Compendium of Plant Genomes
Series Editor: Chittaranjan Kole

Alessandra Gentile
Stefano La Malfa
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The Citrus Genome

Springer

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Series Editor

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Whole-genome sequencing is at the cutting edge of life sciences in the new millennium. Since the first genome sequencing of the model plant Arabidopsis thaliana in 2000, whole genomes of about 100 plant species have been sequenced and genome sequences of several other plants are in the pipeline. Research publications on these genome initiatives are scattered on dedicated web sites and in journals with all too brief descriptions. The individual volumes elucidate the background history of the national and international genome initiatives; public and private partners involved; strategies and genomic resources and tools utilized; enumeration on the sequences and their assembly; repetitive sequences; gene annotation and genome duplication. In addition, synteny with other sequences, comparison of gene families and most importantly potential of the genome sequence information for gene pool characterization and genetic improvement of crop plants are described.

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The Citrus Genome

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This book series is dedicated to my wife Phullara, and our children Sourav and Devleena
Chittaranjan Kole

Preface to the Series

Genome sequencing has emerged as the leading discipline in the plant sciences coinciding with the start of the new century. For much of the twentieth century, plant geneticists were only successful in delineating putative chromosomal location, function, and changes in genes indirectly through the use of a number of "markers" physically linked to them. These included visible or morphological, cytological, protein, and molecular or DNA markers. Among them, the first DNA marker, the RFLPs, introduced a revolutionary change in plant genetics and breeding in the mid-1980s, mainly because of their infinite number and thus potential to cover maximum chromosomal regions, phenotypic neutrality, absence of epistasis, and codominant nature. An array of other hybridization-based markers, PCR-based markers, and markers based on both facilitated construction of genetic linkage maps, mapping of genes controlling simply inherited traits and even gene clusters (QTLs) controlling polygenic traits in a large number of model and crop plants. During this period, a number of new mapping populations beyond F2 were utilized and a number of computer programs were developed for map construction, mapping of genes, and for mapping of polygenic clusters or QTLs. Molecular markers were also used in the studies of evolution and phylogenetic relationship, genetic diversity, DNA fingerprinting, and map-based cloning. Markers tightly linked to the genes were used in crop improvement employing the so-called marker-assisted selection. These strategies of molecular genetic mapping and molecular breeding made a spectacular impact during the last one and a half decades of the twentieth century. But still, they remained "indirect" approaches for elucidation and utilization of plant genomes since much of the chromosomes remained unknown and the complete chemical depiction of them was yet to be unraveled.

Physical mapping of genomes was the obvious consequence that facilitated the development of the "genomic resources" including BAC and YAC libraries to develop physical maps in some plant genomes. Subsequently, integrated genetic–physical maps were also developed in many plants. This led to the concept of structural genomics. Later on, emphasis was laid on EST and transcriptome analysis to decipher the function of the active gene sequences leading to another concept defined as functional genomics. The advent of techniques of bacteriophage gene and DNA sequencing in the 1970s was extended to facilitate sequencing of these genomic resources in the last decade of the twentieth century.

As expected, the sequencing of chromosomal regions would have led to too much data to store, characterize, and utilize with the then available computer software than it could handle. But the development of information technology made the life of biologists easier by leading to a swift and sweet marriage of biology and informatics, and a new subject was born—bioinformatics.

Thus, the evolution of the concepts, strategies, and tools of sequencing and bioinformatics reinforced the subject of genomics—structural and functional. Today, genome sequencing has traveled much beyond biology and involves biophysics, biochemistry, and bioinformatics!

Thanks to the efforts of both public and private agencies, genome sequencing strategies are evolving very fast, leading to cheaper, quicker, and automated techniques right from clone-by-clone and whole-genome shotgun approaches to a succession of second-generation sequencing methods. The development of software of different generations facilitated this genome sequencing. At the same time, newer concepts and strategies were emerging to handle the sequencing of the complex genomes, particularly the polyploids.

It became a reality to chemically—and so directly—define plant genomes, popularly called whole-genome sequencing or simply genome sequencing.

The history of plant genome sequencing will always cite the sequencing of the genome of the model plant *Arabidopsis thaliana* in 2000 that was followed by sequencing the genome of the crop and model plant rice in 2002. Since then, the number of sequenced genomes of higher plants has been increasing exponentially, mainly due to the development of cheaper and quicker genomic techniques and, most importantly, the development of collaborative platforms such as national and international consortia involving partners from public and/or private agencies.

As I write this preface for the first volume of the new series "Compendium of Plant Genomes," a net search tells me that complete or nearly complete whole-genome sequencing of 45 crop plants, eight crop and model plants, eight model plants, 15 crop progenitors and relatives, and three basal plants is accomplished, the majority of which are in the public domain. This means that we nowadays know many of our model and crop plants chemically, i.e., directly, and we may depict them and utilize them precisely better than ever. Genome sequencing has covered all groups of crop plants. Hence, information on the precise depiction of plant genomes and the scope of their utilization are growing rapidly every day. However, the information is scattered in research articles and review papers in journals and dedicated web pages of the consortia and databases. There is no compilation of plant genomes and the opportunity of using the information in sequence-assisted breeding or further genomic studies. This is the underlying rationale for starting this book series, with each volume dedicated to a particular plant.

Plant genome science has emerged as an important subject in academia, and the present compendium of plant genomes will be highly useful both to students and teaching faculties. Most importantly, research scientists involved in genomics research will have access to systematic deliberations on the plant genomes of their interest. Elucidation of plant genomes is of interest

not only for the geneticists and breeders but also for practitioners of an array of plant science disciplines, such as taxonomy, evolution, cytology, physiology, pathology, entomology, nematology, crop production, biochemistry, and obviously bioinformatics. It must be mentioned that information regarding each plant genome is ever growing. The contents of the volumes of this compendium are, therefore, focusing on the basic aspects of the genomes and their utility. They include information on the academic and/or economic importance of the plants, description of their genomes from a molecular genetic and cytogenetic point of view, and the genomic resources developed. Detailed deliberations focus on the background history of the national and international genome initiatives, public and private partners involved, strategies and genomic resources and tools utilized, enumeration on the sequences and their assembly, repetitive sequences, gene annotation, and genome duplication. In addition, synteny with other sequences, comparison of gene families, and, most importantly, the potential of the genome sequence information for gene pool characterization through genotyping by sequencing (GBS) and genetic improvement of crop plants have been described. As expected, there is a lot of variation of these topics in the volumes based on the information available on the crop, model, or reference plants.

I must confess that as the series editor, it has been a daunting task for me to work on such a huge and broad knowledge base that spans so many diverse plant species. However, pioneering scientists with a lifetime experience and expertise on the particular crops did excellent jobs editing the respective volumes. I myself have been a small science worker on plant genomes since the mid-1980s and that provided me the opportunity to personally know several stalwarts of plant genomics from all over the globe. Most, if not all, of the volume editors are my longtime friends and colleagues. It has been highly comfortable and enriching for me to work with them on this book series. To be honest, while working on this series I have been and will remain a student first, a science worker second, and a series editor last. And I must express my gratitude to the volume editors and the chapter authors for providing me the opportunity to work with them on this compendium.

I also wish to mention here my thanks and gratitude to the Springer staff particularly, Dr. Christina Eckey and Dr. Jutta Lindenborn for the earlier set of volumes and presently Ing. Zuzana Bernhart for all their timely help and support.

I always had to set aside additional hours to edit books besides my professional and personal commitments—hours I could and should have given to my wife, Phullara, and our kids, Sourav and Devleena. I must mention that they not only allowed me the freedom to take away those hours from them but also offered their support in the editing job itself. I am really not sure whether my dedication of this compendium to them will suffice to do justice to their sacrifices for the interest of science and the science community.

New Delhi, India Chittaranjan Kole

Preface

Citrus (L. 1753) is one of the most important genera of the *Rutaceae* family. Genus *Citrus* encompasses most of the widespread fruit crops such as oranges, mandarins, clementines, lemons, limes, pummelos, and grapefruits in the Mediterranean and subtropical environments. The wide diversity among such species is accompanied by an overall positive appreciation by consumers, thanks to the outstanding organoleptic characteristics of citrus fruits and, not secondarily, to their health and nutritional value. Citrus was originated in Southeast Asia and diversified during the late Miocene epoch. The genealogy of the different citrus species indicated that they were originated through successive events of hybridization that occurred among the three "true" or "biological" ancestral species: citron (*Citrus medica*), mandarin (*Citrus reticulata*), pummelo (*Citrus maxima*), and/or their hybrids.

Despite the efforts on genetic improvement, citrus species cultivation undergoes several limitations especially for the adaptability to different environmental conditions and for the risks derived from pests and diseases spreading at the global level. In citrus, traditional breeding enabled the setup of several varieties and rootstocks showing improved agronomical traits; however, in these species, conventional breeding techniques are hampered by several aspects such as the long juvenile phase, the high level of heterozygosis, the male and female sterility, and the occurrence of apomixis.

In such a picture, the recent advances of molecular biology and molecular-based breeding techniques greatly helped researchers to speed up the entire breeding process. In particular, the release of the genome sequence of most of the economically important *Citrus* species is facilitating the critical investigation of several aspects spanning from *Citrus* phylogeny, till the study of their complex biological features and the understanding of the genetic basis underlying traits of agronomic interest.

This book is aimed at reviewing with a multidisciplinary approach, the-state-of-the-art of the researches in Citrus, highlighting both novel discoveries and open questions for future works. The book provided an exhaustive overview of the Citrus phylogeny with particular interest in the description of the genetic resources and approaches at the base of conventional breeding of varieties and rootstocks. The rapid increase in efficiency and reliability of the high-throughput sequencing platforms enabled the use of both molecular markers and sequencing data to get novel insights both on the genetic architecture of traits of interests and on the genetic diversity among Citrus species. To this extent, the recent findings on the genetic basis

underlying traits of agronomical interests (either related to fruit quality, tree production, or resistance to biotic/abiotic stress) were presented and discussed. Citrus fruit quality was also addressed from a metabolic point of view highlighting two of the most peculiar aspects of fruits: the color and the presence of essential oils. The book will be a guide for those who are interested in a comprehensive overview of the progress in the scientific research related to Citrus with attention also to the transferability of such findings in the context of breeding. For such reason, these pages will be particularly useful for the scientists, breeders, and students of the universities, public sector institutes that are involved in research for the development of citrus industry for updating the amount of knowledge generated in recent years.

We are grateful to all our colleagues for their contribution. We wish to record our thanks and appreciation to Prof. Chittaranjan Kole, the Series Editor, for his assistance and guidance right from the inception till the publication of this book.

Changsha, China — Ziniu Deng
Catania, Italy — Alessandra Gentile
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Contents

The Citrus Genome: Past, Present and Future

1

Eliezer E. Goldschmidt

Abstract

Within this introductory chapter, I would like to briefly discuss the contribution of the genomic revolution to the science of Citrus. Genomics undoubtedly plays a central role in the ongoing reconstruction of evolutionary history of the Citrus clade, but elucidation of the role played by man in the more recent wandering and distribution of citrus relies on other disciplines, such as Archaeobotany and Historic documentation. As to the current challenges faced by the Citrus Industry, the most serious threat of the 21st century is the pandemic, devastating HLB disease. In this case implementation of the genomic tools has not yet lead to a real breakthrough. Can anything meaningful be said about the future? Two major frontiers are evident; breeding and physiology. Modern plant breeding includes by now techniques for precise editing of the genome, which hold a bright promise for targeted crop improvement. The term 'physiology' means, in this context, an advanced understanding of all the developmental and biochemical traits responsible for the citrus phenotype. This is a tremendous challenge, since the citrus phenome is still rather mysterious, even the genetic basis of the 'Hesperidium' fruit unit is as yet poorly understood.

As already indicated in the title, the genomic era has brought new insights for studies of the past of the citrus tribe, as well as new vistas for the future of citrus as a horticultural crop and industry.

Within this preface, I would like to address the following issue: What did we, as citrologists, or, what did the science of Citrus gain from the recent genomic revolution? I am asking this question as an old-fashioned horticulturist–physiologist who was not personally involved in genomic research, but attempts nevertheless to evaluate the contribution of genomics to our broader understanding of Citrus' universal survival.

Thinking about the general evolution and domestication of fruit tree genera (Janick 2005), *Citrus* appears to be one of the most, if not the most complicated case. Genomics is evidently the principal tool through which the history of citrus can be reconstructed although one must not forget the seminal work of Barrett and Rhodes (1976) who came up with the 'Three Ancestors' theory long before the molecular biotechnological approach took the lead. Within the efforts to reconstruct the history of citrus, one may distinguish between two major research areas: (1) The extensive, ancient botanical evolution scenery which goes back to the previous geological era and must take into account presumed

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continental movements and climate changes. (2) The role played by man in the prehistoric and historic expansion of citrus species from their centers of origin, leading to the current worldwide distribution of citrus and the shaping of the present, extremely diverse population of citrus phenotypes. Several research teams have joined forces during the last decade or so in an attempt to resolve the mysteries of the 1st research area, based on sequencing and comparative analysis of an increasing number of citrus genomes. Although a multidimensional, unifying theory has recently been proposed (Wu et al. 2018), solid evidence with regard to the emergence sites of some of the citrus clades is still fragmentary.

As to the 2nd area mentioned above, this aspect has not received sufficient attention in recent years. Here genomics alone is not enough. The contribution of humans to the spread and diversification of citrus genotypes must be studied by a combination of historical, archaeological and biological methods. A noteworthy example of this kind of research is the archaeobotanical study by Langgut et al. (2013), that provided unequivocal evidence for the presence of the citron (*Citrus medica* L.) in the fourth century B. C., Palestine by fossil pollen found in the plaster of the Persian palace excavated in Ramat Rahel, just south of Jerusalem.

Moving now from the past to the present and future, it seems appropriate to begin by considering the current challenges faced by the citrus industry. It is rather obvious that the most serious threat of the twenty-first century is the pandemic HLB disease, which has destroyed by now most of Florida's citrus and might spread within the foreseeable future to other, as yet unaffected areas. But, this is not the first time that an erupting disease endangers the citrus crop worldwide. In the twentieth century, it was the devastating tristeza viral disease, which appeared in South America and gradually spread to the Mediterranean. Prior to that, in the nineteenth century, it was the phytophthora citrus foot rot, which was discovered in Madeira (1854) and enforced the introduction of grafting on the sour orange rootstock. The assertion that pests are the most troublesome threats for agricultural crops is also valid with other crops, e.g. the nineteenth-century phylloxera French grapevine catastrophe. Turning back to the HLB malady, we may wonder whether the progress in genomics has brought us closer to the solution of the HLB distress; and the answer is, I am afraid, rather disappointing.

The reason is, in my opinion, that deciphering the genomic sequence of the DNA does not in itself tell us the whole story, just some kind of a frame—background. Only a very detailed enquiry of the genetic regulation of every biological trait and biochemical activity (as partially achieved with Arabidopsis) will lead to the desired understanding of the observed phenomena; and pathological phenomena are even more complex since they generally involve interaction of several organisms.

That sequencing the genome does not solve all mysteries can be easily demonstrated by the case of the 'fingered citron' (Fig. 1.1). The 'fingered citron', also named 'Buddha's hand' is widespread in China, where it apparently originated (Karp and Hu 2018). In the 'fingered citron' the locules, which are normally united in the *hesperidium*, develop as separate 'fingers'. This malformation has probably arisen by some kind of mutation (Karp and Hu 2018). This mutation apparently interferes with a very basic step in the formation of the hesperidium fruit unit but, in spite of rather detailed enquiry of the citron genome (Ramadugu et al. 2015) there is as yet no clue as to the genomic site responsible for this mutation. Furthermore, we cannot even be sure that the 'fingered' phenotype is brought about by a change at the DNA level or through other molecular regulatory mechanisms.

How about the future? Although it is dangerous to engage in prophecy, two major frontiers are evident: breeding and physiology. Modern plant breeding includes by now, techniques for precise editing of the genome, such as CRISPR, which hold a bright promise for targeted crop improvement. However, the long time

Fig. 1.1 Fingered citron. Zhaocai fingered citron, Jinhua, Zhejiang, China. (Photo credit: Xulan Hu)

required for the establishment of new, successful citrus cultivars is still a major barrier. The term 'physiology', in my opinion, means an advanced understanding of all the developmental and biochemical processes responsible the citrus phenotype. However, as indicated in the previous sections, the progress in both breeding and physiology areas rests heavily on detailed mapping of the genes and resolution of their regulatory loops. Although the molecular tools are available, will there be sufficient funds and research power to confront these challenges? This leaves us certainly with a great room for concern.

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Citrus Origin, Diffusion, and Economic Importance

2

Guangyan Zhong and Elisabetta Nicolosi

Abstract

Citrus are widely cultivated in more than 140 countries in the world, in tropical, subtropical and Mediterranean climates, in the "citrus belt" between approximately 40° N and 40° S latitude, but their natural distribution areas before domestication must be much smaller. The chapter briefly outlines the issues related to the exact identification of the geographical origin and spread of citrus fruits: these simple questions have bothered citrus breeders and taxonomists for centuries. Nevertheless, we have witnessed a tremendous progress in the past two decades since the introduction of molecular tools in citrus researches, and the release of the first citrus genome has made an unprecedented breakthrough in our understanding of citrus genetics, taxonomy and evolution. Currently, on the basis of genomic, phylogenetic, and biogeographic analyses, scientists from different part of the world agree and haven't doubt that the main centers of origin for the citrus species are the tropical and subtropical regions of south-east Asia, the northeastern India in the Himalayan foothills, Yunnan province of south-west China, northern Myanmar, the Indochinese peninsula, and the Malaysian archipelago, from which Citrus began the spread into the other continents. The genus *Citrus* is the result of a long and complex domestication process and this, together with sexual compatibility between *Citrus* and related genera and the frequency of bud mutations, makes citrus taxonomy and phylogeny very complicated. The chapter focuses on the genetic origin of the main cultivated species such as sweet oranges, clementine, citron, pummelo, grapefruit and mandarins. Finally, the major citrus production areas and the main commercial citrus groups and producing countries are reported.

2.1 On the Origin Center of Citrus

The citrus is certainly one of a few most delicious fruits that are generous gifts of Mother Nature. Most of us will intuitively fall in love with the attractive, fragrant, and delicious fruits at first sight, and wonder where and how they have come into existence. These seemly simple questions, however, have bothered citrus breeders and taxonomists for centuries. Two peculiar reproductive behaviors of citrus should be heavily blamed, first, the citrus is notorious for their sexual promiscuity, and hybrids between species and even genera are not only very easy to produce but also quite normal in fecundity, making it difficult to delineate a clear genealogy

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of them; second, many citrus including mandarins, kumquats, oranges, and *Poncirus* are polyembryonic and propagated apomictically through nucellar embryos of maternal origin that are dominant over sexual embryos, freezing the normal evolutionary clock of the affected species and causing problems for scientists, especially the early taxonomists, who relied heavily on morphological characters to identify hybrids from true species. Additionally, long-distance dispersal should have occurred frequently in the history of evolution and spread of citrus, for their fruits are not only favorite foods of migrating birds and other herbivorous animals including humans but also, with sponge tissues and water-repellant wax skins, floatable for a considerably long time that certainly facilitate their dispersal through rivers that flow across their vast habituating areas, which adds a lot of difficulties to trace their origins. Moreover, fossil evidence is very limited since only one of the three fossil specimen reports described definitely a citrus species. Not to mention that not many wild citrus have been discovered and recognized, and the population sizes of the extant wild species, without exception, are too small to do population genetics studies.

Without enough corroborating evidence from geographical, genetic diversity, wild population, fossil, paleoclimate, and domestication history studies, the questions about the origin, dispersal, divergence, and evolution of citrus are difficult to answer. Nevertheless, we have witnessed tremendous progress in the past two decades since the introduction of molecular tools in citrus researches, and the release of the first citrus genome has made an unprecedented breakthrough in our understanding of citrus genetics, taxonomy, and evolution (Xu et al. 2013).

2.2 Inference from Citrus Domestication History

Though citrus is widely cultivated in more than 140 countries in the world, its natural distribution areas before domestication must be much smaller.

Clearly, the citrus are not native to America since it had not been known by native Americans before the Italian explorer and navigator Christopher Columbus brought the first citrus to North America in 1493, even though the American countries, USA and Brazil, have been the two of the three most important and biggest citrus producers in the past half centuries in the world. Evidently, the birthplace of citrus must be within the borders of the Eurasian continent.

Citron (*Citrus medica*) was probably the first citrus plant known to Europeans. In Africa, citron must be cultivated by ancient Egyptians since it was depicted on the walls of the botanical garden at the Karnak Temple that dates back to 3000 years ago. The Jews might be familiar with citrus as early as the sixth century BC when they were about to depart from Sinai since the "fruit of the beautiful ('hadar') tree" was mentioned in Leviticus, although there was no direct mention of citrus in the Bible. The spread of citron to European countries is believed to be accredited to Alexander the Great and his armies who brought it from the east, probably India, in the late fourth century BC, since Theophrastus who lived in Greece in the same era recorded that citron was already grown in the east (Median and Persia). According to Langgut (2017), citron was brought from Persia to the Western Mediterranean during the early Roman period, and lemon was the second introduced citrus, while Sour orange (*C. aurantium*), lime (*C. aurantifolia*), and pummelo (*C. maxima*) were introduced in the tenth century AD. Sweet orange (*C. sinensis*) and mandarin (*C. reticulata*) were introduced in the late fifteenth century, and the early nineteenth century, respectively. Introduction of them to the west must be via southern trade routes that spread from South China to the west, and in the process, Northern Africa and the Iberian Peninsula served apparently as the relay stations (Ramón-Laca 2003). Considering that the time needed for a citrus species to arrive at a certain location must be correlated to the geographical distance between the location and the origin place of the species and that the reachable distances of the ancient traders were limited by their travel capabilities, it is reasonable to

conclude that Citron must be geographically closer than other citruses to the west. In other words, the later arrivals must come from more distant regions.

Evolution theories tell us that the genetic diversities of a given species are gradually reduced as disperses away from its origin center. In fact, the unique morphological attributes of the citrons also suggest that they must be derived from a geographically marginal population that was distant or isolated from the core citrus population. We know that wild citrons are still grown in valleys at the foothills of the Eastern Himalayas, a region stretched from Northeast India to Myanmar. To the east of this area are China, Japan, Australia, and the Southeastern Asian countries where numerous different native citruses have been reported. The geographical center of the vast area is located in the middle of South China sea if we exclude the northern areas where the winter temperatures can drop to a lower than normal survival level for citrus.

Citrus plants were rarely mentioned in ancient literature in most countries of the aforementioned regions, but abundantly documented in Chinese literature in the past 4000 years. A very ancient book, "Yu Kung 禹贡", mainly documenting the geography of China in Xia dynasty, mentioned that mandarins and pummelos were listed as tributes to the emperor Yu (ca 2205–2197). "Lu Shi Chun Qiu 吕氏春秋", a cyclopedia edited by the prime minister of Qin state in 241 BC, described that the most beautiful and delicious fruits were the mandarins produced in "Jiangpu" and the pummelos produced in "Yunmeng". Qu Yuan (340–278 BC), one of the most famous poets in the history of China, wrote a long piece of poem to eulogize mandarin trees. "Han Shu 汉书", edited between 54 and 74 AD, stated that a person who grew 1000 mandarin trees in Jiangling area (between nowadays East Sichuan and West Hubei provinces) was as rich as a marquis who possessed 1000 households, which indicated that there was large-scale citrus production in the region at that time. In the following centuries, the Chinese Citrus industry had been developed to such a level that permitted Han Yenchi to edit the world first citrus variety atlas, "Chu Lu", in 1127 AD, in which a total of 27 citrus (mostly mandarins and kumquats) cultivars were meticulously documented.

The earlier human activities indicate that the citrus were known, domesticated, and exploited earlier in Southern China than in other regions. The suggestion is that the center of origin of citrus must be somewhere in Southern China or somewhere in the surrounding areas.

2.3 Inference from the Distribution of Extant Wild Citrus

The pattern of a species' natural distribution should provide a valuable clue to where its origin center is. *Citrus* and its close relatives, *Poncirus* and *Fortunella*, are naturally distributed in a vast area bordered by the Yellow River to the north, Japan and Taiwan to the east, Northeastern Australia to the south, and from India down to Indonesia to the west. The geographic center of the areas is approximately in South China. Generally, the center of a species distribution area is the center of origin of the species if it is overlapped with the genetic diversity center. This is because as individuals migrate further away from their distribution center, the chances for them to exchange genetic materials with others with diverse genetic backgrounds are exponentially decreased. Now, where is the center of the genetic diversity of citrus?

There exist Australia limes in Australia. Australia limes, as their names suggest, are indigenous to Australia and nearby Papua New Guinea. These special types of citrus have very distinctive characters such as much smaller and thicker leaves and higher tolerance to drought stress, probably resulted from long time adaptive evolution in Australian desert environments. Though many different species were recognized in Australia limes, they are in fact closely related to each other and most probably only one or two true species since modern molecular genetics studies have repeatedly shown that there are limited genetic diversities among them. Considering that the Australian continent has always been separated from Asian continent by ocean

waters even during the greatest glacial epoch, and that the vast, almost insurmountable waters are definitely natural hindrances preventing against cross-water spread of most land animals and plants, it is conceivable that only a few seeds of citrus happened to arrive at the continent and have since thrived there. In other words, their limited DNA polymorphisms can be best explained by the theory of "founder effect". There might be only one or a few lucky founders (seeds or fruits) brought there by cross-ocean migrating birds or ocean currents flowing between the continents. The cross-ocean dispesal event(s) should have most probably occurred during the greatest glacial epoch(s) when the sea level was very low, and many nowadays sea floors were then drylands, and the waters between the continents were much narrower.

Papedas are mostly uncultivated for their inedible fruits and hence less disturbed by humans and animals. Though named after their characteristic large leaf wings, different papedas do not in fact belong to the same clade when classified by modern molecular taxonomic tools. Ichang papeda is widely distributed in the area that stretches from Northeast Yunan to Southeast Shanxi, approximately along the east margin of the Yunnan–Guizhou plateau where the winter is very harsh and capable of killing other papedas and other citrus species. In contrast, other papedas spread mostly southward and westward from southern Yunan to Indonesia, Philippines, and Malaysia although Khasi papeda can also be found in Northeastern India.

Citrons are native to Yunnan, Tibet, Guizhou, Guangxi, Assam, and Myanmar. As mentioned above, many slightly different types of citrons grow wild in Yunnan and surrounding areas including Northeast India and Myanmar. The citrons are mono-embryonic yet they keep a surprisingly high degree of genetic homozygosity. The high homozygosity can be attributed to their reproductive behavior that their flowers tend to be self-fertilized before opening. There might be other possibilities, anyway. First, the citron's founder population might be separated by high mountains from the main population, which generated a bottleneck effect and as a result, a considerable number of ancient variations of the main population was lost in citrons. Second, the speciation of citrons might have occurred relatively later and there might not be enough time for as many genetic variations as other citruses to accumulate.

Wild kumquats have been naturally growing in southeastern provinces in China, including Guangdong, Guangxi, Hunan, Hainan, Jiangxi, and Zhejiang. The most primitive kumquat is *Fortunella hindsii* (Shan Jin Gan) which is also the bushiest and the smallest fruited citrus fruit. Shan Jin Gan, with inedible fruits, can still be found in the wild although some good-looking trees are occasionally removed for use as pot materials. Other kumquats with larger and fleshier fruits have been domesticated and cultivated for at least more than 1000 years in the southeast of China. Though hybrids can be obtained without any difficulty when kumquats are artificially fertilized with other citruses, some degrees of natural reproductive isolation do exist among them. The barrier comes from the difference in their flowering seasons. Kumquats sprout and blossom later than any other citrus, leaving no chance for natural pollination to occur between them. The late blossom and high tolerance to low temperatures, compared to other *Citrus* species, suggest that they have evolved from an area with a longer and harsher winter season. That is, they may have originated from either a higher latitude, a higher altitude or the colder side (e.g., the north side) of a high mountain.

Mandarins (*C. reticulata*) vary greatly in fruit size, shape, color, and other morphological characters but their fruits are usually smaller and easy to peel. Several wild mandarins have been recognized as true species by different taxonomists. One of them, *C. indica*, that was considered as a primitive mandarin by Tanaka, is in fact a type of citron or a hybrid of citron. The remaining wild mandarins are mostly native to South China except that the *C. tachibana* is a native species of Taiwan and Japan. Various wild mandarins have been discovered in Hunan, Jiangxi, and Guangxi provinces in the past four decades.

A unique citrus, longmengxiangcheng, was discovered on the peak of the Luofu mountain of

Longmeng county, Guangdong province. Its leaves resemble those of mandarins but its fruit looks more like a small pummelo. Its juice pulps are sour and bitter. Studies showed that it should be a true citrus species (Zeng et al. 2014).

Trifoliate oranges (*P. trifoliata*), named after their characteristic trifoliate compound leaves, are widely distributed in a vast area boarded by the yellow river, the east coastal line, the south coastal line and the east Yunnan, and the Yunnan–Guizhou plateau in China. Although only one species and one subspecies have been recognized in *Poncirus*, there are in fact many different types of trifoliate oranges that vary greatly in sizes of fruit and leaves according to our surveys. Most trifoliate oranges, except for fomingzhi, shed their leaves completely in winter, and are thus the most hardy citrus that can tolerate temperatures lower than −10 °C, suggesting strongly their northern origin. Trifoliate oranges open flowers before they expand leaves, and are in fact the earliest in flowering season compared with *Citrus* and *Fortunella*. Early flowering of *Poncirus* isolates them reproductively from *Citrus* and *Fortunella*. But hybridization does occur when their pollens are fallen onto each other's stigmas, and surprisingly, these intergeneric progenies have shown no apparent abnormalities in fertility and many of them are as normal as intraspecific hybrids. Though artificial hybrids such as citranges and citramellos are well known, thanks to the great work of the worldwide renowned breeder W. T. Swingle, natural hybrids between *Poncirus* and *Citrus* were reported in China. Yongshunzhi is, for example, a natural citrange that was found growing in the border area between Hunan and Guizhou. Fomingzhi (*P. polyandra*) which was discovered in Yunan province, is the only evergreen trifoliate orange. It is not clear if fumingzhi is a natural hybrid between *Poncirus* and *Citrus*.

The aforementioned natural distributions of the species of the three close relative genera, *Citrus*, *Fortunella*, and *Poncirus*, which are still inter-fertilizable, suggest in a broad sense that their common origin is somewhere in the Southern China.

2.4 Inference from Genomic Data

Genome sequence is a faithful record of almost all genetic changes that are inheritable. The changes are mostly substitutions of nucleotides which mainly result from errors in the incorporation of nucleotide during DNA replication, deletions/insertions from slipped strand mispairing, and far less frequently large structural rearrangements. Both the single nucleotide substitutions and the small deletions/insertions are regarded as single nucleotide changes (variants). When a single nucleotide change is benign or neutral to the survival of the affected individual, it may spread in the population. When the changed single nucleotide reaches a certain frequency (e.g. >1%), allowing coexistence of two to four alleles (A, T, G, C) at the affected locus in the population, the so-called single nucleotide polymorphism (SNP) can be defined. SNPs can be identified by comparison of genome sequences of the investigated samples. SNPs are more informative for tracing ancestry of any species, hybrid, or individual due to their incomparable abundance. Apparently, the number of individuals carrying an ancient SNP is greater than that carrying a novel SNP. In other words, the number of specific SNPs accumulated in a species is positively correlated to how long the species has diverged from the most recent common ancestor. An evolutionary tree based on SNPs or haplotypes (closely linked SNPs) can thus be established and the chronological order of many key evolutionary events can be determined.

Wu and his colleagues compared the genomes of 58 citrus accessions representing 10 taxonomic groups as well as two related genera, *Poncirus* (*Poncirus trifoliata*) and Chinese box orange (*Severinia*), including some pure accessions of important progenitor species (Wu et al. 2018). By using 362,748 genome-wide ancestry-informative SNPs from nongenic and non-pericentromeric genomic regions, they established so far the most correct evolutionary tree for citrus. Their conclusion was that the center of origin of citrus was the southeast foothills of the Himalayas, a region that includes the eastern area

of Assam, Northern Myanmar, and western Yunnan. The proposed origin center is rich in geographical and climate diversities and should therefore be an ideal cradle for citrus to evolve. However, this area is in the strict sense not the only biodiversity center. As mentioned previously, there may be even more diversified citrus species in Southern China. More importantly, one of the area's indigenous species, *C. manshanensis*, is more primitive than citron, papeda, and pummelo as revealed by genomic studies (Wu et al. 2014a, b). Recently, we found that the Guangdong wild citrus, longmengxiangcheng, should also be a species that diverged as early as citron and pepeda (unpublished data). Moreover, it is well known that the South Central China inhabitants, trifoliate oranges, kumquats, and citrus, have not yet developed a true reproductive isolation mechanism among them although they have been traditionally treated as three separate genera.

More probably, the Central Southern China is the primary origin center of all citrus including trifoliate oranges. As the ancient citrus spread to a much wider area, geographic isolation of them has occurred although not long enough to allow them to develop reproductive isolation. Anyway, two secondary biodiversity centers, i.e., the southeast foothills of the Himalayas and the Southern China areas, have been naturally formed.

2.5 On the Origins of Some Important Citrus Species

2.5.1 Sweet Orange

Sweet orange is economically the most important species and has been planted more than any other citrus species in the world. Why it is superior to other citrus species in fruit quality is a fascinating question that both the breeders and the physiologists have been trying to answer. Undoubtedly some clues could be obtained by deciphering its exact genetic origin.

It was suggested that sweet orange might be the first generation hybrid between pummelo (P) and mandarin (M) (Barrett and Rhodes 1976; Nicolosi et al. 2000; Nicolosi 2007). Analysis of SSRs suggested that the sweet orange should have originated from one or more backcrosses of a pummelo–mandarin (P × M) hybrid to mandarin since there were more mandarin alleles than pummelo alleles in its genome (Barkley et al. 2006). Analysis of more (172) SSR markers assigned 25% of the sweet orange alleles to pummelo and 46% to mandarin with many regions having two alleles derived from mandarin, which strongly suggested that sweet orange was a backcross of a mandarin–pummelo hybrid to mandarin (P × M) × M (Roose et al. 2010). Xu and his colleagues assembled the first citrus genome using sweet orang as material and concluded that sweet orange should be derived from (P × M) × M (Xu et al. 2013).

Wu and Zhong proposed that sweet orange was most probably from [(P × M) × P or P × (P × M)] × M after analyzing 121 randomly selected loci/unigene segments that should have a statistically guaranteed genome-wide representativeness (Zhong 2013; Wu 2016). The involvement of an immediate mandarin parent in the generation of sweet orange was clearly shown by the fact that at least one allele at every analyzed sweet orange locus was from mandarin. In addition, the ratio of P/M loci to M/M loci was 95:26, which suggested that the other non-mandarin parent was most probably from a backcross of a pummelo–mandarin hybrid to a pummelo, i.e., (M × P or P × M) × P or P × (M × P or P × M). Since the sweet orange carries pummelo maternal materials (Luro et al. 2000), the proposal that its maternal parent was a (M × P) × P was invalid. The origin of sweet orange could therefore be expressed as [(P × M) × P or P × (P × M or M × P)] × M. When this model was tested by Markov chain method, the chance to produce a sweet orange like genome was 30%. The (P × M) × M hypothesis was also tested under the extreme condition that no crossover event occurred during gamete formation in the (P × M) parent, but the chance to produce a sweet orange like genome was about 7% which was very slim. The P × M hypothesis is invalid since there is an unignorable large proportion (26/121) of M/M regions in sweet orange, which clearly excluded the direct

involvement of a pure pummelo. A search for the paternal parent of sweet orange was also tried but none of the 69 investigated mandarins was found to be a perfect match when genotyped for the paternal-parent specific SNPs (our unpublished data). The [(P × M) × P or P × (P × M)] × M scheme of sweet orange origin was confirmed by PM haplotype ratio whereas the (P × M) × M model was rejected by the existence of P/P loci, as shown by a more detailed assembly of the sweet orange genome (Wu et al. 2014a).

2.5.2 Clementine

Clementine has been treated as a mandarin. It is one of the two most popular mandarin cultivars (the other one is Satsuma mandarin). Though its leaves are mandarin-like, its fruits are more compact and relatively more difficult to peel than other mandarins. Clementine was thought to be a mysterious Algeria hybrid and was assigned taxonomically to *C. reticulata* by Swingle (1967). Clementine was also suggested to be a hybrid between a mandarin and sweet orange (Bayer et al. 2009), and indeed. Clementine and sweet orange share at least one common allele at all analyzed loci, clearly showing that sweet orange is a parent of clementine. The other parent which is Mediterranean mandarin was easily identified by genotyping related mandarins (Ollitrault et al. 2012; Wu 2016). Thus, it is undoubted that clementine derived from the cross between Mediterranean mandarin and sweet orange in which Mediterranean mandarin acted as the female parent since clementine possesses a different-than sweet orange's chloroplast (Penjor et al. 2010). Genome sequencing result showed more clearly that the genome of clementine mandarin came from a hybridization between Willow leaf mandarin and sweet orange (Wu et al. 2014a).

2.5.3 Citron

Citrons have two morphologically distinct types of fruits, non-fingered and fingered. The non-fingered fruit is usually ovate or oblong with a protruding stylar end. Pulps are often not well developed, mostly taste sour. Some varieties are even pulpless. Rinds are thick and very fragrant. Leaves are more lignified and petioles are often not articulate. Citron is monoembryonic but has maintained a surprisingly high intraspecific homozygosity, probably due to the cleistogamy of its flowers that facilitates self-fertilization in unopened flower buds (Wu et al. 2018). All the morphological characters could be explained by their isolated habitats that should have efficiently prevented them from exchanging genes with other citruses.

Investigations on the genetic diversity of citron have mostly shown that it is a pure species with relatively low intraspecific diversity, which is very rare in *Citrus*. Genome-wide SNP analysis showed indeed that only 0.1% of intraspecific diversity existed in four representative citrons which is significantly lower than those in other citrus species (0.3–0.6%) (Wu et al. 2018). Such a low intraspecific diversity can also be explained by either geographic isolation during their early evolution and/or their cleistogamy of flowers. Nevertheless, two gene pools were still recognizable in 36 citron accessions collected from China by analyzing nSSR marker and SNPs/Indels on six chloroplast genes; the first one included fingered citrons and the wild citrons from Tibet while the other consisted of non-fingered citrons mostly from Yunnan (Yang et al. 2015). Ramadugu et al. (2015) also surveyed the intraspecific variations of 47 citron accessions collected not only from China but also from Mediterranean region by using SSR markers, and SNPs from one nuclear and one chloroplast genes, and confirmed the existence of two groups of citrons in Yunan province of China. They also found a third group that consisted of the citrons cultivated in Mediterranean region and pointed out that the group should have originated from India. Interestingly, both studies observed considerably higher intraspecific variations at some investigated loci although the overall intraspecific variations are low. This is a good example that shows that the high local variation signals can be easily inundated by a way larger number of genome-wide variations.

2.5.4 Pummelo

Pummelos (*C. maxima*) are widely cultivated in China, Thailand, Vietnam, Malaysia, and other Southeast Asian countries while its hybrid, grapefruit (*C. paradisii*), is mostly cultivated in western countries. Mature pummelo fruits are greenish to light orange and very large. Pummelo rinds are very thick, with white or rarely pink sponge-like albedo. Fleshes are mostly white or less frequently pink; taste varies from very sweet to moderately sour. Compared to other citruses, the leaves of pummelo are usually larger and thicker, and with a more prominently winged petiole. Similar to Citron, pummelo is also mono-embryonic. Yet true to type progenies can be normally obtained from seed propagation even though some types of pummelos are self-incompatible and thus prone to cross-pollination, although modern molecular evidence have constantly shown that introgression of pommelo genes into other citruses especially mandarins are quite common.

Pummelo is thought to be native to Southeastern Asia but no true wild pummelo population has been convincingly identified so far anywhere in the world. *Citrus lingcangensis*, the only well-recognized citrus fossil specimen, resembling modern pummelos in that it had a very typical heart-shaped leaf wing, was excavated from Lingcang county of Yunnan province. The fossil leaves were believed to be buried in the late Miocene (Xie et al. 2013).

Taxonomic studies showed that pummelo was related to papedas (Federici et al. 1998). More specifically, it was closely related to Honghe papeda that is still growing in the Honghe river valleys in southern Yunnan province (Zhong et al. 1993; Pang et al. 2003). RAPD, SCAR, and cpDNA data grouped pummelo into the same clade with Khasi papeda, micrantha papeda, and lime (Nicolosi et al. 2000; Nicolosi 2007).

Genome sequencing of two pummelos showed that the overall genome-wide nucleotide heterozygosity was 5.7 heterozygous sites/kb which was not a high number, indicating their ancestors experienced a strong bottleneck ~100,000–300,000 years ago. Analysis of 12 gene segments of more than 20 true pummelos also showed that the nucleotide diversity varied from 0.4/kb to 6.1/kb, and the haplotype diversity varied from 2/locus to 9/locus but there might still be four founder populations that might have contributed to today's pummelo genomes (Wu et al. 2014b). Though different studies told different stories, they were most probably originated from Yunnan (277). The reasons are two fold, first the fossil pommele, *Citrus lingcangensis*, was excavated from Yunan, second Yunan is renown not only for its rich pummelo resources but also for its rich river systems that could facilitate the downstream dispersions of the thick-skinned pummelos that can float on water for a considerablly long time.

2.5.5 Grapefruit

Grapefruit was thought to have originated from Barbados island from a chance cross between a pummelo and a mandarin. Evidence from cpDNA and AFLP further confirmed its pummelo–sweet orange hybrid origin (Nicolosi et al. 2000; Li et al. 2010). Moreover, genome sequencing provided unquestionable evidence that it is a half pummelo half mandarin hybrid (Wu et al. 2018).

2.5.6 Mandarins

Mandarins are worldwidely cultivated and ranked the second, next only to sweet orange in acreages among the cultivated citrus species. Collectively, mandarins are a group of smaller citrus fruits, mostly easy-peelers with darker juice and peel colors in comparison with Citron and Pummelo. Seeds of mandarins are mostly polyembryonic but also occasionally monoembryonic. Morphological variations are larger in mandarins than in any other citrus species, which has caused a lot of confusions and disagreements in studies of their taxonomy. More than 60 mandarin species were recognized by Tanaka whereas only one species and two subspecies were recognized by Swingle. With the

accumulation of molecular data, it is now generally agreed that mandarins are only one species which can be represented by ponkan (*C. reticulata*).

Several wild mandarin populations have been discovered in Nanling mountain and surrounding areas in the past half century (Liu et al. 1990; Wang et al. 2018). A comparative analysis on the genomes of 40 mandarins by Xu's lab showed that there were three distinct wild citrus genomes in the area (Wang et al. 2018). Two of them including Magnshanyeju wild mandarin are true mandarins while the third one, Mangshanyegan (*C. mangshanensis*) is quite different. Their analysis showed that two independent domestication events might have respectively occurred in the north and the south sides of the Nanling mountain. As a result, two different groups of cultivated mandarins have come into existence, the northern group with sourer, deeper colored fruits and the southern group with lighter colored, sweeter fruits (Wang et al. 2018). A comprehensive comparison between the genomes of 28 mandarin accessions, except for Tachibana, showed that there was extensive haplotype sharing among their ancestors, and three types of extant mandarins could be identified (Wu et al. 2018). According to Wu et al., tachibana mandarin, which is indigenous to Taiwan and Southern Japan, was dispersed from the mainland China to its nowadays island areas during the early Pleistocene (around 2 Ma) via the land bridges between the mainland and the surround islands that were exposed by sea level dropping, and has since separated from other mandarins. But a different story that it was a close relative of mangshanyeju and daoxianyeju was told by Wang et al. (2018). Interestingly, both researches noticed the introgression of pummelos into mandarins, which were thought to have helped reduce the acid content, and thus facilitated the domestication, of mandarins.

Mangshanyegan (*C. mangshanensis*) that was discovered in Mangshan of Hunan province was initially considered as a mandarin species for its resemblance to mandarins (Liu et al. 1990). Its habitat is not far from that of the wild Daoxianyeju mandarin. Its fruit is pubescent, round, and with thicker peel and taste bitter for containing oil drops in juice pulps, which contrasts it clearly from other mandarins. Its unique and homozygous β-carotene hydroxylase gene sequence argues that it should be treated as a new citrus species (Zhong 2013). Whole genome analysis by Wu et al. (2014a) also concluded that the *C. mangshanensis* is a distinct species.

2.6 Citrus Economic Importance

2.6.1 Major Citrus Production Areas

The cultivation of citrus fruits involves a large number of countries with great geographical dynamism, so the role of the various states changes rapidly (an important development is currently in Brasil and in China). The expansive trend is constantly growing and we can affirm with certainty that Citrus is the most extensively produced tree fruit crop in the world, with total global production reported to be 124.246 million tons in 2016 (www.fao.org). Nowadays, citrus fruits are grown in more than 140 countries, in tropical, subtropical, and Mediterranean climates, in the "citrus belt" between approximately 40° N and 40° S latitude, where the winter temperatures are suitable for tree survival and escaping freeze destruction, and where there are sufficient water and appropriate soils to sustain tree growth and fruit production. In the most extreme areas it is possible to grow citrus fruits, thanks to the temperatures moderated by ocean winds. The world production is localized both in the northern and in the southern hemisphere; particularly, in the northern hemisphere in the South and East Asian regions such as China (with a total production of more than 32.7 million tons), India (9.7 million tons), Pakistan (1.9 million tons), Indonesia, Japan, and Thailand, in the United States (7.8 million tons), and in Mexico (6.6 million tons), in the Mediterranean regions (totally production around 25.2 million tons) such as Spain (6.8 million tons), Egypt (4.9 million tons), Turkey (3.6 million tons), Italy (3.1 million tons),

Morocco (2.0 million tons), and some other regions. In the southern hemisphere, Brazil leads in citrus production, with more than 16.55 million tons followed by Argentina with 2.8 million tons, South Africa (2.4 million tons), Perù (1.1 million tons) and then Australia (0.58 million tons), and Paraguay (0.43 million tons) (Table 2.1).

Commercially, several species, are considered as a group under the term citrus, including lemons, limes, mandarins, satsumas, clementines, common mandarins and tangerines, oranges, grapefruit, and pummelos. In addition to these species, other citrus fruits such as kumquats, Calamondins, citrons, and many other hybrids are also commercially important. The global citrus industry contribution is immense and it provides employment to millions of people around the world considering all industry chains, starting from production to harvesting, handling, transportation, storage, processing, and market operations. As well known, although many citrus fruits can be eaten fresh, about a third of them worldwide is utilized after processing. In fact there are two types of distinct markets, one linked to fresh fruit and the other one to the processed citrus product, consisting mainly of citrus juice. Sweet orange is the predominant species for both of these markets. As indicated by FAO, in the industrialized countries, the consumption of fresh oranges is reducing while growing in the emergent developing countries like India, Brazil, Argentina, Mexico, and China. Moreover, over the last 20 years, two important market changes are to be recorded, one is that related to the increase of mandarin production, that consist also of tangerines, clementines, and satsumas, to the detriment of oranges; the second important change is related to the increase in juice consumption mainly due to the improvements in quality associated with technological progress and the price reduction.

According to FAO 2016, in order, China, Brazil, India, USA, Spain, Mexico, Egypt, Iran, Turkey, Italy, Argentina, and South Africa are the world's leading citrus fruit-producing countries, representing around 80% of global production (Table 2.1). Spain is the principal producer among the Mediterranean region regarding the citrus fruits produced for the fresh fruit market, which represents the prevailing production, with more than 6 million tons. Production trend indicates that the leading species is orange that contributes significantly to the bulk of world's citrus fruit production accounting for more than 54% of the global citrus output, followed by the group of tangerines, which includes mandarins, clementines, and satsumas, with 26%, the group of lemons and limes (13%), and grapefruit and pummelos comprise roughly 7%.

Although many citrus fruits can be eaten fresh, about 20% of citrus fruit worldwide is utilized after processing and orange juice production accounts for nearly 79% of total processed consumption. Overall, in the last few years, however, the production of juices has decreased compared to fresh citrus fruit (FAO 2016). In particular, Brazil is the leader with 50% of global transformation, followed by USA (around 16%), Mexico and Argentina (7% each), China (5%), and among the Mediterranean region, only Spain has a certain importance contributing with 4% of citrus processing (Table 2.2).

Major fresh fruit exporting and importing countries are shown in Tables 2.3 and 2.4. Fresh citrus fruit exports (about 16 million tons) account for more than 12% of the world production The Mediterranean area is the major exporter of fresh citrus with 55% of the volume and among the Mediterranean region Spain has become the major exporting country with more than 4 million tons of fresh fruit export, around 26% of the total world export. Follow Turkey and Egypt which contribute greatly to exporting with respectively 1.5 and 1.4 million tons (around 9% each). The export main destination from Mediterranean region is toward the European countries, principal to Germany, Netherlands, France, and the Russian Federation. The main exports from United States to other states,

Table 2.1 World citrus production and major producing countries by species. All values are expressed in 1000 tons

	Total	Oranges	Tangerins	Lemons/Lime	Grapefruits
World	*124246.0*	*66974.1*	*32968.5*	*15981.8*	*8321.6*
Northern hemisphere	*97848.9*	*42242.3*	*30609.4*	*12365.6*	*7631.6*
Cina	32705.9	7000.0	19000.0	2405.9	4300.0
Mediterranean region	25216.0	14654.8	6901.9	3034.1	625.1
Spain	6882.0	3642.4	2222.6	950.0	68.0
Egypt	4930.4	3610.4	948.5	369.6	1.9
Turkey	3652.1	1700.0	1040.0	670.0	242.1
Italy	3150.2	1854.9	855.1	434.4	5.8
Morocco	2018.9	925.0	1065.0	28.4	0.5
Algeria	1372.4	1025.5	251.3	93.2	2.4
Greece	1041.5	808.1	165.8	62.6	5.0
Syria	882.4	715.5	–	166.9	–
Israel	476.0	76.0	164.0	67.0	169.0
Tunisia	331.4	127.9	46.0	56.2	101.3
India	9755.8	6850.2	–	2613.8	291.8
United States	7829.0	5371.0	876.0	847.0	735.0
Mexico	6634.0	3535.0	499.0	2270.0	330.0
Iran	4067.6	2717.3	742.4	509.4	98.6
Pakistan	1907.4	1320.1	504.5	82.8	–
Indonesia	1574.8	1574.8	–	–	–
Japan	1143.3	20.3	1115.0	8.0	–
Thailand	1102.1	368.9	137.8	135.1	460.3
Viet Nam	998.7	520.0	–	–	478.7
Southern hemisphere	*26397.1*	*19731.9*	*2359.1*	*3616.2*	*690.0*
Brazil	16555.1	14350.0	910.8	1214.5	79.8
Argentina	2800.7	800.0	350.0	1500.0	150.7
South Africa	2409.2	1560.0	174.2	345.0	330.0
Perù	1112.1	446.9	393.7	265.4	6.1
Australia	584.6	455.0	95.0	27.2	7.4
Paraguay	431.4	323.3	48.7	9.3	50.0
Tanzania	426.8	426.8	–	–	–
Bolivia	371.4	183.5	153.5	25.9	–

Source FAO (2016)

5% of world export, are those toward Canada and the countries of Southeast Asia. In the countries of the Southern hemisphere, only South Africa has a considerable export volume, about 1.7 million tons (10% of global world export). Anyway, other countries of the Southern hemisphere, such as Argentina and Australia, are increasing their export volumes, thanks to the interest of the Northern hemisphere regions to the "out of season" productions.

Table 2.2 Citrus utilized for processing. All values are expressed in 1000 tons

	Total	Oranges	Tangerins	Lemons/Lime	Grapefruits
World	*23538.9*	*18460.9*	*1821.5*	*2469.0*	*787.5*
Northern hemisphere	*9215.9*	*6360.3*	*1374.6*	*941.2*	*539.8*
China	1200.0	600.0	600.0	–	–
Mediterranean region	1925.0	1072.0	356.0	358.2	138.8
Spain	951.9	516.4	204.8	222.6	8.1
Turkey	202.4	115.0	34.9	48.1	4.4
Italy	372.9	237.6	61.9	73.4	–
Morocco	73.1	58.6	14.5	350.0	–
Greece	120.5	120.1	–	–	0.4
Israel	168.9	20.0	27.0	2.0	119.9
United States	3760.0	3080.0	135.0	230.0	315.0
Mexico	1703.7	1225.0	42.7	350.0	86.0
Japan	93.0	–	90.0	3.0	–
Thailand	38.2	38.2		–	–
Republic of Korea	150.9	–	150.9	–	–
Southern hemisphere	*14323.1*	*12100.7*	*446.9*	*1527.8*	*247.7*
Brazil	11791.5	11180.0	244.0	311.0	56.5
Argentina	1670.0	350.0	150.0	1150.0	20.0
South Africa	684.6	436.0	22.8	54.6	171.2
Australia	105.4	100.0	5.4	–	–
Uruguay	71.6	34.7	24.7	12.2	–

Source FAO (2016)

2.7 Main Commercial Citrus Groups and Producing Countries

As well known, on a worldwide basis, citrus fruits are divided into five groups of significant economic importance: sweet oranges (*C. sinensis* L. Osbeck), mandarins (*C. reticulata* Blanco and *C. unshiu* Marc.), grapefruit (*C. paradisi* Macfadyen), lemons (*C. limon* L. Burmann *f.*), and limes (*C. aurantifolia* Christm. Swingle). Other species such as pummelo, citron, *Fortunella* sp., and others have lesser commercial importance although, obviously, they have an economic interest in the places of origin and production.

2.7.1 Sweet Oranges

The principal sweet oranges world producer is Brazil with a production of about 14 million tons (21%), the orange production is concentrated mainly in the state of Sao Paulo. China and India are almost on the same level of production, about 7 million tons each, the second-largest orange producer in the world accounting for more than 10% of the world's production. In the United States, with an orange fruit production of 5 million tons, representing 8% of orange world production, Florida is the major orange-producing state and most of that produce is used for juice processing. Orange fruits from

Table 2.3 Fresh citrus export by principal countries. All values are expressed in 1000 tons

	Total	Oranges	Tangerins	Lemons/Lime	Grapefruits
World	*15912.8*	*7361.6*	*4404.8*	*3055.8*	*1090.6*
Northern hemisphere	*13030.93*	*5857.2*	*3961.7*	*2331.6*	*879.8*
United States	825.1	550.0	40.1	100.0	135.0
Mediterranean region	8842.5	4479.7	2778.3	1226.0	358.5
Spain	4114.1	1870.5	1553.7	624.3	65.6
Egypt	1386.5	1286.4	45.7	34.1	20.4
Turkey	1495.1	403.4	460.0	449.3	182.4
Italy	198.6	123.4	33.2	39.9	2.1
Morocco	524.2	135.0	380.0	8.7	–
Greece	611.3	459.9	127.4	23.0	–
Israel	155.2	5.0	87.0	2.2	61.0
Mexico	693.6	45.0	–	627.4	18.0
Cina	683.1	59.2	445.9	40.4	137.5
Southern hemisphere	*2882.6*	*1504.4*	*443.1*	*724.2*	*210.9*
Argentina	394.3	64.8	49.7	279.3	–
Brazil	126.8	31.1	–	95.7	–
Chile	250.2	75.2	96.6	77.3	1.1
Perù	37.6	10.3	21.0	4.7	1.6
Australia	220.0	165.9	48.9	4.1	1.1
South Africa	1701.3	1064.1	189.7	245.0	202.5

Source FAO (2016)

California, Arizona, and Texas are sold for fresh consumption due to their appreciative quality. The Mediterranean region, Spain and Egypt, with around 3.6 million tons of orange production (5% each) are placed before Mexico and Iran (4% each) and Italy (3%).

Major cultivars in the sweet orange group are classified as "Blonde oranges", "Navel oranges", and "Blood oranges". The main cultivars, with commercial importance in the world, belonging to "Blonde oranges" are as follows: Hamlin, marked fresh in Florida, Brazil, South Africa, and in many other countries; Valencia Late (and its selection), the most widely grown in the world both in the Northern and in the Southern hemisphere, but most of the produce is processed; Shamouti, cultivar from Israel utilized as fresh fruit; Pineapple, grown mainly in USA, Mexico, South Africa, Brazil, and India, appreciate for the high juice content; Salustiana, commercial important seedless cultivar in Spain, similar to Cadenera also grown in Spain; Pera, in Brazil; Belladonna, and Comune widely grown in Italy.

"Navel oranges" fruit is characterized by the presence of a distinctive secondary fruit (navel) at the stylar end of the fruit. Usually are seedless due to complete pollen and partial ovule sterility; generally the fruits are larger than the other sweet orange cultivars. Navel is grown for fresh market. The highest quality fruit is produced in Mediterranean country such as Spain and also in the California coastal region. Washington Navel is the most widely grown important navel cultivar in the world. Its commercial importance is mainly in California, Florida, South Africa, Spain, Australia. Navel in a cultivar is very well established in Spain and Italy; Navelate, is a late maturing bud sport of Washington navel selected

Table 2.4 Fresh citrus import by principal countries. All values are expressed in 1000 tons

	Total	Oranges	Tangerins	Lemons/Lime	Grapefruits
World	*15037.6*	*7011.6*	*4210.4*	*2828.0*	*987.5*
Northern hemisphere	*14858.2*	*6885.5*	*4190.1*	*2803.5*	*979.2*
Canada	450.0	180.0	140.0	90.0	40.0
United States	965.0	160.0	215.0	580.0	10.0
Mediterranean Region	2065.3	981.2	544.2	417.2	122.8
France	1099.2	502.4	365.2	155.9	75.8
Spain	267.4	163.4	10.0	86.1	7.9
Italy	301.3	119.1	60.0	96.7	25.5
Germany	1118.1	477.5	403.6	176.0	61.1
Netherlands	1132.6	539.4	193.5	237.9	161.7
Poland	447.3	153.3	149.7	94.3	49.9
Russian Federation	1455.0	440.0	700.0	210.0	105.0
China	287.0	215.3	–	–	32.4
Hong Kong	341.7	260.0	–	37.6	16.1
Saudi Arabia	688.2	550.0	4.7	100.0	–
UK	796.6	292.4	324.9	147.6	31.6
United Arab Emirates	412.0	215.0	107.0	90.0	–
Southern hemisphere	*179.3*	*126.1*	*20.3*	*24.6*	*8.3*
Brazil	25.4	14.6	9.1	1.4	0.3
Australia	29.8	18.9	3.4	6.5	1.0
New Zeland	17.6	10.7	4.7	1.9	0.2
Paraguay	14.5	12.3	–	1.7	–
Kenya	44.4	44.4	–	–	–
South Africa	10.9	3.5	1.3	2.1	4.0

Source FAO (2016)

in Spain that produces a very vigorous tree; Cara Cara, Powell, Chislett, Rhode Navel, and others are navel oranges grown in several citrus regions.

The third group is that of "Blood or pigmented oranges" so called because of light to deep blood red colored anthocyanin pigments in rind and flesh of the fruit. Red color develops in the Mediterranean climate characterized by warm days and cool nights. In particular are of commercial importance in several Mediterranean countries including Italy, Spain, Morocco, Algeria, and Tunisia. In Italy are widely spread Moro, Sanguinello, and Tarocco, all with several clones. Moro cultivar has fruit subglobose to round with deep red flesh, particularly juicy; Moro is considered the most pigmented variety, at peak maturity, both the pulp and the peel, have an intense red-violet color. Sanguinello fruits are round to slightly oblong with slightly loose rind, deep red-fleshed. Tarocco: it cannot be considered a single cultivar because there are several clones with very different characteristics regarding the ripening period, the shape of the fruits, the pigmentation levels.

2.7.2 Mandarins

Commercially citrus fruit included in this group belong to several separate species, natural hybrids, man-made hybrids, selection, and mutants. The term mandarins is often replaced by

the term tangerines, in fact these two words are used interchangeably and indicate easy-peelers only. The main difference is that tangerines have usually deep orange to reddish-orange color and are smaller in size than standard mandarins.

For tangerines/mandarins production, China leads, being the largest producer with 19.0 million tons, covering 57% of world production, followed at a distance by Spain (7%), Japan, Morocco, Egypt, Turky, and Brazil (around 3% each). In Europe, Spain has had significant success with its seedless clementine varieties. Spain, China, Morocco, Brazil, and Argentina are constantly expanding their production, also due to the economic developments and the changing lifestyle of people in many cultures, and fresh fruit consumption is increasing in the category of "easy-peelers". Moreover, around 90% of total tangerines production is allocated to the fresh market as they are not suitable for juice production because of relatively low juice content, higher harvesting costs, and a tendency for off-flavor juice. As regards taxonomic, the group of mandarins is the most complex of citrus fruits, in any case, today, in all citrus fruit regions, it is well accepted that mandarins are grouped into Common mandarin (*Citrus reticulata* Blanco), Mediterranean mandarin (*Citrus deliciosa* Tenore), King mandarin (*Citrus nobilis* Loureiro), Satsuma mandarin (*Citrus unshu* Marcow.), Natural mandarin hybrids, and Man-made mandarin hybrids.

The most important variety belonging to Common mandarin is Ponkan mainly grown in China, India, and Brazil. Ponkan, also called Nagpur Suntara in India and Batangas in the Philippines, has a very good eating quality even if seeded. In India the Khasi variety is grown extensively; in Australia is largely diffused the Emperor while Clementine and its various clones, are commercially very important in the Mediterranean region; Dancy is an important cultivar in Florida.

Mediterranean mandarin, even if originated in China, is widespread in the regions of the Mediterranean basin. It is also called in the USA Willowleaf mandarin due to its lanceolate leaves like a willow tree. In Italy Avana and Tardivo di Ciaculli are widely diffused.

In the small group of King mandarins, only few varieties assume a certain importance such as the King of Siam, in Cambogia and Viet Nam, and Kunembo in Japan.

Satsuma is the most important and widely cultivated mandarin of Japan and there it is known as Mikan or Unshu Mikan. About 100 varieties, obtained from mutations due to poor genetic stability, are known. They are grouped, according to the ripening period, in very early ripening (*Goko Wase*), early ripening (*Wase*), medium ripening (*Nakate*), and late ripening (*Bansei*). The most important cultivar belonging to *Wase* are Okitsu, Miho, and Miyagawa, and among *Bansei*, the Sugiyama cultivar.

Finally, are so many and constantly evolving the natural and artificial hybrids which are affirmed and are successfully cultivated in all citrus regions, that it is not easy to mention them all. Among the natural mandarin hybrids, most of them are hybrid between mandarin and sweet or sour orange, we report Murcott, also known as *Honey, Honey Murcott*, or *Smith*, Temple, widely grown in Florida and South Africa, Tankan, Ellendale, Ortanique, and more others. Among the artificial or man-made hybrids, in particular, among Tangelos, interspecific hybrids of mandarin or tangerin and grapefruit or pummelo, the following have been affirmed: Orlando and Minneola; arose from a cross between Dancy tangerine and Duncan grapefruit made by Webber and Swingle in 1897 in Florida. Other important man-made hybrids obtained from a cross of Orlando and Clementine are Robinson, Osceola, Lee, Nova, and many others have been obtained from different cross combinations: Page, Sunburst, Ambersweet, Orah, Fortune, Mapo, Cami, and several more recent innovation such as Tango, Orri, Safor, Garbì, and Mor.

