Chapter 3
Citrus

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3.1 Botany of the Genus *Citrus*

Citrus is one of the world's most important fruit crops and has a total world production of 105.4 million tons (FAO 2006). It is also valuable for human nutrition and well-being. The genus *Citrus* belongs to the subtribe Citrinae, tribe Citreaea, subfamily Aurantioideae of the family Rutaceae. Based on leaf, flower, and fruit properties, the genus is further divided into two subgenera, namely, *Citrus* and *Papeda*. A number of studies have been carried out, using their morphological characteristics, on the relationships between genera and species, which have led to the formulation of numerous classification systems. The most commonly used citrus classifications are by Swingle who recognized only 16 species (Swingle and Reece 1967) and Tanaka who recognized 162 species (Tanaka 1977). Most *Citrus* species are diploids with nine pairs of chromosomes ($2n = 2x = 18$), though polyploids have also been reported. Cytogenetic studies have revealed that the citrus chromosomes are small but highly variable (Naithani and Raghuvanshi 1958; Raghuvanshi 1962; Guerra 1984, 1993). Karyotypes based on Geimsa C-banding (Guolu 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. DAPI and CMA staining of citrus metaphase chromosomes showed that several chromosomes contain large blocks of terminal heterochromatin (Miranda et al. 1997). The genome size of citrus is approximately 367 Mb, which is nearly three times the size of *Arabidopsis* genome.

Citrus plants are small to medium sized, spreading, evergreen trees with thorny shoots, growing to about 2–15 m tall with most species being single-trunked with very hard wood but with a relatively thin (<5 mm) bark (Manner et al. 2006; Gmitter et al. 2007; Roose and Close 2008). The stem is green, with unifoliate, alternate leaves. Leaf shape varies from ovate to lanceolate; the size varies from 4 to 10 cm long and may possess more or less broadly winged petioles. Flowers are perfect or staminate due to abortion of the pistil (Roose and Close 2008). They are fragrant, borne solitary, or in short cymes in the axils of the leaves or in small lateral or terminal inflorescences. The sepals are four to five lobed. The petals are five, thick, linear, strap-shaped, and alternate with the sepals (Schneider 1968). The stamens are polyadelphous, cohering toward the bases in a few bundles and are usually four times as many as petals. The yellow, four-lobed anthers surround the pistil at or near the level of the stigma (Spiegel-Roy and Goldschmidt 1996). The ovary is superior and is composed of 6–14 carpels joined to each other and to a central axis (Soost and Roose 1996). The style is prominent but usually deciduous and contains as many tubes as there are cells in the ovary (Gmitter et al. 2007). The fruit is a hesperidium berry whose form and size vary from globose to oblong and oblate. They are highly fragrant and are divided into 10–14 segments consisting of juice vesicles, separated by thin septa, with seeds near the inner segment angle (Manner et al. 2006; Roose and Close 2008). The segments are surrounded by a white endocarp (albedo) and then the rind (flavedo), which has many oil glands and is usually colored orange or yellow at maturity. Seed content is variable among and within species.

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Citrus is grown in the tropical and subtropical regions around the world, primarily between the latitudes of 40°N and 40°S, from equatorial, hot–humid climates through warm–subtropical, and even cooler maritime climates (Spiegel-Roy and Goldschmidt 1996). It can be grown in a wide range of soil types from coarse sand to heavy clay, provided the soils are well drained (Gmitter et al. 2008). The long history of selection and vegetative propagation of citrus has, on the one hand, led to the perpetuation of the "elite" germplasm lines but, on the other hand, to the neglect and disappearance of the progenitor wild types (Krueger and Navarro 2007). This may have reduced the genetic diversity of citrus. While limited information is available on genetic resources/diversity of citrus, no information is available on the frequency of occurrence. With Southeast Asia being the center of origin, greatest amount of diversity of citrus resources may be expected to be found there (Reuther 1977; IBPGR 1982). Wild citrus may rarely be existing as scattered trees in remote areas rather than as pure stands. Even when natural populations are present, it is difficult to determine whether they represent wild ancestors or are derived from naturalized forms of introduced or selected varieties (Krueger and Navarro 2007).

3.2 Citrus Conservation Initiatives

To assess the genetic vulnerability, information on the extent and distribution of genetic diversity is a must. In this regard, limited information is available on citrus due to its wide cultivation. This has made it difficult to assess the magnitude of the actual risk of genetic erosion through the replacement of a large number of unselected citrus varieties by a small number of improved cultivars (Duran-Vila 1995). Citrus germplasm is traditionally conserved in clonal orchards belonging to botanical gardens or scientific institutions (Table 3.1). The accessions of living trees are the source of propagative materials for distribution as well as often being able to serve as resources for a limited amount of characterization and evaluation

Table 3.1 Summary of the number of accessions of citrus conserved in different countries

