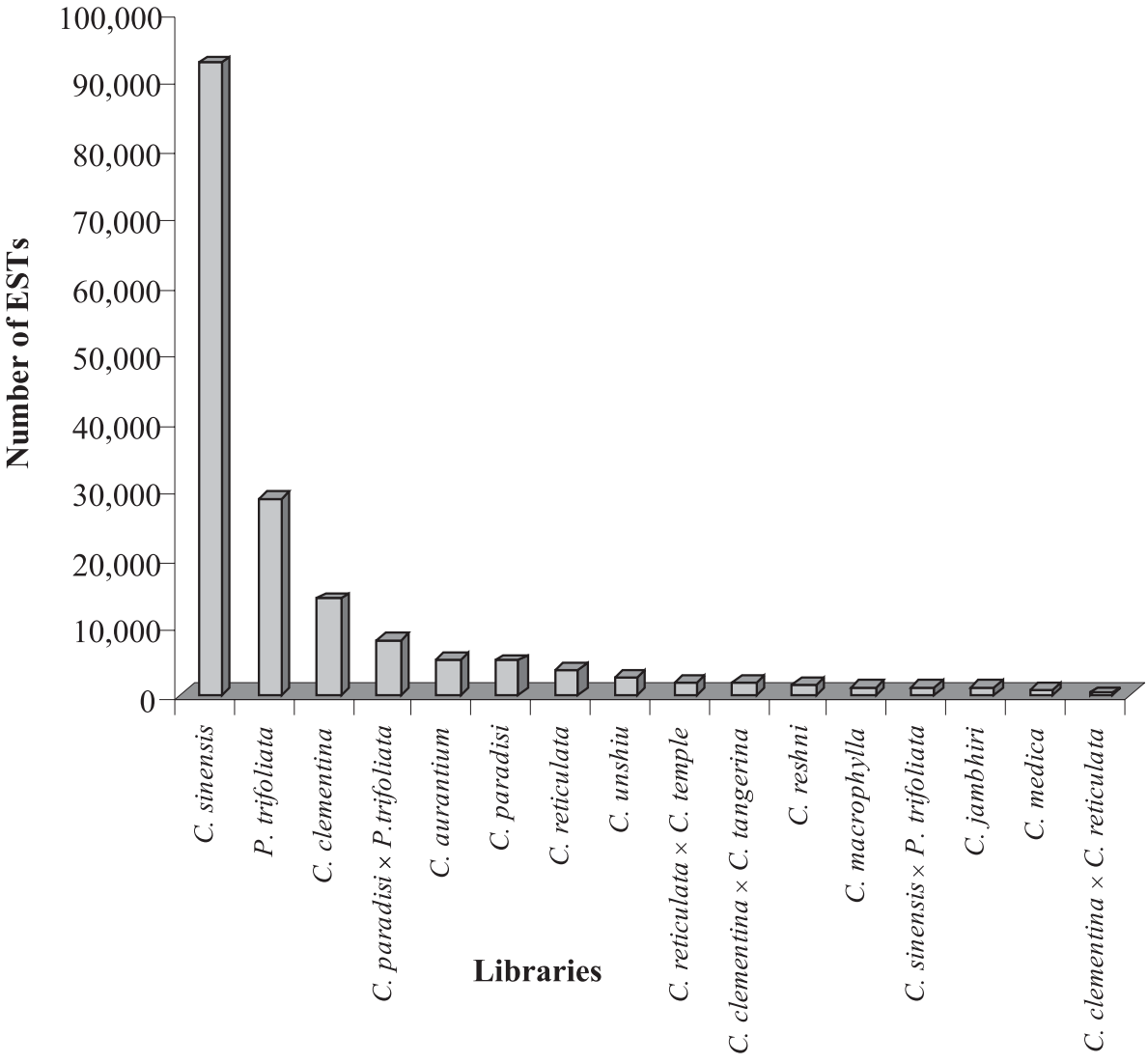

Fig. 5. Current *Citrus* EST entries in GenBank dbEST [Modified from International Citrus Genome Consortium (ICGC)]

flavor, peelability, nutrient content, and phytonutrient value to improve human health), are difficult if not absolutely impossible to approach in any practical sense by conventional breeding strategies. It will be through genomic research that an understanding of fundamental processes can be realized, candidate genes can be identified and cloned, and through some type of genetic transformation these genes and this information will be exploited for the improvement of citrus. Likewise, rootstock improvements will be hastened and maximized through the application of new knowledge and tools developed from it, to make sexual hybridization remarkably more efficient. It is important to keep in mind, however, that the value and utility of new genetic combinations must be demonstrated ultimately by field trials to verify the function and productivity of genetically modified citrus, and the value to consumers of improvements in fruit quality will be proven in the marketplace.

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