2.7.3 Grapefruit

Around 52% of the world grapefruit production is held by China, followed at distance, by the

United States (9%), Viet Nam and Thailand (6% each), Mexico and South Africa (4% each), Turkey (3%) and Israel (2%). Commercially there are white-fleshed, pink-fleshed, and red-fleshed cultivars. Belong to the white-fleshed grapefruit the varieties Duncan, Marsh, and also Oroblanco and Melogold, both triploid hybrids of acidless pummelo × Marsh seedless 4x grapefruit. The main pink-fleshed cultivars are Foster, Henderson, Marsh Pink (Thompson), Red Blush, Ray Ruby while the most commercial cultivars red-fleshed are Rio red, Flame, and Star Ruby.

2.7.4 Lemons and Limes

India is the largest producer of lemons and limes with a production of 2.6 million tons (16% of world production), immediately followed by China (15%) 2.4 million tons, Mexico (14%) 2.2 million tons, and then Argentina (9%), Brazil (7%), Spain (6%), USA (5%), Turkey (4%), and Italy (3%). Lemons are widely produced in temperate climates such as Southern California, which is free of frosts, and for the same reason in Spain, Italy, and Argentina. Lemons are also adapted to drier climates such as Egypt and Iran. Limes are highly sensitive to cold weather and are grown mainly in tropical climates such as in Mexico, India, Brazil, and United States with the main production area in Florida. In the United States are widely diffused Eureka, Lisbon, and recently Genoa while in Spain, Fino and Verna. In Italy, Femminello group represents the most widely grown lemon types with Femminello comune, Femminello siracusano, Femminello Santa Teresa, Femminello Fior d'arancio, and more others. Certainly of minor commercial importance are the limes. Limes are grouped into two major groups that include the acid and acidless lime. The acid limes are further subdivided into "Persian" or "Tahiti" or "Bearss" and "Key" or "West Indian" or "Mexican" limes. Among the sweet limes Palestine lime is known in India as "Mitha Nimbu".

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Genetic Resources of Citrus and Related Genera

3

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Abstract

Genetic resource is the part of the organisms that can reproduce identical copies of the organism with identical genomic profiles. Generally, seeds, pollen, bud wood are considered as genetic resources of *Citrus* and its related genera. Citrus have three basic taxa, pummelos (*Citrus maxima* (L.) Osb.), citrons (*C. medica* L.) and mandarins (*C. reticulata* Blanco). *Citrus* species, cultivar, landraces have evolved by series of interspecific hybridizations, consequently, a huge diversity gained in citrus genus. Recently, Australian limes, Mangshanyegan, Kumquats and *Clymenia* spp. are included in the citrus genus. Due to high economic value of *Citrus*, researchers collect citrus germplasm and maintain them in the gene bank at major citrus producing countries worldwide. Significant progress has been made on the citrus germplasm collection, management and conservation. In this chapter we summarize the present status of the citrus germplasm collection and management, as well as discuss the future path to protect the valuable citrus genetic resources for future use.

3.1 Introduction

In general genetic resource is any part of the living organisms that can reproduce identical copy of the organisms comprised of identical genomic profiles. It is the functional unit of heredity and the most essential basic raw materials to convene the current and future requirements of crop improvement programmes. Normally seeds, pollen, bud wood are considered as genetic resources of citrus and its related genera. In addition, some cases leaves and other vegetative organs of citrus and its related genera were also considered as genetic resources (Khan 2007). Genetic resources collection, proper maintenance, identification and characterization are prerequisites for efficient and sustainable breeding programme. A wide genetic base broadens the horizon of breeding to achieve ultimate goal of the breeders with improved traits of new cultivars. Hence, genetic resources institutes or centers collects and conserves genetic

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resources in a systematic manner as large numbers as possible. In fact, most of the countries established their own national germplasm institute or center, whose main responsibility was to collect and maintain the local genetic resources, most cases important crop plants. Citrus is one of the most common, well known, highly consumed fruit crop worldwide. Accordingly, this fruit species gained lot of attention to plant breeders and scientists worldwide, as a result genetic resources of citrus collected and conserved in a wide range of countries of its major producing regions. A global citrus germplasm network was established in 1997 to monitor the world wide citrus germplasm collection, proper maintenance and ensure their sustainable use in citrus breeding programme and protect citrus genomic resources erosion (Roose et al. 2015).

Citrus belongs to the plant family Rutaceae and it has large diversity. The citrus genera composed with uncertain number of species, landrace or cultivars (Wu et al. 2018). The taxonomy and ancestry relationships among the wild and domesticated species of the citrus genus are complex, even in some of the state very much confusing or unclear. The assessment of the ancestry of modern *Citrus* spp. is relatively complicated due to the apomixis nature and highly cross-compatibility among the related species. Citrus apomixis is sporophytic type, therefore, embryos develop from somatic nucellar cells (Wang et al. 2017). This phenomenon is typically known as 'polyembryony' and it is very stable among modern commercial cultivars like mandarins, sweet oranges, grapefruits and lemons. Polyembryony nature of citrus has both positive and negative impacts on citrus breeding, for example, polyembryony was widely used to propagate large numbers of uniform rootstocks from seeds, while it hampers the cross-breeding programme. The polyembryony remains genetic materials unchanged from generation to generation in citrus species, as a consequence the genetic erosion may reduce.

It is widely believed that citrus genera have three basic taxa viz. pummelos (*Citrus maxima* (L.) Osb.), citrons (*C. medica* L.) and mandarins (*C. reticulata* Blanco) (Nicolosi et al. 2000), from which modern citrus species like, sweet oranges (*C. sinensis* (L.) Osb.), clementines (*C. clementina* Hort. ex Tan.), satsumas (*C. unshiu* (Mak.) Marc.), lemons (*C. limon* (L.) Burm *f.*), limes (*C. aurantifolia* (Christm.) Swing.) and grapefruits (*C. paradisi* Macf.) are evolved by series of interspecific hybridization, consequently, a huge diversity is gained in citrus genus. Apart from these core citrus species, recently discovered some of the species, viz Australian limes, Mangshanyegan, Kumquats and *Clymenia* sp. are included in the citrus genus (Garcia Lor 2013). The commercial rootstock Trifoliate orange (*Poncirus trifoliata*) is the closest relatives of citrus, often they are categorized as citrus. Bayer et al. (2009) suggested that *Oxanthera* sp. from New Caledonia should be transferred to the citrus genus.

The aim of this chapter is to make clear the importance of citrus genetic resources in breeding as well as their world wide present scenario, major challenges, future direction to overcome the hurdle for their better uses.

3.2 Present Status of the Citrus Genetic Resources Around the World

Citrus germplasm is the building block of its varietal improvements, consequently, the citrus research community prefers to collect the citrus germplasm and maintain them in the gene bank. Citrus germplasm collection usually includes primitive landraces, wild species, developed varieties and breeding lines. Major citrus producing countries have huge collection of citrus germplasm in their gene bank. Here briefly we illustrate worldwide present citrus germplasm collection scenario.

Australia: Several citrus related genera like *Eremocitrus* and *Microcitrus* were originated in Australia (Krueger and Navarro 2007). Citrus germplasm collection is maintained in Australia by the State Government, Departments of Primary Industries and the Commonwealth Scientific and Industrial Research Organization (CSIRO). Apart from these, there are several other citrus

germplasm banks (Royal Botanic Garden, Sydney; Brisbane Botanic Gardens; Waite Research Institute, University of Adelaide). Australia mainly follows *ex situ* conservation for wild and cultivated citrus and related genera.

Brazil: Brazil is the major citrus producer in south America (FAO 2018). Brazil initiated citrus germplasm collection in the year 1948. Initially, about 470 citrus accessions were collected from Riverside California, USA and maintained in the Brazilian citrus gene bank; new local accessions were then added to the gene bank and now the total collection reached about 1546 accessions. These collections include nucellar clones, newly introduced cultivars, and new hybrid collections for breeding and rootstocks. Some duplicate accessions have been identified in the Brazilian citrus germplasm collection. Among them some accessions have the same clone with different names, and some have same name but they are from different clones. One plant per accession in the gene bank was maintained under the screen house (Roose et al. 2015).

China: Exploration and collection of Chinese indigenous citrus varieties was initiated in the 1950s; unfortunately the survey was interrupted by the Cultural Revolution of 1967–1972, but was restarted by the Chinese government in the 1970s and 1980s. Conservation of citrus germplasm in PR China is mostly *ex situ* conservation. The national citrus germplasm repository of China was established in 2007 at Beibei, Chongqing, Sichuan province. Beside the national citrus gene bank there are many other sites found in China where wild citrus germplasm has been maintained. Among them Huazhong Agricultural University (HZAU), Huangyan, Zhejiang province; Guiling, Guangxi province; Changsha, Hunan province; Guangzhou, Guangdong province; Jiangjin, Chongqing municipality; Wuzhung province and Hubei province are notable (Krueger and Navarro 2007). HZAU has its own citrus germplasm collection center where more than 400 accessions are maintained, along with 100 embryo callus lines. The HZAU collection includes trifoliate orange, tangerines, kumquat, lemon, lime, pummelo, sweet oranges, papeda, citron, and grapefruit.

France: There are two institutes involved in the citrus germplasm collection and maintenance in France, INRA and CIRAD, both the institutes are located at San Giuliano, Corsica, France. A total of 928 genotypes have been maintained here in the field; among these 340 were mandarins and about 220 sweet oranges (Roose et al. 2015). The origins of these collections are quite diverse, with the highest contribution from California and Florida, and the other from other citrus producing countries around the globe. 60% of the France collection was introduced by budwoods and remaining 40% from seeds. There are no restrictions to import plant seeds in France but live plant materials must go through quarantine check. Therefore, imported citrus budwoods are sent to another location for quarantine check prior to admission into citrus gene bank. Budwood is tested for viruses, viroids and diseases every 1–3 years. France uses cryopreservation of the citrus seeds collection for security backup. The collections are often evaluate by molecular markers (SSR, SNP, Indel markers) and phenotyping. Phenotyping is performed according to IPGRI descriptors. Biochemical traits and abiotic stress tolerance are also evaluated. French collection is available for commercial and scientific uses and no material transfer agreements (MTA) are required for this exchange. Every year in INRA and CIRAD about 25,000 citrus buds are distributed in 22 locations in France and in other Mediterranean countries.

India: Several citrus species are native to India and this country is rich of citrus genetic resources. To protect citrus diversity, NBPGR (National Bureau of Plant Genetic Resources) of ICAR (Indian Council of Agricultural Research) established the 'Citrus Gene Sanctuary' in the Garo hills of Meghalaya in 1981. Around 1500 indigenous and exotic citrus accessions are conserved and maintained in 20 field gene bank at different locations in India. Among them, a large collection of about 600 accessions including *Citrus* spp., *Poncirus trifoliata, Severinia* species are maintained at National Research Center (NRC) Citrus, Nagpur. For long term preservation, Indian collections are also maintained using cryopreservation (Singh et al. 2001; Sharma et al. 2004).

Spain: Spain is one of the major citrus producing countries. The Spanish Citrus Germplasm gene bank is located at Instituto Valenciano de Investigaciones Agrarias (IVIA). Spanish citrus gene bank collects and maintains germplasm from *Citrus* and its related genera of *Aurantioideae* subfamily. Till to date 620 accessions have been introduced in Spanish Citrus gene bank, including 425 accessions from 51 *Citrus* species, 53 from 20 *Citrus* relatives, and 142 intra- and inter-specific hybrids. Spanish germplasm collection is maintained at three states, (a) fields, (b) screenhouses and (c) cryopreservation. All the genotypes are phenotyped according to IPGRI (IPGRI 1999) and UPOV (http://www.upov.int/portal/index.html.en) descriptors and genotyped by 36 SSRs markers. After genotyping and phenotyping duplicate entities are removed from the gene banks and only healthy and pathogen-free accessions are maintained. In vitro shoot-tip grafting technique is also applied to obtained pathogen-free Citrus accessions.

USA: The University of California, Riverside (UCR) first initiated Citrus variety collection in USA in 1910. UCR established Citrus Experiment Station to conserve and maintain Citrus and its relatives. UCR has the most extensive collection of citrus diversity in North America and till to date over 1,000 accessions have been introduced in UCR gene banks. UCR collection includes commercial citrus varieties, breeding line, other citrus germplasm and wild relatives, particularly important for research and extension activities. Apart from UCR collection, USDA ARS National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) has been established in 1987 for collection, evaluation, maintenance, and preservation of citrus and date palms genetic resources. Currently about 400 accessions pathogen-free are available for free distribution on request for research purpose.

3.3 Significance of Citrus Genetic Resources for Breeding

Genetic resources and plant breeding are intimately linked to each other. Genetic resources are the fuel for breeding, without it breeding programme cannot run. The fundamental goal of any plant breeding programme is developing cultivars with high yielding, high quality and quantity, disease resistance/tolerance, pests/nematode resistance/tolerance, increasing adaptation ability to diverse climate and soil conditions. Genetic variation is required to achieve the breeding goals, therefore plant breeders always hunt for the diverse genetic materials for improving the cultivars through breeding. Consequently, plant genetic resources are considered as the treasures in which key genes (traits) are kept stored. For example, wild species are disease resistant but low yielding while cultivated varieties are high yielding but disease susceptible. In that circumstances plant breeders select wild and cultivated species as parent for breeding and subsequently, make a series of crosses between selected parents until getting the most desirable combination of genetic traits. Citrus breeders also follow a similar strategy for high yielding disease resistant cultivar development. Citrus breeders around the world initiated breeding programme in the nineteenth century and the process is still continuing for improving the citrus cultivars to meet the consumption demands (Cooper et al. 1962).

Webber and Swingle hypothesized that the selection of individual from seedlings obtained from crosses may give better results than seedling grove selection. In order to improve citrus varieties they initiated their cross-breeding programme in the spring of 1893. They used sweet orange, grapefruit and mandarin varieties as parents in their hybridization programme (Webber 1894; Swingle 1905). Afterward, a series of cross-breeding and selection programme has been incited by USDA to improve the citrus variety. *P. trifoliata* has been hybridized with other *Citrus* species to produce cold-hardy varieties (Webber 1900). During 1908–14 Webber and Swingle produced 2,500 hybrids from crosses of the *P. trifoliata* with varieties of mandarin, lemon, lime, grapefruit, sour orange, and kumquat. In 1942 Gardner and Bellows made a series of crosses among different citrus genotypes viz. tangors, tangelos, mandarins, and sweet oranges. One of the crosses, Clementine x

Orlando, produced many promising new hybrids; most of them are early-maturing, large in size, sweet taste and have an orange-red rind; but unfortunately they are rather seedy and some of them have prominent navels.

There were several citrus genotypes widely cultivated and maintained in Japan, such as satsuma mandarin, natsudaidai (*C. natsudaidai* Hayata), kunenbo (*C. nobilis* Lour.), kinokuni (*C. kinokuni* Hort. ex Tanaka), iyo (*C. iyo* Hort. ex Tanaka), hyuga-natsu (*C. tamurana* Hort. ex Tanaka), hassaku (*C. hassaku* Hort. ex Tanaka) and yuzu (*C. junos* Sieb. ex Tanaka) (Omura and Shimada 2016). Japanese citrus breeders use these genetic resources to improve cultivated citrus varieties through cross-hybridization and selection. A large scale citrus breeding programme was initiated in Japan in 1937, therefore, several promising citrus varieties have been released and among them Shiranuhi and Kiyomi are very popular for the commercial cultivation (Nesumi and Matsumoto 2000). The first Japanese citrus hybrid was Tanigawa-buntan, which was released in the Taisho period (Omura and Shimada 2016).

China has more than 4,000 years old history of citriculture. Han Yen-Chih, in 1178, firstly recorded and described citrus variety found in Wenzhou, Zhejiang province. Chinese citrus breeders initiated citrus breeding programme to produce seedless citrus variety in the mid twentieth century (Liu and Deng 2007). Their main strategy was bud sport mutant selection to acquire seedless varieties. As a result, until now 150 clones of seedless or less seedy clones were identified. Among them Qianyang, a seedless red sweet orange (Chen et al. 1992), seedless Shatian pummelo (Zhuang et al. 1994), seedless Shatangju (Zi-xing et al. 2006), seedless Ponkan and Xuegan sweet orange (Chen et al. 1997) are the most representative. Similarly, Chinese citrus breeders selected several late-ripening bud mutants, e.g. Yanxi wanlu Ponkan, founded in Fujian, China, whose harvest time is delayed of two months, from late November to February (Zhong and Chen 1994). Another late-ripening citrus variety, Mingliu Tianju, derived from a bud mutation of Chun Tianju tangerine was obtained in Guangdong province (Min 2006). Recently, citrus scientist from the HZAU discover two late-repining bud mutants, Fengjiewancheng and Zaohong navel orange from the Three Gorge Citrus Natural Germplasm Collection center (Li et al. 2006). The result of the citrus breeding programmes over the few centuries demonstrated that the citrus genotypes (germplasm) played a crucial role in the success of the cross-breeding programme.

3.4 Major Challenges for Citrus Genetic Resources

Germplasm represents the genomic treasure that is transmitted down through generations by an unbroken chain (Brown et al. 1989). Genetic erosion is one of the major cases for breakdown of this chain. Many of the plant genetic resources including citrus species facing the challenge for survival. There are several factors that cause the genetic resource erosion, among them population growth, global warming, pollution, deforestation, destroy of the natural habitat of the plants, mono culture, selection and domestication pressure, natural disasters, diseases, etc.

Natural habitat or ecosystem is very important for survival of wild species, destruction or alteration of habitat or ecosystem is the cause for loss of certain gene pools forever. In general, center of origin is the natural habitat of the wild plant species, for example north-eastern Himalayan region and foothills of the central and western Himalayan tracts (Ghosh 1977; Govind and Yadav 1999; Malik et al. 2006) are considered as the natural habitat of the wild citrus species. Citrus species including mandarin and orange (Tanaka 1958; Singh and Chadha 1993; Chadha 1995) originated from this region and their wild progenitors grew in this region from millions of years. Unfortunately, many natural habitats of citrus of this region have been destroyed by urbanization. Consequently, many of the ancient progenitors of citrus and their relatives were extinct and the rest of them are endangered.

Environmental pollution and global warming simultaneously change the global weather vastly, as a result frequently happening cyclone, flooding coastal region, huge rainfall, unexpected draught in the year round. These weather changes have devastating effects on the natural habitats, which may be the cause of citrus germplasm loss. For instance, natural habitat of the citrus are in untraced hill slopes, this kind of landscape practically has not adopted any soil conservation measures, therefore, heavy rainfall washes away the nutrient-rich surface that leads exposure of comparatively less fertile and extremely acidic subsurface and this may increase the risk of genetic erosion of the citrus germplasm. Furthermore, loss of soil fertility, prolonged high rainfall and high humid environment increase the chances of citrus pathogens break out and this could be threatening for citrus germplasm.

Various ancient citrus varieties in the north-east region of India generally have been found in home gardens or backyard gardens since very ancient times. Due to less commercial importance of these ancient citrus species, they are not properly maintained or propagated, consequently, they are at high risk of extinction. Due to consumer demand and economic benefits, citrus growers prefer to grow commercial cultivars like mandarins, sweet oranges, lemon, navel oranges, etc. As a result, they replaced wild and ancient types of citrus varieties from their orchards and replanted commercial cultivars leading to the loss of many citrus genotypes permanently.

3.5 Present Activity and Future Challenges

The genetic diversity of citrus and its relatives are shrinking at alarming rate due to population growth, global warming, pollution, large scale deforestation, selection pressure, diffusion of newly introduced cash crops. Therefore this is the high time for the researchers to explore all the rare and endangered resources of citrus and develop sustainable management systems to prevent their extinction. In this section we briefly discuss the present scenario and the additional needs in order to meet the upcoming challenges of citrus genetic resources conservation.

a. Genetic resources collection and conservation

Plant genetic resources collection and their proper conservation is the first step to prevent the extinction of genetic resources. In order to prevent the extinction of citrus genetic resources and maximizing their utility in the breeding programme, citrus germplasm has been collected and conserved in major citrus producing countries.

The north-east part of India is one of the richest areas of citrus genetic diversity and it is the center of origin of many citrus species. The collection of citrus germplasm from this region intensively began in the early twentieth centuries by Tanaka (1928, 1937) and Bhattacharya and Dutta (1956). During this time, several new citrus species were identified, collected and documented. The comparison of recent survey reports with previous surveys of these regions, shows that some of the citrus species are extinct and some are endangered. For instance, Tanaka (1928) described *Citrus indica* and in the year 1937 he reported this species as one of the wild species found in Now gong district, Khasi hills and Manipur in Assam. Later, the survey report of Singh (1981) demonstrated that this wild *Citrus indica* only found in Naga hills of Nagaland, Kaziranga Reserve Forest in Assam and Garo hills of Meghalaya. Nevertheless, recent survey reports indicated the presence of this species only in the Tura ranges of Garo hills of Meghalaya (Upadhay and Sundriyal 1998; Singh 2003). In order to protect genetic erosion of citrus germplasm, Nokrek Biosphere Reserve has been nominated as Citrus Gene Sanctuary (Singh 1981) and here many of the citrus wild species are conserved. China initiated citrus germplasm conservation establishing the central citrus germplasm repository in 2007 at the Beibei, Chongqing, Sichuan province. Beside this Huazhong Agricultural University established citrus germplasm bank in where more than 400 accessions were collected and maintained. All

these genotypes were collected from the different parts of China as well as around the globe. Similarly Brazil, USA, France, Spain, Italy collected citrus germplasm from the wild, semi-wild and cultivated species from the major citrus growing areas as well as from the center of origin of citrus.

Both in situ and *ex situ* germplasm conservation methods have been adopted for citrus. Among *ex situ* conservation methods, callus culture, cryopreservation, in vitro plant growth, can facilitate the citrus germplasm storage for long time. Tissue culture has been used to preserve disease-free citrus shoots. Also callus from citrus leaf tissue have been maintained on slow growth media for long term preservation. The most important activities for *ex situ* conservation of citrus germplasm have been conducted at the Citrus Research and Education Center, University of Florida, at Huazhong agricultural university, China and at Instituto Valenciano de Investigaciones Agrarias, Spain.

b. **Germplasm phenotyping and genotyping**

Proper phenotyping and genotyping of the germplasm is required for effective plant breeding programmes. Therefore plant breeders and geneticists used markers (both molecular and phenotypic traits) for characterization of the collected germplasm prior to utilizing them in breeding programme. Phenotyping and genotyping provide the opportunity to select best parental combination for the improvement of the cultivars. In addition the information on genetic background of any genotype is required for its proper management as well for its use in research and breeding. During the last few decades citrus breeders and researchers made efforts for citrus germplasm characterization, established phylogenetic relationship among the wild relatives and cultivated species and estimated genetic diversity among the citrus population. These basic information helps to overcome many citrus breeding obstacles. For proper genotyping citrus research community vastly uses molecular markers like SSR (Biswas et al. 2014), ITS, ISSR and SNP. These aspects will be further discussed in Chap. 7.

Although molecular marker-based citrus genotyping techniques are well established, a specific set of molecular marker that can be used for any citrus genotype identification is still missing. Recently, whole genome sequences of the main citrus species have been released (Xu et al. 2013; Wu et al. 2018). The whole genome sequence data could be useful to develop citrus species-specific markers and once developed, those markers could be very useful for the rapid citrus germplasm characterization, identification and their genetic relationships estimation. Eventually, citrus species-specific molecular markers could also accelerate the citrus breeding programmes.

Although many citrus genotypes are well characterized, most of them are rather unknown to young researchers and farmers due to their different name and lack of precise information. So it would be very useful if each unique citrus genotype could be encoded with a universal identifier name. This unique identification of the citrus genotypes may be helpful for a more precise germplasm management.

c. **Strategies for citrus genetic resources management**

Collected germplasm should be maintained and managed in proper way for its efficient use. The richest citrus germplasm collections have been held at national level by the most important citrus countries. A precise plan should be developed for monitoring, supporting, and conserving these collections. Global citrus germplasm network was established in 1997 with the aim of developing a global citrus germplasm management strategy; more than 60 delegates from the major citrus growing countries participate to this network and established a convention. According to this convention citrus germplasm curators and gene bank managers around the globe have been asked for the health

and status of their collection using a specific questionnaire, which was developed and approved by the global citrus germplasm network. The survey report should be submitted to the collaborator at the University of California, Riverside and at the USDA ARS, Riverside. These strategies were implemented by the Global Crop Diversity Trust in collaboration with the International Society for Horticultural Science and several members of the international citrus research community (Roose et al. 2015).

d. Free accessible online information on citrus germplasm

Appropriate information about the germplasm including genetic background, cross compatibility, disease resistance, growing condition, origin, phylogenetic relationship with other members of the taxa, yield performances, agronomic traits etc., are useful for further use. These information can help breeders to take quick decision to design their experiment. In order to easily access information about genetic resources a few online genomic resource database have been developed for plant species. Among them, those concerning banana (https://www.crop-diversity.org/mgis/), tomato (https://tgrc.ucdavis.edu/index.aspx), lily (http://210.110.86.160/Lidb/Lilidb_Genotype.html), rice (http://www.ricediversity.org/proj/germplasm/repositories.cfm) are notable. Banana genetic resources database can be considered a model database for germplasm information for any plant species, since from this database users easily can access the present status of collected banana germplasm, as well as researchers can order genetic materials through the web site for research purposes. In addition this database is very rich of information on the collected banana germplasm and this information accelerate the banana germplasm conservation, management and breeding programme. Unfortunately, such kind of database is lacking for citrus species; therefore, it would be useful to develop a freely accessible online-based citrus genetic resources database in order to speed up the breeding process and to protect germplasm from genetic erosion.

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Conventional Breeding of Cultivated Citrus Varieties

4

Eran Raveh, Livnat Goldenberg, Ron Porat, Nir Carmi, Alessandra Gentile and Stefano La Malfa

Abstract

Citrus species are among the world's most widely grown commercial crops with hundreds of cultivated varieties, some of which with exclusively local importance. Citrus breeding programs concern different species and hybrids, also triploids, such in the case of the development of new mandarins and mandarin-like varieties and, on a smaller scale, the development of new grapefruit-like cultivars with unique fruit-quality traits and the creation of seedlessness. An increasing interest is paid also to the qualitative traits of the fruit and to its nutraceutical properties. So in Italy, and more recently in other citrus regions, the development of new pigmented genotypes, among oranges and mandarin hybrids is a high priority goal of breeding programs. The interest for lemon breeding is currently poor since there are no active large-scale conventional breeding projects. However, in some countries like Italy, Turkey, Greece, the development of new varieties resistant to mal secco disease is of pivotal importance. In Citrus, conventional breeding is hampered by several reproductive biological features, including apomixis, male and female partial sterility, cross- and self-incompatibility, high level of heterozygosity and long juvenile period. However many breeding programs are currently developed in most of the citrus producing countries in order to face different problems and to release novel varieties to accomplish the consumer request. Both conventional and molecular breeding approaches are used with an increasing importance paid to the use of biotechnological tools for marker-assisted selection and for gene function discovery in order to speed up the obtainment of new varieties. In the present contribution, we will discuss the most important achievements for citrus varieties genetic improvement, describing traditional and innovative approaches, and the main results so far achieved for important traits.

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4.1 Aspects of Citrus Biology Related to Breeding

Cultivated species of the genus *Citrus* are among the world's most widely grown commercial crops, with an overall yield of nearly 100 million tons per year (USDA 2018). Current citrus breeding programs are focused mainly on the development of new mandarins and mandarin-like varieties and, on a smaller scale, the development of new grapefruit-like cultivars with unique fruit-quality traits and the creation of seedlessness. In Italy, and more recently in other citrus regions, such as China, Brazil, and Spain, interest is increasing for the development of new pigmented genotypes, among oranges and mandarin hybrids. Regarding to lemon, even if there are currently no active large-scale conventional breeding projects, in some countries like Italy, Turkey, and Greece, the development of new varieties resistant to mal secco disease is a priority goal (Gentile et al. 2007; Gulsen et al. 2007; Nigro et al. 2015; Polat 2018).

Apomixis is a characteristic of many citrus cultivars, discovered by Leeuwenhoek in 1719 (Batygina and Vinogradova 2007), which results in the production of additional somatic embryos to the zygotic one. The seedlings, which are produced by this phenomenon, are 100% identical to the female parent tree. To avoid the production of progeny that are identical to the mother plant, citrus breeders use apomictic varieties only as pollen donors (male parents) in intended crosses.

In the last few years, seedlessness has become a major desired trait in many citrus breeding programs worldwide. Citrus plants have parthenocarpic capability (i.e., they can set fruit without sexual fertilization) and this trait is used to obtain new seedless varieties (Vardi et al. 2008). Some citrus varieties are self-incompatible and when grown in isolated orchards will set seedless fruits. Alternatively, self-incompatible varieties may be grown under insect-proof nets to avoid cross-pollination.

An important trait that makes citrus breeding difficult and time-consuming is the relatively long juvenility period of these plants, as compared with other crops in which backcrossing is often used to introduce new traits into commercial varieties.

4.2 Origin of Cultivated Citrus Varieties

Phylogenetic and taxonomic studies of the genus *Citrus* have revealed that there are three true citrus ancestors—citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), and pummelo (*C. maxima* Merrill)—and all other citrus species actually evolved from crosses between those true original species or other relatives (Scora 1975; Barret and Rhodes 1976; Nicolosi et al. 2000). A recent study by Wu et al. (2018) based on genome sequencing analysis further divided the mandarin group into three types based on genomic purity: type 1 represents pure mandarins; whereas types 2 and 3 represent different degrees of introgression of the pummelo genome in later developed mandarins (Fig. 4.1). According to this analysis, most of the modern cultivated mandarin varieties are actually mixtures of type 2 mandarins and sweet orange (Wu et al. 2014, 2018). Furthermore, pummelo and mandarin are also the ancestors of sweet and sour oranges, grapefruit, and, consequently, of all modern mandarin varieties (Fig. 4.1b). The introgression of *C. maxima* into the genome of *C. reticulata* is probably responsible for the high phenotypic variability found among modern mandarin cultivars (Oueslati et al. 2017; Wu et al. 2018).

Sweet orange (*C. sinensis* L. Osb.) represents a complex hybridization of early admixture mandarin and pummelo. Sour orange (*C. aurantium* L.) is a hybrid of pummelo and pure mandarin (Fig. 4.1b).

Other species were also involved in hybridization. Among those, *C. micrantha* Wester is the parent of Mexican lime and *Fortunella margarita* (Swingle). Most citrus genotypes are apomictic, with the exception of all citrons, pummelos, and some mandarin cultivars (Spiegel-Roy and Goldschmidt 1996; Aleza et al. 2010b). Therefore, it is likely that this trait reached modern mandarin cultivars from their

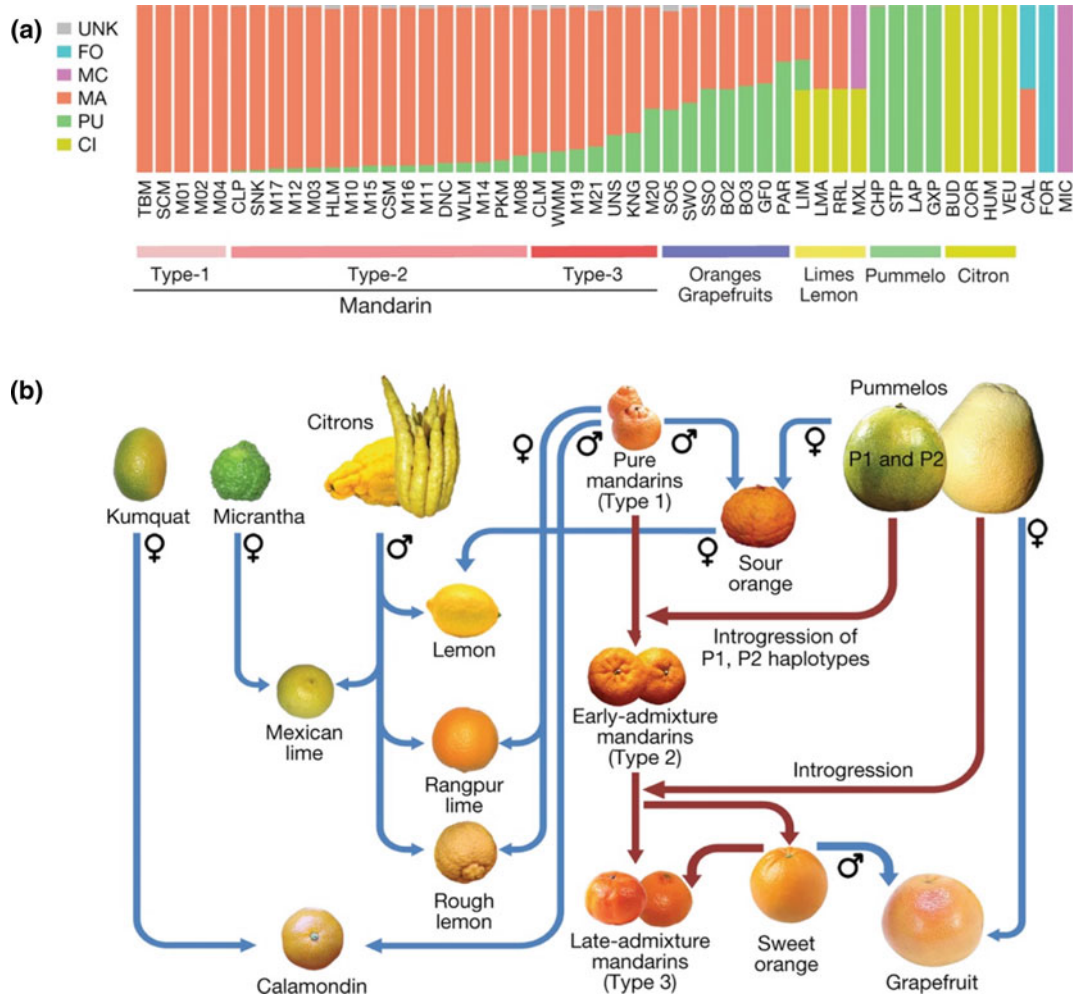

Fig. 4.1 Admixture proportion and citrus genealogy. **a** Allelic proportion of five progenitor citrus species in 50 accessions. CI, *C. medica*; FO *Fortunella*; MA, *C. reticulata*; MC, *C. micrantha*; PU, *C. maxima*; UNK, unknown. The pummelos and citrons represent pure citrus species; whereas in the heterogeneous set of mandarins, the degree of pummelo introgression subdivides the group into pure (type 1) and admixed (type 2 and type 3) mandarins. **b** Genealogy of major citrus genotypes. The five progenitor species are shown at the top. Blue lines represent simple crosses between two parental genotypes and red lines represent more complex processes involving multiple individuals, generations, and/or backcrosses. Whereas type 1 mandarins are pure species, type 2 (early admixture) mandarins contain a small amount of pummelo admixture that can be traced back to a common pummelo ancestor (with a P1 or P2 haplotype). Later, additional pummelo introgressions into type 2 mandarins gave rise to both type 3 (late admixture) mandarins and sweet orange. Further breeding between sweet orange and mandarins or within late admixture mandarins produced additional modern mandarins. Fruit images are not to scale and represent the most popular citrus types. This figure is from Wu et al. (2018) and is used here with permission

mandarin ancestor. Sweet orange is also apomictic; therefore, it has been able to serve as the pollen donor for introgression of new mandarin-like varieties (Fig. 4.1b). All cultivated varieties of sweet orange are originated from spontaneous somatic bud mutations that were selected by growers for various traits, such as ripening season, seedlessness, and fruit color. For example, Valencia orange was selected as a late-ripening summer orange and Shamouti and

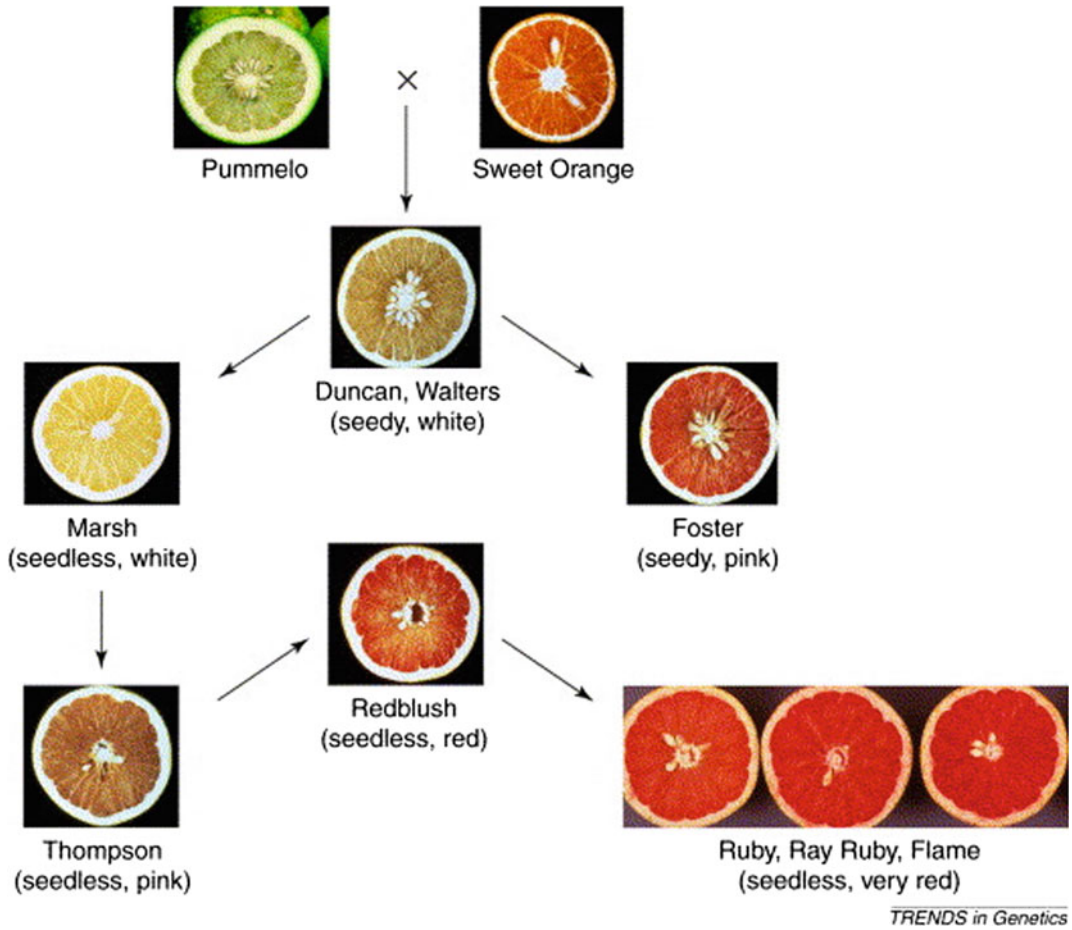

Fig. 4.2 Commercial grapefruit biotypes originated in the Caribbean, most probably through a natural hybridization between pummelo and sweet orange. The fruit of those first grapefruits was white-fleshed and very seedy. All other grapefruit cultivars arose as mutations, selected either for seedlessness or for increasingly red fruit color. This figure is from Moore (2001) and is used here with permission

"Navel" oranges were selected as seedless varieties, following spontaneous mutations that yielded that trait.

As shown in Fig. 4.1, lemon (*C. limon* L. Burm f.) originated as a hybrid between citron and sour orange. *C. medica* was also the male parent for all limes and lemons (Fig. 4.1b).

Grapefruit (*C. paradisi* Macf.) is somewhat unusual within the genus *Citrus* since it was developed in modern times and, therefore, its history has been well documented. The original grapefruit biotype originated in the Caribbean most probably by a natural hybridization between pummelo and sweet orange. As for oranges, the various grapefruit cultivars available today all originated from spontaneous bud mutations and were selected by growers for various commercial traits such as seedlessness and lycopene pigmentation (Moore 2001; Fig. 4.2).

4.3 Breeding Methods

Breeding new varieties requires a large and diverse germplasm collection. A major limitation for citrus breeding, in general, is the existence of a high proportion of apomictic varieties

producing fruit with polyembryonic seeds identical to their mother plant, which are not suitable for use as female parents in cross-hybridizations. In contrast, mandarin varieties are highly heterozygotic, which allows for a great deal of variability among the breeding progeny. The traditional breeding methods, widely used for genetic improvement of citrus species, are hybridization, clonal selection, and induced mutation. Briefly, specific characteristics of these three methods applied to citrus species are described.

4.3.1 Hybridization

Differently from many other vegetal crops, citrus suffers some limitation in the set up of a breeding program based on sexual hybridization. A first drawback is common to all fruit tree species: the long juvenile phase. Once the cross is made, citrus plants underwent a juvenile period of approximately 5 years before the first fruits are produced with direct repercussion in terms of costs and time needed for the selection of a novel cultivar. Moreover, sexual hybridization is limited both by the high heterozygosity of the genome and the complex reproductive biology property of the genus *Citrus* (Cuenca et al. 2018). The high heterozygosity has a strong impact in the set up of a novel breeding program since, in a controlled cross, the offspring will not resemble the parental lines due to a significative reshuffling of the genome. The high heterozygosity is partially due to complex reproductive biology hampering sexual hybridization (apomixis, male or female sterility, and self- and cross-incompatibility).

Despite the above-mentioned limitations, several breeding programs in mandarins are carried out through sexual hybridization. This technique is adopted to increase the genetic diversity within the mandarin-like species even though it comes to a price of a general increase in the seed content. Only the screening of a large amount of plants can guarantee the identification of novel cultivars combining the absence of seed with good quality traits; to this extent, a success example is represented by the seedless mandarin Primosole obtained in Italy from the cross of the Avana mandarin with the Miyagawa satsuma (Tribulato and La Rosa 1993), and by the seedless mandarin Nectar obtained by self pollination of Wilking (Vardi et al. 2008).

The seedless trait can be also achieved through the development of triploid, sterile plants. Triploid hybrid can be obtained through the cross between a diploid maternal line and a tetraploid parental line (Aleza et al. 2012). To this extent, interesting novel varieties such as Safor and Garbì have been obtained in Spain (Aleza et al. 2010a) and Mandared and Sweet Sicily obtained in Italy from CREA (Russo et al. 2004, 2015)

For intended cross-hybridizations, pollen is collected from selected male plants and female flowers are emasculated, pollinated, and covered with insect-proof nets to prevent any unintended cross-pollination events. To encourage blooming, fruits from the previous season should be removed from the trees as early as possible, before new crosses are made. In addition, girdling of branches or trunks can increase fruit set.

Afterward, seeds are collected from the evolving mature fruit and grown in nurseries to produce viable seedlings. Buds of the new varieties are grafted onto rootstocks, which are known to produce high-quality, flavorful fruit (Benjamin et al. 2013).

After vigorous plants have been obtained under greenhouse conditions, the seedlings are planted in the orchard and then screened for various growth characteristics and important fruit-quality traits.

4.3.2 Mutagenesis

The word mutagenesis derives from the contraction of the “mutation” and the Greek word “genesis” (born), it is therefore a process by which a portion of the genome is changed, resulting in a mutation. In its broad sense, the use of mutagenesis can be dated back to the origin of citrus cultivation. Spontaneous mutations in citrus are relatively frequent causing the

occurrence of new cultivars having a genetic background similar to the parent while presenting some new quality traits (fruit size, flesh color) or agronomical traits (harvesting period) of interest. The Washington navel orange (and all other navel oranges) originated through a spontaneous mutation of the orange Selecta in Brazil. The frequency of these naturally occurring mutations can be dramatically increased through the exposition of the vegetal material to various physical or chemical mutagenesis agents (mainly γ ray) inducing novel mutations via point mutations, chromosome breaks, or rearrangements (Ollitrault and Navarro 2012). This method has been widely used since the beginning of the XX century, thanks to its relative inexpensiveness, time-effectiveness, and the fact that the genetic background of the new cultivar remains substantially unaltered compared to the parent. Since mutations induced by irradiation are random events, no prior knowledge on the genetic background underlying a trait of interest is needed, and any trait of agronomical relevance can be improved. On the other side, the random nature of the mutations implies that only in few cases breeders will end up with novel desirable mutations, thus the chance to find such positive mutations is function to the size of the experimental population. Several novel cultivars have been released, thanks to mutagenesis, among those, the most popular are the seedless cultivars Nero and Nulessin both originated from Clemenules clementine, Tango from Nadorcott tangor (Cuenca et al. 2018), and the well-known Star Ruby pink grapefruit originated from Duncan.

4.3.3 Clonal Selection

Once a new source of variation is identified, thanks to spontaneous mutations (frequent especially in sweet orange, clementine, and satsuma), the desired trait can be fixed and propagated through clonal selection. The new plant is therefore grafted, and the stability of the novel trait is evaluated for 2–3 years. After this first evaluation, plants are micrografted to obtain virus-free plants. Virus-free scions are then grafted on different rootstocks and planted in different environments to test the trait stability in relation to such changes. Once the trait stability has been proven and the plants are exempt from virus, the new plant can be vegetatively propagated and commercialized. Among sweet oranges, the frequencies of natural mutation are quite high. For instance, Tarocco orange, the most important blood cultivar in the world is particularly subjected to natural mutation. According to Caruso et al. (2016), Tarocco arose from a spontaneous bud mutation of Sanguinello in the early 1900s in the Siracusae province, Sicily, while the origin of Moro which was cultivated in Sicily in the early twentieth century, is not known.

After their spread into cultivation, an increase in the number of the cultivated selections has been observed; in particular, these selections derived from spontaneous mutations or nucellar selections.

For this reason, Sanguinello, Moro, and Tarocco are to be considered as varietal groups rather than single varieties, composed of a number of clonal selections exhibiting different agronomical features. In particular, the spread of Tarocco resulted in a very high number of vegetative mutations for this variety with interesting traits including ripening period and pulp and peel pigmentation level (Fig. 4.3).

4.4 Important Breeding and Selection for Important Traits

In the following subsections, we will discuss some fruit-quality traits that are important for successful citrus breeding.

4.4.1 Peel Color

The attractive color of citrus peel and pulp is principally governed by the content and composition of its carotenoid pigments (Gross 1987; Alquezar et al. 2008). The color of fully ripe citrus peels ranges from green (i.e., lime) through

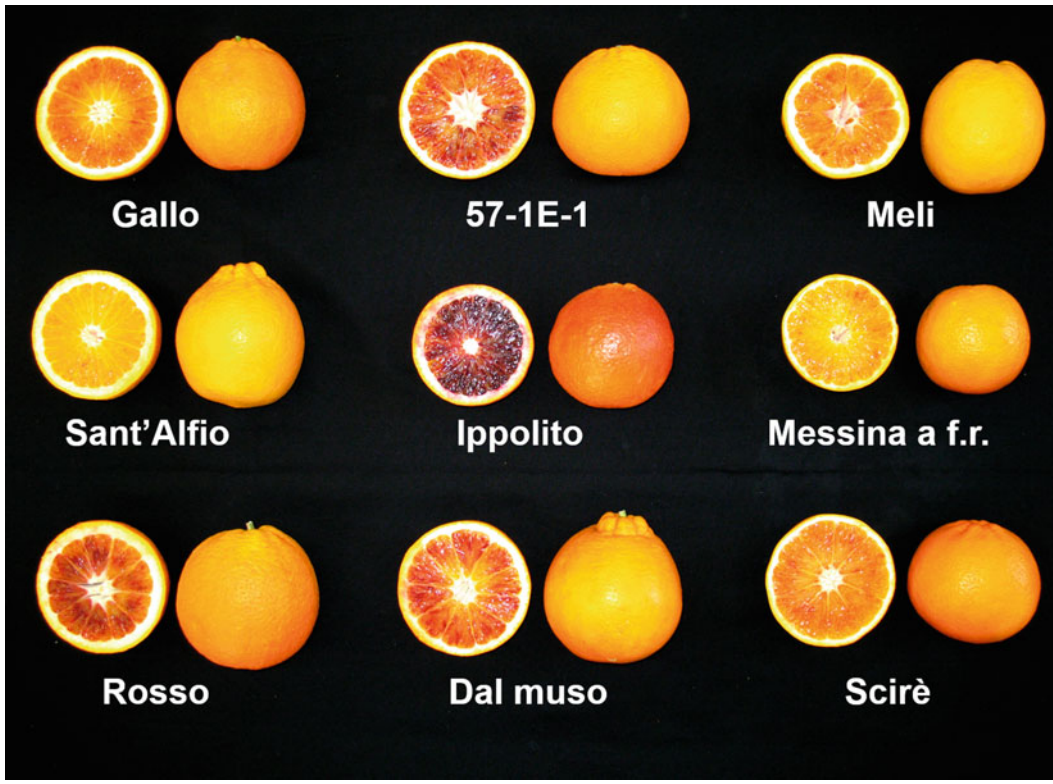

Fig. 4.3 Tarocco clones cultivated in Sicily

yellow (pummelo, grapefruit, and lemon), orange (oranges) and the orange/reddish color, typically attributed to mandarins. In mandarins, peel color is one of the most important fruit-quality traits and many breeding programs are aimed at achieving the desirable orange/reddish color, mainly through crosses between appropriate parents (Gmitter et al. 2016; Goldenberg et al. 2018). According to various previous studies, the main pigments responsible for the orange/reddish peel color of mandarins are the orange carotenoid β-cryptoxanthin and the reddish apocarotenoid β-citraurin (Gross 1987; Goodner et al. 2001; Alquezar et al. 2008). Recently, a study conducted on a Fortune × Murcott F1 population revealed a major quantitative trait locus (QTL) for mandarin peel color that contains the *PDS1* and *CCD4b* genes (Yu et al. 2016). The *CCD4b* gene has been shown to be involved in catalyzing the biosynthesis of β-citraurin (Ma et al. 2013; Rodrigo et al. 2013). This significant discovery will hopefully enable future marker-assisted selection of new mandarin varieties with attractive orange/reddish peel color.

In blood oranges, the pigment responsible for color in the peel and in the pulps are anthocyanins, water-soluble compounds, synthesized in cytosol and stored in vacuoles (Rapisarda et al. 2001) and whose biosynthesis and accumulation is highly variable depending on different climatic, pedological, and agronomic factors. Recently, Butelli et al. (2017) have demonstrated that anthocyanin biosynthesis in different accessions of *Citrus* species and in domesticated cultivars is under the control of the Ruby gene, which encodes a MYB transcription factor.

4.4.2 Fruit Size and Shape

Compared to other citrus varieties, the mandarin group possesses great variability in the size and shape of its fruits. With regard to fruit size, small-fruited mandarins, which are commonly consumed in China and in the Far East, are particularly small; satsumas and clementines are regarded as medium-small to medium size; common and Mediterranean mandarins are medium size; and tangors and tangelos (hybrids of mandarin with orange and grapefruit, respectively) are relatively large (Hodgson 1967; Goldenberg et al. 2018) Regarding fruit shape, mandarins may range from oblate to subglobose (round), to even oblate-necked, similar to the shape of an orange (Hodgson 1967). When commercially handled, oblate mandarin varieties can suffer injuries during rolling on packing house machinery. Therefore, new mandarin varieties should preferably be round rather than oblate. Among the various mandarin varieties, it seems that tangors and tangelos, in addition to being relatively large, are also more round in shape (Goldenberg et al. 2014). Consequently, crossbreeding with tangors or tangelos can result in varieties with larger fruit. In addition, some mandarin varieties have unique characteristic shapes. For example, the Minneola tangelo, often called "Honey Bell", has a characteristic long stem-end neck.

4.4.3 Ease of Peeling

Ease of peeling is an especially important fruit-quality trait in mandarins, since it determines their convenience of consumption. Ease of peeling results from the gradual loosening of the rind from the pulp that normally occurs during fruit ripening. In a study characterizing the variability in the ease of peeling trait among 46 distinct mandarin varieties, we did not find any significant correlations between the ease of peeling and thickness of the albedo, flavedo, or total peel. That finding suggests that the ease of peeling trait is determined solely by the degree of rind separation rather than peel thickness (Goldenberg et al. 2014).

The exact mechanism that controls the degree of peel loosening in mandarins is not yet clearly understood. However, it has been assumed that activation of specific cell wall hydrolases and, especially, pectin hydrolases may be involved in the degradation of the albedo layer during ripening. In previous studies of gene-expression patterns during fruit ripening, rind separation was attributed to several pectin hydrolyzing genes, including pectate lyase, pectin acetylesterase and pectin methylesterase (Fujii et al. 2003), or cell wall-loosening genes, such as expansin, extensin, endoxyloglucan transferase-related protein, and pectin acetylesterase (Kita et al. 2000). Further support for this theory was recently provided by the identification of a specific QTL for ease of peeling in a genome-wide association study conducted on different citrus varieties belonging to both parental and breeding populations (Minamikawa et al. 2017). Within this QTL, the researchers detected a SNP residing in a gene annotated as *callose synthase*, suggesting that this gene is involved in governing peel separation (Minamikawa et al. 2017). Nonetheless, further research is required to identify all of the genetic factors involved in peel separation.

4.4.4 Seedlessness

Another key fruit-quality trait that impacts convenience of consumption, especially in mandarins, is seedlessness. Under strict commercial marketing criteria, a "seedless" mandarin variety is considered to have a total of no more than four seeds in a random sample of 100 fruits, with no single fruit containing more than two seeds (Goldenberg et al. 2018). Seedlessness can occur naturally as a result of genetically inherited traits or the occurrence of spontaneous mutations, or it may result from targeted breeding programs or the application of a wide range of horticultural techniques. In any case, the development of seedless fruit requires a parthenocarpic capability, that is, the ability to set fruit without any fertilization (Vardi et al. 2008; Aleza et al. 2010a). In addition to parthenocarpy, other genetically inherited traits that allow the

production of seedless fruit or influence seed number are male/female sterility and self-incompatibility (Vardi et al. 2008; Distefano et al. 2011; Qin et al. 2015; Goto et al. 2016). Since the induction of mutations results in seedlessness in various citrus varieties, such as pummelo, lemon, and mandarins, parthenocarpy is probably a common trait in the *Citrus* genus.

The main breeding strategies used to produce new seedless mandarin varieties are (1) the creation of triploid varieties, (2) gamma-irradiation mutagenesis, and (3) genetic engineering approaches. Triploid status is usually achieved by cross-hybridization of diploid (2x) × tetraploid (4x) varieties, or by (2x) × (2x) hybridizations between unreduced gametes (Aleza et al. 2016). The resulting triploid varieties are sterile and produce very few viable seeds, due to the formation of abnormal gametes or abortions that occur during the first divisions of the embryo sac (Aleza et al. 2012). However, it should be noted that the triploid breeding approach also has some major disadvantages. Triploid trees tend to be more vigorous, have longer juvenility, and are much thornier than diploids, although the thorns tend to become gradually smaller as the trees start to bear fruit (Vardi et al. 2008; Aleza et al. 2012).

Gamma-irradiation mutagenesis is another common method for the induction of seedlessness and is used in many breeding programs around the world (Vardi et al. 2008; Goldenberg et al. 2014). In this process, young budwoods are exposed to different doses of radiation, which damage their DNA chromosomes and create a wide range of random mutations. Inversions/translocations caused by gamma irradiation often result in diminished fertility, because of the high frequency of pollen or ovule abortion (Vardi et al. 2008). The disadvantages of the gamma-irradiation method for the creation of seedlessness are the long periods of time required, the need for large growing areas and, in some cases, the regeneration of fertility that results in subsequent increased number of seeds (Vardi et al. 2008). Induced mutation often impacts fertility since any mutation occurring in haploid cells (e.g., pollen or ovules) becomes a dominant mutation. Induced mutations have proven to be an important tool for developing seedlessness in several commercial mandarin varieties, such as Orri and Tango (Vardi et al. 2003; Roose and Williams 2007). The buds that develop after the induced mutations process are mainly chimeras (Broertjes and Van Harten 1988). Therefore, hundreds of tree branches need to be screened and selected for seedlessness.

In addition to the conventional breeding methods described above, seedlessness can also be created by genetic engineering and through the use of somatic cybridization. The genetic engineering approach includes overexpression of suicide genes that promote embryo abortion. For example, Li et al. (2002) successfully expressed the barnase suicide gene in embryogenic calluses of Ponkan mandarin, enabling the production of male-sterile mandarins. The cybridization approach includes, for example, the transfer of cytoplasmic male sterility (mtCMS) from the seedless satsuma mandarin to promote seedlessness (Grosser 2004).

4.4.5 Fruit Flavor

Citrus fruits possess unique, delicate, and attractive flavors, which derive from a blend of sweet, sour, fruity, fresh, and earthy notes. The perception of overall citrus flavor is actually the combination of basic taste, aroma, and mouthfeel sensations that are perceived simultaneously by the brain during eating (Goff and Klee 2006).

The taste of citrus fruit is principally governed by the levels of sugars and acids in the juice sacs and the ratio of sugar to acid. The ratio between sugar and acid contents is also termed the total soluble solids: titratable acidity ratio (TSS:TA), or fruit ripening ratio, and is widely used by growers and trading companies as an indicator of fruit maturity. The TSS content of citrus juice comprised mostly of sugars and, therefore, it is commonly used as an indicator of fruits' sugar index (Erickson 1968). Many studies have dealt with the flavor of oranges, and orange juice in particular, due to its importance for the citrus

juice manufacturing industry (Rouseff et al. 2009). However, over the last few years, the increasing importance of mandarins has stimulated research on mandarin flavor, including the factors that influence that flavor. A recent study examining the flavor of 42 mandarin varieties reported that highly preferred mandarins contained average TSS and TA levels of 13% and 1.1%, respectively, resulting in a TSS:TA ratio of ~13 (Goldenberg et al. 2015b).

In addition to sugars and acids, citrus fruits possess a characteristic unique and rich aroma, which can be attributed to the presence of a mixture of dozens of volatile compounds in the pulp (Miyazaki et al. 2012). Many studies have compared and characterized the aroma volatiles of different citrus varieties (Goldenberg et al. 2015a; Zhang et al. 2017; Yu et al. 2018). Alquézar et al. (2017) recently characterized the terpene synthase gene family in oranges and Yu et al. (2017) identified QTLs for the biosynthesis of volatile compounds. The findings of those studies may be useful for the breeding and selection of new citrus varieties with enhanced aroma and flavor.

4.4.6 Blood Mandarins

In the last few years, consumers have been relatively open-minded and appreciative of distinctive fruits with high nutritional value and unique colors. Due to this growing trend, breeding programs in Italy and elsewhere are working on the development of new blood mandarin varieties that contain anthocyanin pigments (Rapisarda et al. 2008; Russo et al. 2015). The ability of citrus plants to synthesize anthocyanins is attributed to the expression of a MYB transcription factor named Ruby, which has been shown to activate anthocyanin production in blood oranges (Butelli et al. 2012). Since the Ruby alleles are defective in mandarins, citrus breeders are trying to create blood mandarins through cross-hybridizations between classic mandarin varieties and anthocyanin-rich blood oranges, such as Moro and Tarocco (Rapisarda et al. 2008; Russo et al. 2015).

4.4.7 Furanocoumarins

Furanocoumarins are natural, sometimes toxic, compounds that are found at relatively high levels in grapefruit and are involved in causing what is known as the "grapefruit juice effect". The toxic effect of human consumption of furanocoumarins is a result of their inhibitory effect on specific cytochrome P450 enzymes involved in the metabolism and detoxification of steroid drugs (Girennavar et al. 2007). In order to produce new grapefruit-like varieties with low-furanocoumarin contents, citrus breeding programs are now using mandarins, which do not contain furanocoumarins, as parents for cross-hybridizations with pummelos (Dugrand-Judek et al. 2015; Fidel et al. 2016). For example, in the Israeli citrus breeding project, cross-hybridizations between pummelo and mandarin parents were used to generate two new grapefruit-like varieties containing low-furanocoumarin levels, which have been named Cookie and Aliza (Fidel et al. 2016). In another breeding project in Florida, the high-furanocoumarin-content Hudson grapefruit was hybridized with the low-furanocoumarin-content Hirado Buntan pummelo. Among the progeny of that cross, the accumulation of furanocoumarin co-segregated in an approximate ratio of 1:1 (Chen et al. 2011).

4.4.8 Yield and Alternate Bearing

High yield is an absolute requirement for commercialization of new varieties, since yield determines profitability for growers. Yield levels depend on various factors, including genetic background, environmental conditions, soil components, rootstocks, irrigation, and fertilization (Iglesias et al. 2007). In addition, the achievement of consistent high yields greatly depends on the tendency of each variety for alternate bearing, that is, the tendency of fruit trees to produce a heavy crop one year (the "on" year) followed by a very small or no crop at all at the following year (the "off" year); (Spiegel-Roy and Goldschmidt 1996). In citrus, alternate bearing is due to a lack of flowering in the spring

following a heavy "on" year. Accordingly, Nishikawa et al. (2012) reported that high levels of fruit bearing suppress the expression levels of the FLOWERING LOCUS T (CiFT) gene, suggesting that future manipulation of this gene could putatively regulate crop yields. Other genes possibly involved in governing alternate bearing in citrus include the SQUAMOSA PROMOTER BINDING-LIKE (SPL), miR156, and other genes that control flowering (Shalom et al. 2012).

4.4.9 Harvesting Season

One of the most important goals for citrus breeders is to extend the harvest and marketing season as long as possible. In a previous study, we showed that the mandarin harvesting season in Israel begins in October and ends in March (i.e., the harvesting season continues over a period of 5 months; Goldenberg et al. 2014).

In Italy, for blood oranges varieties, and in particular for Tarocco orange, the availability of a high number of clones, characterized for different harvesting periods, allows to extend the harvesting season from December to May (Caruso et al. 2016).

As far as we are aware, there are not yet any characterized and available molecular markers for the indication of ripening periods. Currently, to obtain new early or late varieties, citrus breeders just use early- and late-season varieties as parents.

4.4.10 Pest and Disease Resistance

Citrus is subject to numerous diseases, some of which occur only in certain environments, like mal secco disease, which is confined to the Mediterranean Basin, Black Sea, and Asia. Other diseases, like that caused by *Phytophthora*, pose a serious problem in all citrus growing areas. Citrus pathogens include fungal, bacterial, viral, and virus-like agents (Spiegel-Roy and Goldschmidt 1996). Among the fungal diseases, mal secco causes canopy vein chlorosis, leaf wilt, and dryness of trees. Other important fungal diseases of citrus include *Phytophthora*-caused disease, which is characterized by stem and root rot; scab, which involves leaf protuberances and the distortion of shoot apices; and *Alternaria*-caused disease characterized by necrotic blight spots on leaves and stems (Spiegel-Roy and Goldschmidt 1996). Among the bacterial diseases, it is worth noting the canker that causes leaf spots, blemishes on the rind of the fruit and leaf and fruit drop, as well as huanglongbing (HLB, also known as citrus greening disease) blocks the movement of sugar through the phloem and eventually results in the wilting and death of the trees. Among the viruses and virus-like agents, worth noting are the citrus *Tristeza* virus (CTV), which causes stem pitting; psoriasis, which causes tree weakening and decline; stubborn, which is lethal to citrus trees; and the citrus exocortis viroid (CEV), which stunts tree growth and reduces yields.

The degree of resistance/tolerance to various diseases differs greatly among citrus and citrus-related species. For example, Miles et al. (2017) evaluated the sensitivity of various citrus and citrus-related species to HLB and reported that the healthiest trees with the least severe or no HLB symptoms were several distant citrus relatives; whereas within the genus *Citrus*, the healthiest trees with densest canopies, little leaf loss, and greatest growth were those with that included *C. medica* (citron) genetic background. These findings suggest the possibility of including citron and/or citrus relatives as genetic sources for breeding to enhance resistance to HLB.