No. of accessions	Institution/University/Organization where maintained	Country
953	Concordia citrus collection	Argentina
374	EEAOC citrus germplasm collection	Argentina
1,858	Sylvio Moriera citrus collection	Brazil
602	National Research Center for Cassava and Tropical Fruit Crops	Brazil
390	Fruit Crop Research Center	Brazil
96	Universidad Catolica citrus collection	Chile
>1,000	National Citrus Germplasm Repository, Beibei, Chongqing, Sichuan province	China
280 in vivo and 110 in vitro	Huazhong Agricultural University, Huazhong	China
304	Instituto de Investigaciones de Citricos y otros Frutales	Cuba
>600	Garo Hills	India
>600	Eight sites belonging to Agricultural Institutes and Universities at Chetalli, Bangalore, Rahuri, Tirupati, Abohar, Bhatinda, Yercaud, and New Delhi	India
>500	–	Indonesia
>1,200	Fruit Tree Research Stations at Tsukuba, Okitsu, and Kuchinotsu	Japan
>100	Institute of Plant Breeding	Philippines
>500	Three sites including Phichit Horticultural Research Center and Nan Horticultural Research Center	Thailand
412	INIA Salto Grande collection	Uruguay
281	Facultad de agronomia Salto collection	Uruguay
99	Direccion de servicios agricolas collection	Uruguay
>250	Florida Citrus Arboretum	USA
>200	The Texas A&M University, Kingsville Citrus Center	USA
>100	Rio Farms Citrus Variety Collection	USA
865	Citrus Variety Collection	USA
–	National Institute of Agricultural Science and Technology and Phu Ho Fruit Research Center	Vietnam

(Krueger and Navarro 2007). In situ conservation of citrus has been attempted in most of the Southeast Asian countries, where citrus is supposed to have originated. These include China, India, Indonesia, Malaysia, Philippines, and Thailand. However, growing population and other development pressures in these countries have led to deforestation and loss of genetic diversity. This had made ex situ preservation of citrus genetic resources imperative. Ex situ collections also make genetic resources more readily available to users. Outside of the centers of origin/diversity, large ex situ collections are found in Argentina, Australia, Brazil, France, Morocco, New Zealand, South Africa, Spain, Turkey, and the USA (Krueger and Navarro 2007).

To coordinate the interactions between the various entities dealing with citrus germplasm, the Global Citrus Germplasm Network (GCGN) was established under the aegis of the Food and Agriculture Organization (FAO). GCGN is chaired by a General Coordinator and guided by Coordinating Board and will serve to link different initiatives in different parts of the world dealing with citrus genetic resources exploration, conservation, and utilization (Krueger and Navarro 2007). All citrus germplasm banks have a field collection. A duplicated protected collection is maintained by a few germplasm banks to maintain healthy genotypes and to diminish risks of losses of plants due to biotic or abiotic stresses. Usually, collections include two copies of each accession except for genotypes of low use for which only one plant is maintained.

High costs of traditional conservation system have led to the use of cryopreservation for long-term conservation of citrus genetic resources (Perez 2000). Various organs and tissues such as embryogenic callus lines, somatic embryos, shoot tips, ovules, pollen, embryo axes, and intact seeds have been cryopreserved (Mumford and Grout 1979; Bajaj 1984; Marin and Duran-Vila 1988; Kobayashi et al. 1990; Lambardi et al. 2004; Wang and Deng 2004; Malik and Chaudhury 2006) using techniques such as "slow-cooling", "one-step freezing", "encapsulation–dehydration", and "encapsulation–vitrification." Organs and tissues of various species such as *C. aurantifolia*, *C. aurantium*, *C. deliciosa*, *C. limon*, *C. sinensis*, *C. paradisi*, *C. sudachi*, *C. halimii*, *C. madurensis*, *C. latipes*, *C. macroptera*, and *C. hystrix* have been cryopreserved.

3.3 Origin and Evolution of *Citrus*

The lack of sufficient descriptions, specimens, and original habitats has made it difficult for the biogeographers to define precisely the centers of origin and ancestors of citrus. A multitude of natural interspecific hybrids and cultivated varieties, including spontaneous mutants, obscure further the history of citrus (Gmitter et al. 2008). It is considered to have originated from Southeast Asia, especially East India, North Burma, and Southwest China, possibly ranging from northeastern India eastward through the Malay Archipelago, north into China and Japan, and south to Australia (Tanaka 1954; Webber 1967; Scora 1975; Gmitter and Hu 1990; Soost and Roose 1996). The oldest known reference to citrus appears in Sanskrit literature that dates back to before 800 BC followed by descriptions in Chinese, Greek, and Roman literature (Webber 1967; Scora 1975).

Understanding taxonomy, phylogenetic relationships, and genetic variability in citrus is critical for determining the origin, evolution, and genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies or core collections, establishing breeding programs, and registering new cultivars (Herrero et al. 1996). Barrett and Rhodes (1976) carried out a comprehensive phylogenetic study and evaluated 146 morphological and biochemical tree, leaf, flower, and fruit characteristics. They suggested that there were three true ancestral citrus species, namely, *C. maxima* (pummelos), *C. reticulata* (mandarins), and *C. medica* (citrons). Scora (1975) also viewed these three species to be the only true citrus and all other species as introgressions of these ancestral forms. More recently, the phylogeny and genetic origin of the important species of citrus has been investigated using molecular markers (Nicolosi et al. 2000; Moore 2001; Barkley et al. 2006). These studies have supported the hypotheses of Barrett and Rhodes (1976) and Scora (1975), though there may be other species that might also be considered as ancestral. *C. aurantifolia*, *C. micrantha*, and *C. halmii* are also included in the list of "true" citrus species by many researchers. Molecular markers, isozymes, chloroplast, and nuclear genome analysis were utilized to assess diversity, phylogenetic relationships, and parentage in lemon (*C. limon*) accessions (Gulsen and Roose 2001a, b). However, these studies have not

been able to clearly differentiate between all the species. Hence, there is a need for additional taxonomic studies to further clarify the taxonomic distinctions.

3.4 Role in Development of Cytogenetic Stocks and Their Utility

Chromosome addition, deletion, and substitution lines are extremely valuable tools for genome analysis. They allow direct localization of genes to specific chromosomes based on their expression or non-expression in lines carrying the respective added, deleted, or substituted chromosome or chromosome segment (Snowdon et al. 2002). As the chromosomes of citrus are small, it is difficult to study them in detail. This has limited the development of molecular cytogenetic tools in citrus. Another limiting factor is the availability of appropriate flower buds, for studying meiosis, only in the spring (Roose and Close 2008). Studies on chromosome banding in citrus have focused on distinguishing the chromosomes of different species and varieties (Guerra 1984, 1993; Yamamoto and Tominaga 2003). For the detection of cytogenetic variation in citrus chromosomes, fluorescence in situ hybridization (FISH) using repetitive sequence probes (rDNA) has been used (Matsuyama et al. 1996). A combination of chromosome banding and rDNA hybridization has revealed species-specific distinct patterns (Moraes et al. 2007). In situ hybridization of 18S–5.8S–25S rDNA probes labeled with biotin or rhodamine and 5S rDNA probes labeled with digoxigenin have been used to locate rDNA sites on root-tip metaphase chromosomes of Troyer citrange (*C. sinensis* × *Poncirus trifoliata*), Sacaton citrumelo (*C. paradisi* × *P. trifoliata*), and African shaddock × Rubidoux trifoliate (*C. maxima* × *P. trifoliata*). Counterstaining with the fluorochromes CMA3 and DAPI uniquely identified many but not all chromosomes. Counts of 18S–25S rDNA sites on metaphase chromosomes or interphase and prophase nuclei suggested that Troyer had four major and usually one minor site in all complete metaphases examined, Sacaton citrumelo had six sites, and a *C. maxima* × *P. trifoliata* hybrid had five sites in two complete metaphases (Roose et al. 1998). To date, no reports are available on localization of individual gene sequences or large insert clones such as bacterial artificial chromosomes (BACs) onto citrus chromosomes (Roose and Close 2008).

Euploidy in citrus is represented by monoploids, diploids, triploids, tetraploids, pentaploids, hexaploids, and octaploids. Haploids have been obtained by anther culture in *C. madurensis* (Chen et al. 1980) and other citrus species (Germana et al. 1991, 1994; Germana and Reforgiato 1997; Chiancone et al. 2006). Two haploid seedlings of *C. natsudaidai* Hayata were obtained from 3,000 seeds irradiated with gamma rays (Karasawa 1971). Only diploid plants were obtained by in vitro differentiation of microspores of *C. aurantium* (Hidaka and Omura 1989). Improvements in embryo rescue and somatic hybridization methodologies have greatly increased the range of possibilities for development of chromosome addition lines by interspecific and intergeneric hybridization. Tetraploid somatic hybrids have been obtained from intergeneric (compatible as well as incompatible) and interspecific somatic hybridization of citrus for scion and rootstock improvement (Grosser and Gmitter 2005). Attempts have also been made to combine citrus with over 30 sexually incompatible genera, including *Aegle*, *Atalantia*, *Citropsis*, *C. ichangensis*, *C. jambhiri*, *Eremocitrus*, *Fortunella*, *Microcitrus*, *Poncirus,* and *Severinia*, that exhibit desirable rootstock attributes (Mourao Fo and Grosser 1992; Grosser and Gmitter 2005).

3.5 Molecular Mapping in *Citrus*

To increase the efficiency of breeding programs, it is desired to have genetic linkage maps as they include important linkage relationships among molecular markers and genes that breeders wish to manipulate for cultivar improvement. Genetic maps of citrus may provide the basis for early screening procedures, 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) rather than relying on frequently difficult, time-consuming, and inefficient approaches based on phenotypic

Table 3.2 Citrus linkage maps

Mapped parent	Type of cross	Population size	Markers used	Total markers	Map length (cM)	Linkage groups	Reference
C. grandis cv. Acidless × *C. jambhiri* cv. Florida	*Citrus* F_1	35	Isozymes	2	–	2	Torres et al. (1985)
LB 1–21 (*C. reticulata* cv. Clementine × *C. paradisi* cv. Duncan) × *C. reticulata* cv. Clementine	*Citrus* BC_1	65	Isozymes, RFLP	35	314	8	Liou (1990)
C. grandis × *C. grandis*	*Citrus* self F_1	52	RAPD	34	600	7	Luro et al. (1996)
C. auratium	F_1	120	AFLP, RAPD, RFLP	247	1,000	20	de Simone et al. (1998)
C. latipes	F_1	120	AFLP, RAPD, RFLP	92	600	12	de Simone et al. (1998)
C. sunki	F_1	80	RAPD	63	732	10	Cristofani et al. (1999)
C. auratium	F_1	120	AFLP, RAPD, RFLP	247	1,021	10	Recupero et al. (2000)
C. latipes	F_1	120	AFLP, RAPD, RFLP	92	561	12	Recupero et al. (2000)
C. auratium	F_1	80	IRAP, RAPD, SSR	120	442	15	Ruiz and Asins (2003)
C. volkameriana	F_1	80	IRAP, Isozymes, RAPD, RFLP, SSR	97	460	10	Ruiz and Asins (2003)
C. sinensis	F_1	97	EST-SSRs	113	776	11	Chen et al. (2008)