Breeding of scions for disease resistance is a prolonged and complicated process that has to be directed specifically for each variety; whereas the development of disease-resistant rootstocks could provide broad resistance/tolerance to many citrus varieties. Such acquired resistance by grafting on tolerant rootstocks was accomplished for the case of CTV (Bordignon et al. 2004). However, for many other existing pests and diseases, there are not yet any sufficiently

resistant rootstocks. Large-scale evaluations of rootstock/scion combinations under high HLB disease pressure recently revealed a great deal of diversity in disease tolerance under field conditions. This indicates that the selection of proper rootstocks might help to reduce future HLB symptoms in affected production areas (Stover et al. 2016).

Molecular markers for different traits, including disease and pest resistance, are an important tool for citrus breeders, as they may allow the selection of young seedlings that possess the desired traits. For example, Ling et al. (2000) identified molecular markers that are strongly linked to nematode resistance and citrus breeders worldwide are currently trying to identify additional molecular markers for resistance to other pests and diseases. The Israeli citrus breeding program recently identified molecular markers that are strongly linked to resistance to disease caused by *Alternaria*, but those findings still require further evaluation (N. Carmi and D. Ezra, unpublished data).

Finally, disease and pest resistance can also be achieved through the use of various molecular biology techniques. For example, CRISPR/cas9/sgRNA genome-editing technology was recently used to modify the canker susceptibility gene CsLOB1 in Duncan grapefruit, resulting in the creation of resistant lines (Jia et al. 2017).

4.4.11 Long Shelf Life

Although mandarins have an attractive appearance and are convenient to consume, they are much more perishable than other citrus varieties. In particular, they undergo deterioration in sensory acceptability and accumulate off-flavors after harvest (Cohen 1999; Tietel et al. 2011). Previous studies attributed this development of off-flavors mainly to the induction of an ethanol fermentation metabolism and the accumulation of high levels of ethanol and ethyl ester derivatives (Davis et al. 1967; Cohen et al. 1990; Tietel et al. 2010; Obenland et al. 2011). However, in a recent comprehensive study evaluating off-flavor perception in 41 mandarin varieties, we concluded that perception of off-flavor in mandarins is not only due to increases in ethanol and ethyl ester levels, as assumed previously, but also reflects general changes in the profile and composition of fruit aroma volatiles during storage—changes that create an atypical or spoiled flavor (Goldenberg et al. 2016). In varieties that developed strong off-flavors, the proportions of the various chemical classes of aroma volatiles were found to have completely changed during storage; whereas those proportions barely changed in varieties that developed only weak off-flavors after harvest (Goldenberg et al. 2016).

In addition to flavor and aroma deterioration during storage, mandarins may also suffer from chilling injury following exposure to low storage temperatures (Sala 1998). In general, among citrus varieties, lemons, grapefruits, and pummelos are more sensitive to chilling; whereas oranges and mandarins are more tolerant (Eckert and Eaks 1989; Kader and Arpaia 2002). Nonetheless, we noticed a great deal of diversity in chilling tolerance among mandarins as some varieties such as Lee, Orlando, and Cami are very sensitive to chilling; whereas other varieties such as Orri, Ponkan, and Afourer are relatively tolerant of such conditions and can be stored for long periods at low temperatures (L. Goldenberg and R. Porat, unpublished data). Worth noting is the fact that chilling tolerance has recently become a very important trait, as global export often requires exposure to cold quarantine disinfestation treatments.

4.5 Conclusions

Current citrus breeding programs are focused mainly on the development of new easy-peeling seedless mandarins and, to a much lesser extent, the development of new low-furanocoumarin-content grapefruit, as well as some other aspects of citrus development such as seedless lemons. The main goals of current mandarin breeding programs are increased yields, extension of the harvesting season, and improved fruit-quality traits. For mandarins, important fruit-quality traits are attractive orange/reddish color, ease of

peeling, seedlessness, pleasing flavor, and high nutritional value, as well as good postharvest storage performances and long shelf life (Tietel et al. 2011; Goldenberg et al. 2018).

Conventional breeding based on cross-pollination between distinct male and female parents remains the basic tool for the generation of new citrus varieties. Nonetheless, the identification of molecular markers that are closely linked to various desired traits provides an essential and important new tool for citrus breeders. This tool makes the breeding process quicker and more efficient, as it allows for early selection of desired traits at the young seedling stage before the young trees are transferred to the field and without the necessity of waiting until the completion of the juvenility period and fruit setting. To date, several molecular markers have already been identified for various fruit-quality traits, such as peel color, ease of peeling, and different types of disease resistance. In addition, new genetic engineering technologies, especially CRISPR/cas9, will greatly support citrus breeding by allowing the introgression of specific desired genes into commercial varieties without changing their entire genomes, which otherwise would have required many backcrosses (using conventional breeding methods).

Key challenges that the citrus industry has faced in the past and will continue to face in the future include the need to deal with various destructive pests and diseases that pose major threats to citrus crops worldwide. Well-known disasters experienced by the citrus industry include the catastrophic damage caused by CTV in Spain in the middle of the twentieth century (Cambra et al. 2000) and the serious damage currently being caused by HLB in Florida, Brazil, and elsewhere (Blaustein et al. 2018). Dealing with such threats requires the maintenance of diversified germplasm collections of citrus and citrus-related species, which might possess various types of resistance toward different pests and diseases. Overall, dealing with these threats requires the implementation of classical breeding approaches in combination with modern molecular-assisted breeding and genetic engineering approaches.

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Citrus Rootstock Breeding and Selection

5

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Abstract

The use of rootstocks in the evolution of citriculture has played a key role in the success of citrus industries worldwide. Rootstock has a strong influence on yield, fruit quality and tree size, and can provide tolerance to abiotic and biotic stresses. Dominant and emerging rootstocks has been described, reporting their characteristics that allow their utilization in relation to prevailing conditions of soil, climate and disease. Aim of this chapter is also to provide current and future citrus rootstock breeders a practical guide that will facilitate efforts to develop improved rootstocks capable to solve many current issues of citrus production and fruit quality worldwide.

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5.1 Introduction

The use of rootstocks in the evolution of citriculture has been going on for hundreds of years and has played a key role in the success of citrus industries worldwide. In addition to solving issues with juvenility in commercial trees such as thorny, up-right growing trees that are slow to produce, rootstocks can also compensate for deficiencies in the root systems of many important commercial scions (i.e., Phytophthora susceptibility). Rootstocks have a strong influence on yield, fruit quality and tree size, and can also provide tolerance of abiotic (i.e., calcareous soils, salinity, flooding, cold, boron toxicity, etc.) and biotic (Phytophthora, CTV, blight, nematodes, Diaprepes/Phytophthora complex, HLB, etc.) stresses. Historically, several true species or natural hybrids have served as highly successful rootstocks. These include trifoliate orange (very important to the fresh fruit industries of China, Japan and other cold producing areas), rough lemon (formerly very important in Florida before irrigation, and still quite important in India and Pakistan); Rangpur lime (still important in Brazil in non-irrigated groves), Cleopatra mandarin (used widely for fresh fruit); sour orange (formerly the most important rootstock worldwide before the spread of quick decline disease caused by citrus tristeza virus); and Volkamer lemon and Alemow (still widely used for acid-fruit production) (Castle 1987).

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The possibility of fixing a problem with a specific rootstock or combining complementary traits of various rootstocks has made citrus rootstock breeding an important research activity worldwide. However, one common feature of all the above mentioned historical rootstocks is their ability to be propagated via abundant nucellar seed production, a trait that until recently has been a requirement for any successful new rootstock. This genetic characteristic inhibited early rootstock breeding because it is difficult to generate large populations of segregating zygotic hybrids needed for selection when using any of the above rootstocks as a female. Use of trifoliate orange (a source of genes for cold-hardiness, nematode resistance, and high fruit quality) as a pollen parent overcame this problem, as rare zygotic offspring could easily be identified by expression of the dominant trifoliate leaf trait provided by the trifoliate orange parent in hybrid progeny. This led to the production of numerous citrumelos, citranges, and citrandarins (Hutchison 1974). Following extensive testing worldwide, trifoliate orange hybrids have become the dominant rootstocks in many citrus production areas (Castle 1987). In Florida, prior to HLB becoming endemic, Swingle citrumelo was the most widely planted rootstock, with Carrizo citrange the second most widely planted. Swingle use has also increased substantially in Brazil in irrigated groves. Citranges, especially C-35 and Carrizo are widely used for fresh fruit scions, especially in California and Spain. C-22, a citrandarin, is becoming quite popular as a grapefruit rootstock in Texas, replacing sour orange (Louzada et al. 2008). Carrizo citrange is the most important rootstock used in Spain but it is susceptible to salinity and iron chlorosis. Only Cleopatra mandarin is tolerant of this problem but the growth rates of the scions on Cleopatra mandarin are lower than in any other rootstock (Forner-Giner et al. 2003). *C. volkameriana* is very susceptible to *Phytophthora* spp. and Swingle citrumelo is highly sensitive to iron chlorosis. Recently, Forner-Alcaide 5 (Forner et al. 2003), a new citrus rootstock obtained in the citrus rootstock breeding program at the Valencian Institute for Agricultural Research (IVIA), is being very popular and is replacing Carrizo citrange due to its better adaptation to Spanish field conditions. In Italy, sour orange was widely used until CTV spread during the first 2000s. Since then, citranges were almost exclusively used as rootstocks, also for the higher pigmentation that they confer to blood oranges; C-22 appears to be very promising, as it enhances pulp anthocyanin content more than citranges (Continella et al. 2018). In Florida, with Swingle and Carrizo showing susceptibility to HLB, newer rootstocks providing higher levels of HLB tolerance to grafted trees, especially citrandarins are being widely planted. Citrandarin US-942, although it has limited seed supply, is currently the most sought after rootstock in Florida (Bowman et al. 2016). Tissue culture micropropagation and rooted cuttings are being utilized in efforts to make up the shortfall in seed produced liners. Thus, the dominant trifoliate leaf trait has remained as a valuable genetic marker in hybrid rootstock development.

In an effort to expand the germplasm utilized in rootstock breeding, breeders have been crossing monoembryonic females (that produce nearly all zygotic progeny) with polyembryonic pollen parents. This is true for crosses both at the diploid and tetraploid levels. This approach greatly increases the number of hybrids (with much greater genetic diversity) that can be made for targeted selection. If the source of the polyembryony in the pollen parent is from trifoliate orange, generally about 50% of the progeny will be polyembryonic. If the source of polyembryony is from mandarin, the % of progeny exhibiting polyembryony is much less, generally around 10% or less. Partial polyembryony is also a frequent outcome. With the advances being made in alternative methods of commercial rootstock propagation, especially the use of rooted cuttings and tissue culture micropropagation, new rootstock candidates are no longer required to be polyembryonic. Monoembryonic selections with good genetics can now be used either as females or pollen parents in crosses. Through these advances, much greater genetic diversity is now available for rootstock improvement.

The goal of this chapter is to provide current and future citrus rootstock breeders a practical guide/resource that will facilitate efforts to develop improved rootstocks that solve the many current (and future) issues with citrus production and fruit quality worldwide. The screening citrus rootstocks is a long and expensive process, requiring a lot of space for trials and large costs for evaluation. It is essential to shorten the time of evaluation citrus rootstocks to obtain faster and better results.

5.2 Dominant Rootstocks

Until the mid-1800s, citrus was grown mainly as seedlings, even if grafting of citrus was already common in the Mediterranean area, and groves of sweet orange (*C. sinensis* L. Osb.) budded on sour orange (*C. aurantium* L.) were present in Florida (Wutscher 1979). It is after *Phytophthora* root rot spread, first observed in the Azores in 1842 and later affecting Mediterranean countries, that graft propagation became widely used for commercial production, budding scions on resistant rootstocks (Chapot 1975), mainly using sour orange.

5.2.1 Sour Orange

Sour orange (*C. aurantium* L.) is one of the oldest and most widely used rootstocks in almost every citrus growing region of the world. It is a natural hybrid, probably of pummelo and mandarin parentage. Orange and mandarin yields on sour orange are moderate to high, but generally fruit quality is excellent and fruit size is medium to large. Also, fruit can be stored on the tree for longer period than some other rootstocks. Sour orange has many horticultural advantages in terms of abiotic stress as its rusticity permits to well adapt to a broad range of soil types. It grows best on moist, fairly heavy soil, and is highly tolerant of calcareous soils. It is tolerant to *Phytophthora* foot rot and blight, but susceptible to CTV and all citrus nematode races, while exocortis, psoriasis, and xyloporosis are often symptomless (Castle and Krezdorn 1975; Hodgson 1967; Wutscher 1979).

5.2.2 Citranges

They are hybrids of sweet orange and trifoliate orange. The most important among these rootstocks are Carrizo and Troyer citrange, originated from a single hybrid seedling of Washington Navel orange and *P. trifoliata* by USDA in Florida. In 1934, Swingle named the latter for A. M. Troyer, in whose place at Fairhope, Alabama, it first fruited, the other was named because it was developed near Carrizo Springs, Texas. The aim of this breeding made in 1909 was to obtain a cold hard cultivar, combining the good qualitative traits of sweet orange and the cold-hardiness of the trifoliate orange, without success as fruit is not edible. Only much later, they were used as rootstocks, being nowadays among the most diffused in worldwide citrus industry. Troyer was firstly more widely used, especially in California, while lately Carrizo is often preferred in Florida and Europe. They are visually indistinguishable and it is difficult to evidence the superiority of one of them, although a higher resistance of Carrizo for burrowing nematode is reported. Trees on both citranges grow moderately vigorously on a range of soil types, but have poor salt tolerance and are sensitive to calcareous soils and exocortis viroid. Best results with both citranges are achieved in well-drained soils and are unsuitable for heavy clay soils. Troyer and Carrizo citranges are tolerant for tristeza virus and *Phytophthora* root rot but are less tolerant than *P. trifoliata*. They are moderately cold-tolerant, but field experience indicates that trees on Carrizo are generally not as cold tolerant as those on sour orange, Cleopatra or Swingle citrumelo (Castle et al. 1993; Hodgson 1967).

C35, hybrid of Ruby Blood orange and Webber-Fawcett *P. trifoliata*, was bred by the University of California in 1951 and released in 1986 (Cameron and Soost 1986). It is highly resistant to the citrus nematode. Several trials of this rootstock in combination with navels, Valencias, pigmented oranges, grapefruit, and mandarin types showed good performance.

A reduction of the mature tree size 25–30% smaller than other citranges is reported, although this is not limiting for the production due to the high-yield efficiency, i.e., more kilograms of fruit for each cubic meter of canopy volume. Also internal quality and fruit size are good. Reports of tree dieback with Clemenules and Fukumoto on C35 state a certain incompatibility problem (Castle et al. 2016; Roose 2014).

5.2.3 Citrumelos

The term citrumelo indicates hybrids of grapefruit and trifoliate orange [*C. paradisi* (Macfadyen) x *P. trifoliata* (L.) Raf.]. Among all, Swingle originated as a hybrid of Duncan grapefruit and trifoliate orange and has been the most widely spread. It was produced in 1907 in Eustis, Florida. In 1974 it was released with the name Swingle in Florida, but it was sent in California earlier and known also with the code name CPB 4475. Rootstock fruit is seedy (about 90% nucellar) and it confers a good vigor to the grafted plant. Swingle citrumelo is highly productive, especially in loam and sandy loam soils, but performs poorly in heavy and calcareous ones. Most varieties well performed in terms of yield and quality products. It is tolerant of CTV, exocortis, *Phytophthora* foot rot, cold, and blight. Sacaton citrumelo, that was discovered by W.T. Swingle in 1919 in Sacaton, Arizona, is highly productive and has been tested with good performance as rootstock (Castle and Stover 2000; Roose 2014).

5.2.4 Trifoliate Orange

Trifoliate orange [*P. trifoliata* (L.) Raf.] is a deciduous relative of *Citrus*, presents several selections that were classified in three different groups depending on the flower morphology and growth habit: large-flowered types (Pomeroy), tend to be larger and more productive; small-flowered types (Rubidoux and Rich 16-6) show higher yield efficiency; Flying dragon, a distinctive small-flowered type, that is known as the most dwarfing citrus rootstock up to now. Trifoliate orange is now widely used in Japan, China, Uruguay, and Argentina and in cold environments. Seedling is moderately uniform (80–90% nucellar). Trees on trifoliate orange are highly productive with relatively small fruit of very high-quality. Trees propagated on *P. trifoliata* are suitable for planting at high-density. It adapts well to loam, clay, and sandy soil if irrigation is well managed as it suffers water stress.

Trifoliate orange has been used in several breeding programs, besides the good characteristics such as cold-hardiness and resistance to CTV, root rot and nematodes, for the ease in selecting the progeny obtained with sexual hybridization since the trifoliate character is dominant; in this context, as polyembryony is frequent in citrus cultivars, trifoliate trait of the pollen parent is used as a morphological marker for early separation of the putative hybrids (Hodgson 1967; Saunt 1990).

5.2.5 Cleopatra Mandarin

The most common mandarin rootstock is Cleopatra (*C. reshni* Hort. ex Tan.) that presumably originated in India. Citrus trees on Cleopatra have low yields in the early years, but increase as trees mature; fruit quality is good, but fruit size is generally small to intermediate. Cleopatra induces high cold-hardiness in the scion and it is tolerant of CTV, exocortis and xyloporosis. It adapts well to all soils with the exception of calcareous ones and it has one of the highest salinity tolerance among the commercial rootstocks, but is susceptible to *Phytophthora* root rot in wet sites and to citrus nematode (Saunt 1990).

5.2.6 Alemow or Macrophylla

It is probably a natural hybrid originated in Philippines (*C. macrophylla* Wester). It is a lemon-like rootstock and grafted trees are extremely precocious and grow vigorously for a few years. It is highly productive, but fruit quality is poor for the low content in total soluble solids

and less juice percentage. It is often used as rootstock for lemon production. Trees on macrophylla are very susceptible to cold damage. Alemow has good salt tolerance and it adapts well to sandy and sandy loam soils, but it shows a fair performance in heavy soil. It is susceptible to CTV and xyloporosis, but it has good tolerance to *Phytophthora* rot (Roose 2014; Saunt 1990).

5.2.7 Volkamer Lemon

It is apparently a natural hybrid of mandarin (*C. reticulata*) and citron (*C. medica*) and it was named in honor of the German botanist J.C. Volkamer (*C. volkameriana* Ten. and Pasq.). It produces very vigorous and productive trees, fruit quality is poor for the low maturity index (soluble solids/acid ratio). Trees on Volkamer lemon grow well on sandy and sandy loam soils, while have a poor performance on heavy soils; it is well adaptable on calcareous soils and has fair salt tolerance. It is not susceptible to CTV, exocortis and xyloporosis, but it is affected by all citrus nematodes (Castle et al. 1993; Roose 2014; Saunt 1990).

5.2.8 Emerging Rootstocks (What Looks Good Today from Recent and Ongoing Trials)

The success of citrus rootstock is determined by its tolerance to prevailing conditions of soil, climate, and disease, while still producing high yields of quality fruits. Tolerance to biotic and abiotic factors is the leading objective for breeding programs, with special focus for some most common constraints, such as CTV and phytophthora among diseases, and salinity, alkalinity and calcareous soils for environmental aspects. Besides, scion/rootstock interaction and tree size are important for plant development and citrus farming longevity and production costs. Several breeding programs have been carried out so far in all the citrus producing countries, even if screening program requires considerable time. Besides, testing newly released rootstocks is inevitably a continuous activity and on-site evaluation may give useful information for citrus growers, especially in combination with local varieties.

In 1974, the Valencian Institute for Agricultural Research (IVIA) began a program to breed citrus rootstocks by hybridization, and more than 500 hybrids were evaluated to determine their horticultural performance. From among these, a hybrid of Cleopatra mandarin × *P. trifoliata*, specifically Forner–Alcaide 5 (FA-5) was selected for its characteristics, which include high productivity and good fruit quality of the scion cultivar (Forner-Giner et al. 2003; Forner et al. 2003). Furthermore, FA-5 seems to be tolerant of salinity (Forner-Giner et al. 2009; Lopez-Climent et al. 2008) and to calcareous soils (Forner et al. 2003; González-Mas et al. 2009). FA-5 has been used as a commercial rootstock in the European Union since 2005.

Forner-Alcaide 5 is resistant to citrus tristeza virus (CTV) and citrus nematode (*Tylenchulus semipenetrans* Cobb).

Carrizo citrange, the most used rootstock in Spain, frequently shows iron chlorosis and salinity problems. Forner-Alcaide 5 shows resistance to very high salinity, similar to Cleopatra mandarin and it is more tolerant to iron chlorosis than Carrizo citrange. Also, its tolerance to water stress and flooding is good, as well as the *Phytophthora* spp. tolerance.

Agricultural performance of Forner-Alcaide 5 has been evaluated with navel oranges, satsumas, and clementines, compared to the traditional rootstocks used in Spain. This rootstock always shows higher productivity than Carrizo citrange, good fruit size and quality.

Three rootstocks originally obtained by the USDA breeding program at Indio and selected by the University of California, Riverside, were released in 2009 (Federici et al. 2009). Bitters, Carpenter and Furr, tested under the codes C22, C54, and C57, respectively, and sometimes still known with these codes by nurseries, are hybrids of Sunki mandarin (*C. sunki* Hort. ex Tan.) and Swingle trifoliate orange. Their names are

referred to the three researchers that started and monitored the breeding program: John Carpenter and Joe Furr made all three hybrids at Indio, and Professor W.P. Bitters tested the hybrids for Citrus Tristeza Virus tolerance in a trial in Irvine, CA, in 1966 and 1968 (Siebert et al. 2010). Bitters appears to be very promising as it is tolerant to CTV and resulted very tolerant to high pH and calcareous soils of Texas, similarly with C-146, hybrid with the same parent (Louzada et al. 2008); together with C-146, it was also found to be more tolerant of saline conditions than sour orange (Simpson et al. 2014, 2015). It is moderately tolerant to Phytophthora but fairly susceptible to citrus nematode (Freckman and Roose 1995). Bitters produces a highly productive semi-dwarf tree, resulting in a remarkable yield efficiency and suitable for high-density plantings. In combination with pigmented cultivar, it enhanced juice anthocyanin content (Continella et al. 2018). Carpenter produces intermediate-sized trees, and fruit quality of late navels was good; it is moderately tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil. Furr produces medium to large trees, with good yield. It is very tolerant to Phytophthora and citrus nematode, and moderately tolerant of calcareous soil.

US-802, US-812, US-897, and US-942 are hybrid citrus rootstocks released by the U.S. Department of Agriculture (USDA) between 2001 and 2010 (Bowman et al. 2016) and have spread in new citrus plantations of Florida. US-812 is a hybrid obtained by a cross between Sunki mandarin and Benecke trifoliate orange (Bowman and Rouse 2006). It is highly productive of good quality fruit on a moderate-sized tree and exhibits tolerance or resistance to tristeza virus and citrus blight. Valencia trees grafted onto US-812 showed good tolerance to high alkalinity soils (pH 8.1–8.3). US-942, cross of Sunki mandarin and Flying dragon, is reported to be very highly productive and somewhat more tolerant to HLB than other commercial rootstocks (Bowman et al. 2016). US-802 and US-897, the former hybrid of Siamese pummelo (*C. grandis* Osbeck) and *P. trifoliata*, and the latter of Cleopatra mandarin and Flying dragon, resulted with good productivity per tree size; especially US-897 is promising for its semi-dwarfing effect and also for the salinity tolerance (Syvertsen 2012). Recently, some new rootstocks, namely US-1279, US-1281, US-1282, US-1283, and US-1284, were released with improved tolerance to HLB and superior production of good quality fruit is reported (Bowman and McCollum 2015).

X639, developed in South Africa at the Citrus Research International, formerly known as Citrus and Subtropical Fruit Research Institute (CSFRI) by Dr. Hojby in early 1950s, is an hybrid of Cleopatra mandarin and trifoliate orange (Von Broembsen 1985). It is becoming popular in that country for the tolerance to salinity (Syvertsen 2012) and to high pH soil levels (Castle and Baldwin 2006), besides it confers good fruit quality and yields. Some trials in California, Florida and Australia support the suitability of this rootstock to be used, especially in calcareous, poorly drained soils (Stover et al. 2004).

In Italy, the Council for Research in Agriculture and the Agricultural Economic Analysis (CREA-OFA) of Acireale started in 1968 a rootstocks breeding program obtaining a population of 257 hybrids. The monoembryonic species [*C. latipes* (Swing.) Tan.] was used as female parent, while trifoliate orange, sour orange, and Volkamer lemon were used as male parents. Among these, F6P12 was patented in 2014 field trials where several rootstocks were compared in combination with Washington navel orange, SRA 92 clementine and Tarocco orange, F6P12 showed a high cumulative yield, thus maintaining good fruit quality (Reforgiato Recupero et al. 2009).

Since devastating HLB (Huanglongbing or citrus greening disease) has become endemic in Florida, there has been a major emphasis to identify and develop rootstocks that can impart HLB tolerance to grafted commercial scions. Both the UF/CREC and USDA citrus rootstock breeding programs have released several new rootstock selections that impart some level of tolerance. Rootstocks showing some success under improved production systems include US-

942, US-812, US-802, US-897, US-1516, X639, UFR-4, UFR-5, UFR-6, and UFR-17 (Castle et al. 2016).

5.3 Abiotic Factors

5.3.1 Calcareous Soils

Iron is an essential element for plant growth and development since it is fundamental for the proper functioning of numerous metabolic and enzymatic processes. Calcareous soils with restricted iron availability for plants are commonly found in the Mediterranean basin where citrus is the major fruit crop. These conditions affect most citrus cultivated in the Mediterranean basin, which frequently develops Fe chlorosis symptoms, mainly interveinal yellowness in leaves, stunted vegetative growth, worse yields, and poor fruit quality. It has been estimated that calcareous soils are widespread, comprising more than 30% of total land area (Chen and Barak 1982) and in the Mediterranean basin citrus frequently grows in these soils.

The genus Citrus and related rootstocks species are considered to be susceptible to iron chlorosis. Iron deficiency tolerance is determined by the rootstock so citrus trees display differences in susceptibility according to the rootstock they have been grafted on. The Swingle citrumelo is very sensitive to iron chlorosis. The rootstocks trifoliate orange, Carrizo citrange and sweet orange are susceptible to lime-induced chlorosis, while the sour orange and Cleopatra mandarin are tolerant of this deficiency (Castle 1987; Pestana et al. 2005). Cleopatra mandarin is tolerant to iron chlorosis, but grows slowly in the field. Besides the production of fruit is not very high and although the fruit is of good quality, is smaller than that produced on other rootstocks.

A widely applied system to prevent Fe-deficiency in fruit tree crops is to use Fe chlorosis-tolerant genotypes as rootstocks (Jiménez et al. 2011; Ksouri et al. 2006). For this purpose, Fe chlorosis tolerance of citrus rootstocks has been tested in several studies (Castle et al. 2009; Chouliaras et al. 2004; Pestana et al. 2005).

Plant growth (length, dry biomass, root/shoot ratio), Fe nutritional status (in leaves and roots), chlorophyll concentrations (directly or estimated by the SPAD index) and photosynthetic activity are common determinations used to evaluate Fe-chlorosis tolerance in plants (Martínez-Alcántara et al. 2013; Martínez-Cuenca et al. 2013; Pestana et al. 2011, 2005). Since the relative importance of the Fe-acquisition system components seems to differ considerably between plant species and genotypes, some studies have focused on differences in root responses to Fe-deficiency among plant species (Donnini et al. 2009), including citrus (Chouliaras et al. 2004; Pestana et al. 2011, 2005).

Plants have developed certain adaptive mechanisms to increase Fe uptake capacity under Fe-deficiency conditions. Dicotyledonous and non-grass monocotyledonous species like citrus, develop strategy I for Fe mobilization and acquisition (Kim and Guerinot 2007; Marschner and Römheld 1994), which includes the following responses:

(1) The excretion of protons (H^+) into the rhizosphere through the activation of specific plasma membrane-bound Proton-ATPases (H^+-ATPases, EC 3.6.3.6) from the epidermal cells of the roots (Rabotti and Zocchi 2006), which lowers the pH of the soil solution and increases Fe^{3+} solubilization (Rabotti et al. 1995);
(2) An increased Ferric Chelate Reductase (FC-R, EC 1.16.1.7) activity through the induction of a plasma membrane-bound enzyme, which reduces Fe^{3+} to Fe^{2+} at the root surface (Robinson et al. 1999; Yi and Guerinot 1996); and
(3) The stimulation of Fe^{2+}-transport across root cell membranes through the activation of a specific iron-regulated transporter, IRT (Eide et al. 1996).

Genomic tools have contributed to a better understanding of the molecular and metabolic

processes leading to Fe uptake in plants. With respect to proton release, some genes coding for Fe-regulated H^+-ATPases have been characterized (Santi et al. 2005). Thus, in citrus roots, the HA1 gene was induced in Fe-deficient roots, while HA2 did not respond to Fe-deficiency (Martínez-Cuenca et al. 2013). On the other hand, reduction response is encoded by FRO gene family and the main genes have been identified in several species (Jeong and Connolly 2009). FRO2, which is expressed in the epidermal cells of roots, is believed to be primarily responsible for enhancing FC-R activity due to Fe-deficiency and its overexpression confers tolerance to low Fe conditions (Connolly et al. 2003; Martínez-Cuenca et al. 2013). Finally, IRT genes code for family members of zinc transporter proteins (ZIP) in Arabidopsis (Connolly et al. 2002; Vert et al. 2009). Among them, IRT1 gene is localized to the plasma membrane of epidermal cells in roots and its expression is induced by Fe-deficiency, generating the major transporter responsible for Fe uptake from the soil (Martínez-Cuenca et al. 2013; Vert et al. 2002, 2009).

In the citrus breeding program of the Valencian Institute of Agrarian Research (IVIA) the screening methodology of screening new citrus rootstocks under Fe-deficient conditions are done according to several determinations: (Arbona and Gómez-Cadenas 2008) physiological plant responses (biomass, chlorophyll concentration and photosynthesis rate); (Bailey-Serres and Voesenek 2008) absorption and distribution of Fe content in plants; (Baines et al. 1969) acidification response of roots; (Bar-Joseph et al. 1989) activity of the key enzyme involved in the ferrous reduction process; (Bar-Joseph et al. 1983) quantification of Fe content in the ferrous form present in the root system. The assays are carried out in Fe-deficient and Fe-sufficient seedlings of different hybrids, and compared with two of the main rootstocks widely used as citrus rootstocks in Spain, known for their different tolerance Fe chlorosis (Castle et al. 2009): *C. macrophylla* (CM, Fe chlorosis- tolerant genotype) and Carrizo citrange (CC, Fe chlorosis-sensitive genotype) (Martínez-Cuenca et al. 2013, 2016).

5.3.2 Salinity

Salinity is a widespread problem. It is estimated that one-third of the land area is affected due to various degrees of salinity. Originally, salinity problems appeared in arid and semiarid areas, where rain was not enough to wash the salts of the soil. But standard cultural practices used over the years resulted in secondary salinization, which affects some aspects of great importance, such as soil characteristics and vegetation cover. Citrus is a salt-sensitive crop, which, even at moderate salinities, suffers physiological disturbances such as, ion-specific toxicity, nutrient imbalances, altered gas exchange parameters, and adverse water relations, leading to stunted growth. However, there are important differences between species and varieties belonging to the *Aurantiodea* family.

Presently, it is considered that the sensitivity to salinity in citrus species and varieties is associated with the accumulation of excessive concentrations of Cl^- in leaves, which implies a high absorption of this ion by the roots, as well as its efficient translocation (Cooper 1962; Grieve and Walker 1983). In contrast, tolerance to salinity in citrus is related to the ability to restrict the uptake and/or the transport to the shoot (Walker et al. 1983; Zekri and Parsons 1992).

Taking into account that commercial citrus is usually formed by grafting a variety onto the rootstock, the response of trees to salinity depends on the individual behavior of each of the component parts, as well as on the possible interactions scion/rootstock that may occur. However, as demonstrated by numerous studies and cultural practices, it is evident that the component mainly involved in the tree salinity response is the rootstock. Hence, the choice of the appropriate rootstock is crucial to obtain the maximum yield in citrus crops, both in saline and other conditions (Behboudian et al. 1986; Cooper 1962).

The Cl^- and Na^+ exclusion traits in citrus rootstocks are heritable and can be transmitted to breeding progeny (Sykes 1992). This has been observed in hybrids from the Cl^- excluder Rangpur lime (*C. limonia* Osbeck.) and Cleopatra mandarin or from the Na^+ excluder *P. trifoliata* mother plants, selected under glasshouse conditions as well as in artificially salinised field plots (Sykes 1985, 1992), indicating that it is possible to select for salt tolerance in short-term screening experiments.

Many experiments about screening salinity tolerance in citrus have been conducted according to several determinations like physiological plant responses (biomass, gas exchange parameters and leaf damage), ion accumulation in plant organs, water relations in leaves, and osmolytes concentrations in roots and leaves (Forner-Giner et al. 2011, 2009; Rodríguez-Gamir et al. 2012). With this extent nine months old seedlings of different rootstocks were grown in a greenhouse and irrigated over an eight-week period with nutrient solutions to which different amounts of sodium chloride (NaCl) were added, namely 0, 20, 40, and 60 mM. Relative growth, chloride and sodium concentrations and gas exchange parameters were measured.

5.3.3 Drought

The Intergovernmental Panel on Climate (IPCC) projected that the land area affected by drought will increase and water resources in affected areas could decline as much as 30% by mid-century (Christensen et al. 2007). About 42% of the total water used in Europe is allocated for agriculture. However, Southern European countries intercept the major part of total irrigated area in Europe. Water scarcity will be a major limiting factor for agriculture productivity, and it is then needed a better water demand management, achieving higher crop water use efficiency.

Irrigation is commonly employed in citrus orchards in arid and semiarid areas. Water deficit in citrus diminishes vegetative growth and yield, and reduces fruit size, and sometimes quality, causing important economic losses in orchards (Levy 1979; Levy et al. 1978; Romero et al. 2006). Additionally, drought stress reduces CO_2 assimilation, stomatal conductance, and transpiration (García-Sánchez et al. 2007; Syvertsen et al. 1988). Root systems can respond to soil drying by sending signals to the leaves, where stomatal closure is induced to reduce water loss (Davies and Zhang 1991). Moreover, plants have developed other mechanisms to resist droughts, such as increased root development or leaf mass reduction (Lei et al. 2006; Zhang 1989). Also, osmotic adjustment enables plants to maintain the leaf turgor necessary for stomatal opening, thus sustaining photosynthesis and growth (García-Sánchez et al. 2007).

Water relations and abiotic stress tolerance vary significantly between rootstocks. Rootstocks present genetically-determined characteristics that influence plant water relations. These features include root system distribution, water, and nutrient absorption efficiency (Castle and Krezdorn 1975) and vascular element anatomy (Rodríguez-Gamir et al. 2010; Vasconcellos and Castle 1994). These characteristics are associated with differences in root hydraulic conductance (Syvertsen and Graham 1985), which determine the ability of the rootstock to supply water and nutrients to the plant. This ability could be the main factor influencing fruit development in citrus trees, determining the strength of the grafted variety and its tolerance to water stress (Medina et al. 1998; Syvertsen and Lloyd 1994).

Root hydraulic conductivity is considered as one of the main factors controlling water movement through the soil-plant system (Kriedemann and Barrs 1981), and limitations of water supply imposed by the rootstock (Syvertsen and Graham 1985) may exert a marked influence on water relations and transpirational water use (Graham and Syvertsen 1984). Differences in the root hydraulic conductivity of rootstock seedlings could give rise to differences in mass flow of water to the shoots which, in turn, could influence transpiration in leaves. Consequently, the hydraulic capacity of the vascular system could play a key role in regulating stomatal conductance and gas exchange rates (Lo Gullo et al. 2007).

Such flexible, short-term regulation of root water uptake appears to involve aquaporins. Aquaporins are trans-membrane protein channels located in the plasma membrane, tonoplast, and other intracellular membranes, which are abundantly expressed in roots (Javot and Maurel 2002). Aquaporins belong to the superfamily of MIPs (major intrinsic proteins) and can apparently regulate transcellular water passage (Maurel 1997). Depending on their location, aquaporins in plants are classified as tonoplast intrinsic proteins (TIPs) or intrinsic proteins of the plasma membrane (PIPs), or are assigned to one of three other less-studied sub-families: Nodulin 26-like intrinsic membrane proteins (NIPs), basic intrinsic membrane proteins (SIPs) (Johanson et al. 2001) or an uncharacterized subfamily (XIPs) (Danielson and Johanson 2008). Furthermore, the PIPs sub-family is divided into two evolutionary groups based on homologous sequences (PIP1 and PIP2) (Kammerloher et al. 1994), each presenting several isoforms. Concerning water transport through the plant, PIPs are probably more important than TIPs in regulating water uptake by the roots because the plasma membrane is generally much less permeable to water than the tonoplast (Javot and Maurel 2002). In plants, aquaporins often show specificity of expression in tissues and organs (Tyerman et al. 2002), but the role of aquaporins in the roots and stems is more important in regulating plant hydraulic conductance than in other areas of the plant (Luu and Maurel 2005).

In order to determine water deficit, plants were irrigated with four solutions (nutrient solution alone, nutrient solution + 5% PEG, nutrient solution + 10% PEG, nutrient solution + 30% PEG). Plants were irrigated with these solutions at the beginning of the experiment and were maintained for 48 h before determinations. Gas exchange, water relationships, and Kr were determined in independent groups of plants cultured under similar conditions. Three plants were used for ABA analysis in leaves and roots.

The screening methodology in citrus rootstocks is described in Rodriguez-Gamir et al. (Rodríguez-Gamir et al. 2011). Plants were irrigated with two different irrigation doses (normal irrigation and moderate water deficit) during 70 days and leaf water potential (ψs), net CO_2 assimilation (ACO_2), transpiration, stomatal conductance (gs), and substomatal CO_2 concentration (Ci) were measured periodically under both irrigation conditions. Hydraulic conductance (Kr) and PIP1 and PIP2 gene expression levels in fine roots of control plants and plants subjected to water deficit on day 35 of the experiment were determined.

5.3.4 Flooding

Waterlogging generally arises due to poor soil drainage combined with excessive rainfall or irrigation. Soil inundation induces a variety of physiological dysfunctions that alter plant growth, including hormonal imbalances, altered distribution of carbohydrates, deficient nutrient uptake, early senescence of leaves and injury in organs, which sometimes precede plant death. It is known that some species have acquired some characteristics of adaptation to anaerobic conditions (Bailey-Serres and Voesenek 2008). Plant strategies to grow and survive during long periods of waterlogging include biochemical, anatomical, and morphological changes (Colmer and Voesenek 2009).

Although the response is variable among species and cultivars, citrus is considered a flooding-sensitive crop that responds to waterlogging by restricting stomatal conductance to prevent water loss (García-Sánchez et al. 2007; Rodríguez-Gamir et al. 2011). This fact appears to be hormone-regulated and is associated with abscisic acid accumulating mainly in leaves, which induces stomatal closure (Arbona and Gómez-Cadenas 2008; Rodríguez-Gamir et al. 2011). During prolonged soil flooding periods, reduced root hydraulic conductance (Rodríguez-Gamir et al. 2011; Syvertsen et al. 1983) impairs water uptake, which causes leaf wilting and chlorosis (Arbona and Gómez-Cadenas 2008), and then, the rootstock is the main aspect to consider in flooding tolerance.

Flooded plants present reduced water flux under high evaporative demand, which seems to be due to the down-regulation of root hydraulic conductance by anoxia (Rodríguez-Gamir et al. 2011) which has also been associated with stomatal closure (Davies and Flore 1986; Else et al. 1995). Moreover, it is generally accepted that water transport across biological membranes is facilitated by aquaporins. These proteins belong to the major intrinsic proteins (MIPs) family and form water channels that facilitate the passive flow of water through cell membranes and maintain water content in cells and tissues. There is some evidence that, among aquaporins, the subfamily of plasma membrane intrinsic proteins (PIPs) appear to play a critical role in the control of water transport through root tissues, regulating the transcellular pathway (Rodríguez-Gamir et al. 2011). Adaptation of root hydraulic conductance to altered environmental conditions may result from changes in the abundance or activity of aquaporins. In support of this, evidences point to the reduction of the expression of PIP1 and PIP2 genes by anoxia. Furthermore, the root signals and sensory mechanisms that trigger citrus responses to flooding have been described (Arbona and Gómez-Cadenas 2008; Rodríguez-Gamir et al. 2011).

The performance of citrus rootstocks has been tested in several studies, with strong differences in flooding tolerance among citrus rootstocks (García-Sánchez et al. 2007; Ruiz-Sánchez et al. 1996; Syvertsen and Zablotowicz 1981; Syvertsen et al. 1983).

The screening evaluation of new citrus rootstocks under flooding conditions was done with one year-old seedlings of the different rootstocks waterlogged for 35 days and compared with normally watered well-drained plants. Several determinations were determined: (1) physiological plant responses (biomass, chlorophyll concentration, gas exchange, and fluorescence parameters); (2) ABA concentrations in roots and leaves; (3) water relations in leaves; (4) gene expression of the key aquaporins involved in the water transport process in the root system (Rodríguez-Gamir et al. 2011).

5.3.5 Boron Toxicity

Boron (B) is one of the eight essential micronutrients as it is required in important physiological functions for the normal growth and development of all higher plants (Frommer and von Wirén 2002). It participates in the formation of cell wall structure through the borate-diol bonding of two rhamnogalacturonan II molecules. It is also involved in root elongation, carbohydrate metabolism, phenol accumulation, pollen tube growth and integrity of cell membranes (O'Neill et al. 2004).

Among abiotic stresses, B disorders are one of the most severe environmental stresses, impairing plant growth and crop production worldwide. Citrus is classified as B sensitive fruit crop and the range between the critical soil B deficiency and toxicity levels is narrow (Camacho-Cristóbal et al. 2008). Therefore, the regulation that exists between its absorption, mobilization, and removal from the cell is essential for maintaining the general B homeostasis of the plant.

B deficiency is commonly found in acidic and sandy soils that are poor in silt and clays, where boric acid is easily leached below root zones by rainfall or irrigation (Shorrocks 1997). In citrus, B deficiency symptoms start from developing parts and can cause deformations in leaves and fruits, the early death of young shoots, corky split veins in mature leaves, emergence of multiple buds and gum appearance in both cracked twigs and fruits (Xiao et al. 2007). Excess B occurs mostly by irrigation with high B level water, or in arid and semiarid areas where water reaches the topsoil by capillarity and then evaporates to cause B accumulation. B toxicity symptoms usually appear as a leaf tip and margin yellowing on older leaves, which turn to a brownish and burnt appearance and resinous gum spots (Papadakis et al. 2004). Severe B toxicity may induce shorter branches and even twig dieback.

After entering the root, B is transported through xylem vessels, and is mostly bound to cell wall structures (insoluble B pool) or accumulates in apoplastic fluids (soluble B pool), while only another low soluble portion enters

cells (Liu et al. 2013). In plants, B homeostasis seems to be related to the synergic regulation of several genes involved in B uptake, transport, and partitioning in the aerial part. Moreover, vacuolar compartmentation might also play a key role in cell B tolerance.

In line with this, the first B transporter identified in a biological system was *Arabidopsis thaliana* BOR1, an efflux-type B transporter which is essential for the efficient xylem loading of B (Takano et al. 2002). This gene is expressed mainly in root pericycle cells and its overexpression enhances root-to-shoot B translocation, probably as borate, under B limiting conditions (Miwa et al. 2006). Subsequently, its homologous AtBOR4 was characterized (Miwa et al. 2007), and its overexpression markedly improves *A. thaliana* growth under high B conditions through B efflux. In *Oryza sativa*, OsBOR1 loads B into the xylem and also participates in the absorption of this element in roots (Nakagawa et al. 2007). In barley and wheat, HvBOR2 and TaBOR2 activities respectively, lower the B concentration in the roots of tolerant cultivars, which suggests a positive correlation between these genes and the degree of tolerance between different cultivars in these species (Reid 2007). In fruit crops, VvBOR1 and CmBOR1 have been recently characterized as B transporters in Vitis vinifera and Citrus macrophylla, respectively (Canon et al. 2013; Pérez-Castro et al. 2012).

NIP5;1 is a BA channel that facilitates B influx to root cells in *A. thaliana* and *Brassica napus* (Sun et al. 2012; Takano et al. 2006). It is localized to the plasma membrane on the outer side of epidermal, cortical and endodermal cells in roots. NIP5;1 is required for B uptake from the root surface and its accumulation is regulated in response to B deprivation (Takano et al. 2006). Another member of the same subfamily is AtNIP6;1, a BA channel implicated in the proper distribution of B, particularly among young developing shoot tissues (Tanaka et al. 2008). Finally, the vacuolar compartmentation of B has been related to AtTIP5;1 activity, an aquaporin family member localized in the cell tonoplast membrane (Pang et al. 2010). Under B toxic conditions, the AtTIP5;1 overexpression has been reported to lower the cytoplasmic B concentration by accumulating B in the vacuole and to, therefore, confer cell tolerance to toxic B level.

In addition to molecular regulation, some biochemical indicators related to oxidative stress and osmoprotective compounds, notably proline, which have been well-defined in studies of plants subjected to different stress types (Delauney and Verma 1993; Mittler 2002). The possible roles attributed to proline are related with balance of osmotic pressure, preservation of enzymes in the cytoplasm, detoxification of reactive oxygen species (ROS), and protection of membranes against lipid peroxidation (Delauney and Verma 1993).

As citrus rootstocks differ in their susceptibility to B concentration (Gimeno et al. 2012; Papadakis et al. 2004), the analysis of the mechanisms involved in B uptake and distribution in the plant, are essential to better understand B homeostasis in this species, to mitigate the harmful effects on the plant and to improve the management of B stresses in this fruit crop.

5.4 Biotic Factors

5.4.1 CTV

In commercial citriculture, sour orange has been a universal rootstock that is well known for many attributes related especially to yield, fruit and juice quality as well as tolerance to cold temperatures and various soil conditions. However, it has one major weakness, it is highly susceptible to decline by isolates of the citrus tristeza virus (CTV). This virus is an aphid-borne, phloem-limited closterovirus and the causal agent of one of the most important diseases of citrus (Bar-Joseph et al. 1983). The first factor to be considered in screening the new hybrids was their tolerance/resistance or susceptibility to CTV.

There is a wide variety of biological diversity in CTV. As a consequence of that biological diversity interacting with the host, the main symptoms induced by CTV, are the decline and death of trees (e.g., sweet orange on sour orange

rootstock), vein clearing [especially pronounced in *C. aurantifolia* (Christm.) Swing.], stem pitting in different degrees (the most common) and reduction in tree growth (Bar-Joseph et al. 1989). Climatic factors can affect the reaction of some hosts (Calavan et al. 1972). There is also a wide variation between the species and varieties of the subfamily *Aurantioideae* in their reaction to CTV. Salibe (1973) establishes five groups of *Citrus* and related species, according to their reaction to CTV infection, from the "extremely intolerant types", such as sour orange and lemon, to the "resistant or immunes types", such as *P. trifoliata* and some of its hybrids, in that the virus cannot multiply in the tissues of these plants. He also points out that the "extremely intolerant types" can only be successfully used as rootstocks for citrus types belonging to the same class (e.g., trees of lemon varieties on sour orange rootstock). Several methods and criteria have been used to study the reaction of a host to CTV inoculation. ELISA test and screening with markers are the screening method to select tolerant genotypes.

Screening for resistance to CTV was carried out in the greenhouse. Three groups of five-month-old seedlings of each hybrid were inoculated (approximately 10 cm from the ground level) with two bark patches of Mexican lime infected with one out of three CTV isolates: a mild, moderate and severe.

The fourth group of five plants was grown as controls. Once the grafts were taken (approximately one month after inoculation), the upper part of the plants, 10 cm over the bark patches, was cut off. The control plants were also cut off at the same level. New shoots grow and, seven or eight months later (in January or February of the following year), the presence or absence of CTV in the inoculated plants were determined by the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test in the final 15 cm of the new shoots. If only one plant of a group proved positive in the DAS-ELISA test, it was repeated on the other plants of the hybrid, in which the CTV was not detected in the first test. The growth of the control plants compared to the inoculated plants was visually evaluated.

5.4.2 Nematodes

The citrus nematode *Tylenchulus semipenetrans* Cobb. infects citrus worldwide and is associated with poor growth of young citrus trees planted in infested groves and with the poor performance of mature citrus trees (Duncan and Cohn 1990). Most studies estimate yield losses due to this nematode to be in the range of 10–30% depending on the level of infection. Mature trees can tolerate large numbers of these nematodes before exhibiting lack of vigor or decline symptoms; however, young trees grow poorly if replanted into nematode-infested soils (Duncan and Cohn 1990).

Nematode damage to the root system impairs the ability of the tree to absorb water and nutrients necessary for normal growth (Verdejo-Lucas and McKenry 2004).

The host range of *T. semipenetrans* includes all citrus species and most hybrids of citrus with other members of the *Rutaceae* family, such as trifoliate orange (Baines et al. 1969). Nonrutaceous plants, such as grape (*Vitis vinifera* L.), olive (*Olea europea* L.), and persimmon (*Diospyros* spp.) are also hosts of the citrus nematode. The only germplasm source of citrus nematode resistance that has been incorporated into commercially acceptable citrus rootstocks is derived from *P. trifoliata* (Kaplan 1990).

Some selections of *P. trifoliata* have a high level of resistance to populations of *T. semipenetrans*, whereas others are moderately susceptible (Hutchison and O'Bannon 1972; Reddy and Agarwal 1987). Trifoliate orange hybridizes readily with most *Citrus* spp., and many of the resulting hybrids inherit resistance to the citrus nematode (Cameron et al. 1969). The hybrids rootstock Swingle citrumelo, C-35, and Forner-Alcaide 5 are resistant to the citrus nematode (Verdejo-Lucas et al. 2000).

The procedure used to screening citrus rootstocks resistant to *T. semipenetrans* was described by Kaplan (1990) with some modifications (Verdejo Lucas et al. 1997). 12 months old seedlings were infected with nematode inoculum. Plants were inoculated with 1 × 104 eggs + J2 per plant. The infectivity (females/g roots) and

reproductive potential (eggs + J2/g roots) of *T. semipenetrans* were considered as indicators of the response of the rootstocks to the nematode (Verdejo-Lucas et al. 2000).

The experiments were arranged in randomized complete blocks, and each rootstock nematode combination was replicated seven times. The temperature of the greenhouse ranged from 16 to 26 °C during the 6 months of the experiment. At harvest, roots were washed free of soil, weighed, and then frozen at –20 °C until processed. When required, roots were thawed at room temperature, and nematodes extracted from the entire root system by blender maceration (McSorley et al. 1984) using a 0.5% sodium hypochlorite solution. Nematodes collected on a 25-mm screen were subjected to centrifugation and sugar flotation to remove root debris (Jenkins 1964). Additional research is needed to determine mechanisms involved in the identification of molecular markers that link to the gene for *T. semipenetrans* resistance in *P. trifoliata*.

5.4.3 Blight

Citrus blight is a serious disease associated primarily with hot humid citrus growing regions, such as Florida and the hotter regions of Sao Paulo, Brazil (Derrick and Timmer 2000); it is not found in Mediterranean climates. Blight is responsible for an estimated loss of 650,000 trees per year in Florida and about 10 million per year in Brazil. Although the disease is transmissible via root grafts, the actual cause is still unproven. Most hypotheses suggest a virus-like organism(s) as the cause (Derrick and Timmer 2000), but more recently, a pararetrovirus has been suggested (Roy et al. 2014). Disease symptoms include zinc deficiency and xylem plugging, leading to water starvation, reduced production, and eventually tree death. Tree age at which the disease appears and incidence of the disease is known to be affected by rootstock. Most susceptible rootstocks (early and high-frequency infections) include Carrizo citrange, rough lemon, and Volkamer lemon. Most trifoliate hybrids and vigorous citron-derived rootstocks are considered susceptible, and can begin to show blight symptoms as early as 5–8 years after planting. Cleopatra mandarin is considered to be intermediate, and pummelo/mandarin hybrids have proven to be the least susceptible rootstocks, including sour orange, sweet orange, Smooth Flat Seville and Kinkoji. These least susceptible rootstocks generally remain blight free for approximately 20 years, followed by a low increase in disease incidence. The different responses from rootstocks with different genetics suggest that improved tolerance can be achieved through rootstock breeding and selection, with focus on pummelo and mandarin germplasm, including contributions of diluted trifoliate orange germplasm in some cases, as needed to improve resistance to other diseases.

Hybrid production at UF/CREC: Rootstock crosses at the diploid level include selected pummelos with Cleopatra, Shekwasha, and Amblycarpa (Nasnaran) mandarins. Crosses are generally made using a monoembryonic female that produces zygotic seedlings with complementary polyembryonic pollen parents that produce mostly nucellar seed. Tetraploid hybrids, many combining selected pummelos with mandarins, have been produced by somatic hybridization via protoplast fusion (Grosser et al. 2004; Louzada et al. 1992; Medina-Urrutia et al. 2004), and by conventional crosses at the tetraploid level (Grosser et al. 2003). Without a known causal organism, screening to identify tolerant/rootstocks is difficult and very time-consuming. Our approach has been to identify grove sites with severe citrus blight pressure (10–12% loss per year) for screening new rootstock candidates. Test trees were planted in holes where the previous tree was removed due to blight. In an effort to speed up the process, we are not waiting for seed from new hybrids; rather, we are propagating the rootstock hybrids by making rooted two-node cuttings (Sabba et al. 1992). Many hybrids from crosses involving pummelo and mandarin produce high percentages of zygotic seed, which makes them not amenable to seed propagation. With the advance of tissue culture propagation techniques and companies that efficiently utilize them to propagate uniform,

high-quality rootstock liners, abundant production of nucellar seed is no longer a primary criterion for rootstock selection and success. In our initial blight screening efforts, we are testing 6-10 liners per rootstock selection, all grafted with Valencia sweet orange. Valencia on rough lemon trees is being used as the control. In the largest experiment planted to date, 125 hybrid rootstock candidates, all propagated by rooted cuttings (including diploids and tetraploids, mostly pummelo/mandarin hybrids), seven Valencia trees per candidate, are under evaluation. This trial was planted in 2008 east of St. Cloud, Florida (USA) on the property of the Orie Lee Family. Unfortunately, HLB began to move into the trial around 2010, and now 100% of the trees are infected with CLas, the purported cause of HLB. This grove and trial have never received a full-blown psyllid control program and generally have been sprayed for psyllids and other pests just two times per year. Despite these challenging conditions, trees on a few of the rootstocks have continued to thrive showing good tree health and productivity, with the best performers being diploid hybrids of Hirado Buntan pummelo x Cleopatra mandarin. Trees are now just coming into the age where blight is expected to have an impact, so it will be a few more years before we can hopefully identify new rootstocks that can handle both blight and HLB, under a more traditional and affordable IPM spray program. HLB has also confounded all of our other blight screening experiments in the field. There are two available diagnostic tests for citrus blight that can be used to validate the presence of blight in test field trees (Derrick and Timmer 2000). The "syringe injection test" measures the quantity of water that can be injected into the trunk xylem of field trees in a given period of time. Blighted trees have plugged xylem vessels and take up significantly less water than healthy trees. The second test is a serological test based on the presence of a blight-specific pathogenesis-related protein p12, which is capable of separating blight from other tree decline disorders (Derrick et al. 1990).

5.4.4 The Diaprepes/Phytophthora (DP) Complex

The sugarcane root weevil *Diaprepes abbreviautus* (L.) that prefers citrus as an alternative host, is now widespread in most of the major citrus production areas in Florida due to the movement of contaminated ornamental nursery stock, followed by local dispersion by flying adults (Hall 2000). It has become a major problem in Florida citriculture because the larval feeding causes severe mechanical damage to scaffold roots. Gaping wounds resulting from feeding of advanced stage larvae create opportunities for invading soil pathogens, especially *Phytophthora* spp. (Graham et al. 2003), thus resulting in the DP complex. Before the arrival of HLB, the DP complex was considered by many to be the primary flatwoods rootstock problem in Florida. In this area, the two primary problem are *P. nicotianae* and *P. palmivora*, the latter being much more of a problem in poorly drained flatwoods soils. The response of citrus rootstock germplasm to the two different *Phytophthora* spp. is a paradox, as rootstocks resistant to *P. nicotianae* such as Swingle citrumelo and Carrizo citrange are susceptible to *P. palmivora*; and rootstocks resistant to *P. palmivora*, such as Cleopatra, Shekwasha, SunChuSha, and Amblycarpa mandarins are susceptible to *P. nicotianae* (Graham et al. 2003). This should be taken into consideration when designing crosses to generate candidate rootstock progeny that can handle the entire DP complex.

Two-Phase Screening Program (Grosser et al. 2003, 2007): New rootstock hybrids to be tested are propagated by rooted cuttings unless nucellar seed is available. Phase-1 is a replicated weevil larva forced feeding assay, conducted in containers®, to assess differences in root system damage caused by the feeding. Six to ten replicates per rootstock are inoculated with five 1st instar neonate larvae obtained from eggs of wild female weevils. After 6–8 weeks, cuttings are harvested and root systems are rated using the following scale: 1 = healthy roots, no feeding

damage; 2 = visible root injury, minor damage; 3 = visible root injury, severe damage; 4 = severe root girdling, no evidence of Phytophthora; and 5 = severe root girdling, evidence of Phytophthora. A mean ranking score is determined for each rootstock candidate. The number of surviving larvae per container can also be recorded. Phase-2 is a plant recovery assay. Hybrids from the Phase-1 challenge feeding assay, showing a mean score of 3.0 or lower, are replanted in 10-cm deep flats containing typical Florida calcareous soil (pH = 8.0) inoculated with both *P. nicotianae* and *P. palmivora* (as described in HLB section). Plants are watered and fertilized as needed. After three months, plants are removed from the soil and root systems rated as described above from 1 to 5. A Recovery Index score is calculated as follows: for each rootstock candidate: score = (number of replicates) − (average root rating) × (number of surviving plants). The higher the index score, the better the recovery and thus better tolerance of the DP complex. Cleopatra mandarin and Volkamer lemon are good control rootstocks, as they have routinely performed poorly in this assay.

We have used this two-phase assay to screen several hundred diploid and tetraploid rootstock candidates (Grosser et al. 2003, 2007). Thus, so far, three rootstocks that performed well in this assay have been released by UF for commercial use. These include UFR-16, a diploid hybrid of Hirado Buntan pummelo x Shekwasha mandarin; and two tetraploid hybrids UFR-4 and UFR-5. The latter two rootstocks both had the highly DP complex tolerant Nova + Hirado Buntan pummelo (HBP) somatic hybrid as the female parent (UFR-4: Nova + HBP x Cleopatra + Argentine trifoliate orange; UFR-5: Nova + HBP x Succari sweet orange + Argentine trifoliate orange). UFR 4 and 5 have been planted in a trial in a commercial grove (Bowling Green, FL) with heavy DP and HLB pressure, and after four years are performing quite well.

5.4.5 HLB (High Throughput Screening Method)

Citrus Greening Disease or Huanglongbing (HLB) is a very complicated disease, which makes screening for tolerant/resistant rootstocks a real challenge (Bové 2006, 2014). HLB, the most devastating disease affecting citrus worldwide, is putatively caused by the phloem-limited *Candidatus* Liberibacter asiaticus (*C*Las) bacterium and vectored by the Asian citrus psyllid *Diaphorina citri*. The disease was first confirmed in Florida in 2005, and since has spread throughout the state, infecting most producing trees. The disease is also now spreading in Texas and has been discovered in California. Development of a rootstock that can mitigate the disease in the scion is the ultimate solution since it would work with all commercial scions in all environments. Some commercial rootstocks are exceptionally tolerant when ungrafted (i.e., Carrizo, X639, US-897). However, when these rootstocks are grafted with a susceptible scion such as any commercial sweet orange or grapefruit, the tolerance is not necessarily transmitted through the graft union to protect the scion, when trees are grown under a traditional nutrition program. Thus, effective screening has to be done solely with grafted trees. The differential response of rootstocks in commercial trials, mostly rootstocks developed prior to the HLB crisis, demonstrates that there is indeed genetic variation for rootstock response to HLB. This variation can be exploited by further breeding and selection. We (UF/CREC) have observed differential rootstock responses for rate of infection, speed, and severity of symptom development, and rate of recovery. This led to our development of a protocol for high throughput screening of new diploid and tetraploid rootstock hybrids, as necessary to quickly identify the very small fraction of new rootstock hybrids that have potential to significantly prevent or mitigate HLB in grafted commercial trees.

Screening: The evolving "Gauntlet" screening method (UF/CREC) is conducted as follows: seed from rootstock crosses are planted in calcareous soil inoculated with *P. nicotiana* and *P. palmivora*. Liquid *Phytophthora* spp. inoculum is prepared as described in Graham et al. (2003). Robust seedlings are transferred to 4 × 4 citripots. Tops of selected hybrids are used to propagate seed trees via rooted cuttings; thus subsequent seed trees can be planted in the field on their own roots, avoiding grafting to other rootstocks that might compromise their health and survivability. Remaining liners are grafted with 10-inch bud sticks of *Liberibacter asiaticus* (Las)-infected sweet orange (and possibly Las-infected Murcott, a highly susceptible scion, as a rapid indicator plant), which will be forced to flush to produce the entire scion portion of the new tree. The use of large bud sticks rather than smaller buds minimizes the possibility of uninfected escapes. Trees showing little or no disease symptoms and normal growth from the infected sweet orange are then selected for further challenge in a "hot" psyllid house until visible psyllid damage is observed. Promising trees are transferred to a challenging field test site (the current site is the USDA Picos Farm in Fort Pierce, FL; FDACS-DPI, the Florida regulatory agency has already issued a permit to move pathogen-infected material). Rootstocks showing ability to mitigate HLB after three years in the field with minimal psyllid control (HLB symptomless trees with good growth; PCR status monitored yearly) will be propagated by having clean seed trees entered into tissue culture-based propagation schemes for large scale testing. One pathogen-free seed tree of each field-planted gauntlet rootstock selection is maintained in a certified greenhouse until this determination, to avoid the additional two-year meristem-tip culture process used by the state of Florida's Parent Tree Program. Any selections entered into tissue culture propagation using seeds for culture initiation will be genotyped using appropriate SSR markers according to Chen et al. (2008).

Results to date and proof of concept: "Gauntlet" screening (UF/CREC) work is already well underway, as more than 10,000 individual hybrid seedlings have been entered into the screening process. The first set of putatively tolerant trees has now been in the ground more than five years, and a few trees are still thriving despite the *C*Las infection. Seed trees from two of the outstanding rootstocks are producing abundant polyembryonic seed, and large-scale testing is underway. Approximately 40 of 220 hybrid rootstocks that have been in the field for more than two years look quite promising. Genetic patterns are emerging. One cross generating multiple hybrids that are performing well is cybrid tetraploid Volkamer lemon (with Amblycarpa cytoplasm) crossed with allotetraploid UFR-4 (Nova + Hirado Buntan pummelo x Cleopatra + Argentine trifoliate orange) as the pollen parent. Volkamer lemon provides the phloem regeneration capacity associated with lemons infected with *C*Las (Fan et al. 2013), and UFR-4 provides genetics for reduced feeder root loss following *C*Las infection (Evan Johnson and Jim Graham, personal communication). Preliminary data shows that cytoplasm from Amblycarpa improves fruit quality from trees on lemon-type rootstocks. One of the two hybrids producing nucellar seeds mentioned above is from this cross. A second emerging pattern suggests that genetics for abiotic stress tolerance have value in development of biotic stress tolerance needed to overcome HLB. A diploid cross of a monoembryonic hybrid from Hirado Buntan pummelo x Shekwasha mandarin selected for salinity tolerance crossed with a hybrid of Hirado Buntan pummelo x Cleopatra mandarin also selected for salinity tolerance as the pollen parent has also generated multiple hybrids performing well in the "gauntlet" screen. Seed trees of these hybrids are not yet fruiting, so rooted cuttings or tissue culture propagated liners will be used for advanced testing. We expect continued research, with focus on breeding and selection of rootstocks able to mitigate HLB in grafted

commercial scions, to generate new rootstocks an order of magnitude more HLB tolerant than any commercial rootstock available today.

5.5 Tree Size Control

It is widely assumed that the rootstock greatly affects tree size (Tworkoski and Fazio 2016), and in *Citrus*, like in most fruit tree species, dwarfing rootstocks produce small trees, allowing closer tree spacing that shortens the period of unproductivity and increases yield per unit area (Webster 1995). These rootstocks reduce vegetative growth in scions. These "dwarfing" rootstocks are used to plant high-density orchards, in which the small tree size helps reduce costs in some cultural practices, such as pruning and harvesting, and in some cases bringing trees into production earlier.

Until recently, the availability of dwarfing rootstocks in citrus was restricted almost exclusively to Flying dragon (*P. trifoliata* L. Raf var. *monstrosa*), a mutant of trifoliate orange, that reduces tree size when used as rootstock. Thus, with most scions, mature trees on this rootstock are no more than 2.5 m tall, and overall growth may be reduced by 75% as compared to trifoliate orange standard rootstock (Bitters et al. 1979). In other aspects, the behavior of Flying dragon rootstock is similar to that of other *P. trifoliata*, being resistant to the citrus tristeza virus (CTV), *Phytophthora* spp., and citrus nematode (*Tylenchulus semipenetrans*). It is cold hardy and transmits some of this hardiness to the scions budded on it (Cheng and Roose 1995). In addition to these characteristics, Flying dragon induces high-yield efficiency, early bearing, and good fruit quality in most citrus cultivars (Castle et al. 2007, 2010; Gonzatto et al. 2011), and it has been recommended as an alternative dwarfing rootstock for acid soils, being better suited to high-density plantings (Mademba-Sy et al. 2012). However, like trifoliate orange, it is highly susceptible to iron chlorosis, likely limiting its diffusion into commercial orchards across wide cultivated citrus areas.

Fortunately, it can be used as a parent and has been used to develop a few Forner-Alcaide hybrid selections that also confer dwarfing response in grafted scions. In particular, FA 517 and FA 418, whose agronomical behavior has been tested under field conditions (Forner-Giner et al. 2014). Both rootstocks show lower canopy volumes but higher yield efficiency when compared with Carrizo citrange (Forner-Giner et al. 2014). Moreover, they produce good fruit quality and optimal response when cultured in alkaline soils, one of the main factors limiting crops in Spanish soils (Forner-Giner et al. 2014; González-Mas et al. 2009).

It has been suggested that reduced growth, induced by dwarfing rootstocks, is associated to lower leaf and stem water potentials in scions grafted onto them, when compared with those grafted onto vigorous rootstocks; this could be a consequence of their higher hydraulic resistance which may cause water deficit in leaves during periods of high evaporative demand and stomata closure (Martínez-Alcántara et al. 2013). Then, dwarfing rootstocks present a minor ability to transport water from the soil to the stem (Basile et al. 2003). Although the resistance of bud union to water transport and xylem anatomical characteristics, in particular, the number and diameter of vessels, may limit plant growth, carbohydrate distribution is also an important constraint involved in tree response (Forner-Giner et al. 2014; Martínez-Alcántara et al. 2013). The reduced translocation of photoassimilates from leaves to roots limits root development and it also contributes to higher availability of these compounds in the scion, resulting in increasing carbon transport towards the fruits.

Among the rootstocks released by the University of California, Bitters is reported to confer a semi-dwarf habit to grafted scions and this was confirmed in several trials in combination with different citrus species (Roose 2014). The same performance was also observed in Italy when Bitters was grafted with Tarocco orange, monitoring vegetative growth along six years (Continella et al. 2018). Trees on Bitters were less vigorous, i.e., smaller and with shorter

branches. The higher yield efficiency, compared to Carrizo citrange, may allow to asserting the ability to limit vegetative growth, giving at the same time good yields.

Tetraploid rootstocks, both autotetraploids and allotetraploids, have also been shown in general to reduce tree size (Grosser et al. 1998, 2011). Autotetraploids can generally be recovered from large seedling populations by selecting seedlings with thicker, darker green leaves, followed by validation using flow cytometry. Both autotetraploids and allotetraploids can be obtained from somatic hybridization experiments (Dambier et al. 2011; Grosser et al. 1994). More recently, allotetraploid rootstock candidates are being generated from sexual crosses of tetraploids, generally using a monoembryonic female. Hybrid populations produced in this manner have the potential for tremendous genetic diversity. The first tree size controlling rootstock released by the University of Florida citrus breeding program was UFR-6, a somatic hybrid of Changsha mandarin and trifoliate orange 50-7 (Grosser et al. 2011). The evaluation of morpho-physiological parameters in trees grafted onto different rootstocks such as xylem anatomy, hydraulic conductance, water potential, gas exchange responses, and 13C-photoassimilates transport, could improve the understanding of the key processes controlling growth in scion/rootstock combinations. This will also allow the identification of parameters related with dwarfing, in order to provide citrus breeders with useful tools to screen plants at an early stage for candidate rootstocks that could potentially reduce scion growth (Syvertsen et al. 1999, 2000).

5.6 Field Evaluation

Citrus rootstocks can affect many horticultural aspects of the trees. Yield and yield efficiency, tree size, fruit size and fruit quality, organic acid contents, fruit alterations, precocity, etc. All these aspects can affect the economic return of the groves/plantations and can be improved by the correct use of citrus rootstocks, and have to be studied in field trials.

5.6.1 Yield and Yield Efficiency

In general terms, citrus trees yield their first crop two or three years after planting and it rises until the fifth or sixth year when trees generally reach full production. However, there are big differences among rootstocks. Cleopatra mandarin produces a very reduced number of fruits typical from its tendency to grow slowly for the first few years after planting, and trees grafted on this genotype do not reach full production until the 8th year. However, in contrast, trees on *C. macrophylla* produce much earlier and generate large crops.

To estimate the first year of full production, for each rootstock, a statistical comparison has to be done with the means of yields of 3rd to 9th years, considering the full production to be first-year displaying no statistical difference with some of the means of the posterior harvests. The crop of each tree will be harvested and weighed every year to evaluate yield and yield efficiency. Fruit yield can also be measured using a volumetric method. Cumulative yield (kg tree^{-1}) will be calculated for the sum of all the crops. Yield efficiency (kg m^{-3}) will be estimated as the ratio of cumulative yield to canopy volume. Tree size is usually evaluated using the formula V = 1⁄4 0.524 hd^2 that is one-half of a prolate spheroid and where h is the tree height and d the average of two diameters N–S and E–W.

5.6.2 Alternate Bearing Index (ABI)

The analysis of the ABI, which reflects the difference in yield between two consecutive harvests, shows important new information as it normally differs among scions and rootstocks. There are some rootstocks which increase alternate bearing of the yield, which is problematic with existing commercial varieties already presenting this defect.

The alternate bearing index is calculated by dividing the difference between two consecutive harvests by the sum of these two yields × 100. If the index exceeds 50%, this means that the tree is in alternate bearing, while the tree is in regular bearing if this index is below 50%.

5.6.3 Fruit Drop

Fruit drop is an important disorder that comprises fruit yield in citrus orchards, so growers apply treatments to reduce this problem, but these practices increase the cost of cultivation. In addition, the excess of production of some cultivars lengthens the harvest period and an important part of the crop is lost. For these reasons it is an excellent trait for a rootstock to retain the ripened fruit. The tendency of fruits to drop increases with plant age and it is strongly regulated by rootstock influence on scion.

5.6.4 Fruit Quality Variables

In fruits destined for fresh consumption, fruit size, juice content and TSS/TA ratio are of great importance. Rootstock is reported to significantly affect fruit, both external (size, rind thickness, peel color, etc.) and internal (juice content and color, pH, total soluble solids, etc.), quality variables. After the harvest, 25-fruit samples can be taken randomly per tree to determine the physical and chemical characteristics. Fruit weights can be measured with a digital balance.

Color is considered one of the most important external factors of fruit quality, as fruit appearance greatly influences on consumer acceptance. A colored fruit on the tree is always ripe, thus the risk of selecting immature fruit due to color is highly improbable unless they are artificially degreened. There now exists a non-destructive method that can be applied in the field as well as in the industry to accurately show the apparent maturation degree of the fruit in temperate countries. Color index determinations can be made for fruit skin on four opposite faces in the equatorial zone and for juice. The CIELAB L* (brightness or lightness; 0 = black, 100 = white), a* (−a* = greenness, +a* = redness) and b* (−b* = blueness, +b* = yellowness) color variables were measured using a chromatometer. Color index (CI) can be calculated using the following formula CI = 100 a*/L*b*.

Fruit diameter (D), fruit height (H) and peel thickness are measured with an electronic digital slide gauge and fruit shape index (D/H) can be calculated. Analysis of rind thickness is a good parameter to estimate fruit quality, as it is inversely correlated with the amount of juice. Extremes in rind thickness are not desirable as thick rind is normally related with low juice content, while thin rinds are prone to splitting, and are sensitive to peel disorders, which can occur during storage. This parameter is also influenced by the rootstock.

The juice has to be extracted with an electric squeezer and juice percentages (w/w) calculated. The chemical analyses can be determined using three juice samples for each scion/stock combination. The flavor and palatability of citrus fruit vary according to relative levels of TSS and the presence or absence of aromatic or bitter juice constituents. The pH can be measured with a pH-meter. Total soluble solids (TSS) are measured with a hand-held refractometer and expressed as degrees Brix at 20 °C. The method to analyze titratable acidity (TA) is based on neutralization (0.1 N NaOH) to pH 8.1 and values are expressed as g citric acid L^{-1}, since this is the dominant organic acid in orange. The ripening index (RI), which relates the soluble solid content measured in °Brix and the titratable acidity, determined as a percentage of citric acid content in the fruit juice, is the most widely used method to estimate citrus fruit maturity level. The ripening index (RI) is calculated as the ratio of total soluble solids/titratable acidity.

Sugars are the major components of citrus juice soluble solids and the sweetness of orange juice is intrinsic to its sugar composition. Sucrose is the main sugar present in orange juice, more than 55%, followed by glucose and fructose. Bibliography strongly relates sugar content and rootstock influence on the variety. This effect is very important in orange and mandarin fruits. Sugar determinations are analyzed with HLPC.

Pigmentated citrus juices are investigated for the identification and quantification of flavonoids (anthocyanins, flavanones, flavones)

and hydroxycinnamic acid derivatives. Cyanidin 3-O-glucoside, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, and sinapic acid, whilst eriocitrin, neoeriocitrin, narirutin, hesperidin, didymin, and vitexin are the usual reagents used to unambiguously identify the chromatographic signals. Small portions (2 mL) of the juices are put in 15 mL plastic sample tubes and 100 μL of formic acid (98%) added. Samples are sonicated for five minutes, then centrifuged at 4000 rpm for 15 min to separate the solid portion of the juices. 1 mL of the clear supernatant is transferred into 2 mL HPLC amber vial and immediately analyzed. All analyses are carried out in triplicate; results are reported in milligram (mg) of compound per liter (L) of juice.

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Ploidy Manipulation for Citrus Breeding, Genetics, and Genomics

6

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Abstract

Polyploidy appears to have played a limited role in citrus germplasm evolution. However, today, ploidy manipulation is an important component of citrus breeding strategies. For varieties, the main objective is to develop triploid seedless cultivars. For rootstock, the aim is to cumulate interesting traits in tetraploid hybrids and to improve adaptation to biotic and abiotic stresses. This chapter starts with a review of the recent knowledge acquired on the natural mechanisms of citrus polyploidization and tetraploid meiosis. Chromosome doubling of nucellar cells is frequent in apomictic citrus and results in tetraploid seedling production. Unreduced gametes are also frequently produced, mainly by second division restitution for ovules. First division restitution was described for pollen as well as alternative mechanisms for both ovules and pollen. Tetraploid plants display tetrasomic to disomic segregations in relation to their genome structure (autotetraploid versus allotetraploid) and the divergence of the parental species. The implications of the origin of diploid gametes, on the genetic diversity of polyploid progenies, are discussed. The biotechnological tools (haplomethods, chromosome doubling by chemical treatments, somatic hybridization, and cytogenetic/molecular tools for polyploid genome studies) used to optimize ploidy manipulation are presented. The interest of haploid and polyploid genotypes for basic genetic and genomic

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studies is discussed. The research areas reviewed are as follows: haploids and doubled haploids for genome sequencing and haplotyping, centromere mapping from unreduced gametes, marker–trait association study in polyploids, and phenome and gene expression in polyploids with a special focus on polyploidy and adaptation. Finally, we give an overview of the recent advances of concrete polyploid citrus breeding programs in China, Florida, and the Mediterranean Basin.

6.1 Introduction

Polyploidy is a major component of angiosperm evolution (Grant 1981; Soltis et al. 2003; Wendel 2000; Otto and Whitton 2000). Many plant species result from autopolyploidization or allopolyploidization events and polyploidization should be considered as the most common of the sympatric speciation mechanism (Otto and Whitton 2000). Most *Citrus* species and related genera are diploid with a basic chromosome number $x = 9$ (Krug 1943). However, Longley (1925) was the first to identify, in 1925, a polyploid wild form: the tetraploid Hong Kong kumquat (*Fortunella hindsii* Swing.). Tetraploid strains of *Poncirus trifoliata* (Iwasaki 1943), triploid limes (Jackson and Sherman 1975), allotetraploid *Clausena excavata*, tetraploid *Clausena harmandiana*, and hexaploid *Glycosmis pentaphylla* are other examples of natural polyploids found in the Aurantioideae subfamily germplasm. In relation to the objectives of citrus genetic improvement, ploidy manipulation became an important component of citrus genetics and breeding. Indeed, triploidy generally induces a high level of male and female sterility, resulting, when coupled with parthenocarpy, in the production of seedless fruits (Ollitrault et al. 2008). Seedlessness is a very important objective mostly not only for the fresh-fruit market but also for the juice industry. Several agronomical evaluation and physiological studies revealed that tetraploid rootstock displayed interesting behavior, particularly for adaptation and resilience to abiotic stresses (Mouhaya et al. 2010; Podda et al. 2013; Allario et al. 2013; Dutra de Souza et al. 2017; Oliveira et al. 2017; Oustric et al. 2017, 2018). These characteristics are very interesting for citrus adaptation to climate change and increased environmental instability. In this chapter, we make a review of (i) the recent knowledge acquired on the natural mechanisms of citrus polyploidization and tetraploid meiosis and their implications on the genetic structures of polyploid citrus populations, (ii) the biotechnological tools to optimize polyploidy manipulation, (iii) the interest of haploid and polyploid germplasm for basic genetic and genomic studies, and (iv) the recent advance of the concrete polyploid citrus breeding programs over the world.

6.2 Natural Mechanisms of Polyploidization and Their Implications on the Genetic Diversity of Polyploid Populations

For a long time, chromosome doubling was considered as the major mechanism leading to polyploidy and the hypothesis of Winge (1917), considering that allotetraploid species resulted from chromosome doubling of interspecific diploid hybrids was agreed as a general concept. Most authors as Stebbins (1971) considered that meiotic restitution played only a minor role in the evolution of polyploid complexes. However, Harlan and de Wet (1975) argued that spontaneous chromosome doubling must be relatively rare while polyploidization arising from 2n gametes seems much more frequent. These conclusions are now assumed by numerous plant evolutionists (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998, 2002). Interestingly, both mechanisms occur in citrus and contribute to actual breeding programs based on ploidy manipulation. The different meiotic mechanisms susceptible to producing diploid gametes strongly affect their genetic structures and diversity and therefore the breeding efficiency. During the last 10 years, the implementation of molecular tools allowing to infer allele

doses in polyploid citrus resulted in a deep knowledge of these mechanisms and the associated genetic diversity of diploid gamete populations.

6.2.1 Chromosome Doubling in Apomictic Lines

The production of tetraploid plants in polyembryonic citrus seedlings was described by several authors. Lapin (1937) found tetraploid seedlings among eight *Citrus* species (<1 to 5.6%) and *Poncirus* (4%). Russo and Torrisi (1951) detected tetraploid forms of *C. aurantium* and *C. limon*. Cameron and Frost (1968) observed that 2.5% of nucellar seedlings from a broad range of citrus cultivars were tetraploid. From a systematic search of autotetraploids in a wide range of taxa, Barrett and Hutchison (1978) postulated that the ability to produce such autotetraploid seedlings is a variable genetic trait present in polyembryonic *Citrus* and relatives. They also proposed that the rates of tetraploid seedlings are affected by environmental conditions. It was confirmed in a more recent study using seeds of numerous rootstock and cultivars coming from the tropical, subtropical, and Mediterranean area (Aleza et al. 2011). The authors concluded that colder conditions of marginal climatic areas appeared favorable for the expression of tetraploidization and the higher rate of tetraploid seedlings was observed for Spanish seeds of Carrizo citrange. The effect of cold on polyploidization events seems to be a general rule both in plants and animals (Otto and Whitton 2000; Ramsey and Schemske 1998). Chromosome doubling of nucellar cells was proposed as the general mechanism by Cameron and Frost (1968). Indeed, these tetraploid seedlings are homogenous and do not display traits of the pollen parents in controlled crosses. Moreover tetraploid and diploid plantlets can emerge from the same polyembryonic seed and no ploidy chimerism is observed in tetraploid plants (Aleza et al. 2011). The maternal tissue origin was verified by systematic studies with SSR markers in a wide range of tetraploid plants produced by polyembryonic citrus types (Aleza et al. 2011). Chromosome doubling in meristem has been observed and should lead to chimeric shoots and branches. However, very few tetraploid bud sports have been identified probably due to unfavorable competition between diploid and tetraploid cells (Iwamasa and Nito 1988).

6.2.2 Unreduced Gametes

Meiotic aberrations related to spindle formation, spindle function, and cytokinesis can lead to unreduced gamete formation in plants. Up to seven major mechanisms of 2n gamete formation have been cytogenetically characterized as follows: premeiotic doubling, first-division restitution (FDR), chromosome replication during the meiotic interphase, second-division restitution (SDR), post-meiotic doubling, indeterminate meiotic restitution, and apospory (Peloquin et al. 1989; Lim et al. 2004; Dewitte et al. 2012). An FDR 2n gamete contains non-sister chromatids while an SDR 2n gamete contains two-sister chromatids (Bretagnolle and Thompson 1995; Tavoletti et al. 1996). The rate of 2n gametes is under strong genetic control and is often determined by a single locus (Ramsey and Schemske 1998). Sexual polyploidization has been widely used in several cultivated plant breeding programs (Dewitte et al. 2012; Ramanna and Jacobsen 2003), including citrus (Ollitrault et al. 2008; Aleza et al. 2010; Cuenca et al. 2010; Recupero et al. 2005). The origin of 2n gamete greatly impacts the gametic structures and, therefore, the polyploid populations and breeding strategy efficiency. Under FDR, non-sister chromatids retain the parental heterozygosity from the centromere to the first crossover point, and hence the gametes transfer a large part of its parental heterozygosity to the progenies. Under SDR, the two-sister chromatids are homozygous between the centromere and the first crossover (Bretagnolle and Thompson 1995), and the resultant gametes have reduced levels of heterozygosity compared with FDR gametes (Fig. 6.1). Several studies based on genetic markers indicate that FDR gametes

transmit 70–80% of the parental heterozygosity, whereas this is about 30–40% for SDR (Dewitte et al. 2012; Barone et al. 1995; Maas 1998; Crespel and Gudin 2003). Thus, a tighter distribution is expected in FDR-derived populations than in SDR ones because a higher percentage of the parental genome is transferred intact, resulting in a more uniform gamete production (Maas 1998). Premeiotic doubling produces 2n gametes equivalent to the meiosis of doubled-diploid genotypes (see below). In the case of post-meiotic doubling (PMD), haploid gametes undergo an extra round of genome duplication, leading to the formation of fully homozygous 2n gametes (Ramanna and Jacobsen 2003; Cuenca et al. 2015; De Storme and Geelen 2013).

Triploid citrus hybrids arising from 2n megagametophytes were described in the 1970s (Esen and Soost 1971; Geraci et al. 1975). According to the genotypes, the frequency of duplication in the female gametes varies between less than 1% to more than 20% (Iwamasa and Nito 1988; Aleza et al. 2010; Soost 1987). The association of triploid embryos with pentaploid endosperm was a strong indication that triploid hybrids resulted from the fertilization of unreduced ovules by normal haploid pollen (Esen et al. 1979). From cytogenetic evidences, it was proposed that SDR was the mechanism producing 2n ovules (Esen et al. 1979). Cuenca et al. (2011) developed a method based on multilocus half tetrad analysis at the population level. The estimation of allele doses of codominant molecular markers along a linkage group allowed to simultaneously evaluate the better model between SDR and FDR and to locate the centromere on the linkage group, taking into account different models of chromosome interference. Indeed, under FDR or SDR, the parental heterozygosity restitution is a direct function of the crossing over frequency between the considered locus and the centromere (Fig. 6.1). SDR was found as the mechanisms of 2n ovules formation in Fortune mandarin (Cuenca et al. 2011) and in clementine (Aleza et al. 2015). The same method was applied by Aleza et al. (2015) to

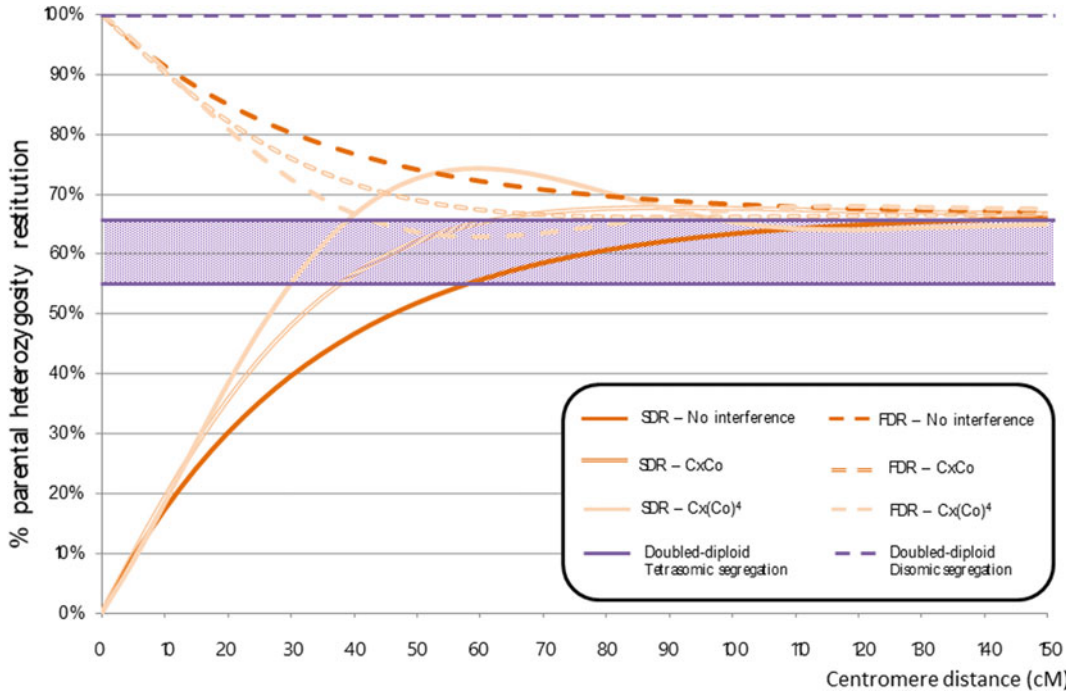

Fig. 6.1 Implication of meiotic mechanisms on polyploid population structure CxCo and Cx(Co)4: two partial interference models (from Cuenca et al. 2011)

locate the centromere on the nine linkage groups of the clementine reference genetic map (Ollitrault et al. 2012a). Taking advantage of the centromere location, a maximum likelihood method based on independent centromeric loci was developed to test the FDR/SDR hypotheses both at population and individual level (Cuenca et al. 2015). Based on this new method, SDR was found predominant for 2n megagametophytes of 19 mandarin varieties (Cuenca et al. 2015; Aleza et al. 2015) while FDR was the major mechanism for the production of 2n pollen in a clementine x sweet orange hybrid (Rouiss et al. 2017a). SDR was also found to be the mechanism underlying formation of the 2n megagametophytes in Nadorcott tangor (Xie et al. 2014a). In lemon, SDR was found as a predominant mechanism for 2n ovule formation associated with a few percents of SDR and post-meiotic genome doubling events resulting in fully homozygous diploid gametes (Rouiss et al. 2017b).

6.2.3 Tetraploid Meiosis

The genetic structure of diploid gamete populations from tetraploid parents and particularly the parental heterozygosity restitution (PHR) depends on the preferential chromosome pairing and the double reduction rate. There are two extreme models, i.e., disomic in allotetraploids and tetrasomic in autotetraploids (Stift et al. 2008; Sybenga 2012; Stebbins 1947). In allotetraploids resulting from the merger of two species genomes, there are two sets of homologous chromosomes. Each chromosome pairs only with its homologous form during meiosis and only bivalents are formed (Stebbins 1947). This results in the disomic inheritance with 100% of the interspecific heterozygosity transmitted by each gamete (Stift et al. 2008). In Aurantioideae subfamily, such pattern was revealed for the tetraploid *Clausena excavata* (Froelicher et al. 2000). In autotetraploids, the presence of four homologous chromosomes instead of two result in equal opportunities to pair at meiosis, leading to multivalent formation and tetrasomic inheritance (Jackson and Jackson 1996). For doubled diploids, it hypothetically leads to 66% restitution of the heterozygosity (Aleza et al. 2016; Sanford 1983) in the absence of double reduction. In cases where parents are divergent but have retained enough homology to prevent exclusive preferential pairing, inheritance patterns intermediate between di- and tetrasomic can be expected (Stift et al. 2008; Stebbins 1947; Sybenga 1996; Jeridi et al. 2012). Stift et al. (2008) developed a likelihood-based approach to evaluate whether disomic, intermediate or tetrasomic inheritances best fitted the segregation of genetic markers and to estimate preferential pairing and double reduction (DR) rates. This method was simplified for doubled-diploids by Aleza et al. (2016) and was applied to analyses of the meiotic behavior of doubled-diploid clementines (Aleza et al. 2016) and Mexican lime (Rouiss et al. 2018). Doubled-diploid clementine largely exhibited tetrasomic segregation. However, three linkage groups have intermediate segregation and one had a tendency for disomy (Aleza et al. 2016). The comparison of the diploid gametes produced by the doubled-diploid clementine and unreduced 2n gametes of diploid clementine revealed complementary genetic variability. Interploid 4x × 2x hybridization were potentially more efficient for developing new cultivars phenotypically closer to the diploid clementine than sexual hybridization through SDR 2n gametes. Conversely, 2x × 2x triploidization has the potential to produce novel products for market segmentation strategies. The doubled-diploid Mexican lime (an interspecific *C. micrantha* × *C. medica* natural hybrid) had predominantly disomic segregation, producing interspecific diploid gamete structures with high *C. medica*/*C. micrantha* heterozygosity and limited effective interspecific recombination (Rouiss et al. 2018). It should allow the efficient transfer of complex traits and multi-traits phenotypes of the parental diploid Mexican lime to triploid progenies. The meiotic inheritance patterns were also evaluated in citrus allotetraploid somatic hybrid between Nova tangelo and "HB" pummelo through cytogenetic observation of meiosis and segregation analysis using 18 SSR markers (Xie et al. 2015). Meiotic analysis of

pollen mother cells (PMCs) showed a high rate of quadrivalents (31.6%) in the tangelo + pummelo hybrid although bivalents were predominant (57.6%). For segregation analysis, the meiotic chromosome pairing behaviour was analyzed by genotyping a triploid population derived from a cross between diploid Nadorcott tangor and this allotetraploid using the microsatellite DNA allele counting—peak ratios (MAC-PR) method. In agreement with the cytogenetic analysis, a mixture of disomic, tetrasomic, and intermediated inheritance patterns was deduced. 23.8% intra-parental and 76.2% interparental heterozygosity was transmitted (Xie et al. 2015). Similar intermediate inheritance was also observed for an allotetraploid *C. deliciosa* + *C. limon* somatic hybrids with restitution of interparental heterozygosity values ranging from 54 to 79% according to the chromosome (Kamiri et al. 2011).

6.3 Biotechnology and Ploidy Manipulation

6.3.1 Gametic Embryogenesis

An interesting kind of in vitro embryogenesis in the plant kingdom is the "gametic embryogenesis", the phenomenon for which an immature plant gametophyte can switch from its normal developmental gametophytic pathway toward a sporophytic one. Because such embryos are derived from the regeneration of gametes (products of meiotic segregation), this occurrence permits to achieve, in a single step, the homozygosity at all loci, and, consequently, this biotechnological technology constitutes a powerful tool in breeding programs of many crops. Resultant haploids (Hs) are sporophytes with the gametophytic chromosome number (n instead of 2n) while the doubled haploids (DHs) are haploids that went through chromosome doubling, in a spontaneous or stimulated manner (Yahata et al. 2015). Haploid technology and gametic embryogenesis are valuable for breeding programs of perennial crops, that cannot be submitted to several cycles of selfing, improving their efficiency and speed. Particularly mutation, in vitro selection during gametic embryogenesis, marker-assisted selection (MAS), gametoclonal variation, genetic analysis, transformation, and genome sequencing can take advantages of them (Aleza et al. 2009a; Germanà et al. 2013). The biotechnological method to reach haploidization can be distinguished in "gynogenesis" (when originates from the female gamete) and "microspore embryogenesis" (when it begins from the immature male gamete, the microspore). After embryo or callus recovery, ploidy analysis (usually by flow cytometry), and control of homozygosity by isozyme analyses (Germanà et al. 1992, 1994, 2000a, b; Germanà and Recupero 1997; Deng et al. 1992a; Ollitrault et al. 1996a), RAPD markers and microsatellites (Germanà and Chiancone 2003; Germanà 2006; Germanà et al. 2005; Cao et al. 2011; Cardoso et al. 2014, 2015; Wang et al. 2015) have to be performed to distinguish between somatic (anther and ovule culture), zygotic (ovule culture), and gametic origins.

The in situ production of an embryo from an egg cell without fertilization can be stimulated by pollination with irradiated pollen (Aleza et al. 2009a; Ollitrault et al. 1996a; Froelicher et al. 2007) (Fig. 6.2), or by in situ or in vitro parthenogenesis triggered by pollen from a triploid plant (Germanà 2012). The irradiated pollen or pollen grains from a triploid plant stimulate the development of haploid embryoids from ovules. Combined with pollination of an in vitro cultured gynoecium of *Citrus clementina* Hort. ex Tan., cv. Nules, it permitted haploid plantlet regeneration (Germanà and Chiancone 2001). Three haploid plants were produced from in vivo crosses of two monoembryonic diploids (clementine and Lee) × a triploid hybrid of Kawano natsudaidai (*C. natsudaidai* Hayata) (Oiyama and Kobayashi 1993). Nine haploid plantlets and two embryogenic callus lines in *C. clementina* cv. SRA 63 were produced by in situ pollination by pollen of Meyer lemon (*C. meyeri* Y. Tan.) irradiated at 300, 600, and 900 Gray (Gy) at the rate of 6 Gy/min from a cobalt 60 source (Ollitrault et al. 1996a). Pollination with gamma-ray-irradiated (150, 300, 600, and

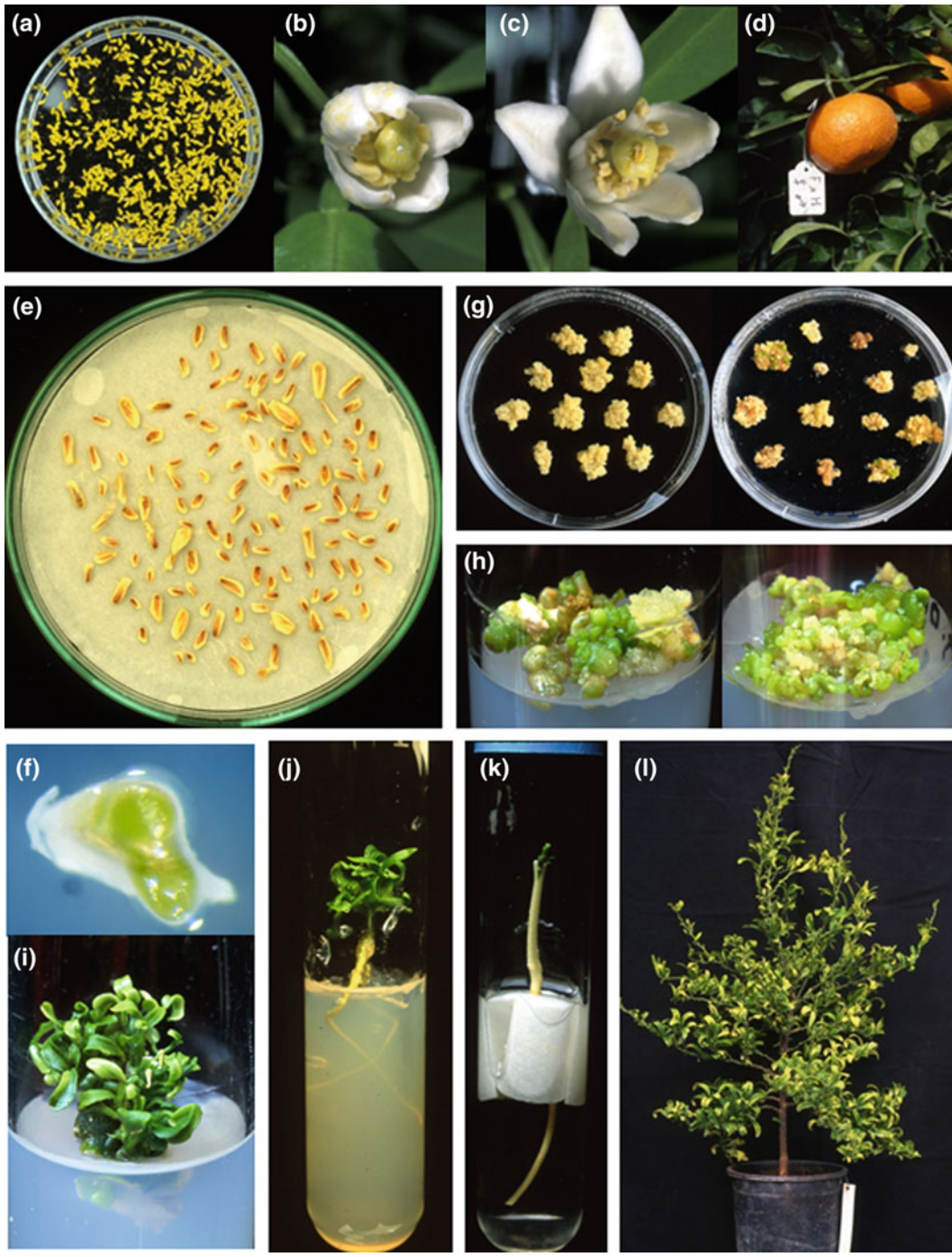

Fig. 6.2 Gynogenesis induced by irradiated pollen in citrus. **a** Irradiated pollen of Fortune mandarin. **b** Receptive flower in anthesis of Clemenules clementine. **c** Flower pollinated with irradiated pollen. **d** Mature fruit ready to collect seeds. **e** Small seeds recovered from pollination with irradiated pollen. **f** Embryo contained in small seeds. **g** Embryogenic calli originated from in vitro embryo culture. **h** Cluster of embryos obtained from embryogenic calli. **i** Shoots produced by embryos regenerated from embryogenic calli. **j** Regenerated plant from direct germination of embryo without a callus phase. **k** In vitro micrograft of haploid shoot. **l** Haploid plant established in the greenhouse

900 Grays) pollen of Meyer lemon, followed by embryo rescue, enabled the regeneration of haploid plants in Fortune (*C. clementina* Hort ex Tan. × *C. tangerina* Hort. ex Tan.) and in Ellendale (*C. reticulata* Blanco × *C. sinensis* L. Osb.) (Froelicher et al. 2007). Aleza et al. (2009) carried out irradiation of Fortune mandarin pollen with a single dose of 500 Gy gamma rays (cobalt 60), followed by pollination of Clemenules clementine and embryo rescue, and obtained a haploid clementine plant.

Anther and isolated microspore culture (Fig. 6.3) can be the methods to induce microspore embryogenesis in the majority of crops. Several descriptions on *Citrus* microspore embryogenesis are reported (among them Germanà et al. 1992, 1994, 2005; Germanà and Recupero 1997; Germanà and Chiancone 2003; Cao et al. 2011; Cardoso et al. 2014, 2015; Wang et al. 2015; Froelicher and Ollitrault 2000; Germanà 2007, 2009, 2017). Using this method, haploid plantlets from *P. trifoliata* L. Raf. (Hidaka et al. 1979) and *C. madurensis* Lour. (Chen et al. 1980) have been regenerated; one doubled haploid plantlet has been achieved from anther culture of a *C. ichangensis* × *C. reticulata* hybrid (Deng et al. 1992a); haploid plantlets and highly embryogenic haploid calli of *C. clementina* (Germanà et al. 1994, 2000a, b, 2005; Germanà and Chiancone 2003); albino embryos of Mapo tangelo (*C. deliciosa* x *C. paradisi*) (Germanà and Recupero 1997); haploid and diploid calli, embryos and leafy structures but no green plants of *C. limon* L. Burm. f. (Germanà et al. 1992); haploid embryos of *Clausena excavata* (Froelicher and Ollitrault 2000) have been produced. However, it should be noted that most of regenerants from clementine anther culture, analyzed by flow cytometry, resulted in tri-haploid, instead of haploid or doubled haploid as supposed (Germanà et al. 2005). Anther culture of sweet orange (*C. sinensis*) yielded short-lived homozygous plantlets for cv. Rhode Red Valencia (Cao et al. 2011) as well as homozygous line from Early Gold and Rhode Red Valencia (Wang et al. 2015). Homozygous callus was obtained from a hybrid between *C. clementina* × *C. sinensis* Hamlin by Cardoso et al. (2014) and, more recently, Cardoso et al. (2015) obtained homozygous and microspore-derived embryos (triploid) from two clementines, specifically Hernandina and Corsica, and somatic embryos from anther culture of three cultivars of Tarocco and Moro. The microspore isolation culture, carried out through the elimination of the somatic anther tissue (by squashing or blending anthers, centrifugating, and filtrating), has many advantages such as the direct control of the process. It required more sophisticated instruments and expertises, and for this reason the studies in *Citrus* are not as much as the ones of anther culture (Chiancone et al. 2015; Cimò et al. 2016; Germanà et al. 1996). Multinucleated calli and homozygous early microspore-derived embryos at different stages were obtained in *C. clementina* cvs. "Monreal Rosso" and "Nules" (Chiancone et al. 2015), in mandarin (*C. reticulata* Blanco), cv. Tardivo di Ciaculli (Cimò et al. 2016) and in *C. sinensis* 2cv. Moro (Germanà, unpublished). Different factors, such as genotype, physiological status of donor plants, gamete developmental stage, pretreatment of the flower buds, culture medium, conditions of incubation, and their interactions, influence the response of the in vitro culture (Atanassov et al. 1995; Smykal 2000; Datta 2005; Germanà 2011a, b; Wang et al. 2000). Moreover, the requirements are variable according to the different cultivars within one species (Germanà and Recupero 1997; Germanà and Chiancone 2003; Germanà 2003, 2006, 2011a, b; Cardoso et al. 2014, 2015; Wang et al. 2015; Chiancone et al. 2006, 2015; Germanà et al. 1996; Chiancone and Germanà 2016).

Although research on gametic embryogenesis and haploid and doubled haploid production in *Citrus* is not very recent, many aspects of the protocols are not clear and conclusive, especially the response linked to the genotype and to the season. For this reason, it is opportune that further efforts will be focused to overcome the bottlenecks of this important biotechnological tool for genetic/genomic studies and breeding programs.

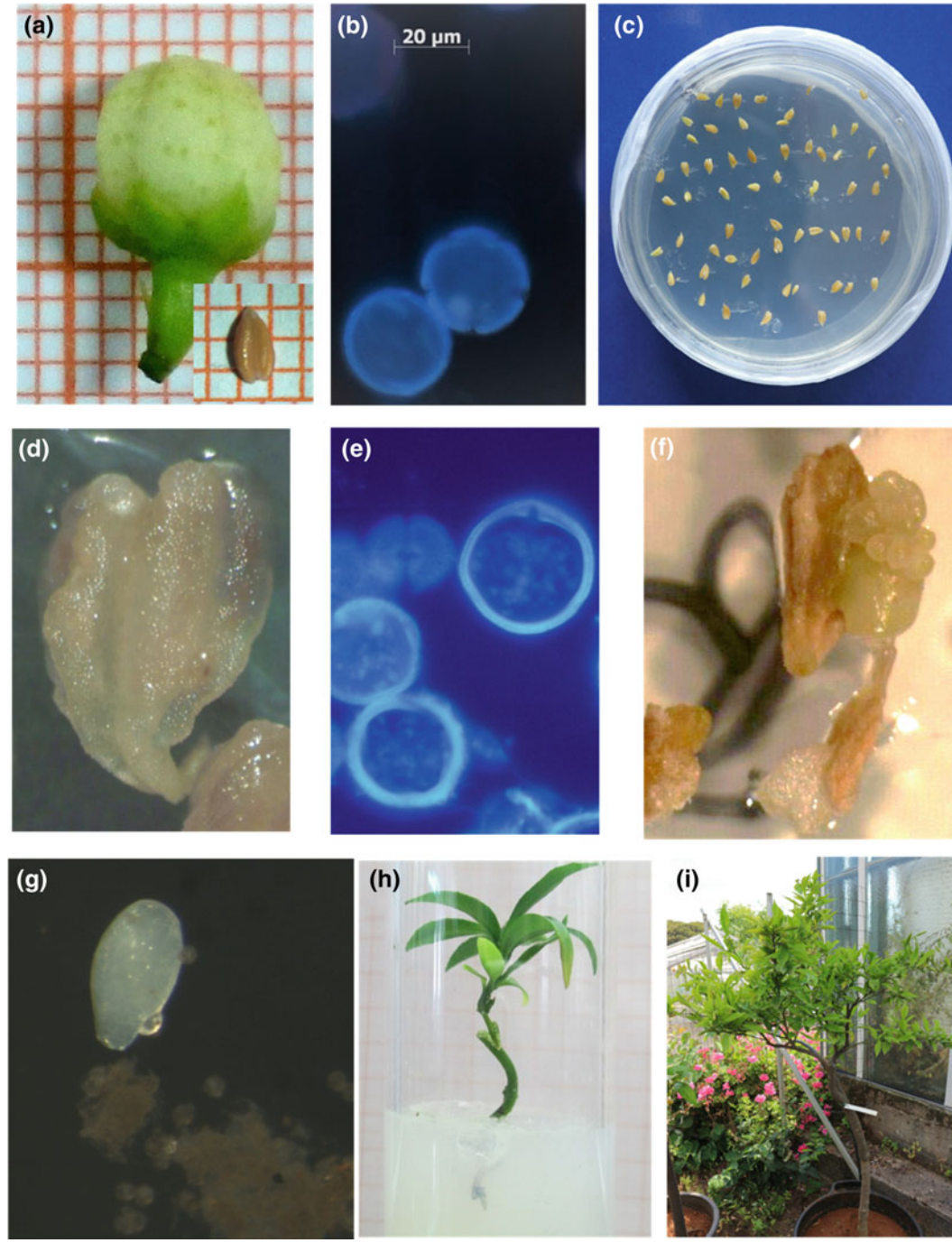

Fig. 6.3 Microspore embryogenesis through anther and isolated microspore in *C. clementina* **a** Flower bud and anthers sizes of clementine, with vacuolated microspores. **b** Vacuolated microspores (DAPI staining). **c** Petri dish with anthers just after the culture. **d** Clementine anther after 1 month of in vitro culture. **e** Multinucleated pollen grains (DAPI staining) after 7 months of culture. **f** In vitro anther culture of *C. clementina* cv. Monreal, with microspore-derived embryos. **g** Gametic embryogenesis, through isolated microspore culture: an early microspore-derived embryo of *C. clementina* cv. Nules, after 11 months of culture. **h** In vitro grafting of small shoot apexes (2–3 mm) of homozygous clementine regenerants onto etiolated 20-day-old Troyer citrange seedlings. **i** Homozygous clementine Nules regenerated in 1998 and grafted in 2000 onto Troyer citrange seedlings

6.3.2 Chromosome Doubling by Chemical Treatments

Besides the exploitation of 2n gametes, the use of tetraploid parents for triploid cultivars generation by interploid crosses (2x × 4x and 4x × 2x) or tetrazyg rootstock breeding required the development of a tetraploid gene pool. Many spontaneous doubled diploids were selected from polyembryonic varieties, however their use as female parents was hampered by the apomixes of the obtained tetraploids. Therefore, several teams worked on the production of non-apomictic tetraploid genotypes. The oldest method used to induce tetraploidization in citrus was the in vivo treatment of buds with colchicine (Barrett 1974; Jaskani et al. 1996; Wakana et al. 2005). However, the majority of the obtained plants were cytochimeras without value for plant breeding. Embryogenic calli obtained from ovules cultured in vitro and treated with colchicine and oryzalin were applied successfully (Wu and Mooney 2002; Gmitter and Ling 1991; Gmitter et al. 1991) to obtain solid tetraploid plants from monoembryonic and polyembryonic genotypes. Autotetraploid pummelo plants were obtained through colchicine treatment of germinating seed (Kainth and Grosser 2010). When applied to seedlings or embryogenic calli such method implied a long juvenile phase of the tetraploid plants before using them as breeding parents. To perform colchicine treatment in adult tissue and limit the chimera problem, Oiyama and Okudai (1986) combined colchicine treatments with shoot tip grafting. This approach was improved by Aleza et al. (2009) who used 0.1% colchicine or oryzalin solution to treat shoot tips 7–10 days after grafting and implemented a de-chimerization procedure assisted by flow cytometry. They successfully obtained stable tetraploid plants of Clemenules, Fina and Marisol clementines, and Moncada mandarin from the direct sprout of the shoot tip micrografted and from segregation of 2x–4x cytochimeras. These tetraploid plants have great value as a female parent for triploid breeding programs and allowed to establish large populations of triploid hybrids (Aleza et al. 2012a). Grosser et al. (2014) also recovered colchicine-induced stable autotetraploid plants through indirect organogenesis from stem sections of in vitro-grown zygotic seedlings of pink/red-fleshed pummelos.

6.3.3 Somatic Hybridization

Somatic hybridization is a powerful tool to manipulate ploidy and to increase the genetic variability of plant species. It allows not only overcoming sexual incompatibility between species but also combining nuclear, chloroplastic, and mitochondrial genomes in new patterns. It appears particularly well adapted for citrus with regards to breeding system constraints (apomixis, sterility, inter-specificity, and high heterozygosity level) and the interest for ploidy manipulation.

In citrus, somatic hybridization takes advantage of a very efficient in vitro regeneration system by somatic embryogenesis from calluses induced from nucellar tissues (Kochba and Spiegel-Roy 1977), derived cell lines (Cabasson et al. 1995), and protoplasts (Vardi et al. 1982; Kobayashi et al. 1983). Several methods have been developed for protoplast fusion including PEG-induced fusion (Grosser and Gmitter 1990), electrofusion (Ollitrault et al. 1996b; Guo et al. 1998), and electrochemical fusion (Olivares-Fuster et al. 2005).

Since the first citrus somatic hybrid between *C. sinensis* and *P. trifoliata* (Ohgawara et al. 1985) citrus somatic hybridization via protoplast fusion has become an integral part of citrus variety improvement programs worldwide. Tetraploid somatic hybrid plants have been regenerated from more than 200 parental combinations. Applications of somatic hybridization to citrus scion improvement include the production of quality tetraploid breeding parents that can be used in interploid crosses to generate seedless triploids (Grosser et al. 1992, 2000, 2010b; Deng et al. 1992b; Tusa and Fatta del Bosco 1997; Viloria and Grosser 2005) and the direct production of triploids by haploid + diploid fusion (Kobayashi et al. 1997; Ollitrault et al. 2000).

For citrus rootstock improvement, somatic hybridization allows adding completely the

nuclear genomes of the two parents. It is a very interesting feature considering the high heterozygosity level of most citrus rootstock resulting in a high level of segregation in sexual diploid progenies and therefore a low probability to combine the favorable traits of the two parents by sexual crossing. It is expected that symmetric somatic hybridization between complementary diploid rootstock (particularly *Citrus* sp. + *P. trifoliata* combinations) will result in tetraploid rootstocks combining the dominant traits of the two parents. Such strategy was successfully developed by several rootstock breeding programs around the world (Grosser et al. 1994, 2007; Mendes et al. 2001; Motomura et al. 1995; Guo et al. 2007; Grosser and Gmitter 2011; Dambier et al. 2011). Another interest of somatic hybridization for rootstock breeding is the potential to combine citrus with sexually incompatible or difficult to hybridize genera that possess traits of interest (Grosser et al. 1990, 1996; Guo and Deng 1999, 2001). It found an increasing interest with the spread over the world of the Huanglongbing disease (HLB, a bacterial disease due to *Candidatus Liberibacter* sp.) with resistance sources found in sexually incompatible genera such as *Murraya koenigii* (Beloti et al. 2018). Following their first flowering, tetraploid somatic hybrids have also been used as parents to generate tetraploid recombining progenies (tetrazyg strategy, Grosser et al. 2003).

6.3.4 Cytogenetic and Molecular Tools for Polyploid Genome Studies

During the last 20 years, the development of new cytogenetic and molecular tools greatly modified our knowledge on the genetics of polyploid citrus and our capacity for polyploidy breeding. The estimation of the ploidy level by flow cytometry (Ollitrault and Michaux-Ferriere 1992; Ollitrault et al. 1994) constituted the first decisive step. It allowed the screening of large populations to identify spontaneous doubled diploids in polyembryonic seedlings (Aleza et al. 2011). It was also the key to the development of intensive triploid breeding programs, based on 2n gametes in 2x × 2x hybridizations (Aleza et al. 2010) and to identify tetraploids in 4x × 2x progenies. It allowed identifying easily ploidy chimeras in programs aiming to develop doubled-diploid lines by colchicine or oryzalin treatments and to drive de-chimerization processes (Aleza et al. 2009b).

The genetics of polyploid species remained during a long time, less well-known than those of their diploid counterparts. Indeed, the estimation of molecular marker allele copy number has long been considered a challenge for polyploid species with polysomic inheritance, while it is essential to assign the allelic configuration of heterozygotes for accurate population genetic studies. Moreover, allelic dosage can affect gene expression and phenotype and its determination is therefore particularly important for marker–trait association studies (De Jong et al. 2003). Several techniques have been used to estimate allele dosage in polyploid genotypes or tissues. Direct co-dominant interpretation of microsatellite loci based on relative polymerase chain reaction (PCR) product intensities has been reported in different plants (Landergott et al. 2006; Martins et al. 2009). The limits of such direct allele doses evaluation are associated with PCR selection caused by differential primer affinity and PCR drift resulting from random events during early cycles of PCR (Wagner et al. 1994). MAC-PR has been proposed as an alternative approach to deal with differential amplification intensities among alleles in polyploid plant species (Esselink et al. 2004). The MAC-PR method requires analyzing all alleles in pairwise comparisons by calculating the ratios between the amplification intensities of two cooccurring alleles. Diploid or tetraploid duplex heterozygous genotypes are suitable to determine the 1:1 ratio that is used as a baseline for allele quantification in the other heterozygous genotypes. This method was successfully transferred in citrus (Cuenca et al. 2011; Ferrante et al. 2010) for the analysis of 2n gamete origin. However, SSR analysis remains relatively costly and time consuming compared with actual SNP genotyping methods. Moreover, with the increasing

availability of expressed sequence tag (EST) databases (Terol et al. 2007) and whole-genome sequences databases (Wu et al. 2014, 2018), SNPs have become the most abundant and powerful polymorphic markers that can be selected throughout the entire genome. Cleaved Amplified Polymorphic Sequences approaches were successfully developed in Japan (Omura and Shimada 2016; Shimada et al. 2014) but remain difficult to apply to polyploid citrus. SNP arrays have been developed for high-throughput studies (Ollitrault et al. 2012b; Fujii et al. 2013) and recently, in California, two Affymetrix Axion SNP arrays with about 1.5 million and 56,000 SNPs were developed (Eck et al. 2016). However, the formats of these arrays are rigid (predefined set of markers, number of sample) and analyses are costly. Efficient SNP genotyping methods have been developed in citrus for scalable experiments using competitive allele amplification (KASPar© technology Garcia-Lor et al. 2013a; Curk et al. 2015). Cuenca et al. (2013b) demonstrate the efficiency of the KASPar SNP genotyping technique, to assign heterozygous allelic configurations within polyploid citrus populations. MAC-PR approach for SSR markers and KASPar technology for SNPs were routinely applied for the study of 2n gamete origin (Cuenca et al. 2015; Aleza et al. 2015; Rouiss et al. 2017a, b), tetraploid meiosis analysis (Aleza et al. 2016; Rouiss et al. 2018; Ollitrault et al. 2016), centromere mapping (Aleza et al. 2015) and marker–trait association in a triploid progeny (Cuenca et al. 2013b). Recently, methods based on re-sequencing of a reduced complexity of the genome were developed in citrus to perform deep scan of the genome in large population. Indeed, methods such as genotyping by sequencing (GBS Oueslati et al. 2017) and DArTseq (Penjor et al. 2014, 2016; Curtolo et al. 2017) are more flexible than array analysis and allow, with a unique step, the discovery of polymorphisms and their genotyping. New pipelines were implemented to decipher the phylogenomic structures of large diploid (Oueslati et al. 2017) and polyploïd (Ahmed et al. 2019) population from GBS data. They should be useful for marker–traits association studies in triploid and tetraploid populations.

6.4 Haploids and Polyploids as Biological Resources for Phenotypic, Genetic, and Genomic Studies

6.4.1 Haploids for Genome Sequencing and Haplotyping

In plants, haploid and doubled haploid lines (H/DH) have been used for physical mapping, genetic mapping (Zhang et al. 2002; Chu et al. 2008; Xu et al. 2015), and for the integration of genetic and physical maps. It is particularly interesting for the new genotyping methodology based on NGS such as RADSeq and GBS. Indeed relatively low sequencing coverage is sufficient without loss of accuracy compared to the coverage necessary for sequencing more common diploid individuals owing to the presence of heterozygous single nucleotide polymorphisms (Zhang et al. 2018; Szarejko and Forster 2007). Haploid and double haploid plants are homozygous and therefore can reveal recessive alleles resulting from mutagenesis experiments or natural mutations (Szarejko and Forster 2007). In citrus, the difficulty to obtain haploid or doubled haploid lines and their frequent weakness precluded their utilization at population level. However they were decisive resources for de novo whole-genome sequencing (WGS) projects. Indeed, due to their complete homozygosity haploid and double haploid plants play an increasingly important role in WGS projects, where homozygosity is a particular advantage. Polymorphism in a whole-genome sequence complicates the assembly process, displays lower quality and assembly contiguity, and completeness is significantly lower than would have been expected in the absence of heterozygosity (Vinson et al. 2005; Huang et al. 2017). Haploid and

doubled haploid plants were used to generate de novo reference sequences of plant and animal species such as banana (D'Hont et al. 2012), apple (Daccord et al. 2017), fugu fish (Zhang et al. 2014), and yellowtail fish (Iwasaki et al. 2016). Commercial citrus varieties being characterized by high heterozygosity (Curk et al. 2015; Garcia-Lor et al. 2013b), the International Citrus Genome Consortium (ICGC) decided to establish the reference sequence of the Citrus genome from a homozygous genotype derived from haplomethods. Among several haploid, doubled haploid and tri-haploid lines of clementine obtained by induced gynogenesis and anther culture (Germanà et al. 2013), the ICGC selected a haploid line from IVIA (Spain). This haploid resulted from in situ gynogenesis of Nules clementine induced by irradiated pollen (Aleza et al. 2009a). A high-quality reference genome assembled in nine pseudomolecules was established from this line combining Sanger and NGS sequencing methods (Wu et al. 2014). In the same period, a Chinese consortium produced a draft genome of sweet orange (Xu et al. 2013) from doubled haploid line derived from the anther culture of Valencia sweet orange (Cao et al. 2011). More recently a de novo assembly of *C. maxima* was performed with a pummelo haploid line (Wang et al. 2017). The sequencing of haploid and doubled haploid citrus lines provides very useful haplotypic data to be used for phylogenomic and functional genomics.

6.4.2 Centromere Mapping from Unreduced Gametes

Centromeres constitute a very important component of sexual reproduction. They mediate chromosome segregation at mitosis and meiosis, provide the proteinaceous kinetochore, promote sister chromatid cohesion, and suppress recombination. Centromere mapping allows the development of improved linkage maps, the identification of chromosome arms, and the analysis of crossover interference. Centromeres can be localized using half-tetrad analysis (HTA) of unreduced (2n) gametes. FDR and SDR mechanisms result in the opposite pattern of parental heterozygosity restitution (PHR). However, for both models PHR is a direct function of the genetic distance to the centromere (Tavoletti et al. 1996; Zhao and Speed 1998). Therefore, molecular marker analysis of 2n gamete population is a powerful means to locate centromeres genetically (Kauffman et al. 1995; Park et al. 2007; Nie et al. 2012). Tavoletti et al. (1996) developed a multilocus maximum likelihood method of HTA assuming complete chromosome interference. Cuenca et al. (2011) proposed an alternative approach to locate the positions of the centromeres in linkage groups, based on functions describing the PHR along a chromosome in relation to locus-centromere genetic distance (derived from Zao and Speed 1998), allowing different chromosome interference models. It was applied to triploid hybrids resulting from unreduced ovule of Fortune mandarin to locate the chromosome II centromere (Cuenca et al. 2011). Aleza et al. (2015) extended the study and genetically located the centromeres of the nine citrus chromosomes by SSR and SNP genotyping of a triploid population derived from SDR 2n ovules of clementine. The inferred physical locations of centromeres revealed one acrocentric, four metacentric, and four submetacentric chromosomes. Identification of the centromere positions paved the way for developing a simple maximum likelihood method to determine the mechanism of unreduced gamete formation in a large range of genotypes using centromeric markers (Cuenca et al. 2015). It was successfully applied for the analysis of the origin of 2n ovules and pollens (Rouiss et al. 2017a, b). The centromere location is also useful to model the inheritance of phenotypic traits controlled by single gene in population issued from unreduced gametes, according to the gene-centromere genetic distance (Cuenca et al. 2013a).

6.4.3 Marker–Trait Association Study: The *Alternaria alternata* Recessive Resistance Gene

Genetic analysis of phenotypical traits and marker–trait association in polyploid species is generally considered as a challenge due to complex segregation, dosage effects, and eventually potential non-Mendelian inheritance associated with epigenetic variations (Osborn et al. 2003; Bottani et al. 2018). The main genetic factor affecting trait inheritance in triploid or tetraploid families is the origin of diploid gametes, with significant differences between the sexual polyploidization approach (2x × 2x crosses with unreduced—2n—gamete formation) and interploid crosses (2x × 4x or 4x × 2x). In sexual polyploidization, two factors affect the transmission of parental heterozygosity to the offspring: the mechanism of 2n gamete formation (i.e., FDR or SDR) and the genetic distance from the locus of interest to the centromere (Cuenca et al. 2011). According to the partial chromosome interference model proposed by Cuenca et al. (2011), PHR is a direct function of the distance between the considered locus and the centromere. Therefore, half-tetrad analysis (HTA) based on 2n gamete genotyping is an efficient means of genetic mapping (Tavoletti et al. 1996; Mendiburu and Peloquin 1979; Douches and Quiros 1987), and may be used to locate genes controlling useful traits. Under the partial chromosome interference model, from the centromere to the telomere PHR vary from 0 to 0.66 and 1 to 0.66 for SDR and FDR, respectively (Fig. 6.1; Cuenca et al. 2011). Recent studies have revealed that SDR is the main mechanism involved in unreduced ovule formation in the majority of citrus cultivars (Cuenca et al. 2011; Aleza et al. 2015; Rouiss et al. 2017b). For interploid crosses, most of the tetraploid parents used in citrus breeding arise from chromosome doubling in nucellar cells of apomictic diploid parents (Aleza et al. 2011). According to the phylogenomic structures of the parental diploid line, the frequency of diploid gametes that receive a locus in heterozygosity from the tetraploid parent varies for autotetraploid with strict tetrasomic inheritance between 0.55 and 0.66, depending on the double-reduction frequency (Marsden et al. 1987). For intermediate segregation to disomic segregation for strict allotetraploids, it should vary between 0.66 and 1. Preferential disomic inheritance such as the one observed for tetraploid Mexican lime (Rouiss et al. 2018) strongly limits the efficient recombination rate and therefore the accuracy of genetic mapping and QTL analysis. The first marker–traits analysis performed in polyploid segregating citrus progeny concerned *Alternaria alternata* resistance gene. Previous results in diploid progenies suggested that resistance was controlled by a single recessive gene (Dalkilic et al. 2005; Gulsen et al. 2010). It was confirmed by the observed frequency of resistant triploid hybrids issued from different interploid hybridizations (Cuenca et al. 2013b). The location of the considered gene was performed in a triploid progeny issued from 2n ovules of Fortune mandarin fertilized by Willowleaf mandarin. Bulk segregant analysis, coupled with genome scan using a large set of genetically mapped SNP markers, followed by a targeted genetic mapping by half tetrad analysis, using SSR and SNP markers, allowed locating a 3.3 Mb genomic region linked to ABS resistance near the centromere of chromosome III. This genomic area was defined by two flanking markers at 3.77 and 1.71 cM of the ABSr locus. A third marker did not exhibit any recombination with the ABSr locus within the analyzed population. These markers have been used together for efficient marker-assisted selection. The authors concluded that it was possible to use susceptible heterozygous parents for the resistance gene to breed resistant triploid varieties. For instance, the susceptible cultivar Fortune, which is a very efficient female parent in producing high-quality triploid hybrids in 2x × 2x hybridization (Aleza et al. 2010), should not be discarded. Indeed, the 39 and 19% of resistant triploid hybrids produced when crossed with homozygous resistant or heterozygous susceptible diploid genotypes, respectively, are acceptable if combined with early selection. They also argued that when

heterozygous susceptible parents are used as producers of diploid ovules, it is much more efficient to integrate them in a 2x × 2x strategy rather than to use them as doubled-diploid parents in interploid crosses. Indeed, the heterozygosity transmission of the ABSr locus (associated with susceptibility transmission to the triploid progeny) should be lower in the 2n gametes than in the diploid gametes produced by doubled diploids, due to its location close to the centromere of chromosome III and the SDR origin of unreduced ovules in most citrus genotypes.