AFLP amplified fragment length polymorphism, *EST-SSR* expressed sequence tag-simple sequence repeat, *IRAP* inter-retrotransposon amplified polymorphism, *RAPD* randomly amplified polymorphic DNA, *RFLP* restriction fragment length polymorphism, *SSR* simple sequence repeat

characterizations (Talon and Gmitter Jr 2008). Several linkage maps of citrus have been published (Table 3.2) since 1980s using isozymes as well as DNA markers such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR), simple sequence repeat (SSR), and intersimple sequence repeat (ISSR) (Durham et al. 1992; Garcia et al. 1999; Roose et al. 2000; Sankar and Moore 2001; Chen et al. 2008). Several of these maps involved at least one parent that is a *Citrus* × *Poncirus* parent to ensure high heterozygosity, but the hybrids obtained have inedible fruit, making these mapping populations of limited use for fruit traits. These maps share few markers making their comparison difficult (Roose et al. 2000). They are also incomplete in that the number of major linkage groups often differs from the number of chromosomes. While such maps are still quite useful, more complete, high-density maps would be more useful for both quantitative trait loci (QTL) analysis and for anchoring BAC and genome sequence (Roose and Close 2008).

Gene tagging has been widely used in citrus. Citrus, being heterozygous, individuals differing for a trait are identified and crossed, and the trait in the progeny is measured and analyzed using bulk-segregant analysis with DNA markers to identify segregating markers

linked to the genes of interest. Several traits of horticultural importance including CTV resistance (Gmitter et al. 1996) from *P. trifoliata*, nematode resistance (Ling et al. 2000) from *P. trifoliata*, fruit acidity (Fang et al. 1997), dwarfing (Cheng and Roose 1995), and nucellar embryony (Garcia et al. 1999; Kepiro 2004; Nageswara Rao et al. 2008) from *C. latipes* × *C.aurantium* F_1 hybrids (Recupero et al. 2000) have been tagged. QTL analysis for tagging regions containing genes that influence salinity tolerance, *Alternaria* brown spot resistance, and several other traits have been identified, but fine mapping of QTLs has not been reported (Tozlu et al. 1999a, b; Dalkilic et al. 2005; Roose and Close 2008). F_1 progeny of *C. sunki* × *P. trifoliata* were evaluated for the detection of QTLs linked to citrus *Phythophthora* gummosis resistance (Siviero et al. 2006). 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 pseudo-testcross population (Weber et al. 2003). Molecular maps, particularly of those with very closely flanking or cosegregating DNA markers with the gene(s) of interest, may be very useful for further genomic manipulation, map-based cloning, and marker-assisted breeding programs (Recupero et al. 2000; Asins 2002; Gmitter et al. 2007).

The physical mapping of complex genomes is based on the construction of a genomic library and the determination of the overlaps between the inserts of the mapping clones in order to generate an ordered, cloned representation of nearly all the sequences present in the target genome (Talon and Gmitter Jr 2008). The construction of BAC libraries containing clones with large DNA fragments is essential for the generation of high-resolution physical maps. The relatively small genome size of citrus makes preparation and analysis of BAC libraries an attractive approach (Roose and Close 2008). Two BAC contigs with integrated fine genetic maps have been constructed from intergeneric citrus and *P. trifoliata* hybrid (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).

3.6 Role in Crop Improvement Through Traditional and Advanced Tools

3.6.1 Traditional Breeding Efforts in Citrus

Citrus is vegetatively propagated. Due to its heterozygous nature, sexual hybridization to create new genotypes results in substantial variation of the characters in the progeny as they produce widely variant sexually derived progeny (Gmitter et al. 2009). It also has a long juvenile period of seedlings (5 or more years) making citrus breeding not only a difficult, but a costly and land-intensive proposition. Little is known about the genetic mechanisms controlling the inheritance of agriculturally important traits with only a few important traits showing single gene inheritance (Furr 1969; Gmitter et al. 1992). Nucellar embryony is also common in citrus. Conventionally, controlled crossing is carried out to combine desirable traits by cross-pollination. After the hybrid fruits have matured, the seeds are extracted from the fruits and planted in the greenhouse. Once the seedlings have attained sufficient size, they are either grafted onto rootstocks or directly planted in the field and evaluated for disease and pest resistance, stress tolerance, and overall growth characteristics during the juvenile period (Davies and Albrigo 1994), and subsequently for fruit characteristics of scions or rootstock traits of interest.