6.4.4 Phenome and Gene Expression in Polyploids

Allopolyploidization greatly affects the transcriptome regulation and phenome expression (Adams et al. 2004; Comai et al. 2000; Wang et al. 2004; Flagel and Wendel 2010). Particularly, nonadditive inheritance of gene expression appears frequent (He et al. 2003; Hegarty et al. 2006; Wang et al. 2006; Flagel et al. 2008; Li et al. 2015). Neo-regulation of parental genome expression in allopolyploid plants would partially explain their higher adaptability and why they often give rise to new phenotypes, exceeding the variability range of the diploid gene pool. Somatic hybrids allow combining genomes without sexual recombination and are interesting models to study the immediate effect of allopolyploidization on the regulation of gene expression and subsequent phenotypic variation. Allotetraploid citrus from somatic hybridization displays certain transgressive morphological vegetative traits (leaf thickness, stomata density, size, etc.) similar to doubled diploids arising from chromosome doubling of nucellar cells, that can be associated with tetraploidy per se (Ollitrault et al. 2008). However, the inheritance of other traits is clearly linked with the parental combinations with codominance or dominance of one or the other parent according to the traits under consideration (Bassene et al. 2009a). Allotetraploid hybrids between *C. deliciosa* (Willowleaf mandarin) and six other citrus species (*C. limon*, *C. aurantifolia*, *C. sinensis*, and *C. paradisi*), *P. trifoliata* and *F. margarita* were used for phenome, proteome, and transcriptome analyses. Leaf volatile compounds of these six allotetraploid hybrids were analyzed by GC–MS (Gancel et al. 2003). Dominance of mandarin traits was observed. Interestingly, the synthesis of molecules was not present in the mandarin, but at high concentrations in the non-mandarin parents, was repressed in all the somatic hybrids that displayed an absence of monoterpene aldehydes and monoterpene alcohols, and very low levels of sesquiterpene hydrocarbons, sesquiterpene alcohols, and sesquiterpene aldehydes (β- and α-sinensals). The leaf proteomes of two allotetraploid somatic hybrids combining *C. deliciosa* with *C. aurantifolia* and *F. margarita* were analyzed by 2D electrophoresis (Gancel et al. 2006). The two allotetraploid hybrids were closer to their mandarin parent than to their other parents in terms of presence/absence of protein spots as well as at a quantitative expression level. Seventy-five percent of the protein spots specific to the non-mandarin parent were silenced in the somatic hybrids. Moreover, 14 and 29% spots of the *C. deliciosa* + *C. aurantifolia* and *C. deliciosa* + *F. margarita* hybrids, respectively, were not encountered in their parental genotypes, suggesting a de-repression of the corresponding genes or/and alternative splicing in the allotetraploids. A gene expression analysis on fruit pulp of a somatic hybrid between *C. reticulata* cv Willowleaf mandarin + *C. limon* cv Eureka lemon with a Citrus 20 K cDNA microarray (Bassene et al. 2010) revealed around 4% of transcriptome divergence between the two parental species. The genes under-expressed in mandarin compared to lemon were also repressed in the allotetraploid. When genes were over-expressed in *C. reticulata* compared to *C. limon*, the allotetraploid genes expression distribution was much more equilibrated with evidence of transgressive overexpression as well. This led to global dominance of the mandarin transcriptome. The potential implication of nonadditive gene expression on the phenotype of citrus somatic hybrids was illustrated with the Willowleaf

mandarin + Eureka lemon combination by Bassene et al. (2009) by analyzing the carotenoid and ABA contents and the expression of the genes of the carotenoid/ABA biosynthesis pathway. Xu et al. (2014) considered citrus somatic hybrid as a system to study rapid structural and epigenetic reorganization in allotetraploid genomes. They found that allotetraploid hybrids mainly have the AFLP and MSAP banding patterns containing specific bands from both parents plus some alterations. The frequency of alteration of the AFLP bands in allotetraploids displayed a range 4.61–7.88%, while from 12.50 to 15.67% of the sites were methylated. In addition, the proportions of callus-parent-specific DNA structure and methylation alterations were much greater than those of leaf-parent-specific alterations in the somatic hybrids.

A few studies analyzed the impact of the tetraploidy per se on the transcriptome and metabolome through the comparison of doubled diploid and their parental diploid line. Allario et al. (2011) observed large phenotypic differentiation in 4x Rangpur lime compared with 2x. Growth of 2x was more vigorous than 4x although leaves, stems, and roots of 4x plants were thicker and contained larger cells than 2x that may have a large impact on cell-to-cell water exchanges. Leaf water content was higher in 4x than in 2x. These phenotypic variations were associated with limited changes in genome expression (<1% of the gene with significant differential expression). Interestingly, five of the differentiated expressed genes were related to water deficit stress response. Similarly, comparative metabolic and transcriptional analysis of a doubled diploid and its diploid *C. junos* cv. Ziyang xiangcheng suggested its potential value for stress resistance improvement (Tan et al. 2015). Tetraploidization induced considerable changes in leaf primary and secondary metabolite accumulation in Ziyang xiangcheng associated with limited effect on the transcriptome (0.8% of differentially expressed genes). Notably, those genes were highly related to stress-response functions, including responses to salt stress, water, and abscisic acid. In order to investigate whether there are common metabolic responses following genome doubling, Tan et al. (2017) performed a comparative metabolomic analysis of mature leaves from doubled diploids and the corresponding diploids of red tangerine (*C. reticulata*), trifoliate orange (*P. trifoliata*), and precocious trifoliate orange (*P. trifoliata*). Polyploidization had a significant but relatively limited influence on the accumulation of metabolites in these citrus species. Primary metabolism takes priority over secondary metabolism in tetraploid plants to relieve the genomic stress encountered during the early stages of genome doubling, probably to promote vitality and growth (Tan et al. 2015, 2017).

6.4.5 Polyploidy and Adaptation

Polyploidy was shown to be a major force of plant evolution (Soltis and Soltis 2009; Chen 2010) that is considered to allow better adaption to environmental constraints. In citrus, polyploidy leads to a wide range of phenotypic differences when compared to diploid. It includes reduced tree size, larger stomata size with lower density, thicker and greener leaves, higher leaf water content, thicker and smaller roots (Allario et al. 2011; Cameron and Soost 1970; Romero-Aranda et al. 1997; Ruiz et al. 2016a, b). The thicker mesophyll in 4x citrus was proposed to result in increased internal diffusive resistances leading to a lower net CO_2 assimilation rate in comparison to 2x plants (Romero-Aranda et al. 1997). Other physiological parameters were shown to be reduced in 4 × such as the transpiration and the stomatal conductance that may thus explain the reduced tree size (Allario et al. 2011; Syvertsen JP 2000). In root, a shorter diameter and a high specific root length lead to greater radial hydraulic conductivities (Lpr). Lpr is considered to be the major limiting factor for water absorption when water is present in the soil, which may, in turn, influence the plant water status and plant growth and development. Thus, in citrus tetraploid, the specific root architecture (less branched roots with lower number of root

tips; thicker exodermis) leads to a more limited value of the root hydraulic conductance (Ruiz et al. 2016a, b, c). Altogether, these specific anatomical and morphological characteristics can contribute to a better tolerance to water stress.

Salt stress is one of the major abiotic stresses which is associated, in citrus, with Cl^- accumulation rather than Na^+ uptake (Moya et al. 2003; Banuls et al. 1997; Hussain et al. 2012). If Cl^- is not restricted at the root level, toxic ions are transferred to the scion through the transpiration stream and cause necrosis and even defoliation (Moya et al. 2003). Tetraploid citrus seedlings were shown to be more tolerant to salt stress than diploid (Mouhaya et al. 2010; Ruiz et al. 2016b, c; Syvertsen JP 2000; Saleh et al. 2008). Several mechanisms were proposed to explain the better tolerance such as greater Cl^- exclusion capacity in root related to morphological and histological traits leading to lower hydraulic conductance and transpiration rate (Ruiz et al. 2016a, b, c). Also, higher deposition of suberin in cell walls of the exodermis cells in sections close to the root apex has been proposed to participate in the better tolerance by limiting Cl^- absorption (Ruiz et al. 2016c). Thus, it is expected that grafted trees in the field will benefit the specific anatomy of tetraploid rootstocks by limiting the Cl^- root to shoot transport.

Tetraploid citrus seedlings subjected to drought showed better traits of tolerance than the respective diploid (Oliveira et al. 2017). In these seedlings, better tolerance was shown to be associated with more limited transpiration and higher leaf water content. When grafted with a diploid cultivar, tetraploid (doubled-diploid) rootstocks were shown to confer better tolerance to drought than their relative diploid, which was associated with upregulation of the expression of specific genes in roots (Allario et al. 2013). Among them, drought-responsive genes, including CsNCED1, a pivotal regulatory gene of ABA biosynthesis led to higher constitutive ABA content in root and better drought tolerance. Other genes associated with osmoticum biosynthesis and detoxification processes were also shown to be upregulated by stress and favor better adaptation to environmental stress (Allario et al. 2013; Tan et al. 2015).

Evaluation in the field of tetraploid Carrizo citrange rootstock grafted with clementine showed that tetraploid genotype may promote better chilling tolerance to the scion thanks to a part of the antioxidant system (Oustric et al. 2017). To end, an enhanced tolerance of tetraploid Carrizo citrange seedlings to boron excess was related to the thicker exodermis in the root which in turn limited the boron uptake capacity and root-to-shoot transport (Ruiz et al. 2016c). Also, a higher tolerance to chromium toxicity in "Kinnow" mandarin trees grafted on 4x rootstock was attributed to chromium sequestration in roots with lower transfer to leaves (Balal et al. 2017). Recently, investigation on seedlings from an intergeneric somatic hybrid (*C. reticulata* + *P. trifoliata*) subjected to cold stress and light stress revealed greater tolerance compared to the diploid parents and the respective tetraploids (Oustric et al. 2018). These results suggest that divergent genome merging in allotetraploid also contribute to a better adaptation of polyploid rootstocks.

6.5 Ploidy Manipulation for Breeding

6.5.1 New Developments on Citrus Polyploidy Breeding in China

In the past years, China has made great progress in this research area. In the Institute of Citrus Science of Huazhong Agricultural University, somatic hybrids were produced, spontaneous autotetraploids were exploited, and haploids were recovered from anther culture; using somatic hybrids and autotetraploids as pollen parents, thousands of triploid hybrids were recovered from over 40 sexual crosses.

Production of tetraploid somatic hybrids: to date, more than 50 interspecific and intergeneric somatic hybrids have been produced via protoplast fusion at the Institute of Citrus Science of HZAU. Based on nuclear and cytoplasmic molecular marker analysis, citrus somatic hybrids were verified as generally possessing the nuclear

genomes from both fusion parents while the mitochondrial genome is preferentially inherited from the callus parent, and the chloroplast genome is randomly inherited from either parent, as it was also observed for cybrids (Guo et al. 2013; Xiao et al. 2014).

Exploitation and evaluation of doubled diploids: spontaneous polyploids are robust germplasm resources for seedless citrus and polyploid breeding. In the Institute of Citrus Science of HZAU program, by simply sowing the seeds, very large seedlings were managed, in which polyploids (tetraploids and triploids) were exploited from 40 citrus genotypes and sexual populations. Polyploids were prescreened based on leaf morphology followed by flow cytometry and SSR marker analysis, which included 100 tetraploid seedlings obtained from 12 diploid rootstock genotypes, 66 tetraploid seedlings from 20 diploid scion genotypes, 30 triploid seedlings from nine diploid scion genotypes, and 14 triploid and 13 tetraploid seedlings from eight sexual crosses. As determined by SSR marker analysis, the genetic origins of the spontaneous polyploids were determined (Guo et al. 2016). Polyploidization produced novel phenotypes that through plant breeding have enhanced the production of biomass and improved the stress tolerance of major crops.

Triploid production and the origin of 2n megagametophyte formation: to obtain triploid hybrids, 11 allotetraploid somatic hybrids and 3 autotetraploids were employed as male parents to pollinate 13 polyembryonic and 9 monoembryonic diploid cultivars at the Institute of Citrus Science of HZAU. From the 58 crosses, 3773 fruits were set from the 14,682 pollinated flowers, with an average fruit setting rate of 25.7%. As many as 43,110 immature seeds from 3193 young fruits were cultured in vitro. After the shoot and root induction, 8931 plantlets were obtained, with 1690 plants proved to be triploids by flow cytometry analysis. SSR marker analysis showed that all of the analyzed triploids were hybrids of both their parents (Xie et al. 2014a, b). Meanwhile, 176 tetraploid plants unexpectedly regenerated from some 2x × 4x crosses, suggesting that 2n megagametophyte might have been formed and pollinated or the occurrence of chromosome doubling of female polyembryonic parents. Twenty-two SSR markers, most of which were mapped to the genome of sweet orange, were used to analyze the genetic origins of 54 tetraploid progenies from four 2x × 4x crosses, with the polyembryonic Murcott tangor as the female parent and four allotetraploid somatic hybrids as the male parents. As a result, 13 tetraploids showed an allelic profile identical to that of Murcott tangor, indicating their doubled-diploid origin. The remaining 41 tetraploids proved to be hybrids between both of their corresponding parents because they exhibited paternal alleles. Genotyping results of these 41 tetraploid hybrids based on polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE) analyses confirmed that all of the tetraploid hybrids were derived from 2n megagametophytes. Because 13 of the 22 markers displayed maternal heterozygosity restitution rates lower than 50%, SDR was inferred as the mechanism underlying formation of the 2n megagametophytes in Murcott tangor (Xie et al. 2014a).

Regeneration and molecular characterization of haploids: Homozygous genotypes, i.e., haploids and doubled haploids (DH), have great potential to facilitate citrus genetic breeding and genomic research. At the Institute of Citrus Science of HZAU, the anthers of seven citrus cultivars at the uninucleate stage were cultured using four previously reported media. Ten haploid lines ($2n = x = 9$), six DH lines ($2n = 2x = 18$), two tetraploid lines ($2n = 4x = 36$) of Early Gold sweet orange, and one haploid line of Rhode Red Valencia sweet orange were obtained, as identified by ploidy, karyotype, and SSR analysis. All of them were confirmed to be fully homozygous for 31 SSR markers distributed evenly on each of the chromosomes. Two of the DH lines of Early Gold sweet orange grew vigorously in the greenhouse (Wang et al. 2015).

In addition, in the Citrus Research Institute of Southwest University, thousands of triploid plants were recovered from small seeds collected from open-pollinated or hand-pollinated diploid

cultivars; hundreds of tetraploids and more than 100 haploid plants were also recovered from over 30 open-pollinated cultivars and sexual crosses. In the Horticulture Research Institute, Sichuan Provincial Academy of Agricultural Sciences, more than 100 triploid plants were recovered from eight open-pollinated cultivars whereas 31 tetraploid plants were selected from ten open-pollinated cultivars.

6.5.2 Ploidy Manipulation for Cultivar Development at the University of Florida's Citrus Research and Education Center (Lake Alfred, FL, USA)

Somatic hybridization techniques were established for Citrus at the UF/CREC in the mid-1980s (Grosser and Gmitter 1990; Grosser et al. 2010a; Omar et al. 2016). Since this time, numerous somatic hybrids that combine complementary elite diploid scion material as tetraploid breeding parents were created (Grosser et al. 2000; Grosser and Gmitter 2011; Ollitrault et al. 2007b). Doubled-diploid breeding parents were also created using tissue culture techniques, including colchicine-induced doubling of chromosomes (Gmitter et al. 1991). The CREC breeding program driven by Grosser and Gmitter continually uses the available population of tetraploids in interploid hybridization schemes to develop seedless, triploid hybrids for mandarin, acid fruit (lemon/lime), and pummelo/grapefruit improvement. Thousands of triploids have been generated and planted in the field. However, citrus greening disease or Huanglongbing (HLB) arrived in Florida around 2005, and spread rapidly throughout the state. This disease ravished the parental and hybrid blocks, making it difficult to get the large majority of the hybrids to flower and produce normal fruit for evaluation before becoming overwhelmed by the disease. Of course this also created a natural field screen for HLB tolerance, allowing identifying hybrids and breeding parents showing more tolerance. Thus, during the past few years, significant efforts were invested to overcome the endemic HLB problem. It was found that the combination of rootstock genetics and enhanced root nutrition can work to now provide adequate tree health so that the majority (not all) of new hybrids can indeed flower and fruit. Secondary and micronutrient mining and translocation are inhibited in HLB-affected trees, causing severe deficiencies, especially in root tissue. Providing a constant elevated supply of these nutrients through the use of controlled release fertilizers (CRF) improves root function and overall tree health (Grosser, unpublished data). More recently, CREC researchers have shown that an overdose of slow-release manganese (4*x* the recommended amount) is therapeutic against HLB, and they are now testing this on their hybrid blocks. Combining such evolving enhanced nutrition treatments with the use of new rootstocks that impart at least some level of HLB tolerance into grafted scions has also improved overall tree health, and appears to be increasing the percentage of trees that are able to flower and set fruit. Successful rootstocks include fast-track releases UFR-1, UFR-3, and UFR-5, along with a few other unreleased experimental rootstocks. The HLB-tolerant UFR rootstocks mentioned are all "tetrazygs" produced from crosses of somatic hybrids produced at CREC; this topic is discussed in more detail in the rootstock breeding chapter. Recent interploid crosses conducted during the past few years have focused on increasing HLB tolerance in progeny by using parents identified to exhibit HLB tolerance; with monoembryonic LB8-9 (SugarBelle) leading the way. As a result, the field evaluation component of the CREC breeding program is now regaining the momentum lost during the initial wave of HLB throughout Florida.

The first seedless triploid mandarin hybrid made available commercially from CREC program was C4-15–19, produced from an interploid cross of LB8-9 with a somatic hybrid of [Nova mandarin hybrid + Succari sweet orange]. This hybrid produces fruit with high Brix and excellent flavor, and trees crop well. Although peelable, it is not considered a zipperskin type.

This is the first released triploid citrus cultivar fathered by a somatic hybrid. The CREC program has also released two other triploid hybrids for commercialization, both fathered by autotetraploid pollen parents. One is a navel orange-like hybrid named RB7-34, produced from a cross of LB8-9 with autotetraploid Hamlin sweet orange. Most fruit have pronounced navels, though navels are absent on both parents. Fruit mature in the same window as Glen navel, but are sweeter (15° Brix) and exhibit much better internal and external color than all navel cultivars grown in Florida. The second triploid hybrid is the red grapefruit-like UF-914, from a cross of a red pummelo with autotetraploid Hudson red grapefruit. Fruit are sweeter and less bitter than traditional red grapefruit, but still maintain excellent grapefruit flavor. In taste panel evaluations, people that do not like grapefruit along with grapefruit lovers both found the fruit desirable. Fruit are also very low in furanocoumarins, the chemicals that cause the "grapefruit" effect prescription drug interactions. Other selections believed to have commercial potential are identified in Table 6.1 (mandarin hybrids), Tables 6.2, and 6.3 (acid fruit hybrids). These selections have all been entered in the State of Florida's Parent Tree Program to generate certified pathogen-free budwood. Although many triploid mandarin hybrids from the early work have good color and flavor, most of these hybrids would not be considered as zipperskins. Recently, a somatic hybrid allotetraploid that transmits the zipperskin trait at a high rate to triploid progeny has been identified, along with good fruit size and fruit quality, namely [Page + (Clementine × Satsuma)]. It is therefore used as a pollen parent in many interploid crosses, with very promising results.

The majority of somatic hybrid breeding parents produced for scion improvement have been from fusions of two polyembryonic parents. In this case, the somatic hybrid can only be efficiently used as a pollen parent in interploid crosses. As mentioned, CREC has produced several thousand triploid hybrids fathered by somatic hybrids.

Table 6.1 Diploid × Tetraploid crosses yielding high-quality seedless triploid mandarin hybrids with commercial potential. All crosses required embryo rescue for triploid recovery

Monoembryonic female	Somatic hybrid pollen parent	No. of selected hybrids
LB8-9 (SugarBelle®)	[Succari sweet orange + Ponkan]	4
Nules clementine	[Page + Ortanique]	1
FallGlo	[Page + (Clementine × Satsuma)]	2
Monreal clementine	[Murcott + LB8-8[a]]	1
LB8-9	[Nova + Succari]	4
Temple	[Page + (Clementine × Satsuma)]	1
LB8-9	[Succari + Murcott]	4
LB8-9	[Nova + Osceola]	4
Clementine	[Murcott + LB8-9]	5
LB8-9	[Page + Ortanique]	1
Clementine	[Nova + Osceola]	2
FallGlo	[Murcott + LB8-9]	1
FallGlo	[Nova + Osceola]	1
Temple	[Nova + Osceola]	1
G-96 (trifoliate hybrid)	[Succari + Murcott]	1
LB8-9	[Page + (Clementine × Satsuma)]	1

[a]LB8-8 is a low acid sibling of LB8-9, both developed by F. G. Gmitter

Table 6.2 Interploid crosses yielding high-quality seedless triploid acid fruit hybrids with commercial potential; Diploid × Tetraploid crosses (required embryo rescue for triploid recovery)

Female	Pollen parent	No. of selected hybrids
Todo el ano lemon	[Key lime + Valencia]	4
Todo el ano lemon	[Hamlin + Femminello]	2
Key lime	[Key lime + Valencia]	1
Lisbon	[Key lime + Valencia]	2
Lakeland limequat	4x Femminello lemon	1
Limoneira 49	[Hamlin + Femminello]	1
Etrog citron	[Hamlin + Femminello]	1
Etrog citron	[4x Femminello]	1

Table 6.3 Interploid crosses yielding high-quality seedless triploid acid fruit hybrids with commercial potential; Tetraploid × Diploid crosses (no embryo rescue required)

Female	Pollen parent	No. of selected hybrids
[Santa Teresa lemon + Lakeland limequat]	Cook Eureka	2
Giant key lime	Palestine sweet lime	1

Interploid crosses utilizing a monoembryonic diploid female parent and a tetraploid male parent require embryo rescue for triploid plant recovery (Shen et al. 2011). Without embryo rescue, triploid recovery is very inefficient due to embryo abortion; embryos do not complete normal development, presumably as a consequence of endosperm failure caused by embryo/endosperm ploidy level imbalance. In contrast, interploid crosses utilizing monoembryonic tetraploid females do not require embryo rescue. Somatic hybrids produced by the fusion of a polyembryonic embryogenic parent with a monoembryonic leaf parent are often monoembryonic. Several such hybrids are now flowering, and we have begun using CREC as females in interploid crosses. Triploids have easily been produced by germinating seeds taken from mature full-term fruit. For mandarin improvement, a key moderately HLB-tolerant monoembryonic tetraploid female has been the somatic hybrid [W. Murcott + UF03-B (Fortune × Murcott)], which is also being used as a pollen parent. For grapefruit/pummelo improvement, three monoembryonic somatic hybrid parents: [Succari sweet orange + Hirado Buntan pummelo], [Murcott + Chandler pummelo sdlg.#80], and [Murcott + Chandler pummelo sdlg. A-1-11] are used, the latter two showing excellent HLB and canker tolerances. For acid fruit improvement, we are using the monoembryonic somatic hybrid [Femminello Santa Teresa lemon + Lakeland Limequat], which produces a bright yellow, pleasantly fragrant fruit. The CREC ongoing somatic hybridization program is now focusing on production of monoembryonic somatic hybrids where at least one parent is highly HLB tolerant. HLB tolerance is now an overriding requirement for any new citrus cultivars to be grown in areas where the disease is endemic.

6.5.3 New Developments on Citrus Polyploidy Breeding in the Mediterranean

Several countries in the Mediterranean area had engaged Citrus polyploid breeding programs aiming to select seedless varieties for the fresh citrus fruit market. Indeed, the development of triploid cultivars is of particular interest to generate sterility and thus, seedless fruits when

coupled with parthenocarpy. Several strategies were developed by Mediterranean breeders to obtain triploid citrus (Ollitrault et al. 2007a, 2008; Aleza et al. 2010, 2012a, b; Starrantino and Reforgiato Recupero 1981; Vardi et al. 2008; Navarro et al. 2015). The low availability of tetraploid parents was an initial limitation in triploid breeding using interploid crosses. Starrantino and Reforgiato Recupero (1981) were the first to exploit the spontaneous polyploidization events by chromosome doubling of nucellar cells to select doubled-diploid lines to be used as parents. Triploid mandarin hybrids (or tangor) were obtained by crossing monoembryonic 2x female parents with a 4x males. Some promising hybrids have been patented like Mandared, Alkantara (clementine 2x × Tarocco sweet orange 4x) and Mandalate (Fortune mandarin 2x × Avana mandarin 4x). The first two varieties have fruit with high anthocyanin content while the last one had the flavor of Avana (Reforigato Recupero et al. 2005). By contrast, spontaneous tetraploidization is not common in non-apomictic genotypes and monoembryonic tetraploid parents were obtained using colchicine or oryzalin treatments (Aleza et al. 2009b) or through protoplast fusion (Ollitrault et al. 2007c). At IVIA (Spain) and CIRAD–INRA (France), many tetraploid parents have been obtained from either apomictic and non-apomictic genotypes (Aleza et al. 2009a, 2011) and provided the opportunity to obtain triploid citrus plants in a very efficient way (Aleza et al. 2012a). At IVIA, interploid hybridizations (2x × 4x or 4x × 2x) resulted in more than 10,000 triploid hybrids from 203 parental combinations.

However, many of the citrus triploid hybrids developed in France and Spain have been recovered by 2x × 2x hybridization. Esen and Soost (1971, 1973) and Geraci et al. (1975) discovered triploid progenies in diploid crosses. The selection of monoembryonic female parents producing 2n ovules, like Fortune and Imperial mandarins, clementine and new monoembryonic diploid hybrids allowed to obtain thousands of triploid hybrids around the Mediterranean area (Ollitrault et al. 2008). At IVIA (Spain), more than 5,000 triploid hybrids have been recovered by 2x × 2x crosses from 130 parental combinations. At CIRAD–INRA (France), more than 6,000 triploid hybrids were generated and entered in participatory plant breeding projects with several partners at the national and international levels. Many triploid obtained by this method have been released, like Winola (Volcani, Israel), Safor, Garbi, Mistral (IVIA, Spain), Florina (CIRAD–INRA, France). In Corsica, one variety is under evaluation on fourteen private farms and in Spain ten varieties are included in the new experimental system involving several private companies.

The development of tetraploid rootstock is also promising to cumulate tolerances/resistances for the abiotic and biotic constraints of the Mediterranean Basin. Improved adaptation and resilience of tetraploid rootstock may help to solve some issues associated with the global climatic change. Promising allotetraploid rootstock has been generated by somatic hybridization in France (Dambier et al. 2011) and Spain (Ruiz et al. 2018). More recently CIRAD initiated a tetraploid breeding project based on the "tetrazyg" strategy using doubled-diploid rootstocks and allotetraploid somatic hybrids as parents of tetraploid sexual progenies. Some of these tetraploid population developed in Corsica are under evaluation in French West Indies for HLB tolerance, to contribute to solve the main issue of the tropical citriculture and to anticipate the potential spread of HLB in the Mediterranean Basin.

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Markers, Maps, and Marker-Assisted Selection

7

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Abstract

DNA marker analysis, combined with linkage and quantitative trait locus (QTL) analyses, has drastically changed the scheme of citrus breeding by enabling the marker-assisted selection (MAS) of a promising scion at the early seedling stage. This technique has greatly enhanced breeding efficiency by minimizing the period of trait evaluation at the orchard. It has also provided remarkable achievements for understanding the classification and pedigree of citrus varieties by molecular phylogenetic analysis. In the past decades, valuable DNA markers have been developed from various crosses undertaken for the selection of fruit traits, apomixis, dwarfism and for resistance against pests, stress, or acclimation. Zygotic embryo selection is a basic but important application of MAS for the cross of polyembryonic seed parents. The advent of next-generation sequencing technologies dramatically enhanced the accumulation of genome sequence data. They were used for the development of codominant DNA marker systems (simple sequence repeat: SSR and single nucleotide polymorphism: SNP) that enabled high-throughput genotyping and high-density linkage map construction. Consequently, they also enabled the identification of a gene or genome sequence responsible for a trait by associating the map with the genome sequence. While these advances provided us with the genomic basis for trait selection and the tools to improve breeding, MAS of a complex trait controlled by many genes having a small effect still remained a challenge due to the difficulty in conducting large scale genetic analysis in citrus. Now, two alternative analysis methods based on genome-wide genotype data, genome-wide association study (GWAS) and genomic selection (GS), have become available for identifying QTLs of any trait of interest without the need to cross for predicting the breeding value of offspring. These new methods facilitate the selection of complex traits as well as shed light on the significance of measuring methods, for these methods to become applicable.

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7.1 Introduction

DNA marker analysis has been widely used as a fundamental tool for the classification and identification of diverse citrus varieties in many laboratories. In citrus, an extended juvenile period and

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the significant effort required for trait evaluation in the orchard have restricted crossbreeding. Identifying a promising seedling at the young seedling stage by DNA marker analysis is, therefore, a valuable approach in citrus breeding. While only a limited number of DNA markers were available for earlier studies, more recently a variety of DNA markers linked to a variety of traits have been detected and successfully applied for marker-assisted selection (MAS). Molecular phylogenetic analysis using DNA markers also deepened the knowledge about the phylogenetic relationships of citrus varieties and proposed many suggestions on the origin of genus *Citrus*. While initial attempts at DNA marker analysis delivered many achievements, further improvement in the stability and speed of genotyping analyses and transferability to other varieties is required. The release of next-generation sequencing (NGS) technology enhanced the accumulation of citrus genome sequence resources and facilitated the development of high-precision markers. Consequently, codominant DNA markers that are associated with a gene or genome sequence have replaced the dominant markers used in early studies. High-throughput genotyping technologies developed in the past two decades also enable the accurate acquisition of large amounts of genotype data in a short time from many samples. Currently, high-density linkage maps associated with whole-genome sequences have been applied for the detection of QTLs for important traits. The improved performance of DNA markers and their transferability to a wide range of varieties also helped to uncover the obscured genealogy of indigenous citrus varieties and the origin of citrus varieties; it also enabled the application of new selection methods for genome-wide association studies (GWAS) and genomic selection (GS). In addition to the increase in DNA markers available for selection, quick and low-cost genotyping methods have become available. The use of MAS in citrus crossbreeding has, therefore, reached the stage of practical use.

7.2 DNA Marker Development

7.2.1 Background

A molecular marker is an identifiable DNA sequence or protein, located on (or encoded by) a chromosome or organelle, that can be used to reveal polymorphisms in genetically different individuals. A marker can be a protein, a gene, part of a gene, or a sequence in a non-gene region. Molecular markers have become standard tools in a wide variety of plant genetic applications, including estimation of genetic diversity, determination of genetic identity, development of linkage maps, detection of markers associated with trait variation, and selection of desirable individuals in a breeding program (Aka Kacar et al. 2006). There are many types of molecular markers for genetic applications in plant science. These systems have advantages and disadvantages for each study that depend on several factors, such as a study's objectives and the crops investigated. Throughout the history of molecular technologies, various types of molecular marker have been developed.

The first molecular marker system, developed before polymerase chain reaction (PCR) technology became available, was the restriction fragment length polymorphism (RFLP) marker system. Thereafter, the advent of PCR technology ushered in a new era of molecular marker technologies. The last three decades have seen great advances in the development of marker systems and respective detection platforms. Molecular marker technologies differ in their ease of use, reproducibility, levels of polymorphism, and amount of information provided. An important property of markers is whether they are dominant or codominant. The codominant marker is more informative than a dominant marker for distinguishing heterozygous individuals from homozygotes because they are able to detect both parental alleles (Aka Kacar et al. 2006).

7.2.2 RFLP Markers

The RFLP marker system, which was developed by Botstein et al. (1980), uses restriction enzymes that digest a DNA molecule at specific sites, called restriction sites, resulting in different fragments of variable lengths. After separation by gel electrophoresis, DNA fragments are transferred to nitrocellulose or nylon filters through Southern blotting, followed by hybridization with a DNA probe and visualization using photographic film (Varshney et al. 2004). The resulting pattern reveals different sizes of restriction fragments in specific parts of the genome. The RFLP markers are abundant and informative and are also found to be moderately polymorphic. The RFLP analysis has the advantage of showing codominant alleles and being reproducible; however, the main drawbacks of RFLP markers are the requirement for large amounts of purified, high molecular weight DNA, and the high costs in terms of labor and materials (Uddin and Cheng 2015).

7.2.3 PCR-Based Markers

The invention of PCR technology resulted in the main breakthrough in DNA-based molecular marker analysis. PCR can amplify any genomic region and reveal polymorphisms in many individuals without the requirement for cloning and isolating large amounts of ultra-pure genomic DNA.

RAPD, ISSR, IRAP, and AFLP

These kinds of PCR-based markers rely on the use of PCR primers that can bind to multiple sites in the genome. This can be achieved by using either short PCR primers (randomly amplified polymorphic DNA, RAPD) or PCR primers that are complementary to repetitive elements such as microsatellites (inter-simple sequence repeat, ISSR) or retrotransposons (inter-retrotransposon amplified polymorphism, IRAP). Alternatively, restriction fragments could be amplified by adding linkers followed by selective amplification (amplified fragment length polymorphism, AFLP). In each case, PCR amplification yields multiple bands that show presence/absence-type dominant variation among individuals. The principal advantage of RAPDs, ISSRs, and AFLPs is that they do not require prior knowledge about primer sequences in the target species (Schlötterer 2004).

Sequence-Characterized Amplified Region (SCAR) and Sequence-Tagged Sites (STS)

These markers are a group of useful marker types that improve their performance by referring to nucleotide sequence information derived from previous marker analysis. For example, a SCAR marker is developed by sequencing a product amplified in RAPD analysis and designing a new primer set to amplify that specific sequence (Deng et al. 1997; Fang et al. 1997). Extended primer length (typically 20–25 bases) improves specificity and reproducible amplification of polymorphic products beyond that achieved in a RAPD analysis. Conversion of AFLP bands to specific markers, however, is often not successful (Shan et al. 1999). STS markers are similar to SCAR markers but are based on nucleotide sequence information from cloned DNA fragments, often RFLP probes (Cloutier and Landry 1994). Both SCAR and STS markers are dominant or codominant markers depending on the nature of the polymorphism (Aka Kacar et al. 2006).

Sequence-Related Amplified Polymorphism (SRAP)

Li and Quiros (2001) developed this marker system that focuses on the amplification of open reading frames (ORFs). This marker system amplifies a region of interest using two primers that are 17–18 nucleotides long. SRAP marker scores the presence or absence of a band of DNA fragments for evaluation. This is a simple but efficient marker system that is widely used in a range of research fields, including linkage map construction and fingerprinting of genomic DNA and cDNA. While SRAP is a dominant marker system, it has been successfully applied to investigate the genetic variations in different taxa (Uzun et al. 2009).

Simple Sequence Repeat (SSR)

This marker is also known as microsatellite or short tandem repeat (STR). It reveals the differences in length of repeated sequences, with each

repeat element consisting of one to six nucleotides or more. The repeated sequences are dispersed throughout the genome and often show a high level of polymorphism because of variation in the number of repeats (Tautz 1989). After identifying the flanking sequences of an SSR, a set of nucleotide primers is designed to amplify it selectively via PCR. Amplified fragments are separated by gel electrophoresis and visualized with ethidium bromide staining, silver staining, or fluorescent tagging. With a high-resolution electrophoresis system, polymorphisms as small as one base pair can be detected. Most SSR markers are generally codominant. Once their performance has been confirmed, SSR markers are efficient markers to run. Conversely, identification of the repeat sequences, primer design, and evaluation of primer performance require considerable initial input.

Single Nucleotide Polymorphism (SNP) and Insertions and Deletions (InDel)

SNP markers evaluate a single-base change in the DNA sequence at which different nucleotides occur in different individuals or populations. InDel markers evaluate insertion or deletion of events that happened at a particular position in the genome among varieties or within a population. Both SNPs and InDels are highly abundant and are distributed throughout the plant genome. DNA markers based on SNPs have rapidly become common in extensive molecular genetic studies during the past decade because of their abundance in the genome and their affinity for high-throughput detection and data handling. Compared to SSR markers, SNP analysis does not require DNA fragment separation by size and is, therefore, appropriate for automation and high-throughput assays. The biallelic nature of SNPs has less discriminatory power than multiallelic DNA marker like SSRs and requires the evaluation of increased numbers of SNPs to obtain a comparable performance. Conversely, it offers a much lower error rate in allele calling and raises the level of consistency between laboratories. These advantages have increasingly resulted in SNP markers becoming the marker of choice for genome-wide genotype identification and diversity analysis in perennial crops (Mammadov et al. 2012).

7.2.4 Application of Genome Information for DNA Marker Development

Mining polymorphisms in the DNA sequence of individual plants is the initial step toward the development and utilization of molecular markers. These polymorphisms are often associated with specific genes; molecular markers detect these polymorphisms and, therefore, act as signposts to those genes (Ahmed et al. 2017). Presently, a number of PCR-based systems for marker development exist and have been reported in the literature for citrus. The development of molecular DNA markers for genetic analysis has greatly increased our understanding of the structure and behavior of plant genomes. Various types of DNA markers, including codominant SSR, InDel, or SNP markers, have been developed and used in a wide variety of genotyping studies (Batley 2015). These molecular markers include: (i) hybridization-based markers such as RFLPs; (ii) PCR-based markers such as RAPDs, AFLPs, and SSRs; and (iii) sequence-based markers such as SNPs. The majority of these molecular markers have been developed either from genomic DNA libraries (e.g. RFLP, SSR, or SNP), random PCR amplification of genomic DNA (e.g. RAPD), or both (e.g. AFLP) (Ahmed et al. 2017; Batley 2015).

7.2.4.1 SSR Marker Development

Availability of an array of molecular marker techniques and their modifications led to comparative studies among them in many crops including citrus. Among these techniques, SSR markers have gained considerable importance in plant genetics and breeding owing to their many desirable attributes, including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage (including organellar genomes), chromosome-specific location, higher affinity to automation, and high-throughput genotyping (Parida et al. 2009; Kalia et al. 2011).

Earlier strategies for SSR isolation, that is, screening small insert genomic DNA libraries or constructing SSR-enriched libraries (Zane et al. 2002), were labor-intensive and time-consuming. However, the release of the genome sequence dramatically enhanced the efficiency of SSR mining within an organism and improved the transferability of markers by considering predicted transcripts based on complete genome sequence. Transcript sequences constitute a rich and special source of informative molecular markers because they represent expressed functional genes. Data mining of nucleotide sequences obtained from expressed genes or genome sequence assemblies provides various types of putative polymorphic regions that could be useful for molecular marker development (Gmitter et al. 2012). Among them, genic microsatellites, or expressed sequence tag-derived simple sequence repeats (EST-SSRs), have found a special place in plant genetics and the breeding of several agricultural plants (Kalia et al. 2011). The origin of ESTs is cDNA that is synthesized from mRNA. Both ESTs and cDNA clones harbor the coding regions (transcriptome) within the nucleotide sequence. Coding region sequences are generally more informative than anonymous markers because they imply a direct association between the molecular marker and the phenotype (Liu et al. 2013). These ESTs are expressed in response to a developmental or an environmental change and can be utilized as markers during selection or for mapping purposes (Palmieri et al. 2007). With the development and improvement of next-generation sequencing technology (NGS), publicly available expressed sequence tags (ESTs) are a very useful tool for gene and marker discovery for use in gene mapping, functional studies, genome annotation, and comparative genomics. Several comparative analyses, using ESTs from a variety of materials, have been conducted to enable a better understanding of gene regulation in response to signals. They have also been used successfully for the identification of genes conferring resistance to pests or pathogens, or genes that are differentially regulated during development (Botha et al. 2004).

In the last 20 years, sets of SSR markers have been developed by international collaboration in the citrus community. Only a limited number of SSR markers isolated from genomic libraries have been published (Kijas et al. 1995; Corazza-Nunes et al. 2002; Novelli et al. 2006; Barkley et al. 2006; Froelicher et al. 2008). The implementation of large EST databases has allowed the development of many more SSR markers. Chen et al. (2006) have published 56 SSR markers derived from the GenBank citrus EST database and more than 200 SSR markers have been developed (Luro et al. 2008) from the 1,600 microsatellite sequences from 37,000 ESTs characterized by Terol et al. (2007). Likewise, Jiang et al. (2006) developed SSR markers from citrus unigenes. Terol et al. (2008) identified more than 7,600 microsatellite sequences from the BAC end sequencing (BES) of Clementine that were used to develop 79 SSRs for direct anchoring of the Clementine genetics and genetic map construction (Ollitrault et al. 2010, 2012a).

SSR regions have been mined from EST sequences and utilized for marker development and linkage mapping in Citrus (Chen et al. 2006; Luro et al. 2008; Biswas et al. 2015; Hong et al. 2008). Similar approaches applied to the assemblies of EST and shotgun sequences from sweet orange identified about 144,000 and 382,000 putative SSR regions, respectively. Over 89.5% of identified SSR regions in expressed genes (EST-SSR) were occupied by SSR sequences with motifs of up to ten nucleotides. Conversely, SSR regions with short motif sizes were abundant (83.6% of total putative SSR region with motifs up to five nucleotides) in genomic SSRs. Putative SSR regions found for both ESTs and genome sequences should be available to design SSR markers that could compensate for the unequal distribution of EST-SSRs or genomic SSRs (Gmitter et al. 2012). Ma et al. (2012) identified 22,403 SSRs with 1- to 6-bp long motifs in 46,339 citrus BESs retrieved from NCBI to extend and saturate the existing genetic map. Accordingly, 323 SSR were selected and polymorphism tests showed that 316 primer-pairs (98%) could be amplified successfully and 173 pairs (55%) were polymorphic. Liu et al. (2013)

identified citrus SSRs in the genome of Clementine mandarin and analyzed their frequency and distribution in different genomic regions. A total of 80,708 SSRs were detected in the genome with an overall density of 268 SSRs/Mb. They also identified 6,834 transcripts as containing 8,989 SSRs from 33,929 Clementine mandarin transcripts. Functional categorization of transcripts containing SSRs revealed that 5,879 (86.0%) of such transcripts had homology with known proteins; GO and KEGG annotation revealed that transcripts containing SSRs were those implicated in diverse biological processes in plants, including binding, development, transcription, and protein degradation. When 27 genomic and 78 randomly selected SSRs were tested on Clementine mandarin, 95 SSRs revealed polymorphisms.

7.2.4.2 SNP Markers

With the large-scale availability of sequence information and the development of technologies for SNP genotyping, SNP markers have been increasingly used for genetic studies. The main advantage of SNP markers is their higher potential for automated high-throughput analysis at a moderate cost. A conventional SNP marker focuses on a single nucleotide variation at a position in the genome. The position of the genome and the variation is prerequisite. Thus, prior information about the allelic variation at given genomic positions is required for SNP marker design (Batley 2015). Various strategies have been pursued to identify SNPs; the most straightforward approach is a survey of ESTs for polymorphic sites (Batley et al. 2003; Nasu et al. 2002). The most comprehensive way to identify SNPs throughout the genome is the generation of whole-genome shotgun sequences using a pool of individuals as donors for the genomic DNA to be sequenced (Schlötterer 2004; Jackson et al. 2011). New alleles in genomes are generated by nucleotide substitution or insertion and deletion events and a part of those mutations will also occur within a genic region (Gregory 2004; Zhang and Gerstein 2003). These alleles are plentiful and scattered throughout the genome of various crop species. Functional SNPs and InDels can directly contribute to phenotypic variation, so such polymorphisms are indispensable for the development of functional markers (Thornsberry et al. 2001; Kage et al. 2016).

An initial attempt by Ollitrault et al. (2012a, b) developed 622 SNP markers selected from 1,457 SNPs that were mined from BES of Clementine (Ollitrault et al. 2012b). They demonstrated the 80.5% transferability of the markers to the whole citrus pool and their availability for the phylogenetic study of wide-ranging citrus accessions. The following study by Curk et al. (2015) developed a set of species-diagnostic SNP markers for the four Citrus ancestral taxa covering the nine chromosomes. These markers were then used to infer the phylogenomic structure of secondary species and modern cultivars. They mined species-diagnostic SNPs from 454 amplicon sequencing of 57 gene fragments from 26 genotypes of the four basic taxa. Of the 1,053 SNPs mined from 28,507 kb sequence, 273 were found to be highly diagnostic for a single basic taxon. Species-diagnostic SNP markers (105) were then used to analyze the admixture structure of varieties and rootstocks. Fujii et al. (2013) developed a SNP genotyping array using Illumina's GoldenGate assay system. Among 1,497 SNPs candidates, 384 SNPs were selected for a high-throughput genotyping array based on the physical parameters of Illumina's BeadArray system and applied for genotyping of a hybrid population consisting of 88 progenies and 103 citrus accessions (Omura and Shimada 2016).

Recently, the chloroplast genome sequences of sweet orange (Bausher et al. 2006) and other major citrus accessions (Carbonell-Caballero et al. 2015) have been released and genome sequences of major citrus varieties are now public (Xu et al. 2013; Wu et al. 2014; Wang et al. 2017; Shimizu et al. 2017). These genome sequence resources enable the design of precision DNA markers (Ollitrault et al. 2012a; Shimizu et al. 2016a, b; Curk et al. 2016).

7.2.4.3 CAPS and Other Markers

To date, numerous DNA markers have been developed and applied to different purposes in citrus. Numerous EST-SSR and genomic SSR

markers have been developed from citrus genome sequences and assigned to the genetic map of the Clementine mandarin (Luro et al. 2008; Ollitrault et al. 2012b). Among these markers, cleaved amplified polymorphic sequence (CAPS, aka PCR-RFLP) markers are generally less polymorphic than the other molecular markers but are convenient for genotyping because they do not require special instrumentation for their analysis. Thus far, 3,562 primer sets have been tested for DNA marker construction and 708 CAPS markers were found to be eligible for genetic analysis (Shimada et al. 2014).

7.3 Molecular Markers for Genetic Analysis

7.3.1 RAPD Marker Analysis

A wide variety of DNA-based markers have been used in citrus to study genetic variation as well as phylogenetic and taxonomic relationships among different genera, genetic mapping, identification of zygotic and nucellar seedlings, molecular characterization of triploid cultivars or somatic hybrids, analysis origin of 2*n* gametes, and also marker-assisted selection. RAPD markers have been used for analysis of genetic diversity in citrus (Federici et al. 1998; Nicolosi et al. 2000; Aka Kacar et al. 2005; Pessina et al. 2011; Coletta Filho et al. 1998; Biswas et al. 2010; Machado et al. 1996), characterization of hybrids (Bastianel et al. 1998), cultivar identification (Deng et al. 1995), phylogenetic analysis, and taxonomic studies (Luro et al. 1994). Moreover, in citrus, a number of horticulturally important features, including resistance to the citrus tristeza virus (Gmitter et al. 1996), nematode resistance (Ling et al. 2000), and dwarfing (Cheng and Roose 1995) have been tagged with RAPD markers. Additionally, most of these markers could be converted into reliable SCAR markers (Deng et al. 1997; Ahmed et al. 2017).

7.3.2 ISSR and SRAP Marker Analysis

In citrus, ISSR markers are well distributed over linkage groups (Sankar and Moore 2001) and a single degenerate primer tends to show an even distribution of markers. Thus, ISSR markers have successfully been used in closely related citrus varieties (Fang and Roose 1997) to determine genetic diversity, characterization, phylogenetic relationships among citrus and related genera (Fang et al. 1998; Marak and Laskar 2010; Shahsavar et al. 2007; Uzun et al. 2009, 2010), and to fingerprint *P. trifoliata* accessions (Fang and Roose 1997). Among the various PCR-based DNA analysis methods, SRAP markers, which preferentially amplify open reading frames (ORFs), represent a simple and efficient marker system (Li and Quiros 2001) that has been used to analyze the genetic diversity and phylogeny of *Citrus* species (Uzun et al. 2009; Kacar et al. 2013).

7.3.3 AFLP Analysis

AFLP is a DNA fingerprinting technique that combines the advantages of RFLP and RAPD (Vos et al. 1995). It has been successfully applied to resolve the phylogenetic relationships in a wide range of taxonomic groups. Pang et al. (2007) studied the phylogenetic relationships among members of the genus *Citrus* and relatives using AFLP markers; Liang et al. (2007) examined citrus cultivars and related species with AFLP markers.

7.3.4 SSR and InDel Marker Analysis

SSR markers have been included in citrus genetic maps (Chen et al. 2008; Lyon et al. 2007; Luro et al. 2007; Ollitrault et al. 2008; Bernet et al. 2010). In addition to genetic mapping, SSR markers have been used for the analysis of

genetic diversity (Corazza-Nunes et al. 2002; Barkley et al. 2006; Kacar et al. 2013; Luro et al. 2001; Polat et al. 2012), discriminating zygotic and nucellar seedlings (Ruiz et al. 2000; Carlos de Oliveira et al. 2002; Nageswara Rao et al. 2007), confirming the origin of plants obtained by induced gynogenesis (Froelicher et al. 2007; Aleza et al. 2009), molecular characterization of triploid cultivars (Aleza et al. 2010) or somatic hybrids (Chen et al. 2008; Bassene et al. 2009), and the analysis of the origin of 2*n* gametes (Gmitter et al. 2012; Luro et al. 2004; Chen et al. 2008; Ferrante et al. 2010).

Recently, Shimizu et al. (2016a) inferred the parentage of indigenous citrus varieties using SSR and InDel markers developed from various citrus genome sequence resources. Nucleotide sequences of expressed genes of citrus were obtained from the public cDNA sequence databases dbEST, RefSeq, and HarvEST (Close et al. 2007). Citrus genome sequence resources in public databases, including BES of Clementine (Terol et al. 2008) and Satsuma (Nakano et al. 2012; Kotoda et al. 2015), and whole-genome shotgun sequences of sweet orange 'Ridge Pineapple' in the trace file repository of Sanger reads were used for DNA marker design. NGS analysis of citrus varieties for mining SSR and InDel regions was performed with a HiSeq 2000 sequencing system (Illumina, CA, USA) in paired-end mode (Shimizu et al. 2016b). Candidate SSR and InDel regions in the re-sequenced data were scored and identified. To design DNA markers for genotyping nuclear genomes, SSR regions of each sequence were mined using mreps (Kolpakov et al. 2003) and then candidate regions with a motif length of between two and six nucleotides were selected. The identified candidate regions found in expressed genes or genomic sequences were used for oligonucleotide primer design with PerlPrimer or Primer3. The developed markers were further evaluated for their consistency using numerous known trios and the selected SSR markers were used to infer unknown parentages.

The phenotype information and molecular marker genotype should be known for each individual in a segregating population to develop target gene-related molecular markers. Methods of analyzing marker genotypes have been developed significantly and the use of NGS is now generally required to analyze biological information. Commercial bioinformatics programs provide simple interfaces for researchers with little experience in this area but filtering and analyzing the data is time-consuming. In the big-data era, analyzing a large amount of data with quick homemade scripts will become an essential skill for developing molecular markers (Yang et al. 2015).

7.3.5 SNP and CAPS Marker Analysis

Significant efforts have been initiated for the development of a new generation of markers showing higher accuracy in the detection of polymorphisms for genetic analysis (Xiong and Jin 1999; Hoskins et al. 2001). These markers, which are known as SNPs, represent differences in a single nucleotide position that is present in some populations where the least frequent allele has an abundance of 1% or higher (Novelli et al. 2004). Jiang et al. (2010) have discovered SNP markers suitable for determining the haplotypes used to distinguish very closely related cultivars and to assess genetic diversity within or between related species of citrus. SNPs and small InDels from ESTs of sweet orange and Satsuma were identified using an in silico SNP discovery strategy. They mined 55,296 EST sequences of sweet orange and 2,575 of Satsuma retrieved from the NCBI repository for potential SNPs and validated them with sequencing approaches using 30 citrus accessions. The selected SNPs mined from EST sequences of sweet orange and Satsuma displayed potential as molecular markers to discriminate inter-species accessions of citrus and demonstrated their availability for genetics research and breeding. A variety of methods have been developed to detect SNPs, many of which use automated high-throughput systems. Among various SNP genotyping methods, CAPS and derived CAPS (dCAPS) have been widely applied. CAPS markers are PCR-based markers similar to SNPs that can identify

polymorphisms at a restriction site where variation is present in one of the two amplified fragments. CAPS markers are useful for genotyping, positional or map-based cloning, and molecular identification studies (Spaniolas et al. 2006).

7.4 Linkage Map Construction and QTL Mapping

7.4.1 Linkage Map Construction, a Brief Introduction

Techniques involving molecular markers have solved some of the limiting problems associated with classical breeding in citrus and other perennial species. Citrus breeding is affected by long juvenility, heterozygosis, gametophytic cross-incompatibility, male sterility, apomixis, seedlessness, and trait stability under different environmental conditions (Omura and Shimada 2016). Since the beginning of the 1990s, molecular markers have been used for evaluation or characterization of active germplasm collections, identification of nucellar hybrid seedlings in progenies of controlled crosses, the study of phylogenetic relationships and genetic divergence, and genetic mapping (Machado et al. 2005). When using a polyembryonic female parent, the identification of zygotic embryos at the seedling stage makes it possible to obtain many progenies that can be used for genetic mapping and to study the heritability of traits.

In the past, studies focused on the progeny obtained from crosses that included *Poncirus trifoliata* as one of the parents because of its importance for rootstock breeding. *Poncirus* was also used because the trifoliate leaf, which is a characteristic with monogenic and dominant inheritance, allowed the selection of zygotic plants in the progeny. The monoembryonic cultivars, like Fortune and Clementine mandarins, were also widely used as the female parent in breeding programs in Spain, France, and Italy (Cuenca et al. 2013). With the advent of molecular markers and the ease of genotyping, many progenies could be obtained even using polyembryonic cultivars as the female parent. Genetic mapping, which is of fundamental importance in breeding programs, has benefited from the improvement of genotyping techniques. A high-density linkage map is fundamental for QTL mapping, marker-assisted selection (MAS), and candidate gene identification within the QTL intervals and gene cloning. Compared with other crops, genetic mapping in citrus is relatively less well developed, but this scenario is changing. In this review section, we will highlight works that exemplify these trends in citrus breeding.

7.4.2 Developed Linkage Maps in Citrus

There are a reasonable number of linkage maps for citrus so far. However, with the accumulation of knowledge and advancement of technologies for obtaining molecular markers, the maps are being continually updated. These maps are becoming increasingly representative, making it possible to compare them with reference genomes or even use them to assist in the assembly or updating of sequenced genomes (Ollitrault et al. 2012a; Xu et al. 2013; Curtolo et al. 2017, 2018. Initially, RFLP, RADP, AFLP, and isoenzyme markers were the most commonly used markers for linkage mapping. From the twenty-first century onward, ISSR, IRAP, SSR, and those markers obtained through high-throughput sequencing were also used to generate the linkage maps (Table 7.1). Currently, the DNA markers derived using high-throughput technology are the most commonly used, allowing several genotypes to be analyzed at the same time and thousands of markers to be generated at once.

Citrus plants are perennial species, so F1 populations are typically used for the construction of citrus maps (Chen et al. 2008; García et al. 1999; García et al. 2000; Ruiz and Asins 2003; Raga et al. 2012) although backcrossing and F2 progeny are also sometimes used. In these cases, linkage mapping can be performed for each heterozygous parental individual separately using single-dose DNA polymorphisms segregating 1:1. Such mating configurations are

Table 7.1 A summary of the reported molecular markers in citrus with their types and numbers, number of linkage groups, and map size

References	Type of molecular markers	Number of molecular markers	Number of linkage groups	Map size (cM)
Torres et al. (1985)	Isoenzymes	5	2	
Liou (1990)	RFLP, isoenzymes	35	8	314
Durham et al. (1992)	RFLP, isoenzymes	52	11	533
Jarrel et al. (1992)	RFLP, isoenzymes	38	10	351
Cai et al. (1994)	RAPD, RFLP, isoenzymes	189	9	1.192
Luro et al. (1996)	RAPD, RFLP, isoenzymes	34 *C. grandis* 95 *Poncirus*	7 12	600 1.503
Kijas et al. (1997)	RFLP, ISSR	48	12	410
De Simone et al. (1998)	AFLP, RAPD, RFLP	247 *C. aurantium* 92 *C. latipes*	20 12	1.000 600
Ling et al. (1999)	AFLP, RFLP, isoenzymes	337	11	1.026
Garcia et al. (1999)	RAPD, RFLP, CAP, isoenzymes	69	3	
Cristofani et al. (1999)	RAPD	63 *C. sunki* 62 *P. trifoliata*	10 8	732 866
Roose et al. (2000)	RAPD, RFLP, ISSR	156	16	701
Omura et al. (2000)	CAPS	120	9	801
Sankar and Moore (2001)	ISSR, RAPD, RFLP, isoenzymes	310	9	874
Ruiz and Asins (2003)	RAPD, SSR, IRAP	48 *P. trifoliata* 120 *C. aurantium*	10 17	
Oliveira et al. (2005)	RAPD	217	15	527
Oliveira et al. (2007)	RAPD, SSR, AFLP	227 Murcott tangor × Pera sweet orange	9	845
Gulsen et al. (2010)	RAPD, ISSR, SRAP, SSR, POGP	215 Clementine mandarin 189 Orlando tangelo	9 9	858 886
Ollitrault et al. (2012a)	SNP, SSR	961 Clementine mandarin	9	1084
Xu et al. (2013)	SNP, SSR	972 Sweet orange	9	980
Shimada et al. (2014)	CAPS	708 for Okitsu 46 gou × Kankitsu Chukanbohon Nou 5 gou	9	990.9
Guo et al. (2015)	SNP, SSR	1563 for Pingshan pummelo and Guanxi pummelo	9	976.58
Ohta et al. (2015)	CAPS	189 Nou-8	10	722.8

(continued)

Table 7.1 (continued)

References	Type of molecular markers	Number of molecular markers	Number of linkage groups	Map size (cM)
Yu et al. (2016)	SNP	189 Fortune 106 Murcott	9 9	681.07 395.25
Curtolo et al. (2017)	DArTseq	661 Murcott tangor × Pera sweet orange	13	2774
Imai et al. (2018)	SNP	442 for Harehime 332 for Yoshida ponkan	9 9	635.8 892.9
Curtolo et al. (2018)	DArTseq	2,778 *C. sunki* 3,084 *P. trifoliata*	10 9	2,446.6 2,411.55

Adapted from Chen et al. (2008)

displayed where the marker is present in one parent, absent in the other, and segregating in the progeny. Grattapaglia and Sederoff (1994) called this mapping strategy 'pseudo-testcross' because the testcross mating configuration of the markers is not known a priori, as in a conventional testcross in which the tester is homozygous recessive for the locus of interest. A two-way pseudo-testcross has been conducted in the F1 population in citrus (Cristofani et al. 1999; Weber et al. 2003). According to Weber et al. (2003), in this design, a map of each parent is constructed by grouping marker alleles originating from each parent for analysis but the collinearity between the maps cannot be determined without an intermediary map or codominant markers in both parents. For F1 populations, with markers that segregate 3:1 (dominant), 1:2:1 (codominant), and 1:1:1:1 (codominant), an integrated map can be built (Curtolo et al. 2017; Carlos de Oliveira et al. 2007). Durham et al. (1992), Cai et al. (1994), Gmitter et al. (1996), Deng et al. (1997), Roose et al. (2000), and Deng et al. (2001) constructed integrated maps considering *P. trifoliata* as one of the parents in population formation. Integration was possible because of the type of cross once F2 and backcrossing were adopted.

The number of markers anchored in the maps reflects the evolution of the technologies for marker production and analysis. Durham et al. (1992), Liou et al. (1996), Luro et al. (1996), Kijas et al. (1997), Roose et al. (2000), Sankar and Moore (2001), Oliveira et al. (2007), and Gulsen et al. (2010) associated at least two types of markers in the linkage analysis. Among them, Ling et al. (1999) gathered information from polymorphisms generated by AFLP, RFLP, and isoenzyme markers, then obtained the map with the highest number of markers (337). With high-throughput genotyping technology, high-density linkage maps were developed for Clementine (Ollitrault et al. 2012a), *P. trifoliata*, and *C. sunki* (Curtolo et al. 2018), for instance. Curtolo et al. (2018), using only NGS-combined diversity arrays technology (DArTseq) markers, obtained the map with the highest number of markers so far (Fig. 7.1).

As the number of markers has increased, the genomic coverage of the maps has consequently increased as well. However, attention must be taken when associating genomic coverage with map saturation. In addition to genomic coverage, one should analyze the degree of density of the linkage groups or the number of markers per unit of recombination (cM). The presence of large gaps between markers can often give the false impression of high genomic coverage. For example, Luro et al. (1996) using RAPD, RFLP, and isoenzymes built a map with 95 markers for *Poncirus*, distributed in nine linkage groups, with genomic coverage of 1,503 cM representing, on average, one marker every 15 cM. With the improvement of genotyping methods, the density of markers in the maps increased. Guo et al. (2015) and Xu et al. (2013) published a dense map for citrus, with around one marker per cM. Curtolo et al. (2018) built the map with the largest number of anchored markers (3,084 for

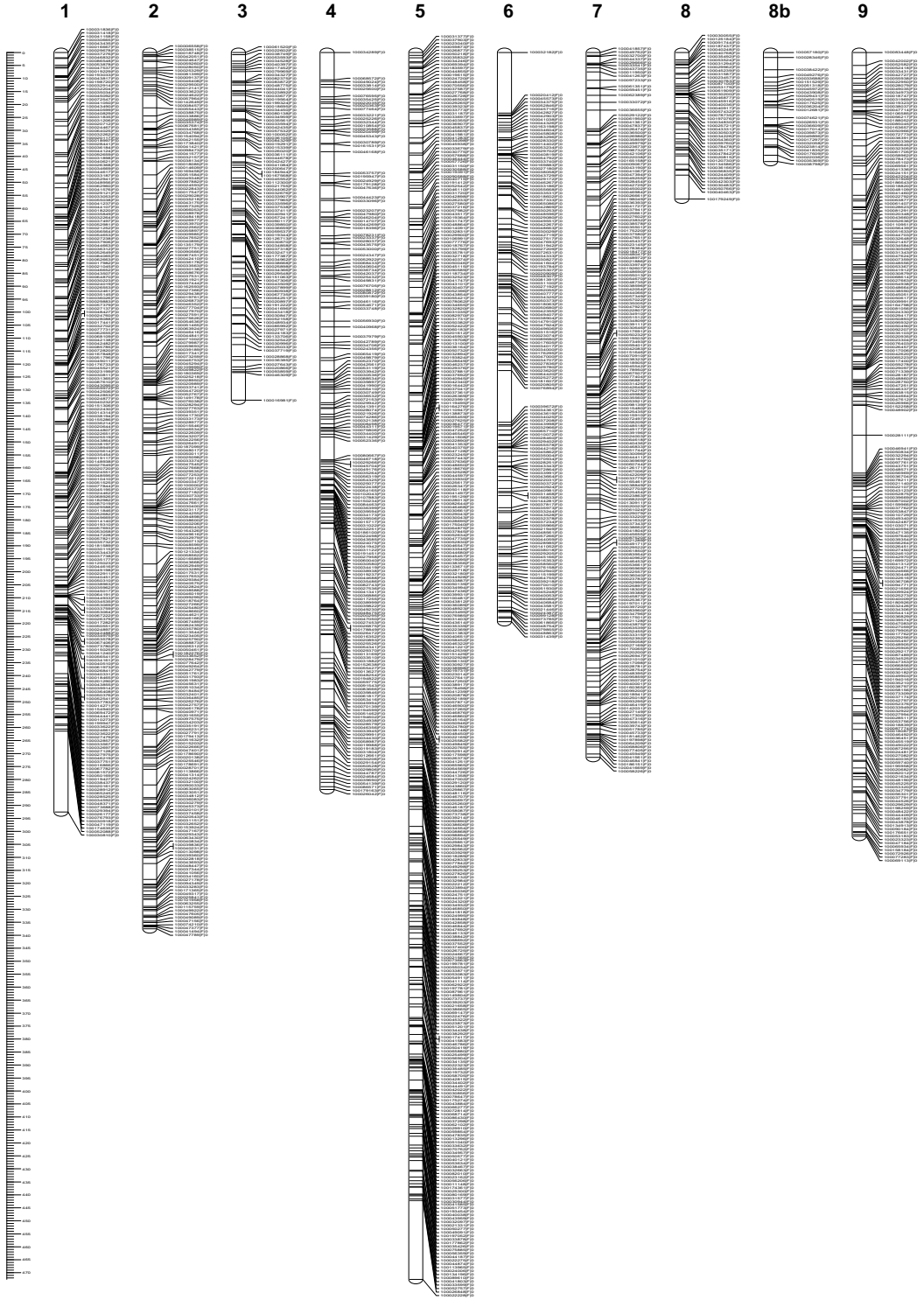

Fig. 7.1 Linkage map of *Poncirus trifoliata* with nine linkage groups, 1,782 NGS-combined diversity arrays technology (DArTseq) loci, and 3,084 DArTseq markers (Curtolo et al. 2018). The numbers on each map represent the linkage groups

P. trifoliata); however, it is not the most saturated map, because some of the markers were positioned at the same loci. While these markers, with recombination frequency equal to zero (Fr = 0), are not genetically informative, this map provides genomic information for candidate gene identification within the QTL intervals.

The number of genotyped individuals in the progeny establishes the maximum level of resolution that can be reached with a saturated number of markers in the genetic map. Curtolo et al. (2017, 2018) used a relatively large population (276 individuals) when compared with the other previous studies: 164 individuals (Gulsen et al. 2010), 151 (Raga et al. 2012), 143 (Bastianel et al. 2009), 124 (Guo et al. 2015), 116 (Yu et al. 2016), 110 (Imai et al. 2017), 97 (Chen et al. 2008), 94 (De et al. 2004), 93 (Ohta et al. 2015), 87 (Shimada et al. 2014; Carlos de Oliveira et al. 2007), 80 (Ruiz and Asins 2003; Siviero et al. 2006), 72 (Luro et al. 1995), 60 (Jarrell et al. 1992), 65 (Deng et al. 1997; Gmitter et al. 1996; Durham et al. 1992), 57 (Kijas et al. 1997), and 52 (Dalkilic et al. 2005). Nevertheless, the number of individuals used in Curtolo et al. (2017, 2018) was not large enough to require the use of a high-throughput genotyping system.

According to Omura and Shimada (2016), chromosome transmission to progeny in citrus tends to result in the inheritance of large linkage blocks and the frequency of recombination in a chromosome is low. To reach higher levels of polymorphism, it is necessary to advance generations through crosses or to work with large population sizes. Both approaches are difficult to apply in citrus because of the biological characteristics of the species. In the map of *P. trifoliata*, 1,782 loci were built using 276 hybrids with 3,084 DArTseq markers (Curtolo et al. 2018) (Fig. 7.1). Compared with the map of Zhou et al. (2008), the number of individuals in the F1 population was three times greater. This demonstrates that increasing the amount of recombination allows the use of fewer individuals on the map. As codominant markers are the most informative, the use of this type of marker can help to minimize the difficulties associated with increasing the population size needed for citrus mapping (Curtolo et al. 2017).

In conclusion, the genetic density of the markers in the map is defined not only by the number of markers obtained but also by the number of recombination events occurring in meiosis, the size of the population, population types, the nature of the markers involved, and the required statistical confidence (Ferreira et al. 2006).

7.4.3 High-Throughput Genotyping

Most of the previous citrus linkage maps suffered from relatively low numbers of analyzed hybrids and the dominant nature of the markers (RAPD, AFLP) without sequence data on the mapped fragments. Most of the maps do not provide enough markers or published sequence information to establish a reference citrus map that can be connected with whole-genome sequence data (Ollitrault et al. 2012a). Several of the more recent maps were generated using codominant markers, particularly SSRs and SNPs (Ollitrault et al. 2012a; Xu et al. 2013; Guo et al. 2015; Yu et al. 2016). With the advent of high-throughput genotyping, new methods of obtaining markers have been developed, whether based on array platforms or sequencing. Numerous SNP and EST-SSR markers have been developed from citrus genome sequences and have been assigned to the genetic map. Lyon (2008) developed a sweet orange genetic linkage map with 988 markers in 972 loci of combined SNP, SSR, GTYPE, and SFP data. Ollitrault et al. (2012b) used the Clementine BES to identify SNPs heterozygous in Clementine, while a GoldenGate SNPs array developed is one of the most representative maps.

With the recent technological advances and a radical decline in sequencing costs, the use of next-generation sequencing (NGS) has grown significantly. Several molecular markers that use NGS technology have been created. Among them, DArTseq (Sansaloni et al. 2011), genotype by sequencing (GBS) (Elshire et al. 2011), and restriction site associated DNA (RAD) (Baird

et al. 2008) markers have been used in many studies. As the costs become less prohibitive and the methods become simpler and more widespread, the markers generated by the NGS platform have been gaining preference over the other markers. For applications that require extensive genome analysis, the ideal technology should offer not only thousands of molecular markers that cover the entire genome but also allow these markers to be obtained preferentially in a single and low-cost experiment. NGS-based genotyping methods have allowed the fast development of sequence-based SNP markers. The NGS-based marker acquisition platform combines advantages in time and cost-effectiveness with high genomic coverage and high-resolution mapping, and that makes it easy to compare genetic maps among mapping populations (Hussain et al. 2017).

Among the various NGS-based technologies, GBS is one method used for the simultaneous discovery and genotyping of SNPs (Hussain et al. 2017; Poland and Rife 2012). The power of GBS in the development of high-density linkage maps and QTL mapping has been explored in several species, including citrus (Imai et al. 2017; Salazar et al. 2017; He et al. 2014). Diversity arrays technology (DArT), in combination with next-generation sequencing platforms (Kilian et al. 2012; Raman et al. 2014), known as DArTseq, is a high-throughput genotyping platform that can develop a relatively large number of polymorphic markers to construct dense genetic maps with a low-cost investment. Complete genome coverage and high-density genetic maps based on DArT array technology have been developed and increase the power of QTL detection (Thudi et al. 2011). Despite their dominant nature, DArTseq markers offer many advantages, including satisfying performance requirements, giving good genome coverage, and high marker density. The DArTseq markers also include genome-wide profiling of a large number of SNPs and detection of insertion/deletion polymorphisms that is easily expandable for genetic scope (Kilian et al. 2012).

The generation of missing allele calls is a problem that can be verified when a large number of lines are multiplexed during sequencing (Poland and Rife 2012; Williams et al. 2010; Fu et al. 2014). The construction of linkage maps can be impaired by the presence of a genotype matrix with a large proportion of missing allele calls (Bajgain et al. 2016). Another drawback of using NGS is the presence of monomorphic markers that are often eliminated from analysis assuming that they are non-informative (Holla et al. 2014). These problems were reported by Yu et al. (2016) who discarded 761 of 1,536 SNPs, including 108 with a high level of missing calls, 521 that were homozygous in both parents, and 132 with no segregation in F1 individuals. Specifically, in comparative studies, NGS-based methods have an additional advantage since they present the nucleotide sequence of each generated marker. The nucleotide sequence allows the comparison of maps previously constructed with reference genomes, as well as comparisons between maps that have sequenced markers from a different study.

Besides improving QTL detection, high-density genetic maps offer a great tool to align the fragmented scaffold sequences into chromosomes to form physical maps (Jackson et al. 2011) and assist in genome assembly because many gaps exist between scaffolds on each chromosome of a reference genome (Zhou et al. 2008). For instance, in the study performed by Curtolo et al. (2018), genomic information of the sweet orange reference genome was used to declare the name of linkage groups for *P. trifoliata* and *C. sunki* maps in accordance with chromosome synteny. All linkage groups showed synteny with a pseudochromosome of sweet orange in the reference genome (http://citrus.hzau.edu.cn/orange/index.php) except markers on linkage group 10 of the *C. sunki* map, markers that were located on chromosome Un (Chr unassigned), and those markers that were not found in the *C. sinensis* genome. This means that these linkage maps can be used to assist in the assembly of the sweet orange reference genome. The existence of regions in maps without the presence of a single marker may hinder the positioning of polymorphic markers close to genes of interest, as well as the identification of QTLs.

7.4.4 QTL Mapping for Particular Traits

Several approaches, including bulked segregation analysis (Michelmore et al. 1991), linkage mapping, and QTL analysis (Iwata et al. 2016), have been used for the development of DNA markers linked to specific traits. The bulked segregation analysis (BSA) approach divides a hybrid population into two groups according to their distinguishable phenotype, and then mines a DNA marker allele that is specific to either one of those groups. BSA is simple and effective for developing a DNA marker for MAS and can be applied to a population too small for linkage mapping. Conversely, application of BSA is limited to simple qualitative traits regulated by a single gene of higher genetic effect. It is also difficult to identify a selectable DNA marker for quantitative loci with minor effects. Furthermore, this method provides no information on the genetic distance between the loci for the trait and mapped position of the selected DNA marker. Linkage mapping analysis and QTL analysis are conventional approaches for the identification of loci linked to a trait of interest. These analyses predict the distance between the trait and mapped DNA marker position and estimate the genetic contribution of the trait. Using a large-sized population or dense DNA markers for the analyses will help to improve the resolution.

A quantitative trait loci (QTLs) mapping using bi-parental populations is a key approach to dissect complex traits and identify genomic regions underlying quantitative traits for breeding purposes. In citrus, efforts have been made over the last two decades to dissect complex traits using a QTL mapping approach. Most characteristics of agronomic interest are controlled by quantitative loci and study of their QTLs allows the identification, mapping, and quantification of their effects. Several factors influence the detection of these regions such as number and frequency of recombination of QTLs, the magnitude of their effects, heritability characteristics, interaction between genes and types of markers, and degree of saturation of the genetic map. The mapping of QTLs has favored breeding programs of several perennial species; in citrus, it was possible to map several characteristics with qualitative and quantitative inheritance.

The identification of QTLs in citrus focused on morphological traits (García et al. 1999, 2000; Roose et al. 2000) as well as resistance to abiotic (Tozlu et al. 1999a, b) or biotic factors (Gmitter et al. 1996; Siviero et al. 2006). The association of molecular markers with citrus characteristics has been previously studied since 1994 with cold acclimation (Cai et al. 1994). Thereafter, genetic maps have been extended to localize important traits such as citrus tristeza virus (CTV) resistance (Gmitter et al. 1996; Cristofani et al. 1999; Fang et al. 1998), fruit acidity (Fang et al. 1997), apomixes (García et al. 1999; Kepiro and Roose 2009; Nakano et al. 2008), nematode resistance (Ling et al. 2000), and *Phytophthora* gummosis resistance (Siviero et al. 2006) (Table 7.2). In some citrus maps, both qualitative and quantitative identification of loci is available. For example, using the same map, CTV (Cristofani et al. 1999) and gummosis of *Phytophthora* (Siviero et al. 2006) resistance loci were mapped. Reviews of QTL mapping efforts in fruit trees and citrus were published recently (Omura and Shimada 2016; Iwata et al. 2016). In the present review section, we would like to highlight the progress of citrus breeding using genetic mapping strategies, focusing on QTLs associated with pathogen resistance and fruit quality.

Recent work on populations of citrus scion varieties, especially mandarins, has focused on fruit characteristics. In these studies, the availability of maps with marker sequences enables the identification of candidate genes within the QTL intervals. The number of studies examining fruit-quality QTLs in citrus is increasing. Curtolo et al. (2017) identified 19 QTL regions for 12 fruit characteristics, including fruit diameter using 278 F1 hybrids from a cross between Murcott tangor and Pera sweet orange. Yu et al. (2016) reported the identification of molecular markers and candidate genes linked to mandarin fruit-quality traits in maps built using data generated from a 1536-SNP Illumina GoldenGate assay in two mandarin parents (Fortune and

Table 7.2 A list of mapped phenotypes and their types of inheritance

Phenotype	Type of inheritance	References
Tristeza resistance	Qualitative	Gmitter et al. (1996)
Tristeza resistance	Qualitative	Cristofani-Yaly et al. (1999)
Nematode resistance	QTL	Ling et al. (2000)
Alternaria Brown Spot resistance	QTL	Dalkilic et al. (2005)
Citrus canker	QTL	Choi et al. (2005)
Citrus leafminer resistance	QTL	Bernet et al. (2005)
Cold acclimation	QTL	Cai et al. (1994)
Cold acclimation	QTL	Weber et al. (2003)
Salinity tolerance	QTL	Tozlu et al. (1999b)
Dwarfing	Qualitative	Cheng and Roose (1995)
Fruit acidity	QTL	Fang et al. (1997)
Apomixes	QTL	Garcia et al. (1999), Nakano et al. (2008)
Alternaria brown spot resistance	Qualitative	Cuenca et al. (2016)
Fruit quality	QTL	Yu et al. (2016)
Fruit quality	QTL	Curtolo et al. (2017)
Fruit quality	QTL	Imai et al. (2017)
Aroma volatiles	QTL	Yu et al. (2017)

Adapted from Chen et al. (2008)

Murcott) and their 116 F1 progeny. Accordingly, they identified 48 QTL regions for eight important fruit-quality traits, including fruit size or weight and flavedo color (Yu et al. 2016). They also used the same population and maps to identify single nucleotide polymorphism (SNP) markers associated with volatile traits and detected a total of 206 quantitative trait loci (QTLs) for 94 volatile compounds (Yu et al. 2017). Some fruit aroma QTLs were identified and the candidate genes in the terpenoid biosynthetic pathway were found within the QTL intervals (Yu et al. 2017). According to the authors, these QTLs could lead to an efficient and feasible MAS approach to mandarin fruit quality improvement (Yu et al. 2016, 2017). In these studies, the sequences that flank the QTL regions were available, allowing comparison between the results of mapping of different studies. For example, Imai et al. (2018) used association mapping and a genome-wide association study (GWAS) of fruit-quality traits in citrus using SNPs obtained by GBS. They found two regions for fruit weight that were common with QTLs in the maps reported by Imai et al. (2017) and one region for pulp firmness that was common with that reported by Minamikawa et al. (2017).

7.5 Marker-Assisted Selection (MAS)

7.5.1 Target Traits for MAS

Evaluating a trait of interest at higher precision is mandatory for estimating its mode of inheritance and heritability toward valuable DNA marker development. A single dominant gene-regulated monogenic trait is a simple but efficient target for MAS. Conversely, many fruit traits are quantitative traits regulated by multiple genes that are often susceptible to the environment. As the bearing position of fruit, girdling, or thinning method all affect citrus fruit quality, the time of evaluation (Verreynne et al. 2004; Freeman and Robbertse 2003; Khalid et al. 2012), orchard management, and evaluated fruit should be carefully selected. Among various fruit traits,

machine-measurable traits such as fruit weight, Brix content, and acidity will give a continuous value that makes it possible to estimate their significance by statistical analysis. Traits for fruit shape, however, rind smoothness, peelability, aroma, taste, or flesh firmness are evaluated by their appearance and generate categorical data (Fig. 7.2). It would be possible to obtain seed number and thickness or firmness of the rind as continuous data but measuring these traits will require considerable effort.

Many fruit traits exhibit large variation across different varieties, within the population, and between populations. Iterative evaluation suppresses interannual variations or environmental variations and improves the accuracy of estimation. For example, Combrink et al. reported the wide distribution of fruit weight and shape (Combrink et al. 2013) and rind color (Combrink et al. 2015) within and between half-sib families of Kiyomi tangor. Evaluation across two years confirmed similar trends for those traits, but the observed distribution varied and was less reproducible. Conversely, estimating the mode of inheritance and anticipated contribution explained by genetic effect for the target trait will help render the trait suitable for MAS. The heritability of a quantitative trait is a primary measure of evaluation; the heritability of *Phytophthora gummosis* resistance (Siviero et al. 2006), *Citrus leprosis virus* resistance (Bastianel et al. 2006), and male sterility of Satsuma mandarin (Goto et al. 2016) have been reported so far. Of those, Goto et al. (2016) reported high broad sense heritability for the number of pollen grains in anthers and the maturity of pollen grains in offspring populations of Satsuma mandarin that represent male sterility. Very recently, they reported the development of DNA marker available for MAS of male sterility (Goto et al. 2018). Furthermore, Kaminuma et al. (2013) deposited a database of broad sense heritability and narrow sense heritability data that cover a range of reported organisms, including citrus.

Despite the efforts needed in evaluating such traits, the chemical content of fruit has also been subjected to DNA marker development for use in the MAS of such a complex trait. Among these

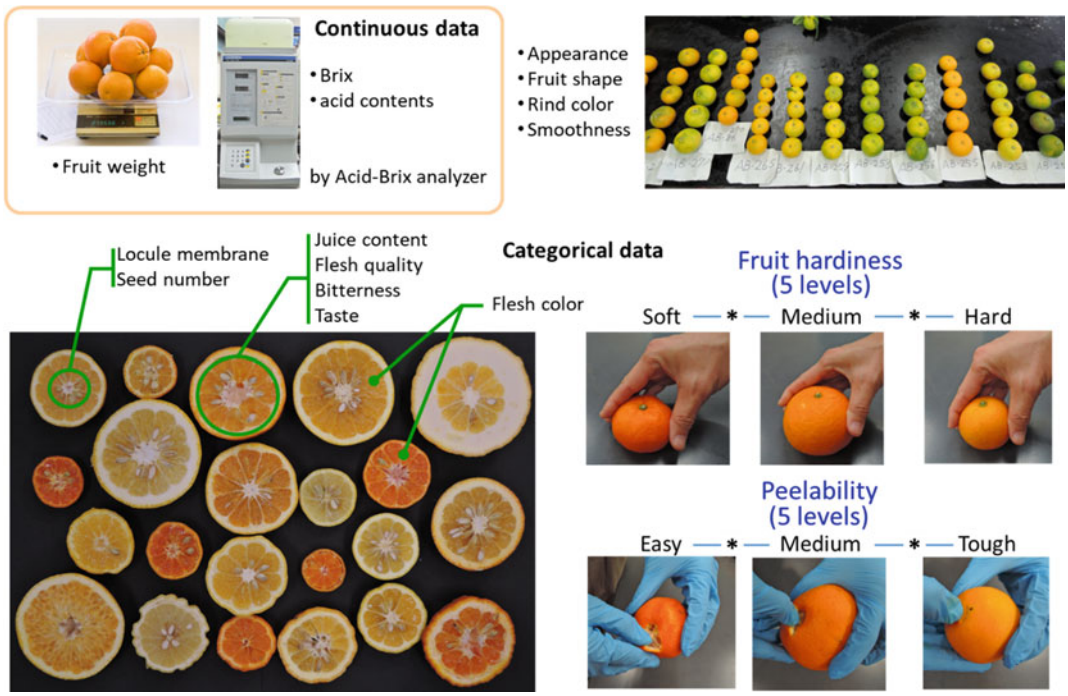

Fig. 7.2 Evaluation scheme of a fruit-related trait

complex traits, aroma is an important factor in determining the preference of consumers (Rouseff et al. 1994). However, evaluation of aroma has relied on subjective impression and has faced difficulties in both quantitative and qualitative representation. Therefore, an objective measure of aroma is required for MAS of this trait. The aroma of citrus is known to consist of various combinations of many chemical components (Zhang et al. 2017; Miyazaki et al. 2011). The contribution of certain chemicals to the flavor of a particular variety has been previously reported (Miyazaki et al. 2012). Yu et al. (2016) reported the detection of 148 volatile compounds by GC/MS analysis in the F1 population derived from Fortune × Murcott. They subjected the detected compounds to QTL analysis that identified 206 QTLs from 94 volatile compounds. Furthermore, they confirmed that 25 QTLs were consistent over more than two harvest times. Among those detected QTLs, they demonstrated that several QTLs corresponded to multiple compounds and identified four candidate genes, geranyl diphosphate synthase 1(*GPS1*), terpene synthase 3 (*TPS3*), terpene synthase 4 (*TPS4*), and terpene synthase 14 (*TPS14*), associated with some of those QTLs (Yu et al. 2017). Though the subjective impression of aroma is hard to describe, resolving it to each component and then evaluating the level of that component in the fruit is a reasonable approach to allow the use of MAS of such complex traits.

Gene expression level is another measure used to identify QTLs for MAS. Sugiyama et al. (2010) evaluated the accumulation of carotenoids by isolating the genes involved in their metabolism and reported the correlation between expression levels of zeaxanthin epoxidase genes to the alleles for those genes. Their subsequent paper reported the detection of QTLs associated with the expression level of four genes involved in carotenoid biosynthesis, zeaxanthin epoxidase (ZEP), beta-ring hydroxylase (HYb), phytoene synthase (PSY), and phytoene desaturase (PDS), determined by expression QTL (eQTL) analysis (Sugiyama et al. 2014).

7.5.2 Available DNA Markers for MAS

QTL detection for various traits in citrus has been reported and valuable DNA markers for the selection of CTV and ABS resistance and polyembryony have been developed. Polyembryony (apomixis or nucellar embryony) is an attractive trait of citrus that enables asexual propagation of seeds (Kepiro and Roose 2007). QTL detection for polyembryony was first reported by Garcia et al. in 1999 when they developed dominant DNA markers associated with loci for polyembryony (García et al. 1999). Nakano et al. (2008) reported the development of DNA markers for polyembryonic loci using a CAPS marker system, while Kepiro and Roose (2009) also reported another marker development study using AFLP markers. In a subsequent study, Nakano et al. (2012) isolated BAC clones that corresponded to the polyembryony loci identified previously, revealed the nucleotide sequence of those clones and predicted 70 candidate genes involved in polyembryony in the region. In 2017, Wang et al. reported the identification of the gene for polyembryony by evaluating trait-to-genotype association for citrus varieties using high-throughput sequencing technology (Wang et al. 2017). The identified gene for polyembryony, *CitRWP*, was a homolog of an *Arabidopsis thaliana* RKD family gene (Wang et al. 2017); they also revealed the point of mutation in this gene. These identified genes and the revealed polymorphisms between monoembryonic and polyembryonic varieties will contribute to developing a DNA marker useful for the selection of monoembryonic seedlings by MAS.

In addition to the demand for varieties with improved yield and fruit quality, demand is increasing for varieties resistant to multiple diseases to help decrease the cost associated with spraying agrichemicals to control these diseases (Machado et al. 2011; Rauf et al. 2013). Resistance to the physiological damages caused by low-temperature exposure is also anticipated to minimize damage during production. Many

studies have, therefore, attempted to develop valid DNA markers for MAS to select candidate seedlings resistant to disease and abiotic stress. Although most characteristics of agronomic interest are controlled by quantitative loci, there are citrus characteristics whose inheritance is known as monogenic, including citrus tristeza (CTV) and Alternaria brown spot (ABS) resistance. Tristeza resistance involved the use of *P. trifoliata* as one of the parental lines and required mapping of this monogenic characteristic with the aim of isolating the gene for future work on the transformation of scion varieties (Gmitter et al. 1996). An attempt at the fine mapping of the loci thought to confer CTV resistance in trifoliata orange selected a 282 kb region (Yang et al. 2003). Further study by Rai (2006) placed the loci within a 121 kb region and suggested candidate *R* genes. In 2012, Asins et al. reported the synteny of the CTV resistance loci among *C. grandis*, *C. aurantium*, and *P. trifoliata* by comparing their linkage maps with common DNA markers (Asins et al. 2012). Ohta et al. (2015) developed DNA markers that linked to the CTV resistance and could be used for MAS of distant hybrids. In the study for Alternaria brown spot (ABS) resistance, Cuenca et al. located the loci for ABS resistance in chromosome III using a bulked segregation analysis (BSA) approach (Cuenca et al. 2013). They revealed that the ABS resistance is a recessive trait and localized it to within 3.3 Mb near the centromeric region of chromosome III by assigning linkage map and genome sequences using DNA markers. They consequently identified a candidate gene by referring to gene annotations detected in that region and located a SNP marker (SNP08) that mapped 0.4 cM from a region containing the so-called *ABSr* locus to Alternaria brown spot resistance (Cuenca et al. 2016). The selected SNP marker is currently used for the selection of ABS resistant genotypes in breeding programs carried out at IVIA (Spain) and CIRAD (France).

As described, the development of DNA markers valid for MAS with higher discriminative power that result in certain trait improvements has already reached a level that renders them useful in the breeding program. However, development of valid DNA markers for MAS of citrus canker, citrus black spot, and Huanglongbing disease (HLB) has not yet been accomplished and requires further work. Although the development of valid DNA markers for MAS of various fruit traits is in demand, this is difficult to achieve using conventional linkage mapping and QTL analysis as many fruit traits are quantitative traits in which multiple genes are involved.

7.5.3 Practical Procedures of MAS in Citrus Breeding

Many factors, including the type of DNA marker for MAS, genotyping method, the number of seedlings to be evaluated, the time taken to complete the analysis, accessible equipment, and total usable budget, restrict the use of MAS in breeding. In this review section, a typical scenario for conducting MAS in citrus breeding is introduced.

7.5.3.1 MAS for Polyembryonic Seedlings

Many citrus varieties except pummelos and certain mandarins display polyembryony (Ueno et al. 1967). Using polyembryonic varieties as female plant reduces the chance to obtain zygotic seedlings that are difficult to be distinguished from nucellar seedlings by their appearance. The use of MAS to identify the zygotic seedlings is a simple and effective strategy to increase diversity at a minimal cost. The probability of distinguishing a zygotic seedling among the nucellar seedlings depends on the genotypes of the parental varieties (Fig. 7.3). A single DNA marker is sufficient for MAS of zygotic seedlings when the parental alleles are homozygous with the parental varieties carrying different alleles (case 1) or where they are heterozygous and not overlapping (case 6). The possibility will vary from 0.5 to 0.75 in other cases that depend on the genotype combination of the parents (case 2–5). In such cases, applying several markers improves the probability of identifying the candidate genotypes. The application of MAS for the

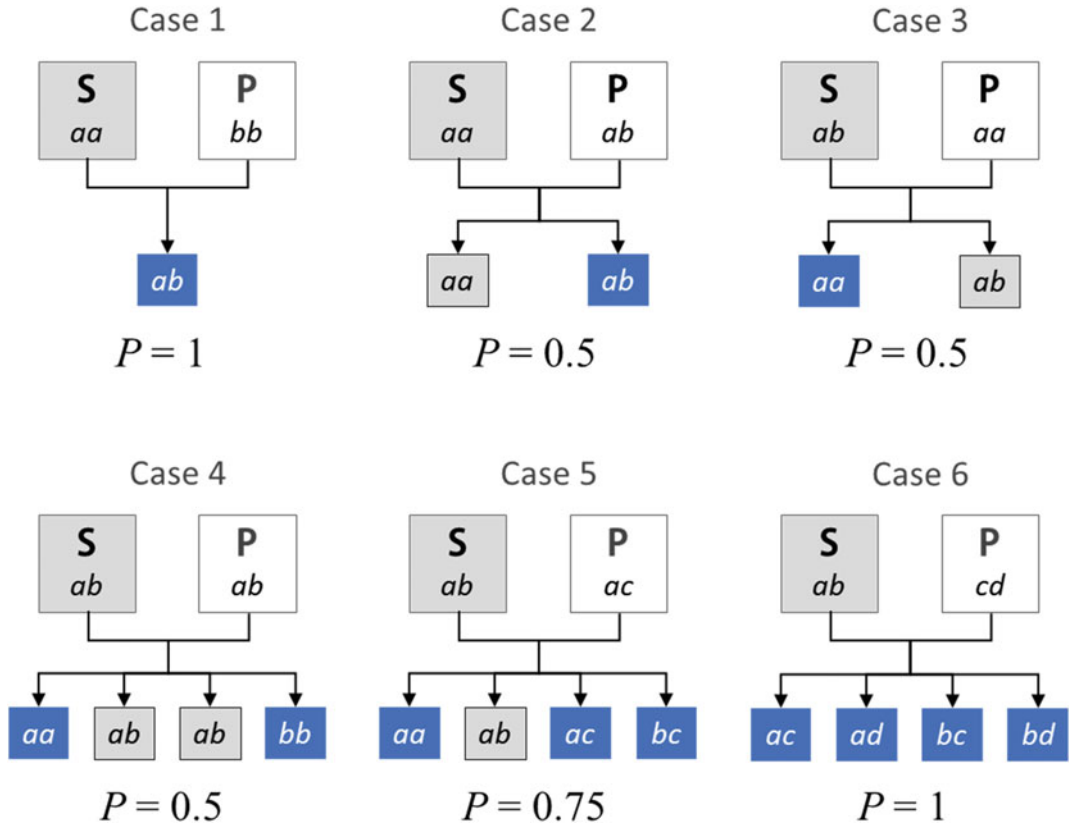

Fig. 7.3 Changes in the probability to distinguish a sexual zygote from nucellar embryo depending on the parental genotypes. S: seed parent, P: pollen parent, *P*: represents a probability to distinguish a zygote from nucellar seedlings

zygotic seedling selection allows an early discrimination of the offspring population obtained from a cross. The use of accurate DNA markers is also beneficial to confirm the parentage of the seedlings at the early stages of breeding.

7.5.3.2 Fast and Low-Cost Genotyping

The steps for DNA extraction and genotyping primarily determine the total cost, throughput rate, and time required to accomplish MAS. Of the available codominant DNA markers, SNP markers require particular equipment for detection in small-scale analysis but are less polymorphic because they only represent up to two alleles. Conversely, SSR markers require a DNA sequencer for high-precision analysis but are more discriminative than SNP markers because of their multiallelic nature. Hard citrus tissue often requires special care for tissue disruption to extract DNA that increases the total cost and time associated with MAS. Many DNA marker analysis applications also require highly purified DNA samples to obtain an accurate result so that the purification of DNA samples would further increase the cost.

It is beneficial for MAS to apply a particular type of Taq polymerase that is less susceptible to impurities as the purification step can then be avoided, thereby reducing the cost. It is also possible to improve the reproducibility by selecting a DNA marker as short as the size of the amplified product because the amplification of longer products tends to fail in degraded DNA samples and impurities in the sample can also inhibit amplification. A typical DNA extraction protocol will yield plenty of DNA for several PCR reactions as required for the first selection of MAS. A fast and cost-effective genotyping

Fig. 7.4 A brief outline of the toothpick method for rapid genotyping of SSR markers without DNA purification. **a** Prick a leaf 30 times on clean filter paper. **b** The pricked leaf. **c** The toothpicks are immersed in an aliquot of solution and then used for PCR after heat denaturation. Compiled from Ohta et al. (2013)

protocol for MAS that extracts DNA with a toothpick was reported (Ohta et al. 2013). The amount of DNA sample obtained by this protocol is sufficient for several analyses and the use of a robust type of Taq DNA polymerase suppressed the inhibition caused by impurities (Fig. 7.4). The obtained product could be used for gel electrophoresis and DNA sequencing analysis, thereby expanding the choice of DNA marker for the analysis. Conversely, application of a high-throughput genotyping analysis using SNP markers or an NGS approach requires sufficient amounts of highly purified intact DNA. From a cost perspective, therefore, selecting a possible candidate by the toothpick method and then evaluating genome-wide genotypes of the selected sample using highly purified DNA may be a cost-effective solution for MAS of citrus.

7.5.4 Quality Control of Genotype Data

Verifying that the obtained genotypes are detected as expected is essential to perform high-precision DNA marker analysis (Pompanon et al. 2005). DNA marker analysis often fails for a variety of reasons, so the incorrect genotype can lead to incorrect conclusions. Analysis failure can be caused by insufficient purity or degradation of the template DNA, sample contamination, inappropriate analysis protocols or use of equipment, and human error in data handling (Utsuno and Minaguchi 2004). MAS typically involves the use of DNA samples that occasionally contain impurities or lower DNA concentrations that could produce a faint amplification product and incorrect genotype. Likewise, the use of degraded DNA samples will give shorter products for a longer amplified product, also resulting in the failure to obtain the correct genotype (Goodwin et al. 2011). Modifying the experimental design to reduce such failures by using multiple genotyping analyses and optimizing the protocol for those samples is effective but will increase the total cost for MAS.

Aside from experimental failures, a DNA marker itself could cause a genotyping error; this would, however, be difficult to detect even with continuing analyses. The type of genotyping error caused by DNA markers is known as the allelic drop-out and is critical in codominant markers. Unexpected polymorphisms within a primer binding site of a particular variety will result in insufficient amplification of the product and it often causes an allelic drop-out (Pompanon et al. 2005). A genotype will show false homozygous when it comprises a null allele. Such genotyping errors are caused by a particular combination of plant variety and DNA marker. The use of whole-genome sequences of a wide range of varieties for DNA marker design would suppress this error but will not avoid the error in unexamined varieties.

Estimating the null allele frequency of a DNA marker from the allele frequency in a broad

population (Oddou-Muratorio et al. 2009; Jones and Ardren 2003) or evaluating the deviation from the Hardy–Weinberg equilibrium (Chen et al. 2017) could detect plausible genotypes but is not a definitive solution. Evaluating the conflict of genotypes with a known set of parent–child trios is an alternative approach. Even though this approach does not approve the validity of the genotype data, it allows for examination of the accuracy of already designed DNA markers. Shimizu et al. (2016) evaluated the SSR marker genotypes with 59 known trios and then applied the selected markers to the parentage estimation.

7.5.5 Current Constraints on MAS

The loci detected by conventional linkage mapping or QTL analysis are valid for selection in a population obtained from the same parents; special care must be taken when applying them to different hybrid combinations. MetaQTL and FlexQTL are alternative methods for identifying common QTLs in diverse varieties. MetaQTL (Veyrieras et al. 2007) integrates the results of multiple QTL studies to estimate the actual QTLs among them by the Akaike criterion (Veyrieras et al. 2007; Goffinet and Gerber 2000). FlexQTL is a pedigree-based QTL estimation method for full-sib families (Bink et al. 2014). Application of both MetaQTL and FlexQTL for identifying fruit traits were demonstrated in apple (Bink et al. 2014; Costa 2015). However, it is hard to evaluate multiple hybrid populations to examine the availability of DNA markers for MAS because of limits in the available orchards, tree size, and the extended juvenile period.

Two alternative analysis methods, genome-wide association study (GWAS) (Oraguzie et al. 2007) and genomic selection (GS) (Meuwissen et al. 2001), are an attractive way to avoid constraints of usual QTL analysis (van Nocker and Gardiner 2014). GWAS detects a genomic region to show correlation with a trait by evaluating linkage disequilibrium observed among wide varieties (Hall et al. 2010; Soto-Cerda and Cloutier 2012; Balding 2006). GWAS evaluates a set of diverse varieties other than a hybrid population for the detection of QTLs. It has the power to detect QTLs that could be missed in a particular population when examined using conventional QTL analysis. Furthermore, GWAS improves the resolution of QTL detection by using varieties with introgressed genomes rather than a hybrid population. Conversely, GWAS is susceptible to the genetic structure of a population and could result in false positive correlation (Balding 2006). Minamikawa et al. (2017) used GWAS analysis to evaluate 111 citrus varieties and 676 individuals from 35 full-sib families using their phenotype data on 17 traits with genotypes for 1,841 SNP markers. They detected multiple genomic regions that show a strong correlation with 11 fruit traits by integrating the genetic structure of those samples to suppress the false positives.

Though GWAS has benefits for the detection of QTLs common to a range of varieties, it has less power for the detection of multiple loci with small genetic gain. Another approach, genomic selection (GS), can be used to predict the performance of a trait in progeny by modeling the correlation between the whole-genome genotype and a phenotypic value (Meuwissen et al. 2001; Meuwissen 2003). In GS analysis, a multivariate correlation prediction model is developed from the genome-wide genotypes of a set of samples and their observed phenotypic values. The model can then be used to estimate the phenotypic value from a given genotype data. As GS does not estimate a genomic region of significant effect on the trait, it is not available for DNA marker development for MAS of a trait. Conversely, GS is useful to estimate the phenotypic value of a trait in which many loci of a minor genetic gain participate. In citrus, Gois et al. (2016) evaluated the prediction accuracy of GS on a hybrid population obtained from Murcott tangor × Pera sweet orange for 10 fruit traits and 5,287 DArTSeq marker scores (Gois et al. 2016). The predicted scores obtained by rrBLUP (random regression best linear unbiased predictor) based prediction equations ranged from 0.59 to 0.91. They also demonstrated that the proportion of genetic variance explained by markers increased in relation to the number of markers.

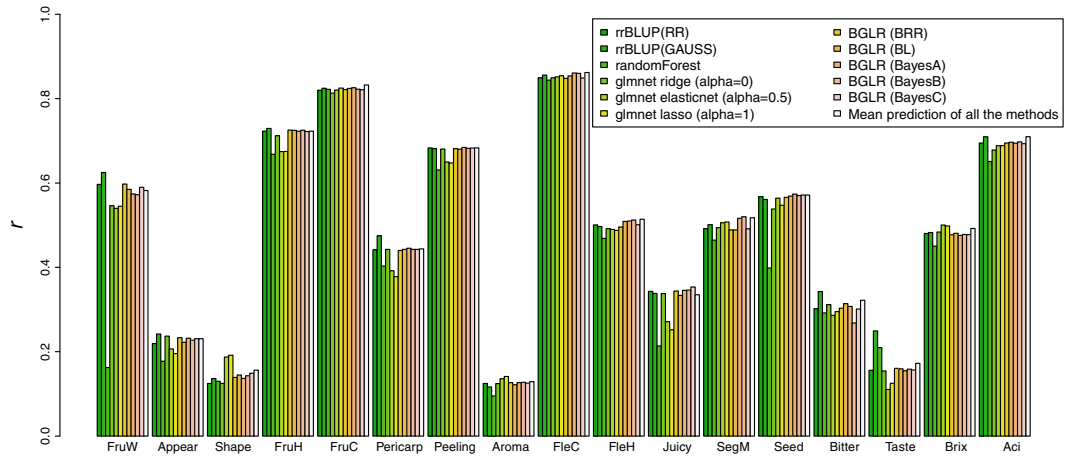

Fig. 7.5 Comparison of prediction models using parental populations. Twelve methods (RR: ridge kernel regression, GAUSS: Gaussian kernel regression). Prediction accuracy was measured as the Pearson's correlation coefficient (r) between predicted genotypic values and phenotypic values (Minamikawa et al. 2017)

The first report of GS proposed by Meuwissen used a single family to build the regression equation (Meuwissen et al. 2001) and Gois et al. (2016) also evaluated a single family for regression modeling. Single-family-based prediction can be extended to integrate multiple family data by integrating phenotypic data and genotype data obtained from multiple families and existing varieties for regression model building. Minamikawa et al. assessed the prediction accuracy for 15 fruit traits by building prediction equations from phenotype and genotype data of 111 existing varieties and 35 full-sib families; then demonstrated the availability for the prediction of three fruit traits (fruit weight, rind color, and flesh color) at high accuracy (>80%; Fig. 7.5) (Minamikawa et al. 2017). They examined the performance of 12 regression models for their prediction, but no apparent differences were observed for those models on individual traits. Meanwhile, they demonstrated that increasing the number of individual for the modeling improved the accuracy of each trait. The effect of the sample size was evident for traits with lower prediction accuracy. The traits evaluated with the categorical data gave lower predictions scores. This suggested that it was possible to improve the accuracy by changing the measuring method for these traits. However, their results demonstrated the advantages of GS for predicting the performance of a trait that is difficult to determine using conventional methods by integrating sufficient amounts of data.

7.6 Future Perspectives

NGS analysis has become a common technique in citrus studies and can now be used to detect a polymorphism at a one-base resolution in the genome sequence. NGS technology also enables the generation of a high-density linkage map in minimum time, thereby drastically changing the identification of QTLs and linkage analysis for various traits. Both GWAS and GS analyses demonstrate the power-of-selection for citrus breeding by estimating trait-to-genotype regression from high-density genotype data obtained from a large sample set. Additionally, attempts to estimate the performance of a variety as a breeding parent from its genome-wide genotype are in progress. The known breeding history of crossbred varieties and recently uncovered history of indigenous varieties would provide a basis for such studies. While genome-wide analysis provides many achievements, MAS using a small number of DNA markers is still a valid technique for selecting promising seedlings

at minimum cost. However, DNA markers for MAS developed from conventional linkage analysis had constraints in terms of their availability for particular cross combinations. A GWAS study is a prospective approach to avoid these constraints by considering a wide range of varieties. Further study into the development of valid DNA markers for MAS is highly anticipated. Also, practical applications of GS as a method of selecting complex traits involving many genes are also anticipated. Conversely, considerable numbers of citrus traits such as fruit shape, tree vigor, and tree form have been measured by their appearance or evaluated as categorical data. Restructuring the measuring methods for these traits is required to develop a valid selection technique. In future, increasing the measuring accuracy of complex traits and applying the genome-wide association technique for these traits are expected to assist in the selection of more exclusive and difficult traits like alternate bearing or aroma.

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Citrus Genomes: From Sequence Variations to Epigenetic Modifications

8

Qiang Xu and Mikeal L. Roose

Abstract

Genome platform is critical to the basic molecular biological research and to the molecular marker assisted breeding. Genomics data of citrus species have accumulated rapidly after the first publication of sweet orange in 2013. With the cost of sequencing decreased dramatically, each individual research group can afford to sequence many genomes. This chapter summarizes methods for genome assembly and annotation, how we identify genes by comparative genomics, linkage mapping and association analysis, how we apply SNP markers in cultivar identification and breeding, and the current knowledge on epigenetic regulations in Citrus. This chapter also anticipates future genomic researches in Citrus.

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8.1 Introduction

A genome is all the genetic information of an organism, including genes encoding proteins and noncoding genes. Physically, a genome sequence is a complete list of the nucleotides (A, C, G and T). Genome sequencing is fascinating because of its capability to reveal the sequence and localization of millions of genes, showing a global view of the gene map and genetic diversity in a species. In the last 10 years, citrus genomics has developed rapidly and provides a genetic framework for both basic and applied studies. It is now relatively easy to identify genetic polymorphisms and potential key genes, particularly those which control interesting and specific traits in fruits by using genomics and transcriptomics strategies. It is also easy to develop molecular markers which are associated with important traits to assist citrus breeding. These aims have been rapidly facilitated by the low cost of next-generation sequencing technology (particularly the Illumina platform) and the recently emerged single-molecule sequencing technology (e.g. PacBio). This sequencing-based approach is favored by citrus geneticists since it is convenient, efficient, fast, and space-conserving when compared with classic genetic studies. The authors believe that a sequencing-based approach will be more popular in citrus basic researches, particularly as a direct route to a gene-level understanding of the molecular basis of interesting traits. This review will summarize recent

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researches on genome assembly and annotation, transposable elements, cytogenomics, comparative genomics, population genomics (GWAS), transcriptome, miRNA, epigenome, and will also discuss the prospects of citrus genomics.

8.2 Genome Assembly

Genome survey is usually employed to estimate the overall genome complexity and characteristics of a species to be sequenced. The survey mainly includes the estimations of genome size, level of heterozygosity, proportion of repeat elements and GC contents. Based on the survey data, an optimal strategy for sequencing and assembly of the genome can be selected, such as the sequencing platform, the minimum depth/data of sequencing and the insert-size of libraries. Repeat elements and heterozygosity levels are the two most challenging factors in a de novo genome assembly project. Usually, a pilot survey is necessary to have an initial assessment on the potential effects of these two factors. The repeat elements are more difficult to correctly assemble from short-read sequencing, such as Illumina-based sequencing, while the long-read sequencing technologies, such as PacBio, are more powerful to gain more information of repetitive elements. The heterozygosity level can be significantly reduced when using successive inbreeding strategy or double haploid strategies. The latter is actually complete homozygous genotypes, which greatly reduce the genome complexity and facilitate genome assembly. In the published genome papers, sweet orange (Xu et al. 2013), clementine mandarin (Wu et al. 2014), apple (Velasco et al. 2010), melon (Garcia-Mas et al. 2012), strawberry (Shulaev et al. 2011), banana (D'Hont et al. 2012) and peach (Verde et al. 2013) are dihaploids; tomato (Consortium 2012), grape (Jaillon et al. 2007), papaya (Ming et al. 2008), and watermelon (Guo et al. 2013) are inbred lines (Fig. 8.1).

Whole-genome shotgun strategy and next-generation sequencing (NGS) technology is an efficient and cost-effective way to assemble a genome, and this strategy is widely applied to draft genome assembly. Sequencing libraries can be constructed with small insert-size from 180 to 500 base pair (bp) and long insert-size with 2, 5, 10, 20 kb, and even longer sizes, and paired-end (PE) reads can be sequenced by Illumina sequencing platforms.

Third-generation sequencing (TGS) strategies are recently adopted more for genome assembly projects. The PacBio RS single-molecular real-time (SMRT) sequencing technology can be used to generate single-molecule long reads. In our case to assemble the pummelo genome (Wang et al. 2017), we constructed a 10 kb SMRTbell™ library and sequenced 10 SMRTcells P6C3 data. Finally, a 9.2 GB fastq file was generated with 4,882,948,654 bp in total, comprising 60x genome coverage, which produced a high-quality genome.

The TGS platform has the major advantage of producing long reads which are more effective to cope with repetitive elements, the shortcoming of NGS data. However, TGS introduced more sequencing errors than the NGS data. A combinatory strategy using both TGS and NGS platform would be more useful for genome assembly.

To avoid the sequencing reads with artificial bias (i.e. low-quality paired reads, which mainly result from base-calling duplicates and adapter contamination) and other possible risk factors, the following type of reads should be removed:

(a) Reads with $\geq 10\%$ unidentified nucleotides (N);
(b) Reads with >10 nt aligned to the adapter, allowing $\leq 10\%$ mismatches;
(c) Reads with >50% bases having phred quality <5;
(d) Putative PCR duplicates generated by PCR amplification in the library construction process (i.e. read 1 and read 2 of two paired-end reads that were completely identical);
(e) A kmer-based method to correct all sequencing data using Jellyfish (Marcais and Kingsford 2011) and Quake (Kelley et al. 2009);

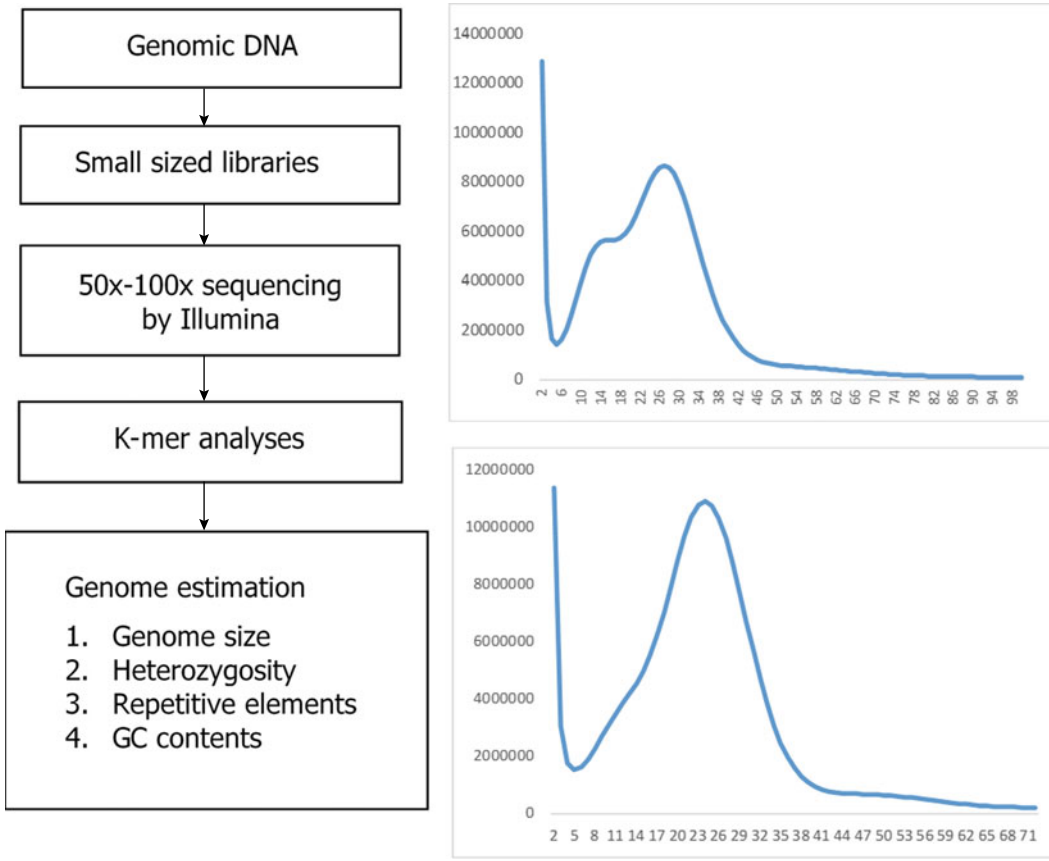

Fig. 8.1 Genome survey of citrus genomes. The left panel shows the procedures for genome estimation. The right panel indicates a highly heterozygous individual (above) and low heterozygosity (below)

(f) In order to eliminate the negative effect caused by chloroplast in genome assembly, all sequencing reads that align to the chloroplast genome should be filtered from the mapped reads with Bowtie (Langmead and Salzberg 2012).

The assembly process used for the pummelo genome (Wang et al. 2017) is exampled by Fig. 8.2. The raw PacBio reads should be error-corrected and assembled using hierarchical genome assembly process (HGAP) in SMRT Analysis v2.3.0 (Chin et al. 2013). The workflow involves three main steps:

(1) Generate long and highly accurate pre-assembled sequences;
(2) Assemble the genome based on the overlap-layout-consensus (OLC) algorithm using Celera;
(3) Use Quiver to polish the draft assembly.

Then perform another round of polishing to further improve the quality of the assembly to produce the original contig file. Next, mate-pair reads can be used to construct scaffolds with package SSPACE-STANDARD (Boetzer et al. 2011). In this step, BWA (Li et al. 2009) can be used to align reads to contigs to extend them for scaffolding. After that, package Gapcloser (Luo et al. 2012) can be used to fill the gaps in scaffolds. Then, the package SSPACE-STANDARD was used again to further extend the scaffolds. Finally, the NGS data can be included to correct

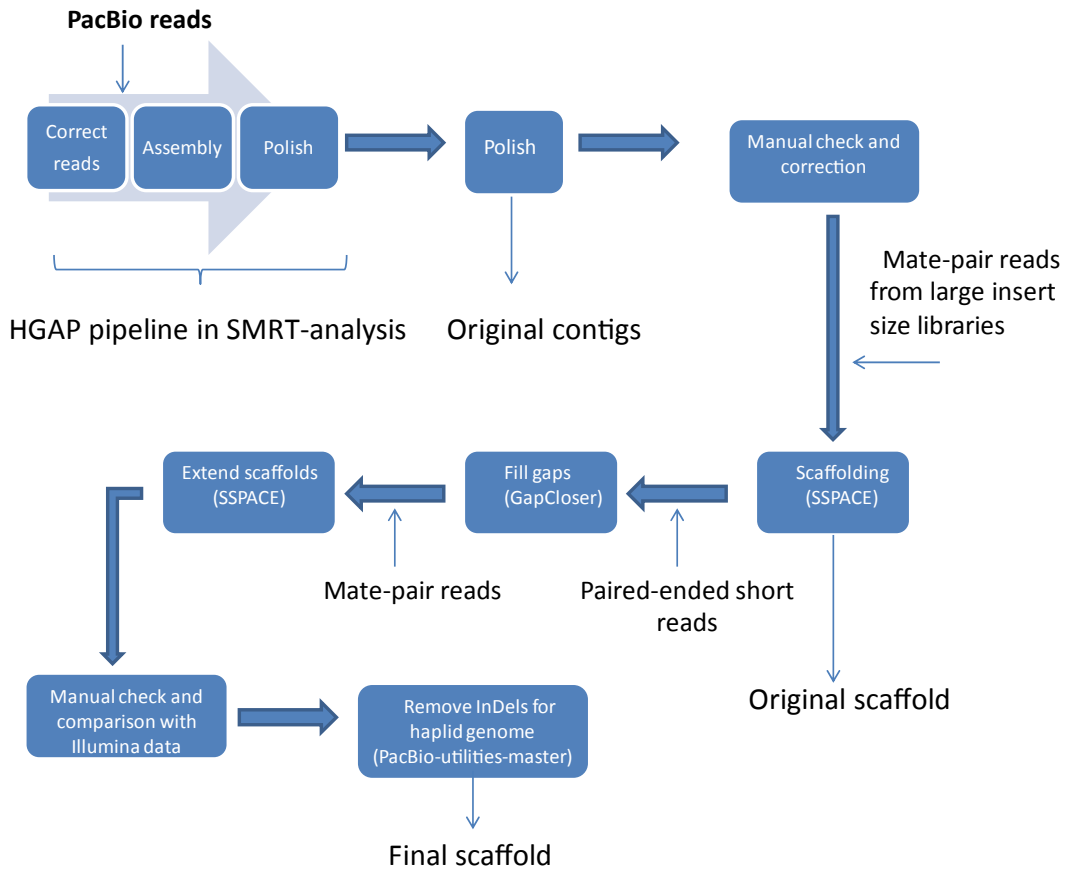

Fig. 8.2 The workflow for the genome assembly of *C. maxima* (*C. grandis*). The PacBio reads used are from 30 SMRT cells. The mate-pair reads are from libraries with insert-size of 2, 5, 8, 10, and 20 kb, respectively. The paired-end reads are from libraries with insert-size of 200, 300, and 500 bp, respectively

small InDels errors in assembly. All NGS data was mapped to scaffolds from last step using BWA, and the generated SAM files were converted to BAM files by SAMtools (Li et al. 2009). Then the package PacBio-utilities-master (https://github.com/douglasgscofield/PacBio-utilities) used the information in BAM files to correct small InDels and we obtained our final assembly.

De novo assemblies of citrus genomes have been published in six citrus species by February 2018, including sweet orange (Xu et al. 2013), clementine (Wu et al. 2014), pummelo (Wang et al. 2017), citron (Wang et al. 2017), Ichang papeda (Wang et al. 2017) and *Atalantia* species (Wang et al. 2017) (Fig. 8.3). The sweet orange genomes sequenced and assembled was a dihaploid line using an Illumina platform by Huazhong Agricultural University, China, and a heterozygous line by Joint Genome Institute and the University of Florida, USA. The clementine genome was assembled mostly by Sanger sequencing reads. The pummelo genome was mainly assembled by the PacBio single-molecule sequencing technology (Table 8.1). The results suggested that the single-molecule sequencing technology is more powerful to assemble a genome with a moderate cost. The pummelo genome constitutes a high-quality genome which could be used as a genetic frame for citrus genetic and genomic studies.

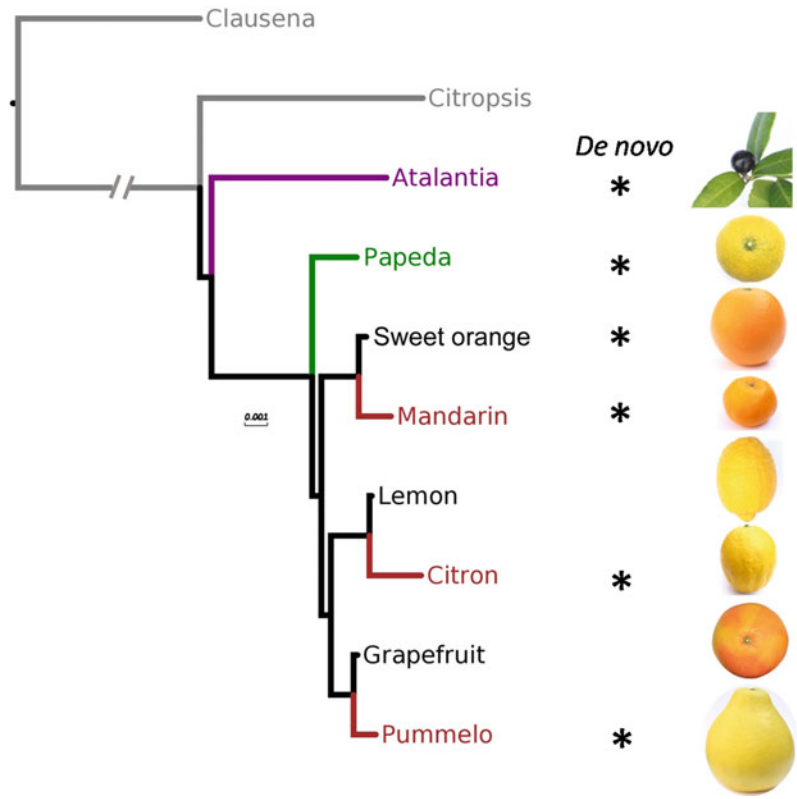

Fig. 8.3 Citrus phylogeny based on published genome assemblies of citrus species. De novo assemblies were indicated by star

8.3 Genome Annotation and Databases

Genome annotation can be conducted by de novo predictions on the repeat-masked genome and then integrate this with evidence-based predictions using PASA (Fig. 8.4) (Haas et al. 2003). Gene finders such as FgenesH, GeneID, Genscan and GlimmerHMM (Majoros et al. 2004) with parameters trained for *Arabidopsis* can be used. Protein sequences of all *Chlorophyte* species derived from UniProt database can be included to align to the genome using TBLASTN, and the homologous genome sequences then can be identified from spliced alignment against the matching proteins using GeneWise (Birney et al. 2004) (Score >60). Transcriptome data such as RNA-seq reads can be aligned to the genome using two splice junction mappers, TopHat (Illumina reads) (Haas et al. 2003) and BioScope (SOLiD reads) (Trapnell et al. 2010) and the alignments can then be used as input for Cufflinks (Trapnell et al. 2010) for transcript assembly. All the gene structures predicted by the methods described above can be combined into consensus gene models using EVM. Gene models produced by EVM can then be updated by PASA assembly alignments.

Putative gene functions could be assigned according to the best hit of alignment using BLASTP (*E*-value $<10^{-5}$) to SwissProt and TrEMBL (Bairoch and Apweiler 2000) databases. Gene models with no match in these databases were labeled with "hypothetical proteins". The pathway in which the gene might be involved was derived from the matched genes in KEGG (Kanehisa and Goto 2000) database. Gene ontology (GO) term assignments, motifs and domain of genes can be extracted by

Table 8.1 Statistics for eight citrus genome assemblies and annotations

	Sweet orange	Sweet orange	Sweet orange	Clementine mandarin	Pummelo	Citron	Ichang papeda	Atalantia
Sequencing strategy	TGS + dihaploid	NGS + dihaploid	NGS of diploid	Sanger + haploid	TGS + haploid	NGS	NGS	NGS
Chromosome number (2*n*)	18	18	18	18	18	18	18	18
Total size of assembled contigs (Mbp)	337.8	301	252.2	295.2	344.8	368.5	334.7	288.4
Largest contig (Kbp)	7,530.2	323.3	119	1,230	10,624	409.9	772.9	196.1
Contig N50 (Kbp)	1,803.1	49.9	6.6	118.9	2,182.5	46.5	76.6	23.9
Contig N90 (Kbp)	122.2	11.2	2.2	24.7	70	4.1	7.4	4.1
Total size of assembled scaffolds (Mbp)	338.4	320.5	319.2	301.4	345.7	404.9	357.2	315.8
Longest scaffold (Mbp)	13.3	8.16	5.93	30.5	14.3	2.44	2.98	7.16
GC content	35.46	34.06	34.6	35.0	34.92	32.00	32.08	30.72
Number of gene/transcript models	29,301/45,016	29,445/44,387	25,379/46,147	24,533/33,929	30,123/42,886	32,579/47,506	32,067/43,103	28,420/65,507
Mean transcript length (bp)	2,161	1,817	1,762	1,708	1,572	1,758	1,563	2,242
Mean coding sequence length (bp)	1,307	1,255	1,245	1,242	1,141	1,154	1,106	1,232
Percentage of transposable elements	51.9	20.5	31.1	44.67	45.83	43.80	39.31	43.55
Data source	Unpublished	Xu et al. (2013)	Wu et al. (2014)	Wu et al. (2014)	Wang et al. (2017)	Wang et al. (2017)	Wang et al. (2017)	Wang et al. (2017)

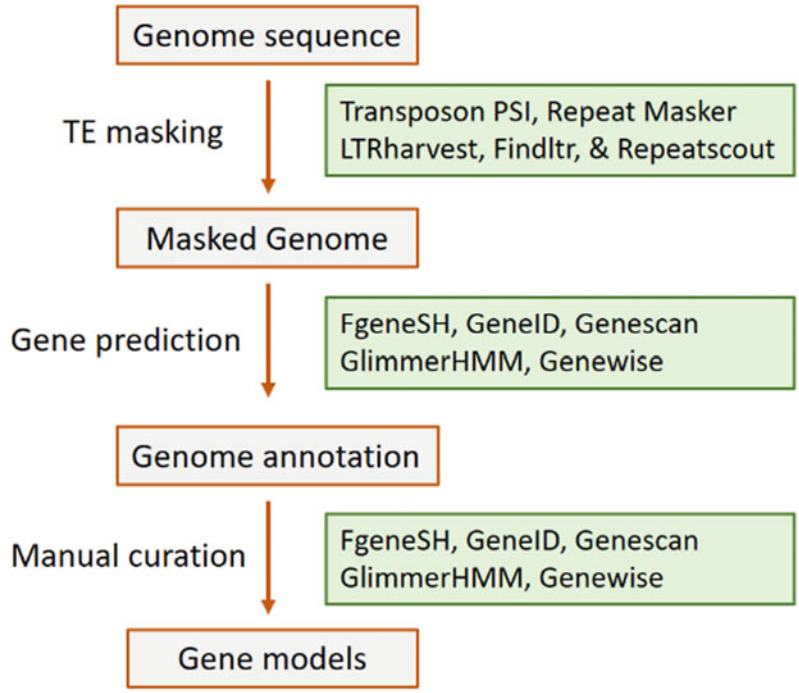

Fig. 8.4 The procedures for genome annotation. This pipeline included repetitive element identification, protein-coding gene annotation and manual curation. The related softwares for each step are indicated in green boxes

InterProScan (Zdobnov and Apweiler 2001) program, which analyzes peptide sequences against the member databases of InterPro (Zdobnov and Apweiler 2001), including Pro-Dom, PROSITE, PRINTS, Pfam, PANTHER, and SMART. Putative signal peptide sequences can also be identified using SignalP and TargetP (Emanuelsson et al. 2007), and transmembrane regions could be predicted by tmHMM (Krogh et al. 2001).

We can employ a combination of ab initio gene prediction programs, homology searches and RNA sequencing (RNA-seq) analysis to annotate the protein-coding genes in *C. sinensis* genome. Take the sweet orange genome as example. Four ab initio gene finding programs were used to analyze the masked genome. Furthermore, 958,121 *Citrus* ESTs, 1,259,935 proteins from UniProt database and 964.6 million RNA-seq reads were generated or collected as homology-based evidence. Totally, we identified 29,655 protein-coding loci with 44,645 transcripts. Public citrus genome databases are listed in Table 8.2.

Table 8.2 List of public databases for citrus genomes

Database	Source	Main functions
Citrus annotation project	http://Citus.hzau.edu.cn	Gene search, BLAST, G-browser, Protein–protein interaction prediction, Pathway enrichment, Downloads
Citrus genome database	http://www.citrusgenomedb.org	G-browser, synteny, Mapviewer, BLAST, Downloads, Search
Phytozome	http://phytozome.jgi.doe.gov	Sequences, annotation files, BLAST

8.4 Transposon Elements in Citrus Genome

Structure-, homology- and ab initio-based methods and relevant algorithms can be used to identify transposable elements in the genome. For long terminal repeat (LTR) retrotransposons, structure-based softwares, LTRharvest (Ellinghaus et al. 2008) and findltr (Rho et al. 2007) can be used to identify full-length candidates in the genome. The GetORF program from the EMBOSS package (Rice et al. 2000) could be further used to find open reading frames (ORFs) in retrotransposons. Then the hmmsearch program from HMMER package could be used to scan retrotransposon domains based on the following profiles: Reverse transcriptase (RT) by PF00078 and PF07727, integrase (INT) by PF00665, PF00552 and PF02022, RNaseH (RH) by PF00075, group-specific antigen (gag) by PF03732, and aspartic proteinase (AP) by PF00026 and PF00077.