Though it takes only minutes to effect pollination, the difficult nature of citrus breeding lies in the elimination of undesirable hybrids and the evaluation of selections (Sykes 1987). The major objectives of rootstock breeding are aimed to control the tree size and to improve resistance and tolerance to biotic and environmental stresses such as citrus blight, CTV, *Phytophthora*, citrus exocortis viriod (CVC), nematodes, cold, drought, salinity, and flooding. Reduction of tree size without affecting yield or scion health is also desirable (Soost and Roose 1996). Most of these attributes are present in *P. trifoliata*, making it a valuable resource for the genetic improvement of citrus rootstocks (Gmitter et al. 2007). A number of breeding programs have focused on obtaining intergeneric hybrids between *C. sinensis* × *P. trifoliata*, *C. paradisi* × *P. trifoliata*, and *C. reticulata* × *P. trifoliata*

for use as rootstocks. Rapid growth and lack of branching are other desirable characters for convenient and economical nursery production of rootstock seedlings (Soost and Roose 1996). Mutation breeding programs have also been established for the genetic improvement of citrus. Mutations have been induced by gamma rays and chemicals and the mutants have been analyzed for the desired traits (Hensz 1977; Hearn 1984; Deng and Zhang 1988; Deng et al. 1993; Gulsen et al. 2007).

3.6.2 Ploidy Manipulation in Citrus

Anther culture may provide the breeders and geneticists with the ability to recover haploid plants that would facilitate the recovery of useful recessive alleles or mutations useful for citrus cultivar improvement and genetic studies (Gmitter et al. 1992). Citrus anthers have been cultured in attempts to produce haploid plants and colchicine has been used to obtain homozygous diploids (Chen et al. 1980; Germana et al. 1991, 1994; Germana and Reforgiato 1997; Chiancone et al. 2006). Anther culture of *C. madurensis* gave rise to haploids (Chen et al. 1980). Haploid seedlings of *C. natsudaidai* have also been obtained by irradiation (Karasawa 1971), after in situ parthenogenesis induced by irradiated pollen (Ollitrault et al. 1996) and through gynogenesis induced by in vitro pollination with pollen from a triploid plant (Germana and Chiancone 2001). The production of diploid plants by culturing anthers of tetraploid somatic hybrids may also provide breeders with greater access to these unique genetic combinations (Gmitter et al. 2009). Diploid regenerants have also been obtained from anthers of diploid plants of *C. aurantium*, with nuclear fusion observed during the routes of microspore development being the suggested cause of diploidy in these regenerants (Hidaka and Omura 1989).

Triploids have been regenerated by in vitro culturing of hybrid endosperms of *C. sinensis*, *C. paradisi*, and *C. grandis* (Gmitter et al. 1990). However, this method has not been adopted as a breeding strategy because it is species- and cultivar-dependent, and is far less efficient than creating triploid offspring by interploid hybridization (Gmitter et al. 2009). Intergeneric and interspecific somatic hybridization has been attempted to produce tetraploid somatic hybrids (Grosser and Gmitter 2005). These tetraploids are used either as rootstock candidates or as parents in breeding programs (Cameron and Soost 1969; Cameron and Burnett 1978; Grosser et al. 1998). Crosses of diploid and tetraploid parentage using monoembryonic seed parents have given rise to tetraploid progenies that might be due to non-disjunction leading to the doubling of chromosome number of one of the parents during cell division. *Aegle*, *Atalantia*, *Citropsis*, *C. ichangensis*, *C. jambhiri*, *Eremocitrus*, *Fortunella*, *Microcitrus*, *Poncirus*, and *Severinia* are some of the sexually incompatible genera, which exhibit desirable rootstock attributes, used for somatic hybridization with citrus (Mourao Fo and Grosser 1992; Grosser and Gmitter 2005). A seedless tetraploid "*Shiyueju*" tangerine was created by treating the shoot of diploid "*Shiyueju*" with colchicine (Chen and Ou 1985). When diploid *Fortunella japonica* was treated with colchicine, it gave rise to seedless tetraploid *F. japonica* (Li and Zhang 1988). Tetraploid plants have also been recovered by the incorporation of colchicine in standard tissue culture media (Gmitter and Ling 1991; Gmitter et al. 1991).

3.6.3 Tissue Culture Regeneration

Application of plant cell and tissue culture has the potential to unlock an entirely new round of genetic improvements for citrus crops, most of which cannot be achieved by any other means (Gmitter et al. 2008). Citrus is amenable to regeneration via organogenesis and somatic embryogenesis. Organogenesis has been induced in vitro from various explants such as shoot meristems of *C. aurantifolia* and *C. sinensis*, nodal explants of *C. junos*, *C. sinensis*, and *C. aurantifolia*, stem internodes of *C. sinensis*, leaf sections of *C. sinensis* and *C. aurantifolia*, root tissues of *C. medica* and *C. aurantifolia*, mature and immature embryos of *C. limon*, and thin sections of mature stem segments of *C. sinensis* (Maheshwari and Rangaswamy 1958; Chaturvedi and Mitra 1974; Sauton et al. 1982; Barlass and Skene 1986; Moore 1986; Duran-Vila et al. 1989; Omura and Hidaka 1992; Al-Khayri and Al-Bahrany 2001; Kim et al. 2001; Kotsias and Roussos 2001; Huang et al. 2002; Cavalcante-Alves et al. 2003; Kobayashi et al. 2003; Mukhtar et al. 2005; Soneji et al. 2007; Perez-Tornero and