DNA transposons and long interspersed elements (LINEs) could be searched using TransposonPSI (http://transposonpsi.sourceforge.net), a software that is based on sequence homology to diverse transposable element families by position-specific scoring matrices (PSSMs).

RepeatScout (Price et al. 2005) that calculates k-mer frequencies could be applied to discover novel transposable element families that are missed by signature-based and homology-based methods. Any sequences highly repeated in the genome could be identified to build consensus repeat candidates. However, to avoid false positives reported by RepeatScout, members known to be functional genes of large gene families and members belonging to simple tandem repeats should be removed for further analysis.

In citrus, the repetitive elements account approximately 40% of the genome. Class I long terminal repeat (LTR) retrotransposons which occupy over two-thirds of the total transposable elements and 30% of the genome belong to 198 Copia families and 130 Gypsy families based on structure analysis. Similar to other small plant genomes such as *Arabidopsis thaliana* (Du et al. 2010) and rice (Gao et al. 2004), some of the LTR family showed large number of members (Table 8.3). Estimation of insertion time indicated that >80% of intact elements have been amplified in the last 2 million years, with 10%

Table 8.3 The top 15 retrotransposon families in sweet orange genome (Zhu 2012)

Family	Sequence in genome (kb)	Count	Avg length (bp)	Avg insertion date (mya)
Cs_RLG1	11537.8	11563	997	1.52
Cs_RLC1	7798.9	8403	928	1.49
Cs_RLG2	1768.2	1804	980	1.29
Cs_RLG3	1294.6	633	2045	1.51
Cs_RLG5	1074.2	1184	907	0.55
Cs_RLG24	1024.4	1253	817	2.46
Cs_RLC70	855.8	1188	720	0.02
Cs_RLC64	812.4	1468	553	1.38
Cs_RLC28	780.1	1484	525	1.49
Cs_RLG14	734.2	979	749	0.84
Cs_RLG8	728.9	970	751	0.98
Cs_RLC48	549.1	999	549	0.86
Cs_RLG9	525.5	584	899	1.27
Cs_RLG6	507.9	708	717	1.11
Cs_RLG5	493.5	1184	416	0.55

younger than 50,000 years. In contrast, DNA transposons only comprise ~8% of the citrus genome. Among them, miniature inverted-repeat transposable elements (MITE) occupied approximately 60%. Interestingly, a new type of MITE, named MiM (MITE inserted in microsatellite), can be identified in the citrus genome (unpublished data). MiM is similar to Micron in that both of them are inserted in microsatellites with TA repeats, but they have no sequence similarity with each other, even in their terminal regions.

One interesting topic is the activity of the transposable elements (TEs) and their causal role in phenotypic variation. Detecting of TE activity can be performed by gene expression analyses, copy number evaluation and characterization of genetic polymorphisms. Some studies have clearly shown that the TE activities caused parthenocarpy in apple (Yao et al. 2001), the activation of anthocyanin biosynthesis in grape and citrus (Kobayashi et al. 2004; Butelli et al. 2012), unusual abnormal development of the inflorescence in grape (Fernandez et al. 2010) and apomixis in citrus (Wang et al. 2017). TEs are also hotspots which show genetic polymorphisms more easily than other genomic regions, thus constitute a good source for development of molecular markers.

8.5 Citrus Cytogenomics

Cytological approaches are increasing in significance in genomic studies. With the advent of molecular cytogenetic techniques, genetic linkage maps can be integrated with individual chromosomes. The most unequivocal way to assign genetic linkage groups with specific chromosomes is the localization of molecular markers by fluorescent in situ hybridization (FISH). With the development of FISH, chromosome identification became more versatile and accurate by using repetitive sequences and large-insert clones as probes. For instance, the application of FISH with rDNA probes and several tandem repeat sequences has enabled the construction of the karyotypes of *Fragaria vesca* and *Malus* (Lim 2004; Schuster et al. 1997), and easy identification of all somatic metaphase chromosomes of cucumber (Han et al. 2008). FISH with rDNA probes was performed in *F. vesca* to gather cytogenetic information that illuminates genomic divergence among different taxa at multiple ploidy levels, as well as to explore the evolution of ribosomal RNA genes (Liu and Davis 2011).

Bacterial artificial chromosome (BAC) clones are another probe resource to be integrated with genetic maps at high density and resolution (Jiang et al. 1995). In *Poncirus trifoliata*, CMA/DAPI double staining followed by in situ hybridization using 45S rDNA and 24 BACs (BAC-FISH) acquired first BAC-FISH map which gives a general framework for comparative genome structure in *Citrus* and *Poncirus* (Moraes et al. 2008). In papaya, a pachytene chromosome-based high-resolution karyotype that encompasses group-specific BACs was developed using FISH, and the 12 linkage groups were integrated into the nine papaya chromosomes (Zhang et al. 2010). Chromosome markers, including simple sequence repeats (SSRs), retrotransposon and chromosome-specific BACs, from the genome could be assigned to physical chromosomes. The application of cytogenetics in fruit trees will be mainly influenced by further technical improvements which have been successfully used in several plant species, such as fiber-FISH (Walling and Jiang 2012), multi-FISH (Szinay et al. 2008), microdissection and microcloning (Zhou and Hu 2007) and ChIP-seq (Chumsakul et al. 2013) (Fig. 8.5).

8.6 Citrus Unique Genes by Comparative Genomics

Comparative genomics enables us to identify species-specific genes, which are likely to be important for the unique phenotype of each species. The sweet orange genome project identified 1,691 candidate citrus-specific genes based on the genome of sweet orange and its comparative analysis with 22 other plant species is available in December 2011. Annotation of this set of specific genes showed that the most

abundant domains are protein kinase, leucine-rich repeat and NB-ARC domain, possibly suggesting that kinase and disease-resistance genes are among the fastest evolving gene families.

As a result of the rapidly increasing number of genomes published, the data of citrus-specific genes (CSGs) will be updated. In a study with the newly released genomes, citrus genes were searched against 41 genomes and 273 transcriptome sequences (Fig. 8.6). This study yielded 296 citrus-specific genes with no significant sequence similarity to sequences from the Plant Kingdom except those from citrus family. The average exon number per gene of the CSGs was significantly smaller than that of the conserved genes. In the genome, the CSGs showed preferential distribution on some particular regions of the chromosome. Most of the CSGs can be detected as transcripts. According to the RNA-seq data, expression profiles for different CSGs varied significantly among the different tissues. For example, 45 CSGs were preferentially expressed in callus, 19 in the leaf, 19 in the flower and 22 in the fruit. These genes may play specialized roles in the development of the corresponding tissues and are potential genes for further functional analysis. Some CSGs may play important roles in adaptation to extreme environmental conditions. Among the nine CSGs which were up-regulated under heat stress (42 °C) condition, the gene Cs8g13286 expressed dramatically higher than the others, which may indicate that it is likely to be associated with high temperature stress response. Three CSGs were significantly induced by ultraviolet light (UV). Surprisingly, the gene Cs8g08420 was simultaneously up-regulated by both the temperature and UV stress treatments. This result further suggested that some of the CSGs may play vital roles in stress tolerance.

8.7 SNP Marker in Citrus Genome

Single nucleotide polymorphisms (SNP) provide high-density molecular markers across the genome, and the number of SNPs for a species can be millions, which revolutionized the scale, number, and efficiency of molecular markers in broad aspects. Many reports proposed that SNP markers are more appropriate than SSRs as genetic markers in linkage analysis due to their abundance and suitability for automatic allele calling (Novelli et al. 2004; Selmer et al. 2009). As for citrus, the authors believed that the SNP and SSR have different advantages at current stage. SSR markers do not need a bioinformatic platform and sequencing data, and require only PCR reactions and size separation. The SSR marker is convenient for the wetLab work and

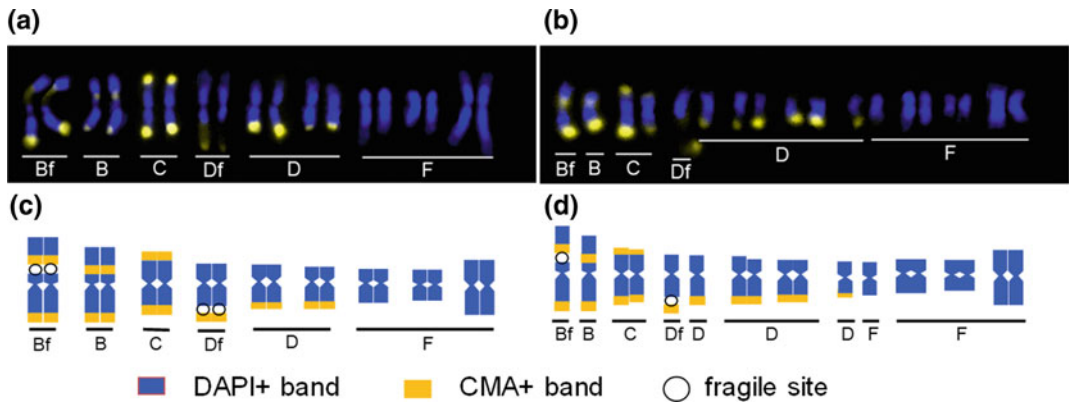

Fig. 8.5 The karyotype of sweet orange chromosomes (adapted from Xu et al. 2013). Double-haploid lines (**a**, **c**) and heterozygous diploid (**b**, **d**) of Valencia sweet orange. The chromosomes were named as type-Bf, B, C, Df, D and F according to DAPI banding region (blue color), CMA banding region (yellow color) and fragile site (circle) according to Guerra (1993)

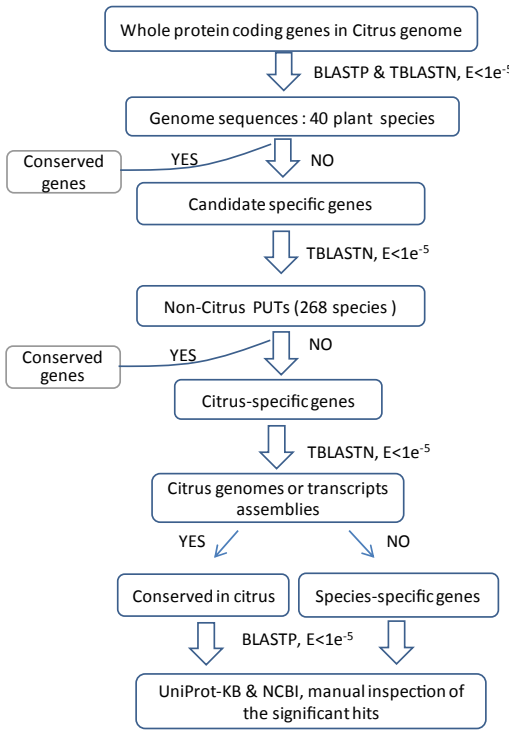

Fig. 8.6 The procedures to identify citrus-specific genes and species-specific genes

can produce results for a modest number of markers very efficiently. Remarkably, reduced cost of next-generation sequencing provides an opportunity to develop genome-wide SNP markers and utility for researches and finally for breeding. SNP markers have been widely used for phylogenetic and population structure determination of citrus germplasm (Fujii et al. 2013; Novelli et al. 2004; Garcia-Lor et al. 2013; Curk et al. 2016). A reference genetic map of clementine mandarin was established from segregating populations, which were genotyped with single nucleotide polymorphism (SNP), simple sequence repeats (SSR) and insertion–deletion (InDel) markers (Ollitrault et al. 2012a). Of the markers mined from clementine, 80.5% were successfully transferred to the whole *Citrus* gene pool (Ollitrault et al. 2012b). Several SNP arrays are now available to genotype specific sets of SNP markers. The high-density SNP arrays currently publicly available are Axiom (ThermoFisher) arrays for citrus with about 1.4 million and 58,000 SNPs. Many citrus genomes have been resequenced at modest (20–30X) depth in order to discover SNPs and characterize specific cultivars. A genome browser view of some of these is available at citrusgenomedb.org.

Species-diagnostic SNP markers have been used to comprehensively dissect the genetic architecture of citrus germplasms or to identify cultivar/variety (Xu et al. 2013; Wu et al. 2014; Curk et al. 2015). Typical heterozygous SNP and homozygous SNP (homozygous but different from reference genome) density in citrus species are summarized in Table 8.4. The density of heterozygous SNP of mandarin × pummelo hybrids is 16–17 heterozygous SNP per kb, which is useful information for genetic background analyses. In comparative analyses, the fixed SNP sites in each basic species of citrus relative to other species can be used as species-specific genetic markers. According to different pairwise combinations of diagnostic SNP markers in the genomes of the six basic citrus genomes, we can identify the genetic origins of the marked genome fragment. It is easy to obtain the admixture pattern by a maximum likelihood algorithm which can be applied to the whole genome.

SNP markers can be developed from the in silico data to a wetLab-based marker, including dCAPS, KASP and high-resolution melt (HRM) analysis. In our experience, HRM analysis easily and accurately detects SNP polymorphisms, and distinguishes allelic mutations from the homozygous background (Fig. 8.7a, b). SNP markers can also be developed in a further step to identify a most similar species by homolog searching the database of known species with SNP information. We developed a web-based algorithm, named as CitrusID, to characterize the genetic characteristics and identify the cultivar/variety. It aimed to confirm whether a tested sample is a new variety or not, and to identify the type or cultivar group or species to which it belongs and show the genetic components by providing the genetic contributions of each basic species (Fig. 8.7).

Table 8.4 Typical SNP density in intraspecies and interspecies hybrids

	Homozygous SNP density (per kb)	Heterozygous SNP density (/kb)
Mandarin	16–17	3–5
Pummelo	3–4	6–7
Citron	17–19	3
Ichang papeda	13–14	3.5
Kumquat	15	4–6
Poncirus	20	3–4
Mandarin × pummelo	4–5	16–17
Mandarin × ichang papeda	11	13
Mandarin × citron	10–11	16
Pummelo × citron	4.6	17

8.8 Citrus Population Genomics

The factor that genome size of citrus is relatively small (~400 Mb) and the rapid development of sequencing methods make it practicable to study citrus population genetics at the whole-genome level. Compared with the study on individuals, population analyses take advantage of statistical methods to analyze populations to infer genetic maps or phylogenetics relationships. In a broad sense, genetic mapping can be done through both linkage analysis and association analysis. Linkage mapping is regarded as the golden standard for genetic mapping, and association analysis can be an efficient and complementary approach by using next-generation sequencing and GWAS for natural populations. Moreover, more recent studies have adopted comparative population analysis to discover domestication- or selection-related genes after population genomics expedition and dividing group by the different environments they are in or the different artificial selection on them.

8.8.1 Bulk Segregant Analysis (BSA) Sequencing

When conducting linkage mapping in citrus, some characteristics of citrus hinder the usage of conventional genetic mapping methods which typically involved constructing hybrid populations after several generations of inbreeding. The first inevitable factor is nucellar polyembryony, in which the embryos develop from somatic nucellar cells. For polyembryonic citrus, like mandarin and its derivatives, there are many nucellar embryo besides the zygotic embryo within a single seed, which caused problems for generating offspring from sexual crosses. And citrus plants have long juvenile phase, that is to say, if the trait is about citrus flowers or fruits, and phenotype can be judged only after getting flowers or fruits, about at least 5 years will be needed for just one generation of citrus. Inbreeding is also difficult in some citrus because of self-incompatibility and inbreeding depression. As being woody plants, citrus population need large area of field to be planted in. Considering the costs of labor, money and time, bulk segregant analysis (BSA) sequencing acts as a high-efficient method for preliminary genetic mapping in citrus.

BSA-seq is a method used to identify genetic markers associated with a phenotypic trait by DNA sequencing of the two offspring pools displaying opposing phenotypes for a trait of interest. The BSA-seq for citrus polyembryony provides an example to demonstrate the pipeline for BSA-seq in citrus. Study of citrus polyembryony is of breeding value, as it is widely employed in citrus nurseries and propagation programs to generate large numbers of uniform

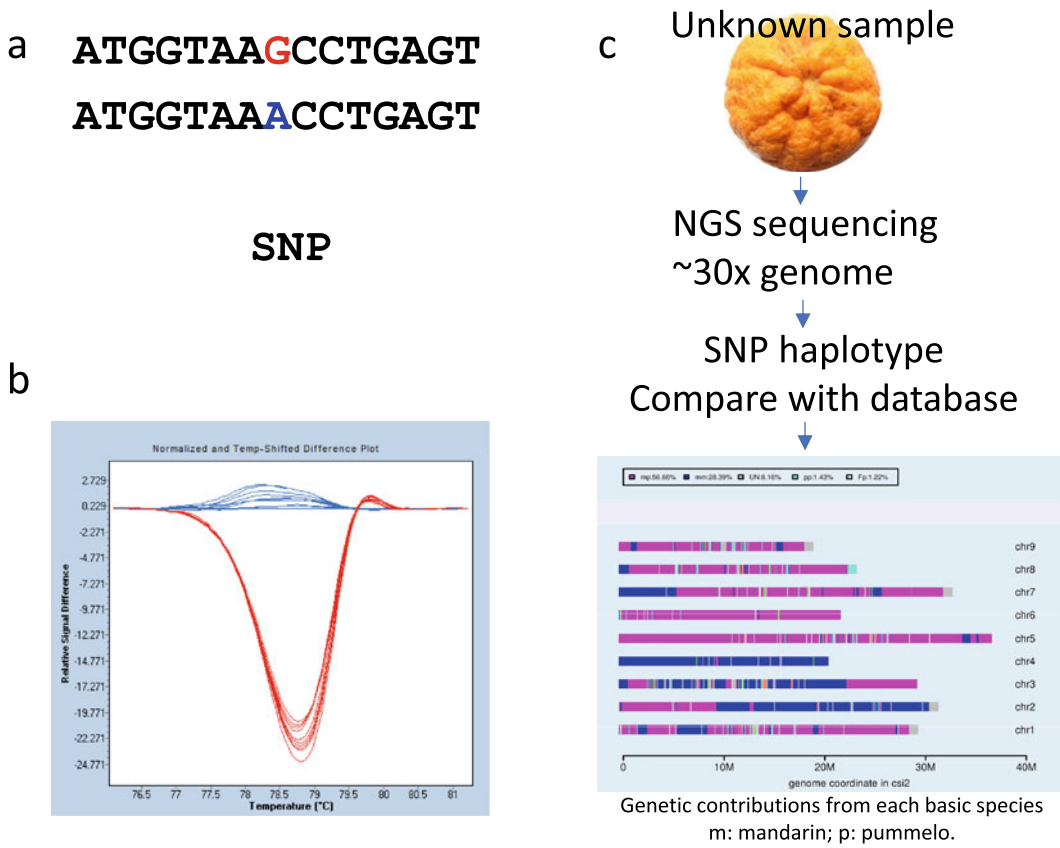

Fig. 8.7 Genotyping of SNPs and application in citrus variety identification. **a** SNP marker; **b** HRM analysis for sweet orange. Normalized melt curves for fluorescent signals from DNA strand dissociation (up panel) and difference plot of genotypes' fluorescence normalized to wild-type samples (down panel). **c** The procedures to identify citrus variety based on SNP markers

rootstocks from seeds and to permanently fix valuable traits and hybrid vigor despite the problems caused for breeding that required sexual crosses.

First, a segregating population was constructed, which was derived from a cross between monoembryonic female parent and polyembryonic male parent. And we scored the polyembryony phenotypes of 124 fruit-yielding progeny for 5 years in a row. Then a monoembryonic pool and a polyembryonic pool that exhibited contrasting phenotypes were constructed. DNA sequencing was performed on the Illumina platform at about 30-fold and 60-fold depth for parents and offspring pools, respectively.

After generating the sequencing data, reads from the two parents and monoembryonic and polyembryonic pools were separately mapped to the reference genome using BWA. SAMtools software was used for SNP calling. According to the genotypes of parents, the parent-specific allele frequencies were calculated. The absolute value of their difference was calculated as Δ (SNP-index) for each site. By sliding window approach, the average Δ(SNP-index) and max-min normalized SNP density were calculated in 250-kb sliding windows with 10-kb steps size. The final parameter to measure the correlation level was the product of Δ(SNP-index) and SNP density. After the above BSA-seq analysis, a region of 1.96 Mb was mapped for the citrus polyembryony (Fig. 8.8). It is worth noting that the BSA-seq and related methods such as PNome (Dardick et al. 2013) have a major advantage

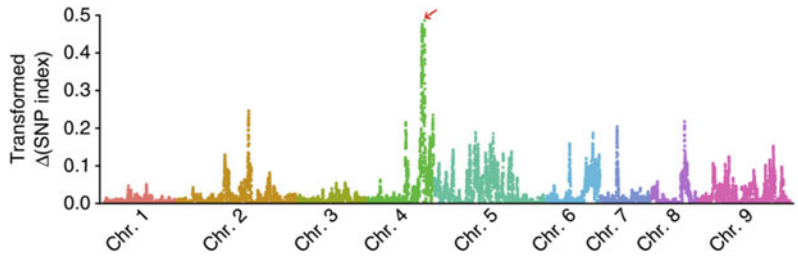

Fig. 8.8 Bulk segregant analysis (BSA) of citrus polyembryony (adapted from Wang et al. 2017)

over BSA with PCR markers in that they are quantitative rather than qualitative. In conventional BSA, it is critical to correctly phenotype every individual included in the bulks because a single misclassified individual can obscure the difference between pools. Sequencing allows read counts to be used, so the effect of a single misclassified individual is greatly reduced.

8.8.2 Genome-Wide/Region-Based Association Analyses

Based on the diversity pattern of extensive citrus resources measured with SSR markers, representative citrus accessions are sampled to construct a proper natural population for the GWAS. This GWAS population represents the genetic diversity of citrus. For example, a natural pummelo population consisting of 191 pummelo accessions has been constructed for GWAS after systemic screening in the widely collected pummelo accessions. For each accession in the population, DNA sequencing was performed with an average depth of 5-fold. During the natural population construction as well as aforementioned hybrid population construction, it is the processes of phenotyping and genotyping that are crucial. After all, the purpose of genetic mapping is to find associations between genotype and phenotype. The accuracy of genotyping could be guaranteed by the control of analysis quality, such as sequencing depth and quality, the mapping quality and the genotyping quality. For phenotyping, the accuracy can be affected by the plant-growing environment or the developmental stages and so on, and the accuracy of phenotyping will be improved if the phenotyping is done under the unified conditions and has biological repetitions. On the other hand, expression of some traits varies with the environment, so it is possible to discover associations that are environment-specific.

Reads from DNA sequencing of citrus accessions were separately mapped to the reference genome with BWA. The alignments were filtered, and then duplications were removed using SAMtools. After combining the mapping results of the accessions, population-based SNP calling was conducted using SAMtools and imputation for the missing SNPs was performed using SHAPEIT software (Delaneau et al. 2011) assisted with reference panels from deep DNA-seq data. The SNP datasets were used to conduct association analysis with linear mixture model and/or general linear model with FaST-LMM software. For the accuracy of GWAS results, it is necessary to control the influences from population structure and kinship between individuals. There are three parts of basic but important population analysis to consider and reduce these influences, including constructing a population phylogenetic tree, principal component analysis (PCA) for population and population structure analysis.

In other case, when a preliminary mapping has been conducted, and/or obvious population structure for the natural population exists, region-based association analysis will be an efficient method to fine map the candidate locus for the trait of interest.

To fine map the polyembryony locus, a local association analysis within the 1.96-Mb region among natural population was performed. A total

of 108 citrus accessions were used in this region-based association analysis, which included 45 polyembryonic accessions and 63 monoembryonic accessions. Sequencing reads of these 108 accessions were mapped to the corresponding genome interval with BWA. Then the reads of each accession that mapped to the candidate polyembryony locus region were obtained from the mapping results described above using the bam2fastq software. Based on the target-captured reads, iCORN2 software (Otto et al. 2010) was used to revise SNPs and small InDels (1–3 bp) in gene sequences in the candidate region for each accession. In the candidate region, 292 gene sequences from each accession were obtained by three iterations in iCORN2. After detection of polyembryony-associated sites using Fisher's exact test adjusted using the FDR correction for multiple testing, the distribution of correlation scores along the region demonstrated a peak in an 80-kb region with strong association to the citrus polyembryony trait (Fig. 8.9).

Among the 11 genes in the polyembryony-related region, CitRWP—whose homologous gene in *Arabidopsis* was reported to serve as a regulator of egg cell-related genes, showing the highest association level with the polyembryony phenotype—was highly expressed in ovules and specifically expressed in polyembryonic citrus cultivars but not in monoembryonic citrus cultivars. Furthermore, an insertion of a MITE (Miniature Inverted-repeat Transposable Element) in the promoter region was identified after sequence alignments of this gene from polyembryonic and monoembryonic citrus and is linked to the polyembryony locus by experimental validation (Wang et al. 2017). Therefore, all of these are the evidences for the accuracy of association results.

8.8.3 Comparative Population Analyses

During the process of evolving or domestication from primitive/wild to the cultivated, if there is selection for a particular phenotype in a population, the genetic diversity in the selected regions in the population decreased and differentiation between the two populations increased, which will increase linkage disequilibrium in the selected region. These changes were regarded as signals of selection during the comparative population analyses.

For genetic diversity and selection signature analysis, SNPs of the whole genome after

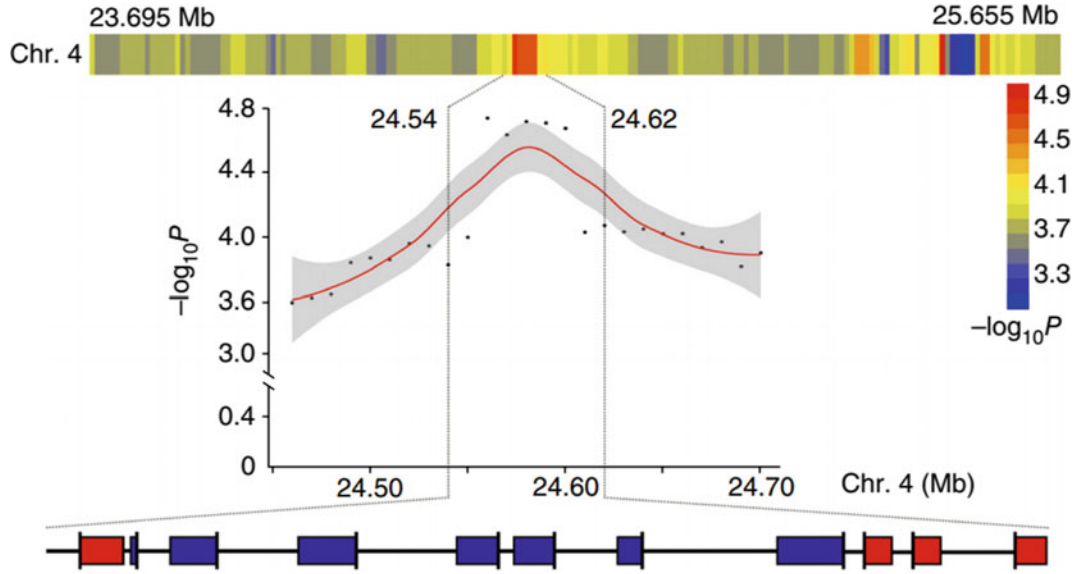

Fig. 8.9 Genome-wide/region-based association analyses of citrus polyembryony (adapted from Wang et al. 2017)

population-based SNP calling were used, and a sliding-window approach (50-kb windows sliding in 10-kb steps) was employed to quantify genetic differentiation (FST) and nucleotide diversity (π) for each citrus population using the VCFtools software (Danecek et al. 2011). To detect the selection signatures, the log10 π ratio was calculated. The regions with significantly high FST values (in the 5% right tail of the empirical distribution of FST values) and significant reduction in diversity (in the 5% right tail of the empirical distribution of log10 π ratio) were considered to be under selection.

For instance, the results of comparison between primitive *Atalantia* species and cultivated pummelo population indicated that the distribution of such regions was uneven along the chromosomes. The genes in such regions were likely under selection. Among these genes, there are many energy-associated genes, such as genes related to ATP synthase and mitochondrial proteins. And among these genes, there are many reproduction-associated genes, like the gene encoding the flowering time control protein FPA. FPA has a critical role in the regulation of flowering time in *Arabidopsis*. This finding is consistent with the observation that *Atalantia* has a prolonged flowering season, from May to December, and that in contrast, the flowering season is synchronized in cultivated types, such as mandarins, pummelos and sweet oranges.

As we know, primitive and wild citrus are monoembryonic. In many cultivated citrus there emerged polyembryonic phenotype. Therefore, it seems that there was selection for polyembryony from monoembryony. Regarding the nucleotide diversity and selection signatures in the 80-kb polyembryony-related region in citrus, the values of FST and π in the sliding windows were calculated with VCFtools and the haplotypes in the region were examined using Haploview in combination with PLINK software (Purcell et al. 2007). The results displayed that the genetic diversity of monoembryonic accessions is higher than that of polyembryonic accessions. The population differentiation between the polyembryonic and monoembryonic accessions was substantially higher for this 80-kb region than the surrounding regions. Because of the selection for polyembryonic citrus, the linkage disequilibrium among polyembryonic accessions was larger than the LD among monoembryonic accessions. These results indicate that a selection signature is present between monoembryonic and polyembryonic citrus in the polyembryony-related region (Fig. 8.10).

BSA-seq using a biparental population was used for preliminary mapping. A natural population with wide genetic background was used for fine mapping. These two parts need flexible application and combination, as well as custom bioinformatics analysis approaches, especially for more complex and quantitative traits. Furthermore, gene expression data were useful to pinpoint the candidate genes. Molecular markers were useful to confirm the association and for breeding purpose. And the comparative population analysis demonstrates the evolving of genes of interest from phylogenetic points of view. The successful case of genetic mapping for citrus polyembryony using citrus population genetic analyses provides practicable references for genetic mapping of agriculturally important genes in citrus. In our case of citrus polyembryony, it is a single dominant gene trait which made it easy to narrow down the target region and the candidate gene. For complex trait, approaches and populations may need to be polished.

8.9 Transcriptome Analyses

Transcriptome represents a first investigation of gene function as it characterizes qualitatively and quantitatively the set of genes that are transcribed into RNA molecules in a particular tissue and environment. Various technologies have been developed to deduce and quantify the transcriptome, including hybridization- or sequence-based approaches. Hybridization-based approaches typically involve incubating fluorescently labeled cDNA with custom-made microarrays or commercial high-density oligonucleotide microarrays. In contrast to microarray methods, sequence-based approaches directly determine

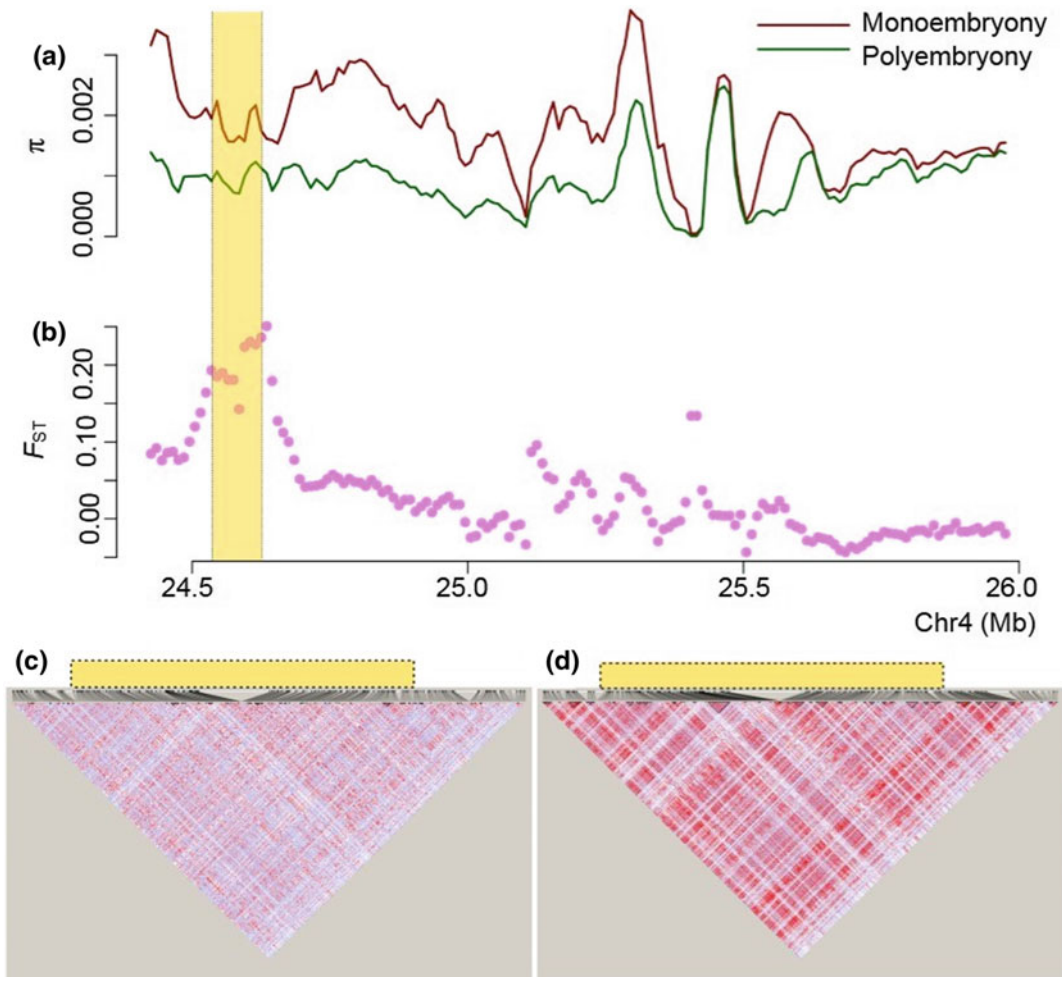

Fig. 8.10 Comparative analysis of citrus mono- and polyembryony populations (adapted from Wang et al. 2017)

the cDNA sequence and its abundance. Initially, Sanger sequencing of cDNA or EST libraries was used, but this approach has relatively low throughput, expensive and generally not quantitative (Wang et al. 2009). The NGS sequencing methods have provided a new approach for both mapping and quantifying transcriptomes. This method, termed RNA-seq (RNA sequencing), has clear advantages over existing approaches and is expected to revolutionize the manner in which eukaryotic transcriptomes are analyzed. Fruit crops have complex genetic backgrounds and highly heterozygous nature. Transcriptome analysis, by reducing complexity from genetic background, can provide much information about traits of interest to researchers by exquisite experimental design.

Transcriptome studies have experienced several stages, including differential display analysis (DDA), cDNA libraries or suppression subtractive hybridization (SSH) cDNA libraries, microarray and RNA-seq. For example, gene expression was studied for citrus fruit developmental and environmentally regulated processes through differential display techniques (Lang et al. 2005; Pasentsis et al. 2007). Genes involved in many processes were identified by suppression subtractive hybridization (SSH) cDNA libraries which enriched differentially expressed transcripts (Ge et al. 2012; Liu et al.

2009; Liu et al. 2012; Peng et al. 2012; Zhang et al. 2008).

RNA-seq is rapidly becoming the main approach nowadays in transcriptomic researches because it can provide a more direct and thorough representation of the absolute transcript population, is more sensitive to genes expressed at low levels and does not require prior gene sequence information. Earlier exploration of RNA-seq in fruit crops demonstrated its advantages (Xu et al. 2009; Zenoni et al. 2010). RNA-seq was widely applied in gene expression studies of different tissues or treatments. It is interesting to understand fruit biology by RNA-seq data, particularly those genes highly expressed in fruit (Table 8.5). From the transcriptome data, it is clear that the genes expressed in developing citrus fruit are strikingly distinct from those of other well-studied crops such as tomato, suggesting that the non-climacteric fruit and climacteric fruit experience distinct genetic programming of the gene expression. Several studies have characterized differences in transcriptomes between citrus trees infected with Huanglongbing, and noninfected controls indicate that genes involved in carbohydrate and nitrogen metabolic processes, transport, defense, signaling and hormone response were overrepresented in the HLB response network (Zheng and Zhao 2013; Martinelli et al. 2012; Aritua et al. 2013).

8.10 MicroRNAs

microRNAs (miRNAs), 20–24 nucleotides (nt) in length, regulate gene expression by cleavage target mRNAs or inhibit their translation. miRNA were discovered using traditional cloning, sequencing methods and computational prediction of pri-miRNA from expressed sequence tags (ESTs) or whole-genome sequence in a wide variety of fruit crops. miRNAs are various negative regulators in plant development and plant stress response, such as miR156, miR159 and miR172 involved in flower development, particularly in flowering time regulation and phase changing from vegetative growth to reproductive growth (Chuck and O'Connor 2010; Garcia 2008; Lu et al. 2005). Compared with miRNA studies in plant species, miRNAs from citrus were first identified from trifoliate orange (*Poncirus trifoliata*) by prediction from expressed sequence tag (EST), and 27 conserved miRNAs were confirmed by Northern blotting. With the development of NGS sequencing, the study of citrus novel miRNAs was carried out in sweet oranges. A set of 85 known miRNAs belonging to 48 families and 12 novel miRNAs by the presence of mature miRNAs and corresponding miRNA*s in the sRNA libraries were identified (Xu et al. 2010). Comparative analysis showed that nine novel miRNAs are differentially expressed between the common sweet

Table 8.5 The genes highly expressed in the sweet orange fruit

Gene	Location	Callus	Leaf	Fruit	Annotation
Cs2g08070	chr2:6214204-6216350	80.7	2113.4	2564.9	Maturase K
Cs3g26060	chr3:29761632-29762039	59.1	469.4	2115.2	Hypothetical proteins
Cs9g03210	chr9:2313276-2314373	428.5	94.1	1715.5	Late embryogenesis abundant protein Lea5
Cs7g06330	chr7:7538354-7538891	0.2	42.8	1003.2	Metallothionein-like protein
Cs5g22180	chr5:25524469-25528741	108.0	207.5	819.9	Cysteine proteinase RD21a
Cs4g09980	chr4:7194402-7200515	0.0	0.3	689.0	Agamous-like MADS-box protein AGL9
Cs1g17790	chr1:18337289-18342840	10.0	3.6	452.7	Vacuolar-processing enzyme

(continued)

Table 8.5 (continued)

Gene	Location	Callus	Leaf	Fruit	Annotation
Cs1g10090	chr1:7776247-7779570	58.4	13.9	447.0	Thiazole biosynthetic enzyme
Cs2g19110	chr2:16807946-16809398	98.6	0.0	406.1	hypothetical proteins
Cs8g17360	chr8:20993803-20994946	12.8	0.7	389.4	17.4 kDa class I heat shock protein
Cs5g23820	chr5:26674936-26675538	5.7	81.6	284.6	Hypothetical proteins
Cs2g01180	chr2:819535-821417	0.2	13.2	282.0	Hypothetical proteins
Cs9g09380	chr9:7702203-7704175	0.1	0.0	262.9	Legumin B
Cs5g08470	chr5:13471884-13473031	36.4	140.3	238.8	Protein ycf2
Cs1g10150	chr1:7827960-7830827	77.0	20.3	224.8	Polyubiquitin-A
Cs9g06670	chr9:4780623-4781599	0.9	21.1	223.0	Early light-induced protein
Cs9g15170	chr9:15594634-15596723	49.5	35.7	203.2	Hypothetical proteins
Cs6g00850	chr6:1331286-1332975	8.1	0.7	195.8	Hydrophobic protein LTI6B
Cs2g30080	chr2:30434065-30434982	0.3	10.8	185.1	Hypothetical proteins
Cs2g19010	chr2:16733013-16734634	0.1	1.9	183.2	Hypothetical proteins
Cs1g10130	chr1:7812129-7813189	2.5	0.0	181.1	2S albumin
Cs2g19780	chr2:17684445-17686913	0.0	8.9	180.9	Nitrate transporter
Cs3g05600	chr3:4103957-4106742	0.5	7.1	173.7	Probable pectate lyase
Cs2g00050	chr2:29159-32196	66.6	33.0	167.8	Zinc finger
Cs8g17020	chr8:20763153-20764015	2.4	0.3	142.5	17.6 kDa class I heat shock protein
Cs9g03630	chr9:2594586-2596827	0.0	2.0	132.5	Protein SRG1
orange1.1t02556	chrUn:39069371-39074989	13.5	3.2	127.9	Probable proteasome inhibitor
orange1.1t00566	chrUn:6690782-6694886	15.2	56.3	127.2	Granule-bound starch synthase 1
Cs3g32320	chr3:34370379-34371207	36.5	11.0	122.2	Indole-3-acetic acid-induced protein ARG2
Cs3g09820	chr3:7645578-7647525	6.4	0.5	120.8	Expansin
orange1.1t01847	chrUn:29226696-29227968	9.9	34.5	113.5	Hypothetical proteins
Cs2g10800	chr2:8833319-8837162	1.6	5.1	101.1	MOSC domain-containing protein 2
Cs3g25130	chr3:28986082-28988201	2.3	0.6	101.0	NAC domain-containing protein 29
Cs3g08880	chr3:6797247-6799599	1.3	8.5	98.6	7-ethoxycoumarin O-deethylase
Cs5g14540	chr5:19609107-19613630	18.0	1.1	96.0	Acyl-[acyl-carrier-protein] desaturase
Cs9g05270	chr9:3809282-3812994	6.4	33.4	89.8	Stem-specific protein TSJT1
Cs9g15640	chr9:16122313-16125504	0.0	0.0	88.3	UDP-glycosyltransferase 76C4
Cs1g06100	chr1:4513376-4515378	0.5	23.5	88.1	Hypothetical proteins
orange1.1t02242	chrUn:34057910-34058682	38.6	0.0	85.5	Hypothetical proteins
Cs7g17540	chr7:21528490-21530405	37.8	2.6	84.7	Inorganic pyrophosphatase 2

orange and red-fleshed orange. By the NGS sequencing power, Song et al. (2010) identified 42 conserved and 10 novel miRNAs from the flower and fruit of trifoliate orange; and validated four target genes including disease resistance genes. By using a precocious trifoliate orange, sRNA sequencing and transcript sequencing was used to target the miRNA and target genes involved in the developmental response to stress of this early-flowering (short juvenile stage) genotype (Sun et al. 2012). By cold treatment, Zhang et al. (2014) found 36 conserved miRNA and 5 novel miRNA responded to the low temperature stress. To dissect whether miRNA involved in the citrus male sterility, a seedless ponkan (*Citrus reticulata*) landrace and wild-type ponkan from China were compared by sRNA and degradome sequencing. A total of 156 known miRNA and 24 novel miRNA were identified, and 138 target genes of 44 miRNA were also characterized. The miRNA profiling indicated that 71 conserved miRNA and 11 novel miRNA were differentially expressed between the seedless and common ponkan. Further experiments indicated that the miR156 and its target gene squamosa-promoter binding protein-like (SPL), miR167 and its target gene auxin response factor (ARF) are involved in the anther development and made a foundation to decipher the mechanism of male sterility. To study the citrus somatic embryogenesis (SE), stage and tissue-specific modulation of ten conserved miRNAs and their targets was investigated during somatic embryogenesis of Valencia sweet orange. By NGS sequencing, 50 conserved miRNA, 45 novel miRNA, 130 miniature inverted-repeat transposable elements (MITEs) derived siRNAs, 94 other siRNAs, 235 phasiRNAs and 203 target genes were investigated. The gene expression and experimental evidence suggested that conserved miRNA and its suppression on the target transcription factor genes are important mechanism to inactivate the postembryonic growth, and thus to maintain normal SE. The miRNA and siRNA-mediated silencing was probably under sophisticated regulation in citrus SE (Wu et al. 2015).

To date, the major miRNAs of citrus, as appeared from miRBase (http://www.mirbase.org/), are annotated by bioinformatics, sequencing and gene expression analysis. The functions of family-specific miRNAs and species-specific miRNAs in citrus remain largely unknown. One recent work characterized a function of miRNA and its regulatory network with phasiRNA and transcription factors. miR3954a is a trigger of phasiRNAs from sweet orange. The miR3954a targeted a NAC transcription factor and two noncoding transcripts, and produced phasiRNAs from targeted transcripts. PhasiRNAs can further target the NAC gene and additional NAC homologous genes. This miR3954a–lncRNAs–phasiRNAs–NAC transcription factor pathway is citrus specific, and constitutes a model of the miRNA–lncRNA–TF regulatory network in citrus. Expression and functional analyses of miR3954a showed a specific expression in flower of citrus species. Interestingly, the overexpression of miR3954a leads to an early flowering in citrus (Liu et al. 2017). In future, tissue (organ)-specific miRNAs may be key miRNAs which need to be functionally studied.

8.11 Epigenome

Epigenome relates to the modifications of DNA and associated histones (Henderson and Jacobsen 2007). In contrast to the genome, epigenome is dynamic and will change according to the tissues, developmental stages and environmental factors. Epigenetic regulation in plants is actually a complicated regulatory network from the chromatin to gene expression. The plant methylomes have been well studied, including the single-base resolution analysis of tomato (Zhong et al. 2013) and apple (Daccord et al. 2017). Genome-wide histone modifications have important influence on gene expression and plant growth and development, such as H3K4me3, H3K9ac, H3K9me3, H3K27ac, H3K36me3 have reported to activate gene expression, while H3K9me2 and H3K27me3 may suppress gene expression (He et al. 2011).

DNA methylation of citrus has been studied in longtime in vitro cultured calli, somaclonal variation, fruit ripening, regulation of secondary metabolism, and response to the growth conditions and environmental stress (Hao and Deng 2002; Hao et al. 2004; Kaeppler et al. 2000; Kaity et al. 2008; Li et al. 2002; Peraza-Echeverria et al. 2001; Valledor et al. 2007). Earlier studies used methylation-sensitive and -insensitive restriction enzymes and found that there are wide DNA methylation variations during long time of in vitro propagation of citrus calli (Hao et al. 2004). Analysis of DNA methylation in 24 navel orange and 57 sweet orange cultivars demonstrates that the DNA methylation changes frequently in different varieties of navel oranges and may involve in somatic mutation (Hong and Deng 2005; Kalisz and Purugganan 2004; Fang et al. 2010).

DNA methylation pattern is dynamic during citrus fruit development. Analysis of genomic DNA methylation levels in different fruit tissues and developmental stages of sweet orange indicated that it is stable, followed by an increase in the peel, while decrease followed by an increase in the flesh during the fruit development (Xu et al. 2015). Methylome, transcriptome and metabolite changes were combined to dissect the effect of application of the DNA methyltransferase inhibitor 5-azacytidine (5azaC). 5azaC treatments induced carotenoid degradation by strong activation of carotenoid cleavage dioxygenases 1 (CpCCD1) and led to a genome-wide demethylation effect. In *Rosaceae* crops, several findings implicate that DNA methylation also plays an important role in genetic regulation of fruit plant secondary metabolism. The promoter of anthocyanin regulator gene MYB10 is highly methylated in green stripes of apple peel (Telias et al. 2011) and red skinned pear (Wang et al. 2013). We would believe that many more epigenomes will be unraveled along with the NGS sequencing rapid development. The report on the function of histone modifications in fruit crops is few and it is promising to dissect their functions in future.

8.12 Prospects in Genomic Studies of Citrus

Multiple omics-based researches will form a next wave in near future of citrus researches, particularly as the number of sequenced citrus genomes increases rapidly. The dramatically accelerated chemistry and sequencing power, such as third-generation technologies, including PacBio and Nanopore, will form a huge impetus to citrus researches in the near future.

Many de novo, high-quality and 3D genomes will be necessary for the genomic studies, such as hybrid citrus, wild citrus, or genotypes with high economic value. More genomes are important for comparative genomics to understand both the evolution and the identification of lineage-specific genes and linking with the phenotype difference. Moreover, finished genomes are necessary for an ultimate effort to fully understand the genomic architecture. In some of the particular genotypes, for example chimera cultivars, 3D genomes may be an alternative scope to understand the mechanism from whole-genome level.

Genome-wide association analysis will be a powerful tool to link the genome data with phenotype. An international effort to collect the typical citrus germplasm, particularly the germplasm during citrus dispersal from Southeast Asia to the Middle East, to West Africa, to the Mediterranean region, and to the Western Hemisphere should be as much as possible for the GWAS studies. In China, many (>1000) primitive, wild, sour and landraces of citrus have been collected and sequenced. Some of the ecotypes, such trifoliate orange, Ichang papeda, wild mandarins, sour oranges, wild limes will have important value for the genetic analysis, and meanwhile more important is to exploit some germplasm, including short juvenile time, dwarfing, baby citrus for functional genomics and highly efficient genetic studies. Moreover, the combination of GWAS with the metabolomic data will be very useful and provides high throughput to study the molecular basis of fruit quality, the disease resistance response and postharvest quality.

We would anticipate that many agriculturally important traits will be dissected by genomics and functional studies. Biological questions that are unique to citrus, that is could not be addressed in model plants such as *Arabidopsis*, will be particularly worthy to be investigated. The potential interesting topics vary from plant to plant; herein we list several that may have general interests: the disease resistance/susceptible mechanisms including to *Citrus* Huanglongbing (greening) and Canker, the transition from juvenile to reproductive phase, the formation of fruit quality (color, aroma, flavor and texture), variation of fruit size and structure, fruit development and ripening, the mechanism of graft union formation, the origin and stability of chimeras, the molecular basis of somatic mutations and synthesis and regulation of human health-beneficial secondary metabolites. Highly efficient gene editing platform, particularly a transgene-free system, is important for the ultimate use of the gene and elucidating molecular mechanisms.

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Citrus Reproductive Biology from Flowering to Fruiting

9

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Abstract

Citrus reproductive biology is characterized by peculiar processes such as self-incompatibility, parthenocarpy, and nucellar embryony. The understanding of citrus flowering mechanism is quite important since it will lead to control of production quality, efficiency, and timing of crops. In cultivation and breeding programs of citrus, the flowering behavior is strictly related to juvenility and alternate bearing. In citrus fruit, the failure of the sexual reproductive process (e.g., female and/or male sterility) in parthenocarpic cultivars results in seedless fruits. In contrast to other fruit crops, sterility can be considered a benefit to avoid the presence of seeds, which is one of the main quality parameters for fresh citrus fruit consumption. Here we review the current state of knowledge about the genetic control of reproductive biology in several citrus and its closed related species.

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9.1 Genetic Regulation of Flowering Induction in Citrus

The transition from leaf meristem into the reproductive floral meristem is one of the most fundamental developmental steps. This transition relies on flower bud differentiation in response to environmental stimuli and endogenous signals such as light, temperature, nutrients, and plant age (Khan et al. 2014). Citrus floral differentiation is certainly influenced from its nature as tropical-subtropical evergreen species, not showing a true dormancy (Spiegel-Roy and Goldschmith 1996). However, a close-related species as Poncirus (*Poncirus trifoliata* L. Raf.) has a deciduous habit, probably due to its origin from temperate zones of north-eastern Asia (Monselise and Goldschmidt 1982). During fall and winter in open field conditions, citrus trees never go dormant like deciduous trees, but show a metabolic slowdown due to low temperature regimes. Several works indicates that floral induction occurs during fall and the chilling winter temperatures play a key role independently from the photoperiod (Spiegel-Roy and Goldschmidt 1996; Nishikawa et al. 2013). Water stress condition seems to be the major flower-inducing signal in semi-tropical area. On the whole, the

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environmental stimuli described above prevail in the natural control of citrus flowering. Floral transition is one of the most drastic changes occurring during the life cycle of a plant. In citrus, flowering induction is strictly related to juvenility and alternate bearing (Nishikawa et al. 2013). The first phenomenon is caused by suppression of flowering in young plants showing a long juvenile period lasting several years after germination or grafting. The alternate bearing mainly results from suppression of flowering by heavy fruit production and late harvest in one year, reducing the flower number as well as the fruits on the tree in the following year.

In recent years, several research groups spent a lot of efforts to resolve these problems studying the genetic regulation of flowering. A complex network of genetic pathway is involved in its regulation process. Based on intensive studies of genetic and molecular mechanisms of *Arabidopsis*, an intricate regulatory network of several major genetic pathways that control the floral transition has been revealed (Khan et al. 2014). Peña and colleagues (2001) demonstrated as LEAFY(LFY) or APETALA1 (AP1) genes involved in flower induction in *Arabidopsis* resulted in a reduction of juvenile traits and early flowering in transgenic citrus. Specifically, the ectopic expression of Arabidopsis AP1 or LFY caused early flowering and fruiting in transgenic citranges, promoting the flower bud development after 12–20 months, instead of five years as reported for non-transformed plants. In the last decade, several genes associated with citrus flowering have been investigated. In particular, citrus expressed sequence tag (CitEST) database displayed sequence homology with known elements of flowering-related genes characterized in *Arabidopsis* (Dornelas et al. 2007). On the basis of sequences data compared to those of *Arabidopsis*, citrus orthologous genes of LEAFY (LFY), APETALA1 (AP1), TERMINAL FLOWER 1 (TFL1), SUPPRESSOR OF OVEREXPRESSION OF COSTANS 1 (SOC1) and FLOWERING LOCUS T (FT), have been identified and characterized (Pillitteri et al. 2004a, b; Endo et al. 2005; Nishikawa et al. 2007; Tan and Swain 2007). Other MADS-box genes were isolated from *P. trifoliata* (Sun et al. 2016). Liu and colleagues (2017) characterized a miRNA triggering phasiRNAs generation, miR3954 that leads to early flowering in transgenic citrus and illustrated a model of the regulatory network of miR3954-lncRNA-phasiRNAs-NAC, which may be functionally involved in flowering time control. Homologues for LFY (CsLFY) and AP1 (CsAP1) in navel orange (*C. sinensis* L. Osbeck) have been identified with more than 65% identity with *Arabidopsis* LFY and AP1 in the deduced amino acid sequences. Transgenic *A. thaliana* plants, over-expressing CsLFY and CsAP1, showed early flowering phenotypes (Pillitteri et al. 2004a). On the other hand, a late flowering effect has been observed for the Terminal Flower (TFL1) identified in citrus (CsTFL) as well as reported in *Arabidopsis* (Pillitteri et al. 2004b). Genes expression analysis displayed as juvenility in citrus was positively correlated with CsTFL transcript accumulation and negatively correlated with the floral regulatory genes, LEAFY and APETALA1. Two SOC1-like genes isolated in navel orange and named CsSL1 and CsSL2, were able to shorten the time taken to flower in *Arabidopsis* (Tan and Swain 2007). In addition, some MADS box genes are also involved in the low-temperature regulation of flowering and bud dormancy in trifoliate orange, typically PtFLC and PtSVP (Zhang et al. 2009a; Li et al. 2010).

Most of the above-mentioned genes showed alternative splicing (AS) in differential expressed genes involved in development processes in plants, such as flowering. Many flowering-related genes such as FLOWERING TIME CONTROL (FCA) and FLOWERING LOCUS C (FLC) showed AS events involved in floral inductive in trifoliate orange, highlighting a functional significance related to their role in the floral transition from juvenile to mature trees (Zhang et al. 2009a; Ai et al. 2016). Several studies suggested that genes above mentioned are also key regulators of flowering time and flower development in citrus (Tan and Swain 2007; Zhang et al. 2009a, b; Li et al. 2010; Chica and Albrigo 2013a).

First, citrus FT homologues (CiFT) have been reported as three paralogues CiFT expressed genes in satsuma mandarin (Nishikawa et al. 2007). Comparing the FT genes to the full genome sequence of clementine, Samach (2012) reported as two of the three FT citrus sequences seem to be encoded by the same gene (Ciclev10013731m, renamed CiFT1) and the other is encoded by a different FT (Ciclev10012905m, renamed CiFT2). A CiFT3 encoding gene (Ciclev10012629m), was found in the genome data set. The CiFT genes have been used in genetic engineering for speeding up genetic studies and breeding programs. The ectopic expression of CiFT genes has been used for rapid evaluation of transgenic citrus flowers and fruits in early flowering phenotype (Endo et al. 2009). CiFT expression level has been used to predict the number of flowers at different locations and over several years (Nishikawa et al. 2017). An innovative biotechnological tool to promote transition from the vegetative to the reproductive phase, in juvenile citrus plants by transient expression of the *A. thaliana* or citrus FLOWERING LOCUS T (FT) genes, was obtained using a citrus leaf blotch virus-based vector (Velázquez et al. 2016).

Several physiological and environmental factors are involved on the expression of flowering-related genes in citrus. Several works reported the changes in gene expression in response to fruit bearing in mandarin and mandarin-like genotypes. In particular, among the different genes analyzed, the higher or lower expression of CiFT genes seems to be strongly associated, respectively, to high and low fruit load in Moncada hybrid [Clementine Oroval (*C. clementina* Hort. ex. Tan) × Kara mandarin (*C. unshiu* Marcow. × *C. nobilis* Lour.)], in Murcott (*C. reticulata* Blanco) and satsuma mandarin (*C. unshiu*) (Muñoz-Fambuena et al. 2011; Nishikawa et al. 2012; Shalom et al. 2012). The works mentioned above shed light on the role of the excessive fruit amount and the delay in the harvesting time on the suppression of flower induction. Chemical treatment with Gibberellic Acid (GA) reduced CiFT expression in buds of mandarin and in leaves of sweet orange (Muñoz-Fambuena et al. 2012; Goldberg-Moeller et al. 2013). In sweet orange, the treatment with GA biosynthesis inhibitor, increased the number of flower by enhancing CiFT expression, indicating the role of endogenous GA in the suppression of CiFT expression (Muñoz-Fambuena et al. 2012). Citrus flowering-related gene expression is affected by seasonal periodicity. In particular, CiFT, CsLFY, CsAP1 and CsTFL expression showed a different mRNA accumulation in fall-winter or spring-summer seasons (Nishikawa et al. 2007; Shalom et al. 2012). On the contrary as reported in satsuma, in which the CiFT expression increased during fall and winter, in sweet orange and pummelo, Pajon and colleagues (2017) reported that high expression level of CiFTs in leaves occurs at the end of spring and after flowering has taken place. This result suggests as CiFTs expression is probably involved with shoot apex differentiation and flower bud determination rather than be associated with dormancy interruption and succeeding flower bud development.

An increased expression level of CiFT instead of CsTFL genes was observed in response to cool temperature treatment, respectively, in citrus adult and young trees (Nishikawa et al. 2007) suggesting their role in flowering suppression in plants. CiFT responds rapidly to floral inductive low-temperatures, photoperiodic stimuli and water deficit during induction in sweet orange (Chica and Albrigo 2013a, b). Furthermore, the expression patterns of the different CiFT alternative splicing transcripts (CiFTα and CiFTβ) acted as a floral inducer during floral inductive water deficit in lemon (Li et al. 2017).

9.2 Flower and Gametophytes Development

Flower formation involved a common group of genes identified using various homeotic mutants of *Arabidopsis*. Based on conservation of protein sequence and function, homologous genes were isolated from a wide range of different plant species such as citrus. Degenerate primers were used to identify homologous MADS-box genes involved in floral organ development (Tan and Swain 2007). In particular, one APETALA3-like (CitMADS8), two SOC1-like (CsSL1 and

CsSL2) and a WUSCHEL homologue (CsWUS), functional equivalent of *Arabidopsis* WUSCHEL, from sweet orange have been isolated.

CsSL1 and CsSL2 are expressed in citrus floral organs and their ectopic expression of either CsSL1 or CsSL2 in *Arabidopsis* WT plants is likely to be involved in determining flowering time, in the onset of floral organ senescence and in the development of petals flowers into sepal-like structures. On the whole, they may be required for maintaining floral organ identity and function in citrus. CsWUS plays a central role in promoting and safeguarding the identity and structural integrity of shoot and flower meristems (Tan and Swain 2007). CsWUS is expressed in active meristem of shoot and flower as well as in dormant winter buds, tissues that contain either vegetative meristems or a mixture of inflorescence and floral meristems (Lord and Eckard 1987). Also a gene isolated in Poncirus named SHORT VEGETATIVE PHASE (SVP) during apical meristem determination is involved in meristem development. Ectopic overexpression of PtSVP in wild-type *Arabidopsis* and tobacco produced additional trichomes and floral defects, such as flower-like structures instead of carpels and coflorescence with additional florets, respectively (Li et al. 2010).

The PISTILLATA (PI) and APETALA3 (AP3) nuclear genes involved in nucleic acid binding and response to hormone synthesis and metabolism, genes required for floral bud identification and expressed in particular floral whorls in male sterile cybrid of pummelo, have been identified (Zheng et al. 2012).

9.3 Self-Incompatibility System in Citrus

To better adapt to the wider range of environmental conditions, many angiosperm, including citrus, adopted the self-incompatibility system to improve their rate of polymorphism. This system prevents inbreeding and promotes outcrossing through rejecting cognate pollen (De Nettancourt 2001). This specificity of recognition is genetically determined as a single polymorphic locus, termed the self-incompatibility S-locus, of which it is consisted of a polymorphic locus with at least two tightly linked genes, the female and male determinant. There is no doubt that the identification of S glycoproteins in citrus is more difficult because of the long juvenile period. The citrus flowers in 5–8 years. In contrast, *Solanaceae* can flower in few months. The long juvenile phase hinders the confirmation of phenotypes and the construction of populations. Nevertheless, some progresses have been made in the self-incompatibility of citrus. The S genotype of Banpeiyu pummelo was defined as S1S2 (Ngo et al. 2010), which was used to produce self-fertilized seedlings (S1) with homozygous genotypes (S1S1 and S2S2) (Wakana et al. 2004). Although there is an ever-growing list of self-incompatible (SI) citrus accessions, the reported S genotypes of these SI varieties are limited.

The SI varieties of citrus are mainly pummelo and some seedless species (Ngo et al. 2001, 2010; Yamamoto et al. 2006). As the *Solanaceae*-type SI, the incompatible pollen of citrus is arrested within the style, rather than on the stigmatic surface (Newbigin et al. 1993). For example, in *Nicotiana*, the growth of the pollen tube is inhibited in the upper and lower parts of the transmitting tract (Lush and Clarke 1997). Shatian pummelo is a traditional cultivar of the *Citrus* genus that exhibits self-incompatibility. In self-pollination experiments with this cultivar, the pollen tubes penetrated the stigma and were rejected after growing through the top one-third of the style (Liang et al. 2017). The growth of self-pollen on Comune clementine exhibited the same behavior (Distefano et al. 2009a). The inhibition site for the self-pollen tubes of Kagzi Kalan lemon was in the middle of the style (Kakade et al. 2017).

S-RNases share conserved sequence motifs and a similar topology with RNase T2, although their amino acid sequences diverge substantially. A large number of S genotypes in *Rosaceae* were identified based on these conserved motifs

(Ushijima et al. 1998; Sonneveld et al. 2003). Many researchers tried to clone the homologous S genes from citrus using the same method, but none of the genes identified with this approach exhibited tissue-specific expression (Chai et al. 2011a, c; Miao et al. 2011a).

In the past, constructing Suppression Subtractive Hybridization (SSH) cDNA libraries provided an effective method for isolating differentially expressed genes. For this reason, researchers constructed the SSH libraries of self- and cross-pollinated styles from Shatian pummelo (Qin et al. 2008), and the self-compatible (SC) Shatangju and its SI mutant Wuzishatangju (Miao et al. 2011b). But most of these differentially expressed genes might function downstream of SI or perform functions that are related to the SC response.