Porras 2008). Somatic embryogenesis has been induced in cultured nucelli of *C. sinensis* and *C. limon*, undeveloped ovules of *C. limonimedica*, nucellar embryos of *C. limonia* and *C. unshiu*, endosperm-derived callus of *C. grandis*, *C. paradisi*, and *C. sinensis*, juice vesicles of *C. unshiu*, anthers of *C. madurensis*, *C. natsudaidai*, and *C. aurantium*, styles of *C. aurantium*, *C. deliciosa*, *C. paradisi*, and *C. sinensis*, and pistil thin cell layers of *C. deliciosa*, *C. limon*, *C. madurensis*, *C. medica*, *C. tardiva*, and *C. sinensis* (Rangan et al. 1969; Starrantino and Russo 1980; Chaturvedi and Sharma 1985; Gmitter and Moore 1986; Gmitter et al. 1990; Nito and Iwamasa 1990; Carimi et al. 1995, 1998, 1999; Calovic et al. 2003; Chiancone et al. 2006; Jajoo 2010). The identification of valuable somaclonal variants holds great promise for cultivar improvement especially for those citrus species that are difficult to manipulate by sexual hybridization (Gmitter et al. 1992) and is being exploited to identify clones with improved traits such as fruit quality improvements across an extended season of maturity and disease resistance (Nadel and Spiegel-Roy 1987; Grosser et al. 2003). Plant regeneration systems are also potentially useful for obtaining genetic change through cell transformation or mutagenesis.

3.6.4 Genetic Engineering

For the introduction of specific traits into citrus without altering the genetic background, genetic engineering may be an efficient alternative. Genetic engineering has been applied for a number of traits in citrus (Table 3.3). CTV-derived genes such as the coat-protein gene, *p23 ORF*, have been introduced into citrus cultivars such as *C. aurantifolia*, *C. aurantium*, and *C. paradisi* in efforts to produce resistance to CTV (Gutierrez et al. 1997; Dominguez et al. 2000; Ghorbel et al. 2001, Fagoaga et al. 2006; Ananthakrishnan et al. 2007). CTV R genes, derived from the CTV resistant *P. trifoliata* genome, have also been introduced into *C. sinensis* (Soneji et al. 2007). The cDNA of the *Xa21*, a *Xanthomonas* resistance gene, has been introduced into citrus in efforts to produce resistance to citrus canker (Omar and Grosser 2007). *Arabidopsis* genes such as *LEAFY* or *APETALA1* that alter the growth habit, reduce juvenility, and regulate vegetative and other behavior have been introduced into citrange, a hybrid of *C. sinensis* × *P. trifoliata* (Pena et al. 2001). Genetic transformation and regeneration of mature tissues of citrus, which could bypass the juvenile phase, has also been attempted (Cervera et al. 1998). For salinity tolerance, a gene from yeast has been introduced into citrus (Cervera et al. 2000). Genes involved in the metabolic pathway regulation such as the carotenoid biosynthetic genes have been introduced in *C. paradisi* (Costa et al. 2002). Ethylene biosynthesis genes have also been introduced in Carrizo citrange (*C. sinensis* × *P. trifoliata*), *C. sinensis*, and *P. trifoliata* (Wong et al. 2001). Attempts have also been made to introduce juice quality-related pectin methylesterase gene for enzyme associated with the "cloud separation" into citrus (Guo et al. 2005).

3.7 Genomics Resources Developed

Expressed sequence tags (ESTs) are useful in identifying gene transcripts, making them instrumental in gene discovery and sequence determination. The first set of ESTs was reported from citrus seeds and mature fruits in rapid cell development phase (Hisada et al. 1997). Since then several groups have contributed to the sequencing and development of ESTs from reproductive and vegetative organs and tissues at different developmental stages and challenged with biotic and abiotic agents, and elicitor and hormonal treatments (Bausher et al. 2003; Shimada et al. 2003; Talon and Gmitter Jr 2008). At present, around 494,484 ESTs developed from various citrus species have been deposited in the National Center for Biotechnology Information (NCBI) EST database (http://harvest.ucr.edu) (Table 3.4).

Microarrays have also been developed for citrus for transcript profiling. Construction of a cDNA microarray to monitor expression of mRNA from 2,213 genes during fruit development was the first transcript profiling data to be reported (Shimada et al. 2005). A spotted array of 6,875 clones obtained from 22,000-EST collection was used to investigate gene expression patterns during fruit ripening and other traits (Forment et al. 2005; Cercós et al. 2006). Many higher density citrus microarrays consisting of 12,000 unigenes or 24,000-element cDNA array containing 20,000 unigenes, based on nearly 90,000 high-quality sequences generated from 52 different cDNA libraries

Table 3.3 Summary of genetic transformation of *Citrus*

Genotype used	Explant used	Method used	Gene(s) introduced	Reference
Citrus sinensis cv. Trovita	Protoplasts	PEG-mediated	*npt*II	Kobayashi and Uchimiya (1989)
C. jambhiri	Protoplasts	PEG-mediated	*cat, npt*II	Vardi et al. (1990)
C. sinensis cvs. Washington Navel and Trovita	Cell suspensions	*At*-mediated	*hpt, npt*II	Hidaka et al. (1990)
C. reticulata cv. Ohta Ponkan	Protoplasts	Electroporation	*uidA*	Hidaka and Omura (1993)
C. sinensis cv. Hamlin	Protoplasts	Electroporation	*gfp*	Niedz et al. (1995)
C. sinensis cv. Pineapple	Greenhouse internodal stem segments	*At*-mediated	*npt*II, *uidA*	Pena et al. (1995)
C. reticulata × *C. paradisi*	Cell suspensions	Particle bombardment	*npt*II, *uidA*	Yao et al. (1996)
C. aurantifolia	In vitro internodal stem segments	*At*-mediated	CTV-*CP*	Gutierrez et al. (1997)
C. aurantium	In vitro internodal stem segments	*At*-mediated	CTV-*CP*	Gutierrez et al. (1997)
C. aurantifolia cv. Mexican	Greenhouse internodal stem segments	*At*-mediated	*npt*II, *uidA*	Pena et al. (1997)
C. sinensis cv. Washington navel	In vitro epicotyl segments	*At*-mediated	*npt*II, *uidA*	Bond and Roose (1998)
C. sinensis cv. Pineapple	Greenhouse internodal stem segments	*At*-mediated	*npt*II, *uidA*	Cervera et al. (1998)
C. sinensis cv. Tarocco	In vitro epicotyl segments	*At*-mediated	*npt*II, *rolA, rolB, rolC*	Gentile et al. (1998)
C. aurantifolia cv. Mexican	In vitro internodal stem segments	*At*-mediated	*npt*II, *uidA*	Perez-Molphe and Ochoa-Alejo (1998)
C. paradisi cv. Duncan	In vitro epicotyl segments	*At*-mediated	*npt*II, *uidA*	Luth and Moore (1999)
C. aurantifolia	In vitro epicotyl segments or greenhouse intermodal stem segments	*At*-mediated	*npt*II, *gfp*	Ghorbel et al. (1999)
C. aurantium	In vitro epicotyl segments or greenhouse intermodal stem segments	*At*-mediated	*npt*II, *gfp*	Ghorbel et al. (1999)
C. aurantifolia cv. Mexican	Greenhouse internodal stem segments	*At*-mediated	CTV-*CP*	Dominguez et al. (2000)
C. sinensis cv. Itaborai	Protoplasts	PEG-mediated	*gfp*	Fleming et al. (2000)
C. aurantium	Greenhouse internodal stem segments	*At*-mediated	CTV-*CP*	Ghorbel et al. (2000)
C. paradisi cv. Rio Red	In vitro epicotyl segments	*At*-mediated	*npt*II, *uidA*, unCTV-*CP*, *gna*	Yang et al. (2000)
C. sinensis cv. Pineapple	Greenhouse internodal stem segments	*At*-mediated	*P R-5*	Fagoaga et al. (2001)
C. aurantifolia cv. Mexican	Greenhouse internodal stem segments	*At*-mediated	CTV-*p23*	Ghorbel et al. (2001)
C. sinensis	In vitro epicotyl segments	*At*-mediated	CS-*ACS1*	Wong et al. (2001)
C. paradisi cv. Duncan	In vitro internodal stem segments	*At*-mediated	*CitPSY*, *CitPDS*, *CitLCY-B*	Costa et al. (2002)
C. aurantifolia cv. Mexican	Greenhouse internodal stem segments	*At*-mediated	CTV-*CP*, unCTV-*CP*	Dominguez et al. (2002a, b)
C. reticulata cv. Ponkan	Embryogenic callus	*At*-mediated	*bar*, *pAT29-barnase*	Li et al. (2002)
C. sinensis cv. Hamlin	In vitro epicotyl segments	*At*-mediated	*npt*II, *gfp*	Mendes et al. (2002)
C. sinensis		*At*-mediated	*npt*II, *uidA*	

(*continued*)

Table 3.3 (continued)

Genotype used	Explant used	Method used	Gene(s) introduced	Reference
	In vitro epicotyl segments or greenhouse intermodal stem segments			Almeida et al. (2003a, b)
C. limonia	In vitro epicotyl segments	*At*-mediated	*npt*II, *uidA*	Almeida et al. (2003a)
C. sinensis cvs. Valencia, Hamlin, Natal and Pera	In vitro epicotyl segments	*At*-mediated	*PMI*	Boscariol et al. (2003)
C. paradisi cv. Duncan	In vitro internodal stem segments	*At*-mediated	ntCTV-*CP*	Febres et al. (2003)
C. sinensis cv. Itaborai	Protoplasts	PEG-mediated	*gfp*, CTV-derived sequence	Olivares-Fuster et al. (2003)
C. sinensis cv. Valencia	Embryogenic callus	*At*-mediated	*bar*, *pAT29-barnase*	Li et al. (2003)
C. sinensis cv. Hamlin	Protoplasts	Electroporation	*egfp*	Niedz et al. (2003)
C. aurantifolia cv. Mexican	Greenhouse internodal stem segments	*At*-mediated	CTV-*p23*	Fagoaga et al. (2005)
C. aurantium	Greenhouse internodal stem segments	*At*-mediated	CTV-*p23*	Fagoaga et al. (2005)
C. sinensis cv. Valencia	Protoplasts	PEG-mediated	*gfp*, *T S P M E*	Guo et al. (2005)
C. paradisi cv. Duncan	In vitro internodal stem segments	*At*-mediated	*Ac*	Trainin et al. (2005)
C. macrophylla	In vitro epicotyl segments	*At*-mediated	ds(*p23* + *3′UTR*)	Batuman et al. (2006)
C. sinensis cv. Hamlin	In vitro epicotyl segments	*At*-mediated	*att A*	Boscariol et al. (2006)
C. sinensis cv. Pineapple	In vitro epicotyl segments or greenhouse internodal stem segments	*At*-mediated	*ipt*, *R*/*RS* recombinase system	Ballester et al. (2007)
C. sinensis cv. Bingtang	In vitro epicotyl segments	*At*-mediated	*gfp*	Duan et al. (2007)
C. paradisi cv. Duncan	In vitro epicotyl segments	*At*-mediated	*nptII*, *gfp*, CTV R genes	Chen et al. (2007)
C. sinensis cv. Hamlin	Protoplasts	PEG-mediated	*gfp*, *Xa21*	Omar and Grosser (2007)
C. sinensis cv. Midsweet	In vitro epicotyl segments	*At*-mediated	*nptII*, *gus*, *gfp*, CTV R genes	Soneji et al. (2007)
C. sinensis cv. Navelina	Greenhouse internodal stem segments	*At*-mediated	*npt*II	Rodriguez et al. (2008)
C. sinensis cv. Pineapple	Greenhouse internodal stem segments	*At*-mediated	CPsV-*CP*	Zanek et al. (2008)

or 22,000 oligoarray containing 21,495 independent ESTs, have also been developed (Talon and Gmitter Jr 2008). An Affymetrix citrus GeneChip containing 30,264 probe sets (22 probes each) for measurement of citrus transcripts and 5,023 probe sets (56 probes each) to serve as single nucleotide polymorphism (SNP) markers for 3,219 genes has also been designed from the HarvEST:Citrus database and is commercially available (Close et al. 2006). Transcript profiling has been carried out using genes from various species such as *P. trifoliata*, *C. latipes* × *C. aurantium*, *C. sunki* × *P. trifoliata*, *C. volkameriana*, and *C. grandis* × *P. trifoliata* for fruit growth and ripening involving traits such as ethylene production, anthocyanin and flavonoid biosynthesis, carbohydrate buildup, acid reduction, and chlorophyll degradation for responses to pathogenic infections such as leaf spot, mold, post-bloom fruit drop, root rot, canker, citrus variegated chlorosis, Huanglongbing or greening, CTV, and citrus leprosis virus, and for environmental stresses such as cold temperatures, drought, flooding, salinity, and high and low soil pH (Romero-Aranda

Table 3.4 Current *Citrus* EST entries in GenBank

Citrus species/hybrids	No. of ESTs
Citrus sinensis	208,909
Citrus clementina	118,365
Poncirus trifoliata	62,344
Citrus reticulata	55,980
Citrus unshiu	19,066
Citrus aurantium	14,584
Citrus × *limonia*	11,045
Citrus latifolia	8,756
Citrus aurantiifolia	8,219
Citrus limettioides	8,188
Citrus × *paradisi*	8,039
Citrus × *paradisi* × *Poncirus trifoliata*	7,954
Citrus reticulata × *Citrus temple*	5,823
Citrus reshni	5,768
Citrus sunki	5,216
Citrus macrophylla	1,929
Citrus sinensis × *Poncirus trifoliata*	1,837
Citrus clementina × *Citrus tangerina*	1,834
Citrus limon	1,505
Citrus medica	1,115
Citrus jambhiri	989
Citrus nobilis × *Citrus kinokuni*	645
Citrus tamurana	358
Citrus natsudaidai	202
Citrus hassaku	154
Total	558,898

Source: International Citrus Genome Consortium

et al. 1998; Moya et al. 2003; Lahey et al. 2004; Shimada et al. 2005; Cercós et al. 2006; Tsukuda et al. 2006; Fujii et al. 2007; Gandia et al. 2007).

3.8 Conclusion

Citrus is grown throughout the world in the tropics and subtropics. It is vegetatively propagated by grafting or budding. Limited information is available on the extent and distribution of genetic diversity of citrus, making it difficult to understand the actual risk of genetic erosion. Hence, concerted efforts are required for efficient conservation of citrus germplasm. Though citrus breeding is very challenging, different breeding programs throughout the world have made significant progress in the application of conventional and modern approaches for genetic improvement and cultivar development. Advances in plant cell and tissue culture also have major impacts on genetic improvement of citrus. Efforts have been made to produce haploids via anther culture as they are highly beneficial in breeding programs and in understanding citrus genetics. For the production of seedless cultivars, triploids have been generated by fusion of haploid with diploid protoplasts and/or endosperm culture. Use of somatic hybridization for the recovery of tetraploids has also expanded the range of germplasm available to citrus breeders.

Linkage mapping and tagging will aid in identification and cloning of candidate genes. These candidate genes can then be introduced into citrus via genetic engineering and exploited for the improvement of citrus without altering its integrity. In recent years, citrus genomics has progressed rapidly. The development of BAC libraries, an extensive EST collection, microarrays, and dense, sequence-based maps will be of immense importance in whole genome sequencing of citrus and will aid in elucidation of gene function, regulation, and expression. Advances in genomic science will have a great impact on citrus improvement and will continue to provide new information and gene targets for manipulation.

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