After the introduction of next-generation sequencing technologies, RNA-seq became more popular than the construction of SSH cDNA libraries because RNA-seq provides a high throughput approach for identifying differentially expressed genes. Recently, an increasing number of transcript profiles on citrus SI were carried out, such as the transcriptomes of the styles from non-, self- and cross-pollinated Xiangshui lemon (Zhang et al. 2015), the differentially expressed genes in SI variety of citrus and its SC mutant (Distefano et al. 2009b; Caruso et al. 2012; Ma et al. 2017), and a transcriptome dataset based from seven tissues from Shatian pummelo (Liang et al. 2015). Moreover, the protein expression profiles of three developmental stages of Hyuganatsu styles were obtained using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry (Uchida et al. 2012).

In summary, past researchers used mainly two kinds of methods to identify the S-determinants of the citrus self-incompatibility system: homologous amplification and identification of differentially expressed genes. However, to date, there is no direct proof of a *bona-fide* S-RNases in Citrus. In *xylophyta*, SI and SC mutants are also valuable to identify the S determinants. The first female factor of *Rosaceae* self-incompatibility was identified utilizing a mutated Japanese peer (Nakanishil et al. 1992). Several pairs of SI and SC mutants are known in citrus, Shatian pummelo and Ziguishatian pummelo (Chai et al. 2011b), Shatangju and Wuzishatangju (Miao et al. 2011a, b), and Comune and Monreal clementine (Caruso et al. 2012). Additionally, in vitro culture systems and antisense oligonucleotide technologies are the most effective means to test the function of candidate genes in *xylophyta*. These types of experiments will provide information on the processes of SI in citrus and lay a solid foundation for determining the molecular mechanism of SI in this genus.

9.4 Seed Development and Nucellar Embryony

In citrus, nucellar embryony coexists with sexual reproduction pathway. The sexual reproduction in citrus experiences the embryo sac development by a typical *Polygonum* way (Fig. 9.1). Nucellar embryos develop from maternal nucellus tissue surrounding the sexual embryo sac, which is a kind of apomixis found in many citrus cultivars. Apomixis is generally defined as asexual reproduction by seed with or without fertilization and is widely variant mechanism in angiosperm (Koltunow and Grossniklaus 2003; Ozias-Akins 2006). Most cultivars in *C. paradisi*, *C. sinensis*, and *C. reticulata* are capable to reproduce by nucellar embryony except for some cultivars, such as Kiyomi Temple and Amber. Several types of citrus with ancient origins always produce monoembryonic seeds, such as *C. medica, C. grandis, C. mangshanensis*, and *C. ichangensis*. Most cultivars in *Poncirus* and *Fortunella* are mostly polyembryonic, except for *F. margarita, F. hindsii* and *F. japonica.* Some citrus relative genera also undergo nucellar embryony, such as *Zanthoxylum*, *Clausena*, and *Ruta* (Carman 1997).

The offspring derived from nucellar embryony possess the same genetic constitution as the female parent. Therefore, it greatly benefits the production of offspring for true-to-type rootstock with good unity and identical to the mother tree. Propagation by polyembryonic seeds is more

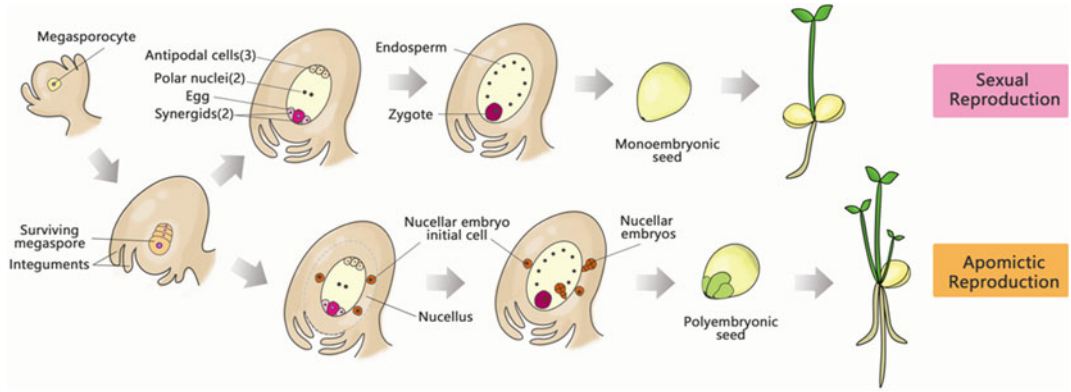

Fig. 9.1 Citrus sexual and asexual seed development. In the sexual reproduction pathways, the archesporial cell go through meiosis and produce four haploid cells with only one survived. The megaspore divides to form the embryo sac by mitosis. It contains three antipodal cells, two polar nuclei, one egg cell and two synergids. After double fertilization, the zygote (2n) and the endosperm (3n) were formed. In apomictic ovules, nucellar initial cells (orange) appear during the formation embryo sac. Several nucellar embryo initial cells develop into nucellar embryos in the nucellus tissue surrounding the sexual embryo sac. The polyembryonic genotype undergoes apomictic nucellar embryogenesis and sexual embryogenesis simultaneously. The monoembryonic genotype reproduces only by sexual embryogenesis

economic and effective to yield virus-free seedlings in citrus industry. Therefore, investigating the mechanism of nucellar embryony in citrus will provide deeper insight into the processes controlling apomictic reproduction and facilitate the transfer of apomixis into other crops for the propose of fixing elite trait (Spillane et al. 2001). On the contrary, the nucellar embryony hinders advance and efficiency for the scion breeding in citrus. The alternative way is to use monoembryonic cultivars as female parents. As only a few monoembryonic varieties with excellent features are frequently chose as maternal parents in cross breeding, resulting in a narrow genetic background of current commercial hybrid varieties. A possible approach to change the difficulties may be silencing the gene responsible for nucellar embryony by transformation.

Parlevliet and Carmenon (1959) proposed that the initiation of nucellar embryony is controlled by a major dominant gene in trifoliate and absent in pummelo. Other works suggest that modified genes complicated the ratios of segregating phenotypes (Iwamasa et al. 1967; Cameron and Soost 1979). García et al. (1999) evaluated nucellar embryony of the interspecific hybrid population derived from *C. volkameriana* × *P. trifoliata*. Six quantitative trait loci (QTLs) with both positive and negative effects were identified by genetic mapping. The QTL Apo2 may be the major gene controlling nucellar embryony, but other multiple QTLs also affect the level of multiple embryos. Raga et al. (2012) identified two molecule markers CR14, 290 and TAA15 closely linked to the nucellar embryony and useful for the early selection of polyembryonic rootstocks. Kepiro and Roose (2010) used AFLP markers to construct the genetic maps of the progenies of pummelo and Poncirus and found that five molecular markers may be associated with nucellar embryony and a marker EMB-6P links to the major loci controlling nucellar embryony in Poncirus. Nakano et al. (2008a, b, 2012) established genetic maps and haplotype-specific physics maps for nucellar embryony loci using several F1 and BC1 segregating populations derived from the monoembryonic cultivar *C. unshiu* × *C. sinensis* cv. Kiyomi and its polyembryonic parents *C. unshiu* Miyagawa wase and *C. sinensis* Trovita. The trait nucellar embryony was mapped to a region spanning 380 kb containing 70 predicted ORFs.

Nakano et al. (2013) performed suppressive subtractive hybridization studies on the ovule

tissues of two pairs of citrus varieties and screened out two polyembryonic and three monoembryonic specific genes. Kumar et al. (2014) conducted differential gene analysis of the ovules and leaves of polyembryonic and monoembryonic sweet oranges (Temple) by microarray, indicating that transcriptional regulation of nucellar embryony in sweet orange is related to stress. A conjoint analysis of mRNA and microRNA sequencing to analyze two pairs of citrus varieties indicate that a new small RNA, miRN23-5p, was up-regulated in two polyembryonic cultivars and its target gene was down-regulated (Long et al. 2016).

An integrated analysis of genetic and genome-wide association analysis in citrus showed that *CitRWP* is cosegregated with nucellar embryony in 786 citrus accessions, which is reported to be a transcriptional factor regulating zygotic embryo pattern formation and promoting somatic embryogenesis (Wang et al. 2017; Jeong et al. 2011; Waki et al. 2011). *CitRWP* is specifically expressed in polyembryonic ovules and its expression may depend on a miniature inverted repeat transposable element insertion in the promoter region. Recent studies showed that loss function of *CitRWP/CitRKD1* by antisense-overexpression resulted in failure to generate polyembryonic seeds in transgenic sweet orange (Shimada et al. 2018). These results indicate that *CitRWP/CitRKD1* may play a critical role in nucellar embryony.

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Genomics of Citrus Fruit Ripening

10

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Abstract

Ripening evolves most dramatic transformation in the life span of the fruit, from external an internal coloration to sugar accumulation, acid degradation, aroma formation, to provide the fruit appearance, organoleptic and nutritional quality for fresh or juice consumption. The advent of modern, large-scale transcriptomic technology in combination with available mutants affected in the harvesting time or some ripening-related processes is generating new and relevant information to understand the biochemical and molecular mechanisms regulating the process. The involvement of environmental, nutritional and hormonal signals in the initiation of ripening, key steps of most important biochemical pathways (acids, sugars, carotenoids, anthocyanins, etc.), identification of transcription factors and the characterization of new varieties and mutants are being addressed to elucidate the complex metabolic network operating in *Citrus* fruit maturation. It is likely that these genomic strategies are providing the basis for future citrus breeding to improve quality, postharvest performance and the nutritional and health-promoting benefits of *Citrus* fruits.

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10.1 Introduction

Citrus fruit is botanically defined as a modified hesperidium with a leather external layer (composed by the exocarp or flavedo and the mesocarp or albedo) and a variable number of internal carpels filled of elongated vesicles, forming the edible portion of the fruit (flesh or pulp). This internal structure is unique among the fruit of the different plant species and allows accumulation of the juice, providing the characteristic juicy palatability of citrus fruit and the properties for juice extraction (Tadeo et al. 2008). The development of *Citrus* fruits is generally divided in three stages: cell division, cell enlargement and cell maturation. In the last stage, cell growth is arrested and fruit initiates most the changes and transformations characteristic of maturity, such as sugar accumulation, color development, loss of acidity, aroma formation, among others, conferring the fruit their organoleptic and nutritional values (Bain 1958). The acquisition of these characteristics are genetically regulated and modulated by environmental and agronomically factors, to finally determinate the quality of the fruit of each particular variety, the time of harvest and, in last instance, their marketability and the consumer's preference. Moreover, Citrus fruit is an important source of bioactive compounds (carotenoids, flavonoids, anthocyanins, etc.), vitamins and minerals that accumulate progressively during fruit maturation, then, understanding the ripening process is of relevance for

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human nutrition and health (Ladaniya 2008; Terol et al. 2019a). The particular structure of Citrus fruit determined that the external peel and the flesh follow different spatial and temporal pattern of changes during maturity. These transformations in each tissue are not correlated and, in general, the flesh ripes earlier than the peel without the participation of light signals, indicating that the ripening process of both tissue are subjected, at least in part, to different regulatory mechanisms and transcriptional modifications (Tadeo et al. 2008; Terol et al. 2019a).

The metabolic changes occurring during fruit ripening and their biochemical basis have been the subject of intensive investigation over the years (Ladaniya 2008; Terol et al. 2019a; El-Otmani et al. 2011). Early studies were initially aimed to dissect changes in gene expression during fruit ripening, initially by small-scale molecular approaches, as 2-D gel of in vitro translation products (Alonso et al. 1992), subtractive hybridization of cDNA libraries constructed from ethylene and air-treated orange fruit (Alonso and Granell 1995; Jacob-Wilk et al. 1999), that allowed the isolation of only few genes, and some of them of unknown function. The advent of EST-sequencing projects allowed the construction of different generation of custom cDNA-microarrays and their use to analyse gene expression profiling (Talon and Gmitter 2008) during fruit ripening or changes induced by different hormonal treatments (Shimada et al. 2005; Fujii et al. 2007) or in the flesh of mandarin fruits (Cercos et al. 2006). More recently, exhaustive RNAseq and proteomic strategies have been used to identify large-scale transcriptomic changes during ripening of Clementine mandarins (Terol et al. 2019b), sweet oranges (Liu et al. 2009; Xu et al. 2009; Wang et al. 2017), Ponkan mandarin (Lin et al. 2015) and grapefruits (Patel et al. 2014). Massive information of the changes in gene expression have been generated allowing the characterization of putative regulatory elements and revealing the complex network of metabolic pathways taking place during the ripening of the peel and pulp of Citrus fruits. Although this information is still fragmentary, the function of many genes are unknown, and in many instances there is not convincing evidence to ascribe the function of a gene or regulatory element with a specific biochemical process, considerable progress has been made that is helping to elucidate key mechanisms operating during ripening and their regulation. Therefore, the objective of this chapter is to provide an update and critical revision of relevant genomic strategies designed to understand key metabolic changes occurring during Citrus fruit ripening as well as to delineate the complex network of molecular signals regulating the initiation and the progress of the process.

10.2 Environmental and Hormonal Cues Regulating Fruit Maturation

For many years is widely recognized that nitrogen and sugars are the main nutritional factors affecting on-tree ripening and fruit colouration (Tadeo et al. 2008; Alós et al. 2006). In vitro studies on grapefruit pericarp established an inverse correlation between nitrogen and sugars controlling colour changes. Peel degreening is induced under low nitrogen content and increasing sugars, and is highly dependent of the temperature (Huff 1983, 1984). Under field conditions, fruit coloration is stimulated at temperatures below 10–13 °C and high differences between day/night temperatures, which in turn reduced nitrogen translocation. Maturing fruit are a strong sink of photosynthates and favoured the transition from chloroplasts to chromoplasts (Terol et al. 2019a; Gambetta et al. 2012).

Massive transcriptional analysis is providing evidences linking environmental signal and nutritional status with changes in gene expression. A number of transcriptions factors are differentially expressed during fruit ripening and in comparison with late-ripening mutants, and many of them have been related to stress responses (*MYB77, MYB62, RD26*, *bHLH* factors, *ERFs*), light perception (*GATA7*, *MYR2, C2H2*) and sugar responses (*MYB21*) (Zhang et al. 2014; Ding et al. 2015; Wu et al. 2016). Whether these TFs may coordinate and translate

environmental- and nutrient-related stimuli into the transcriptional and biochemical changes accompanying fruit maturation deserve further investigation.

The plant hormones ethylene, ABA and gibberellins are known to be regulators of Citrus fruit maturation. Ethylene is long known to accelerate colour change in detached Citrus fruits, inducing the expression of carotenoid biosynthetic genes and many other ripening-related metabolic steps (Fujii et al. 2007; Wardowski et al. 2006; Rodrigo and Zacarías 2007). However, Citrus are non-climacteric fruit evolving very low level of ethylene during natural maturation. A detailed analysis of the expression of ethylene biosynthetic and signalling genes during the whole reproductive development of Valencia orange fruit revealed a shift in ethylene regulation (Katz et al. 2004). Ethylene production is high in young fruitlets, probably related to June drop, and ethylene stimulated its own synthesis (autocatalytic) similarly to climacteric fruits. However, maturing fruits produce low ethylene levels and the hormone exerts a negative feedback regulation of its synthesis. Expression of ethylene biosynthetic genes is coordinately with these patterns of regulation, supporting the non-climacteric ripening of *Citrus* fruits (Katz et al. 2004). It appears, then, that Citrus fruits have lost the ability to regulate ethylene production during maturation in an autocatalytic manner (Paul et al. 2012), which is the key factor of ripening in climacteric (Alós et al. 2019). Consistent with these previous results, massive transcriptional analysis during maturation in fruit of several Citrus species have identified the presence of ethylene biosynthetic enzymes, ACC synthase (ACS) and ACC oxidase (ACO) among the genes differentially expressed between late-ripening mutants compared with their corresponding parental genotypes (Terol et al. 2019a; Ding et al. 2015; Wu et al. 2014). In the genome of Clementine mandarin 7 *ACS* and 4 *ACO* genes have been identified and gene expressions of some members of these families were lower in fruit of the late-ripening mandarin than in the early-harvesting, in agreement with ethylene production (Terol et al. 2019a; Wu et al. 2014). The expression of *CsACS1* and *CsACO1* also increased during post-ripening and shelf-life conditions (Ding et al. 2015). Together, transcriptomic evidences provide support to the motion of non-climacteric behaviour of Citrus fruit maturation, evolving very low levels of ethylene production and gene expression but playing a role regulating the rate of fruit maturation.

The involvement of ethylene in the ripening of Citrus fruits is also exemplified by the expression of ethylene receptors and signalling genes, some of which increase during ripening (Wu et al. 2016). However, the expression of these genes not always correlated with the late-ripening phenotype of some mutants, indicating that post-transcriptional and post-translational modifications may operate in ethylene signalling. Moreover, many transcriptional studied performed in fruit of diverse *Citrus* species have identified large number of ERFs that have been implicated in several ripening-related processes in climacteric fruits. Since the ERFs identified are related to multiple metabolic functions in both peel and pulp, it is likely the ethylene regulates the complex network of events taking place during Citrus maturity (Xie et al. 2014). How ethylene action is involved in the regulation of these metabolic processes, despite the low levels of ethylene involved during ripening, is currently unknown and this question is a major gap in non-climacteric fruits (Paul et al. 2012; Alós et al. 2019). Current evidences suggest that the participation of ethylene in the ripening of Citrus fruits may be through changes in the sensitivity to the hormone (Alós et al. 2014).

The involvement of abscisic acid (ABA) in the Citrus fruit maturation is complex but physiological and molecular evidences clearly indicated an important role for this phytohormone. ABA content in the peel increased concomitantly with color development and carotenoid biosynthesis, as ABA is an end product of the pathway (Rodrigo et al. 2003). Expression of ABA biosynthetic and signalling elements also increased during natural fruit maturation (Rodrigo et al. 2006; Romero et al. 2012) and massive transcriptomic analysis identified genes

of ABA signalling and other ABA-regulated genes (Zhang et al. 2014; Ding et al. 2015; Wu et al. 2014, 2016). Supporting these evidences is the fact that exogenous ABA accelerated fruit colouration in Ponkan mandarin (Wang et al. 2016) and the ABA-deficient mutant of sweet orange, Pinalate, displayed a lower rate of fruit degreening (Rodrigo et al. 2003). In this ABA-deficient mutant, GAs and ethylene application have a reduced effect (unpublished results), indicating that the action of these hormones may be partially mediated by ABA. The promoting effect of sugar in peel degreening appears to be also mediated by ethylene (Iglesias et al. 2001). This complex hormonal interaction in which ABA appears to have a central-intermediate role is further supported by transcriptomic results that identified elements regulated by ABA, sugars and JA differentially expressed during fruit ripening (Zhang et al. 2014). Moreover, ABA-related transcription factors are differentially expressed between late-ripening and ordinary oranges, such as *ABR1, RD26, DREB26, MYB77, MYB61, MYB62*. These MYB TFs have been shown to respond to the stress-induced ABA, whereas *RD26* is an activator of ABA signalling. Other TFs, as *bHLH* and *MYB* were related to JA signalling, and *MYB21* is part of a module regulated by glucose and fructose (Wu et al. 2016). These results reinforce the role of ABA in the regulation and signalling of ripening-related processes in Citrus fruits. An attractive hypothesis is that ABA may mediate the response of the fruit to environmental signals (water and cold stress, or nutrients) stimulating Citrus fruit ripening.

10.3 Acid and Sugar Metabolism; Key Components of Fruit Quality

Changes in internal maturation of Citrus fruit are critical factors determining the harvest time and the organoleptic and nutritional quality of the fruit for fresh consumption or juice production. Acidity and sugar content are the two key parameters of internal fruit quality, that decrease and increase, respectively, during maturity and their ratio determine the harvesting and marketing time.

In fruits of most citrus varieties, citric acid is the most abundant organic acid in the pulp and the major contributor to acidity. Citric acid may account between 70–90% of total acids followed by malic acid (10–15%) and minor proportions of succinate and isocitrate. Its accumulation in the pulp is initiated during the first phase of stage II to reach a maximum when the fruit has reached 50% of the final size. The relevance of citrate metabolism to citrus fruit quality has encouraged extensive research in recent years and transcriptomic and proteomic approaches are providing new insights into the biochemical mechanisms and its regulation during fruit ripening (Cercos et al. 2006; Katz et al. 2011; Etienne et al. 2013; Hussain et al. 2017; Terol et al. 2010). Citrate accumulation in the vacuole depends on the balance of citrate synthesis, membrane transport and degradation or utilization (Hussain et al. 2017). Expression of the genes encoding for citrate synthase (CT) and phosphoenolpyruvate carboxylase (PEPC) was up-regulated during maturity in fruit of different cultivars and no clear association with the changes in citrate concentration could be established (Lin et al. 2016; Guo et al. 2016). Moreover, differences in citrate accumulation between acid and acidless varieties did not correlate with citrate synthase gene expression and activity (Sadka et al. 2000; Albertini et al. 2006). A tonoplast citrate/H+ symporter involved in citrate efflux from the vacuole has been characterized (Shimada et al. 2006). Citrate is released to the cytosol and transcriptomic analysis indicated that is metabolized to glutamate and then processed through the gamma-aminobutyrate (GABA) shunt. Two glutamate dehydrogenase genes (*CsGAD1* and *CsGAD2*) have been identified in the Citrus genome, and their expression increased during fruit maturation, as well as GAD activity, in parallel with the reduction in acid content (Liu et al. 2014). Moreover, ATP-citrate lyase gene expression decreased during maturation, indicating that citrate is consumed during the process (Cercos et al. 2006). This enzyme is encoded by

three genes and their expression was up-regulated during maturity in the juice sacs of oranges and mandarins (Hu et al. 2015). These results are supported by the observation that inhibition of aconitase by citralmalate increased citrate content and induced amino acid synthesis and GABA shunt (Degu et al. 2011). Together, these observations may explain the important reduction in citrate content and acidity taking place during maturation.

Isomerization of citrate to isocitrate is catalized by aconitase (*Aco*). Aconitase gene expression was first studied in lemon fruits (Sadka et al. 2000) but genome-wide analysis of aconitase revealed the presence of 3 members, two of them increased in parallel with the decline in citrate content in mandarins and oranges. The fact that acid and acidless lemons did not follow a similar pattern suggests that other additional mechanism may exist in other citrus species (Terol et al. 2010). Proteomic analysis of Navel orange juice vesicles corroborates the results on the changes in gene expression, indicating that the GABA shunt is active and that accumulation of organic acids and amino acids shifted to sugar synthesis during fruit maturation (Katz et al. 2011). In agreement with previous observation, analysis of 11 genes involved in citrate accumulation during ripening of Ponkan mandarins indicated the up regulation of *CitPEPCs, CitCSs, CitAco3* and *CitGAD4* which correlated with the increase in citrate content induced by low temperature (Lin et al. 2016).

Citrate is transported to the cytoplasm and should be rapidly metabolized or stored into the vacuole to maintain a neutral cytoplasmic pH (Etienne et al. 2013). Vacuolar acidification in plants is caused by proton pumps and it is envisaged that differences in acid content between Citrus species and cultivars may be potentially caused by activity of these pumps. Among the different types of vacuolar ATPs contributing to vacuolar pH, the plasma membrane H+-ATPase (P-type) is composed by eight different gene members (*CsPHs*) in Citrus that have a tissue-specify pattern of expression. Transcript accumulation differed between pummelo and oranges and between varieties with low and high acidity, but only *CsPH3* and *CsPH8* decreased concomitantly with the reduction of citrate concentration, suggesting that these gene products participate in the reduction of citrate influx into the vacuole (Shi et al. 2015). Two other families of vacuolar H+-ATPase (VHA) and the vacuolar proton pump: H+-pyrophosphatase (VHP) have been characterized in the genome of *Citrus* and their expression studied during maturity. VHA is a large gene family composed of 20 members coding for different protein subunits, whereas VHP is only composed by four members. Transcriptomic analysis of the expression of these genes revealed complex pattern of expression, with different levels in different tissues, but a limited number of genes showed a temporal expression consistent with the reduction of citrate concentration in the juice sacs (Guo et al. 2016; Shi et al. 2018). Comparative gene expression analysis of the members of these two families in the flesh of the lycopene-accumulating mutant Hong Anliu, which contains reduced citrate content, identified specific genes that correlated with the pattern of acid accumulation. Moreover, treatment of the mutant pulp with ABA increased citrate content and modified the expression of some genes that were expressed at reduced level (Guo et al. 2016; Shi et al. 2018). Then, these results demonstrated the contribution of the proton pump activity in the transport of citrate into the vacuole, and that down-regulation of the genes encoding for specific transporters is likely responsible for the low acid accumulation of some varieties (Hussain et al. 2017).

Sugar content in the pulp is an important determinant of fruit quality. Sucrose is the main form of photoassimilate for export from the source to the sink tissues. The source/sink relationship is important during the transition from cell enlargement (stage II) to fruit maturation (stage III), as developing and maturing fruits act as carbohydrate storage sink. Sucrose is metabolized in plant tissues by two main enzymes, sucrose synthase (SUS) which form fructose and UDP-glusose, and invertase (INV) that hydrolizes sucrose in glucose and fructose (Koch 2004). Sucrose content increases progressively

during fruit maturation; a lower increase concerns also glucose and fructose. In later stage of ripening, glucose and fructose increase more rapidly than sucrose, indicating sucrose degradation. Then, accumulation of sugar is the result of a fine balance between synthesis/degradation reactions (Lin et al. 2015). SUS activity is thought to play a potential role in the regulation of sugar accumulation in juice sacs, since it is presumed to participate in phloem loading and unloading (Tomlinson et al. 1991; Etxeberria et al. 2005). Genes encoding for sugar metabolizing enzymes have been identified and their expression analysed in juice sacs during maturation in fruit of different *Citrus* species and varieties. Early work in Satsuma mandarin identified SUS genes; two were up-regulated during fruit maturation but a third member is expressed at early stages of fruit development and declined thereafter (Komatsu et al. 2002). Large-scale transcriptomic analysis and searching in the *Citrus* genomes databases identified four genes coding for sucrose-phosphate synthase (*SPS*) involved in sucrose synthesis, 5 *SUS* genes, 10 invertase (5 acidic and 5 neutral) 2 fructokinase (*FRK*) and 5 hexokinase (*HXK)*. Early increase of these two later gene members may be responsible for the early increase in sugar and a latter stimulation of INV gene would participate in sucrose degradation during latter stages of maturity of Ponkan mandarin (Lin et al. 2015). Other studies reported six *SUS* genes in Satsuma mandarins, being *CitSus1* and *CitSus2* predominantly expressed in juice sacs, whereas other genes showed variable pattern of expression and in different tissues, indicating a complex regulation of the expression of this gene family member in different Citrus tissues (Islam et al. 2014).

An exhaustive analysis of the changes in proteome during fruit development in Navel oranges identified several members of sugar metabolizing enzymes (Katz et al. 2011). Most of these enzymes were up-regulated highlighting the role of glycolysis in sugar utilization during cell division and maturation. Other sucrose degradation enzymes also accumulated, indicating a post-transcriptional regulation of sucrose-metabolizing enzymes, and that the balance between sucrose synthase/invertase plays a key role in the regulation of sucrose content in the juice sacs (Katz et al. 2011). Genomic and transcriptomic analysis of INV gene family revealed the presence of 10 members in the Citrus genomes (Ding et al. 2015; Deng et al. 2019). *CsINV2* were upregulated during fruit maturation indicating a role in sucrose metabolism, whereas other members were up-regulated in the ring of the fruit. Moreover, two INV inhibitors were also identified and the *CsINH1* displayed a pattern of expression parallel to that of *CsINV2* (Deng et al. 2019). Moreover, sugar transportation has been also claimed to play an important function in loading, uploading and determining cellular homeostasis (Koch 2004). Three sucrose transporter genes (*CrSUT*) were initially identified in Satsuma mandarin and more recently up to 10 different sugar transporters have been shown to be differentially expressed in the rind and the pulp of different Citrus fruits (Ding et al. 2015). These observations suggested that the transport of hexoses from the apoplastic space to the cytoplasm is important in sugar homeostasis and pointed out that in the source/sink balance may operate a transfer of nutrient from the flesh to the rind, but not in the opposite direction (Ding et al. 2015).

10.4 Citrus Fruits as a Source of Carotenoids, Anthocyanins and Vitamin C

Citrus fruit and juice are highly recognized by their nutritional and health-related properties. Among the different bioactive compounds of the fruits, the pigments carotenoids and anthocyanins are of special relevance, since contribute to both the appearance and the nutritional quality. Moreover, Citrus fruits are worldwide distinguished by the vitamin C content and are one of the main sources of this compound in the human diet. The biosynthetic pathways of these compounds have been studied in detail and in recent years, considerably progress has been made in

the understanding of their transcriptional regulation.

Carotenoids are isoprenoids-derived pigments responsible for the typical coloration of the peel and pulp of fruits of most Citrus species. Carotenoids are important determinants of fruit quality and consumer acceptance, and are also essential components of the nutritional and health-related benefits of the fruits, because of their potential antioxidant activity and the provitamin A capacity of specific carotenoids (Rodriguez-Concepcion et al. 2018). During fruit maturation and the transition from chloroplast to chromoplasts, carotenoids composition changes from those typical of green tissue (lutein, α- and β-carotene, *all-E*-violaxanthin and neoxanthin) to β-β xanthophylls, being 9-Z-violaxanthin the main carotenoid in oranges, and β-cryptoxanthin and *9-Z*-violaxanthin the preponderant in mandarins and their hybrids (Gross 1987).

In last decade, intensive research in the transcriptomic changes related to carotenoids metabolism and their regulation has been carried out (Rodrigo et al. 2013; Ikoma et al. 2016; Wei et al. 2014). The general picture emerging from these studies indicates that regulation of carotenoid biosynthesis is rather complex but there are several steps in the pathway that are essential to control the metabolic flux and the accumulation of specific carotenoids. The first enzyme of the pathway, phytoene synthase (PSY) is highly stimulated at the onset of fruit maturation, mainly *PSY1*, that appears to control the general flow of metabolites to the formation of downstream products. A second gene, *PSY2,* is also expressed in both flavedo and pulp but to a lower level than *PSY1* (Wei et al. 2014). Other genes from early steps of the pathway, phytoene desaturase (PDS) and as ζ-carotene desaturase (ZDS) are also stimulated during maturation. Several genes from these steps (2 for PDS and 11 for ZDS) have been identified in the genome of sweet orange (Wei et al. 2014). In green fruits an elevated expression of the genes from the ε,β-branch of the pathway, enabling the syntheses and accumulation of α-carotene and lutein, carotenoids characteristics of green tissues has been demonstrated. At the onset of ripening, the expression of the genes from this branch of the pathway are progressively reduced, particularly *ε-LCY*, while *β-LCY* slightly increases and the chromoplast-specific *β-LCY2* gene is highly induced, shifting the flux of the pathway into the β, β-branch (Alquézar et al. 2009; Zhang et al. 2012). Subsequently, the expression of the gene responsible for the hydroxylation of β-carotene (β-carotene hydroxylase, CHX) is also stimulated, leading to the massive accumulation of violaxanthin and other xanthophylls (Wei et al. 2014). Carotene hydroxylase (CHX) is also composed by a multigene family of at least five members in the orange genome (Ma et al. 2016). It is interesting to remark that in fruits of most *Citrus* species the expression of carotenogenic biosynthetic genes are substantially lower in the pulp than in the peel, in agreement with its reduced carotenoid content (Ikoma et al. 2016; Alquézar et al. 2008).

The xanthophyll β-cryptoxanthin is the main contributor of mature fresh Citrus fruits to vitamin A capacity, and is much higher in mandarins and hybrids than in oranges (Fanciullino et al. 2006; Liu et al. 2012). The metabolic steps of the synthesis of β-cryptoxanthin in plants have not been elucidated yet. This xanthophyll is an intermediate in the formation of zeaxanthin from β-carotene catalyzed by CHX. Comparison of the expression of genes of the pathway and β-cryptoxanthin content in mandarins and oranges suggest that this xanthophyll may accumulate as a result of the balance between activity of early steps and degradation of β-carotene. Thus, mandarin fruits have low expression of CHX than oranges and probably a less efficient capacity to convert β-carotene to zeaxanthin allowing accumulation of the intermediate β-cryptoxanthin (Ikoma et al. 2016).

Accumulation of lycopene in the pulp is an unusual feature in citrus fruits, being grapefruit (*Citrus paradisi*) and pummelo (*Citrus maxima*) among the *Citrus* species in which a large number of red mutants has been identified, or few sweet orange mutants, as Hong Anliu (Liu et al. 2007) or Cara Cara (Alquézar et al. 2009; Lee 2001). During maturation of pummelo fruit with contrasting pulp colouration, differences in

the accumulation of some transcripts of carotenoids biosynthetic genes (*ZDS*, *LCYb2* and *CHX*) were observed (Liu et al. 2016; Yan et al. 2018). However, reduced transcripts levels of the chromoplast-specific lycopene-cyclase gene *LCYb2* has been detected in the pulp of red grapefruit compared with the pulp of either oranges or white grapefruit (Rodrigo et al. 2013; Alquézar et al. 2009). In the sweet orange 'Hong Anliu' mutant, accumulation lycopene has been associated with the down-regulation of both *LCYb* and *LCYb2* (Xu et al. 2009; Yu et al. 2012), indicating that *LCY2B* appears to play a key role in the regulation of carotenoid biosynthesis and that alterations in the expression of the gene or its functionality may be responsible for the abnormal lycopene accumulation (Wei et al. 2017). Comprehensive transcriptional and proteomic analysis in the flesh of the 'Hong Anliu' mutant has been done using different large-scale technologies (Liu et al. 2009; Xu et al. 2009; Pan et al. 2009, 2012). The general picture from these exhaustive screening revealed profound transformations in the pulp of the lycopene-accumulating genotype: monoterpenes, gibberellins, sterol, photosynthesis, glycolysis, starch and sucrose, are among the most significant metabolites and processes that were stimulated, whereas ABA, fatty acids or acid metabolism are down-regulated. Moreover, several enzymes of the antioxidant system were up-regulated, suggesting an enhancement of the oxidative stress in these cells (Pan et al. 2012). Together, these results do not explain the molecular and biochemical basis of the mutation, although the expression of some MYB transcription factors was also altered. Alternatively, it is conceivable to speculate that these metabolic rearrangements may be a consequence (not the cause) of lycopene accumulation in the cells that may change the redox status and produce other metabolic transformations compared with the wild-type cells that develop their metabolic activity in the absence of lycopene and with a completely different carotenoid complement. Similar metabolic consequences have been reported in fruit of an orange-pericarp mutant of pummelo (*Citrus grandis*) which accumulates β-carotene in the peel. Expression of down-stream genes of carotenoids biosynthesis was lower in the mutant than in the wild-type. Mutant fruit reveals alteration of several metabolic pathways, such as sucrose metabolism, different compounds (asparagine, fatty acids) and diverse transcription factors (3 MYBs, 2 NACs, 1 ERF, WRKY and MUTE). These results illustrate that alteration of the carotenoid complements may produce important metabolic rearrangements and modification, but whether these changes are cause or consequence of the mutation, remains to be determined (Guo et al. 2015).

The peel of coloured oranges and mandarins is able to accumulate genus-specific C30 apocarotenoids (β-citraurin and 8-β-apocarotenal), conferring the intense reddish colouration. A new carotenoid-cleavage dioxygenase (CCD4) responsible of the conversion of β-cryptoxanthin and zeaxanthin into the apocarotenoids has been identified. The corresponding *CCD4* gene is expressed during fruit coloration of the peel, just before the massive accumulation of β-citraurin, and it is stimulated by ethylene, then playing a role in the colouration of the peel (Rodrigo et al. 2013; Ma et al. 2013).

Anthocyanins are a second group of pigments responsible for the particular coloration of a group of *Citrus* fruit called 'blood oranges'. Anthocyanins are water-soluble pigments belonging to the flavonoids family, providing red, purple and blue coloration to flowers, fruits and vegetables of many species. They also play important functions in plants and have recognized health-related properties (Winkel-Shirley 2001).

Blood oranges constitute a group of varieties (Tarocco, Moro and Sanguinello) characterized by the red-purple colouration of the pulp and also the peel by accumulation of anthocyanins. The relative amount of these pigments depends on the cultivars and is highly dependent on environmental and agronomical factors. The main anthocyanins present in blood orange are cyanidin-3-glucoside and delphenidin-3-glucoside although more than 10 different structures have been identified. Moreover, environmental stimuli such as light, osmotic stress and

cold temperature, either on the field or under postharvest storage, are known to stimulate accumulation of anthocyanins. Anthocyanins distribution, biological activity, biosynthesis and their regulation in *Citrus* have been the subject of comprehensive reviews (Lo Piero 2015; Ballistreri et al. 2019).

Anthocyanins biosynthesis mechanisms in blood oranges have been elucidated and are similar to those operating in other plant tissues and most structural genes for enzymes responsible for each step have been isolated (Ballistreri et al. 2019). Briefly, the phenylpropanoid pathway is initiated by phenylalanine ammonia lyase (PAL) which catalyzes conversion of L-phenylalanine to trans-cinnammate. The first committed step for anthocyanins biosynthesis is catalyzed by chalcone synthase (CHS), which condenses malonyl-CoA and 4-coumaroyl-CoA to form tetrahydroxy chalcone. After a series of reactions, anthocyanin 3-O-glycosides are synthesized from dihydroflavonols by consecutive reactions catalyzed by dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose flavonoid glucosyl transferase (UFGT). Each particular tissue may undergo specific metabolic steps to form different anthocyanins but the general consensus is that anthocyanin levels are regulated at the transcriptional level (Winkel-Shirley 2001). In blood oranges it has been observed a coordinated activation of the expression of anthocyanin biosynthetic genes (*CHS, CHI, F3H, DFR, ANS, UFGT*) during natural ripening and under cold stress, concomitantly with the increase in the concentration of anthocyanins (Lo Piero 2015; Marocco et al. 2004; Licciardello et al. 2008; Crifò et al. 2011). Interestingly, in ordinary blond oranges the three early genes of the pathway were expressed at similar levels, but downstream genes failed to be stimulated (Butelli et al. 2012) suggesting that only blood oranges have the capability to activate, by several stimuli, anthocyanin genes expression. Large-scale transcriptomic analysis of differential expressed genes in blood oranges revealed activation by cold stress of a numbers of transcripts involved in defence responses, oxidative damage, osmoregulation and lipid desaturation and several associate transcription factors (Licciardello et al. 2008; Crifò et al. 2011). These results indicate that blood oranges have acquired the capability to express genes of the anthocyanin biosynthetic pathway under inductive conditions. Moreover, a comparative transcriptomic and proteomic study between the blood Zaohongan and ordinary Washington navel sweet oranges revealed more than 900 genes and 94 proteins differentially expressed in the pulp during fruit maturation. Anthocyanin, flavonoids, sugars, citrate, ABA and ethylene are among the metabolic pathways differential induced in both genotypes, as well as diverse stress-related genes or transcription factors. These results are not unexpected, and indicate that the presence of high content of anthocyanin in the juice sacs of blood oranges predisposes different metabolic networks and redox status respect to the tissue of ordinary oranges and also different responses to stress conditions (Wang et al. 2017).

Biosynthesis of anthocyanins in plants is known to be regulated by MYB transcription factors which activate the expression of anthocyanin genes (Winkel-Shirley 2001; Butelli et al. 2012). A major breakthrough in the understanding of anthocyanin synthesis in blood oranges has been the finding of the so called 'Ruby Gene'. This gene is a MYB transcription factor responsible for the synthesis of anthocyanins in blood oranges and whose expression is closely associated with the concentration of pigments in different varieties of blood oranges under cold tress. The gene was not expressed in ordinary oranges under the same conditions. Blood oranges have in their genome a retrotransposon inserted close to the 'Ruby Gene' which is regulated by cold. This retrotransposon causes a gain-of-function since it activates the expression of the 'Ruby Gene' and initiates the synthesis of anthocyanins (Butelli et al. 2012). Interestingly, the recombination and transposition of these genetic elements are not the same in blood oranges of different origin, indicating that variations in this kind of mechanism have generated much of the genetic diversity during domestication (Butelli et al. 2017). A novel gene *Ruby2*

which is adjacent to *Ruby1* has been recently identified (Huang et al. 2018). Different *Ruby2* alleles may exist in the genome of the different *Citrus* species that may function as a transcriptional repressor. Then, the complex of both genes exhibited subfunctionalization and it has been hypothesized that mutations in the different alleles of both two genes during evolution and domestication have originated the diversity in anthocyanin pigmentation (Huang et al. 2018).

Citrus fruits are generally considered as one of the major source of vitamin C (ascorbic acid, Asa) for human nutrition and health. In general, the content of vitamin C, as of many other bioactive compounds and phytonutrients, is considerably higher in the peel than in the flesh or pulp. Although the content reported in different studies is very variable, the pulp contains about one-fourth of the vit C content of the peel (Martí et al. 2009). Vitamin C in citrus fruit increases during development and maturation. However, analysis of the concentration throughout the process indicate that it is not a ripening-related event, since major increments may occur during the cell-enlargement phase and are not coordinated with other maturation processes as color change or sugar accumulation (Magawaza et al. 2017).

The metabolic pathways involved in vit C synthesis in plants are very complex since four biosynthetic routes have been shown to be operative in coordination with degradation and recycling pathways (Mellidou and Kanellis 2017). The emerging conclusions obtained from different vit C-accumulating fruits is that each species may regulate in a specific manner the metabolic pathways conducing to ascorbic acid and that this pattern may change during development and in the different fruit tissues (Gest et al. 2013). A comparative analysis of the expression of genes involved in ascorbic acid biosynthesis and recycling during ripening of Satsuma mandarin (low Asa content) and navel oranges (high Asa content) indicate a differential regulation between peel and pulp and that L-galactose pathway plays a key role in the accumulation of ascorbic acid in these fruits (Alós et al. 2014). In fruits of several *Citrus* species, as grapefruit, Clementine mandarin or Navel oranges, it has been shown that light avoidance reduced drastically Asa content in the peel (Lado et al. 2015), suggesting that light exposure may be a trigger factor responsible for the high level of Asa usually found in the peel respect to the pulp. Transcriptional analysis of a large number of genes involved in Asa synthesis, recycling and degradation revealed that the isoforms *GalUR8* or *GalUR12* from the L-galacturonic acid pathway were significantly repressed, in parallel with the reduction of Asa concentration (Lado et al. 2015).

10.5 Transcription Factors and Ripening-Related Mutants, and Epigenetics

The use of massive of RNAseq technology to decode the molecular mechanism regulating *Citrus* fruit maturity in combination with available mutants (bud sports) altered in the rate of ripening (early- or late-harvesting) is allowing the identification of transcription factors (TFs) potentially involve in the regulation of the process. Many TFs belong to large gene families and only correlative evidences of the participation in the ripening have been obtained. However, there are relevant examples of TF regulating the transcription of genes involved in specifying ripening-related events.

Comparative analysis of genes differentially expressed between the orange Fengjie 72-1 and its spontaneous late-ripening mutant Fengwan revealed more than 600 genes and 130 proteins. Many of these changes are not unexpected since are metabolic processes delayed in mutant fruits, as ABA, carotenoids, sugar, acid metabolism and cell-wall degradation enzymes. Several heat-shock proteins (HSP), including the HSP90, were also differentially accumulated between both genotypes, indicating the kind of changes accompanying the general remodelling processes occurring during orange pulp ripening (Wu et al. 2014). The involvement of ABA and jasmonic acid (JA) synthesis and signalling has been also detected in the transcriptomic analysis of other late-ripening orange mutant (Jinchen), as well as

sugar and acid metabolism, revealing that this hormonal interaction may be part of the complex changes in fruit colouration (Zhang et al. 2014). Analysis of this mutant identified more than 159 transcription factors involved in the ripening processes, and many of them were differentially expressed in the late-ripening mutants (Wu et al. 2016). These transcriptions factors belong to different families, as C2H2, Dof, bHLH, ERF, MYB, NAC and LBD, being the ERF family the most highly represented. Co-expression analysis revealed a close association of RD26, NTT, GATA7 and MYB21/62/77 with ABA, acids and sugar. Moreover, TFs that have been related to different metabolic processes, as ethylene and auxins synthesis and signaling, responses to salicylate, light and sugar responses, etc., were also differentially expressed, indicating the complex network of metabolic interaction during orange ripening. An high-throughput transcriptomic analysis of the changes occurring in fruits with thin peel (Ponkan and Satsuma mandarin) in comparison with those of thick peel (orange and pummelo) indicated that more than 11,000 genes were expressed during ripening and senescence (around 50% of the *Citrus* genome) which exemplified the dramatic and general rearrangements in those cells (Ding et al. 2015). MYB transcription factors were expressed in the four genotypes, indicating their participation in general ripening-related processes. Interestingly, some TFs were more differentially expressed in either the loose or tight-skin. Thus, TF highly expressed in loose-skin belong to AR2/ERF or NAC families, whereas in the tight-skin were more highly represented TF of the WRKY family. This selective expression appears to be in relation with the kind of stress occurring during ripening and senescence in both types of fruits: water stress and ABA-related responses in the loose-skin fruits, in comparison with responses to pathogen and biotic stresses in thick-skin fruits (Ding et al. 2015).

The AP2/ERF (APETALA2/ethylene response factor) superfamily of transcription factors are among the largest and well conserved regulatory elements in plants. Different members of this superfamily act downstream ethylene and influenced many developmental processes, interactions with plant hormones and stress responses to biotic and abiotic stresses. Because the influence of ethylene in the control of ripening in climacteric fruits, the involvement of these TFs in the transduction of ethylene action and in the regulation of many ripening-related processes have been extensively studied (Xie et al. 2014). The AP2/ERF superfamily is divided in several groups attending to the structure and regulatory motifs and may act as both transcriptional activators or repressors.

The AP2/ERF superfamily in *Citrus* has been studied and the members of the different subfamilies identified in the citrus genome database (Xie et al. 2014; Ito et al. 2014). The number of total members of AP2/ERF TF varied from 108 to 126, a number that in general is similar to that found in *Prunus* species but much lower than that present in other species, such as tomato, grapes or Arabidopsis. Most of the AP2/ERF members (91 or 102) belong to the ERF family, which include the DREB2 which is involved in drought responses, CBF/DREB1 implicate in cold tolerance, and other elements related to developmental processes and ethylene responses. An in silico analysis of gene expression pattern, based on EST databases of *Citrus*, revealed that 67% of the ERFs are expressed in during development of different tissues, whereas 14% are expressed in stressed tissues. Interestingly, 25% of the ERFs were expressed in fruit tissues and many of them are homologous to ERF expressed in tomato fruit during fruit ripening that modulate ethylene biosynthesis and postharvest life of the fruits (Ito et al. 2014). A detailed analysis of the ERFs genes during development and maturation of Newhall oranges revealed that most of them exhibited an expression pattern stage-specific. In particular, four *CitERFs* genes were induced at the initiation of fruit maturation whereas other member (*CitERF5, CitERF18, CitERF24, CitERF41, CitERF53, CitERF91*) were clearly expressed in mature fruits, indicating a potential role in the regulation of fruit ripening (Xie et al. 2014).

The direct regulatory role of ETRs on several ripening-related processes is now started to be elucidated, and there are examples demonstrating

a direct binding of several *CitETR* to key genes of important metabolic pathways. During peel degreening of orange fruit, the expression of *CitERF13* was inversely correlated with the breakdown of chlorophyll. A detailed molecular and biochemical analysis of this TF revealed the binding to the promotor of the *CitPPH* gene, which encode pheophytin pheophorbide hydrolase, an important enzyme in chlorophyll degradation. *CitERF13* was up-regulated by ethylene and accelerated peel degreening, and its function was demonstrated to be conserved in other plants (Xie et al. 2014). Another ERF, homologous to the Arabidopsis *AtERF017*, was demonstrated by transient luciferase assay and the yeast two-hybrid system, to interact with a specific sequence of the promotor of the vacuolar ATPasa *CitVHA-c4*, and its expression correlated with that of other four different vacuolar ATPase from the pulp of Ponkan mandarin (Li et al. 2016). A similar experimental approach was used to demonstrate the transcriptional regulatory activity of *CitERF71* on the expression of the gene *CitTPS16* which encode a terpene synthase responsible for the formation of *E*-geraniol in sweet orange (Li et al. 2017). All these evidences strongly support the involvement of the AP2/ETF family members in the regulation of Citrus fruit maturation, that may be or not mediated through the ethylene action.

Massive analysis of small RNAs and RNAs degradome tags in sweet orange fruits have revealed around 130 miRNA and more that 225 target genes. Only 15% of the miRNA are expressed in fruits in a ripening-related pattern. Some miRNAs and targets genes are homologous to those found in other plants, such as csi-miR156k, csi-miR159, csi-miR166d, csi-miRN21, *GAMYBs*, *SPLs* and *ATHBs*, and in general are related to hormone signaling (ethylene, ABA or auxins) and other diverse metabolic processes (Wu et al. 2016).

A comparative transcriptional study of the changes during ripening in fruit of three mandarins with different rate of fruit degreening and harvest time (Arrufatina, early-harvested, Clementine de Nules, middle season and Hernandina, late-harvesting) identified more than 5,300 genes differentially expressed among these genotypes (Terol et al. 2019b). Among these differentially expressed genes, 600 corresponded to TFs and after a more restrictive analysis, 40 potentially implicate in fruit ripening were identified. Most abundant families are C2H2, AP2-EREBP, MYB-HB-like, WD40-like, PHD, MADS-MIKC, bHLH and NAM/NAC families. Many of these TFs showed sequence homology with genes from other plants, like tomato, involved in several ripening-related processes (ethylene synthesis and signalling acid metabolism, sugar content, etc.). These results are in well agreement with those obtained in fruits of oranges, indicating the conservation of metabolic pathways in the ripening processes of fruits in different *Citrus* species (Wu et al. 2016). It is relevant the number of TFs identified with sequence similarity to factors acting as repressor of diverse metabolic steps, as *ERF3b*, which has been proposed to repress ethylene functions, or *SlZFP2* (a C2H2 zinc-finger protein) and *SlAP2a* which are negative regulators of ABA or fruit ripening in tomato, respectively (Terol et al. 2019b). The most outstanding contribution of this study is the identification of a MAD box (Ciclev10021357), homologous to tomato *SlMADS1*, that showed a lower expression level in the early cultivar and higher in the late cultivar. The tomato *SlMADS1* was demonstrated to affect the ripening time (Dong et al. 2013). Then, mandarin MADS1 may function as a repressor of ripening in *Citrus* fruits, potentially modulating ethylene synthesis. Interestingly, MADS1 was also located in the region of the genome that was deleted in the early-harvesting Nero mandarin, reinforcing its role in precocity of mandarin fruits (Terol et al. 2015).

The involvement of other TFs in several ripening-related events was also established. In particular, three different regulatory elements participate in the regulation of carotenoid biosynthesis. *CubHLH1* is a member of the helix-loop-helix (HLH) family of TFs that is expressed during ripening of Satsuma mandarin, stimulated by ethylene and repressed by gibberellins. This gene is homologous to the Arabidopsis ATBS1 which is s negative regulator of

brassinolide signalling. Overexpression of this factor in tomato produces dwarf plants and increase lycopene content in mature fruits, suggesting its involvement in carotenoid and ABA metabolism in Citrus fruit (Endo et al. 2015). Other TFs showed a direct binding activity with genes of carotenoid biosynthesis. The *CrMYB68* is a MYB transcription factor that by different assays has been demonstrated to interact with the *CrBCH2* and *CrNCED5*, acting as a negative regulator. It was suggested then that this factor may control the α- and β-branch of the carotenoid biosynthetic pathway and also indirectly through modification of the ABA (Zhu et al. 2016). As mentioned previously, MADS proteins are one of the largest TF families in plants, and several members are implicated in the regulation of several processes of tomato fruit ripening (Lu et al. 2018). A MADS element isolated from orange (*CsMADS6*) has been shown to be expressed co-ordinately with *PSY, PDS, LCYb1* and *CCD*. Yeast one-hybrid assay and ectopic expression in tomato reveals the binding to the promotor of these carotenogenic genes and its function as a positive regulator. This factor may also interact with other TFs, then acting at multiple steps in the regulation of carotenoid biosynthesis in Citrus fruits (Lu et al. 2018).

DNA methylation is a main epigenetic mark that is associated with genome stability, inactive transcription, developmental regulation and environmental responses (Law and Jacobsen 2010). In tomato fruit, general loss of DNA methylation by an active DNA demethylation has been reported during the ripening process. In a recent study, the single-base resolution DNA methylomes were investigated in sweet orange fruits, in order to understand the relevance of this regulatory process during citrus fruit ripening (Huang et al. 2019). A comparison of DNA methylomes between immature *versus* ripe orange fruits showed that gained DNA methylation at over 30,000 genomic regions and lost DNA methylation at about 1,000 genomic regions, indicating a significant enhancement of DNA methylation during orange fruit ripening. This increase in DNA methylation was correlated with decreased expression of DNA demethylase genes. Moreover, the use of a DNA methylation inhibitor delayed fruit ripening, strongly suggesting the relevant role of DNA hypermethylation in ripening of sweet orange. Interestingly, the DNA hypermethylation was associated with the repression of several hundred genes, such as photosynthesis genes, and also with the activation of hundreds of genes, including genes involved in ABA responses. Other independent work also analysed the role of methylation-related genes during development and ripening in sweet orange fruits, and characterized three types of DNA methyltransferase (*CsMET1, CsCMT3* and *CsDRM1*), a chromatin-remodeling gene (*CsDDM1*) and a demethylation gene (*CsDME1*). The expression of *CsMET1*, *CsCMT1*, *CsCMT2*, *CsDRM1* and *CsDME*s were higher in the peel than in the flesh during fruit ripening and it was concluded that the changes in the global DNA methylation level could not be explained by the expression of a single gene (Xu et al. 2014). These works point out that dynamic change in DNA methylation is a complex and a key process regulating citrus fruit ripening and deserves further investigations.

Although the understanding of the genetic control of *Citrus* ripening is still limited, much progress has been done in recent years and the application of massive transcriptomic strategies, the availability of genome sequences in combination with the diversity of mutants and varieties with distinctive alterations in ripening-related processes, is opening new challenges to decipher this process and to improve the postharvest performance and the organoleptic, nutritional and health-related properties of *Citrus* fruits.

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Pigments in Citrus Fruit: Mutants, Compounds, Genes, and Beyond

11

Chunxian Chen

Abstract

Pigments in citrus fruit include chlorophylls, carotenoids, and flavonoids. While chlorophylls color green citrus fruit, carotenoids and flavonoids are synthesized predominantly in citrus fruit after the color breaker stage. Various carotenoids are responsible for a spectrum of characteristic red, orange, and yellow colors for mature citrus fruit. Lycopene is the carotenoid producing pink to red in some sweet orange and grapefruit mutants while other carotenes and xanthophylls contribute to commonly seen orange to yellow colors. Anthocyanins, a subgroup of colored flavonoids, yield additional blood red colors only in blood oranges. Citrus fruit mutants rich in red lycopene or anthocyanins that generate additional organoleptic attraction and marketing appeal have been extensively used in various pigment studies and comparisons. Most biosynthetic genes and some regulatory genes in the carotenoid and anthocyanin pathways have been cloned and characterized. Pigmentation and gene expression changes during citrus fruit postharvest degreening and storage were also gradually uncovered. Genetic knowledge of citrus fruit pigmentation facilitates continuing research on and variety improvement for beneficial pigment compounds. Dietetics of some citrus pigments has been studied from both nutritional and preventive medicinal perspectives, with emphasis on lycopene and anthocyanins due to the predominant content in popular red-fleshed cultivars, special marketing appeal, and potential health benefits. Nutraceutical and medicinal benefits of consumption of citrus fruit (juice) include substantially improved health biomarkers for lipid profiles, low risk of obesity and cardiovascular disease, and potential suppression of some cancers. Regular moderate consumption of fresh citrus fruit and/or orange juice should be encouraged as part of a daily healthy diet.

11.1 Introduction

Citrus is one of the most valued and widely planted fruit commodities in the world. As one popular and healthy diet component, citrus fruit and juice are rich in pigments, vitamins, micronutrients, and other beneficial compounds. The color of citrus fruit flesh and peel is an extremely important characteristic that helps distinguish fruit types and impacts market values. Main citrus fruit types include sweet orange (*Citrus sinensis*), mandarin (*C. reticulata*), pummelo (*C. maxima*), grapefruit (*C. paradisi*), lemon (*C. limon*), lime (*C. aurantifolia*), citron (*C. medica*) and other assorted

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hybrids. Each type includes cultivars in subgroups that share similarities in certain fruit characteristics and/or domestication origins (Gmitter et al. 2012). Spontaneous/induced mutation and natural/artificial sexual introgression are the main avenues to diversification of these subgroups. For example, sweet oranges may be conveniently divided into six subgroups: common sweet oranges (sometimes called blonde orange when compared to blood orange or red orange), Valencia oranges (ripening in next spring to summer, sometimes called summer orange), acidless oranges (little acidity, sometimes called candy orange), navel oranges (containing an internal small fruit like a navel), red oranges (pigmented by lycopene), and blood oranges (pigmented by anthocyanins). The latter five subgroups are spontaneous mutants of the first one (common sweet orange) that ripens in the fall, has no navel, and retains typical acidity and orange pigmentation (Fig. 11.1). Mandarins include common pure mandarins (e.g., Nanfengmiju, an ancient non-hybrid mandarin originated in China), Satsumas (*C. unshiu*, an ancient mandarin and pummelo introgression originated in China), Clementine (*C. clementina*, a spontaneous Willowleaf mandarin and sweet orange hybrid originated in Mediterranean), tangerines (orange- to reddish-skinned mandarin hybrids marketed in the United States), tangelos (hybrids between tangerine and grapefruit or pummelo), and other hybrid subgroups, each of which has a distinct natural or artificial origin.

Orange is the typical color in the flesh and peel of fully mature citrus fruit of most types, but a wide spectrum of red, pink, orange, yellow, greenish, and whitish color is not rare, and more often seen in flesh than in peel (Chen et al. 2015). These colors are determined by the composition and concentration of various pigments, including chlorophylls, carotenoids, and flavonoids (Lee and Castle 2001; Nogata et al. 2006; Matsumoto et al. 2007; Melendez-Martinez et al. 2007; Dhuique-Mayer et al. 2009; Chen 2015a; Chen et al. 2015). Chlorophylls, primarily capturing light to fuel photosynthesis in green leaves, also produce the green colors of immature citrus fruit and the peel of ripening citrus fruit prior to the color breaker stage. Once that stage starts and peel coloration begins in citrus fruit, carotenoids are massively synthesized while chlorophylls are gradually degraded (Kato et al. 2004). Carotenoids are the primary pigments that generate a range of characteristic red, orange, and yellow colors for fully mature citrus fruit (Pupin et al. 1999; Lee 2001; Matsumoto et al. 2007; Melendez-Martinez et al. 2007; Chen et al. 2015). Lycopene, predominantly present in red-fleshed citrus cultivars, is the carotenoid displaying a spectrum of color from pink to red in the flesh of citrus fruit. Most other xanthophyll and carotene carotenoids produce orange to yellow colors in the flesh and peel of citrus fruit. Anthocyanins, one group of colored flavonoids, are the blood-colored pigments only cold-induced and synthesized in fully ripe blood orange cultivars (Chandler 1958; Mondello et al. 2000; Lee 2002; Hillebrand et al. 2004; Lo Piero et al. 2005; Butelli et al. 2012).

This chapter summarizes research advances in pigments in citrus fruit, with much coverage of pigmented mutants, pigment compounds, biosynthesis genes, postharvest alterations, and potential health benefits, and considerable emphasis on red-colored lycopene and anthocyanins. It is worth mentioning there are reviews on citrus fruit pigments with different coverages and emphases, such as carotenoid accumulation mechanism (Kato 2012) and biosynthesis genomics and dietetics (Chen et al. 2015). Additional reviews and books also covered broad aspects of carotenoids, flavonoids, and other pigments, to list a few (Gross 1987; Cunningham and Gantt 1998; Winkel-Shirley 2001; Koes et al. 2005; Cazzonelli 2011; Walter and Strack 2011; Bowen et al. 2015; Chen 2015b; Parihar et al. 2015).

11.2 Pigmented Mutants

Flesh color is a trait mostly used for selection of pigmented mutants and cultivars. Figure 11.1 showed a few sweet orange mutants with accumulated lycopene or anthocyanins. Figure 11.2 illustrated the historical path of some important

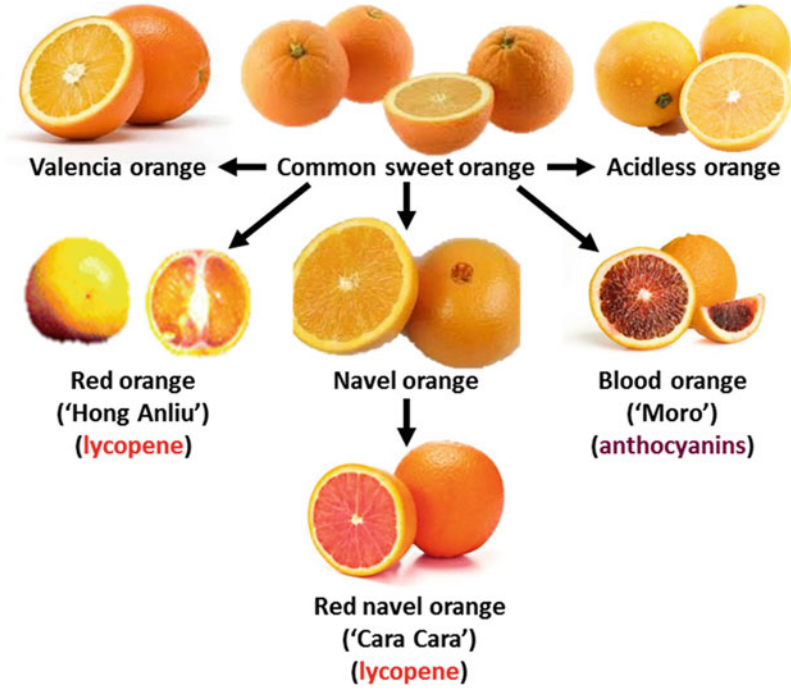

Fig. 11.1 Common sweet orange (*Citrus sinensis*) and five mutant subgroups with examples of red-pigmented cultivars. Common sweet oranges ripen in the fall, have no navel, and retain typical acidity and orange colors. Each mutant subgroup has at least one distinct fruit characteristic: Valencia orange (aka. summer orange) ripening in next spring to summer; acidless orange having little acidity; navel orange containing an aborted second tiny orange (resembling a human navel) at the opposite end from the stem; red orange producing lycopene for the red flesh (e.g., Hong Anliu, a bud mutant from Anliu sweet orange; and Cara Cara, a bud mutant from Washington navel orange); and blood orange producing anthocyanins for the additional blood-colored flesh (e.g., Moro, a bud mutant of Sanguinello Moscato sweet orange)

Floridian and Texan grapefruit selections (most were mutants) that were well documented (da Graça et al. 2004), in which lycopene pigmentation was gradually augmented and seed numbers were reduced to zero in the last few deeply-colored cultivars, such as Flame, Rio Red, and Star Ruby. Those red-pigmented cultivars have been extensively used for citrus fruit pigment quantification and studies, such as Cara Cara navel orange (Lee 2001; Tao et al. 2005, 2007, 2012; Alquezar et al. 2008; Wang et al. 2011), Hong Anliu sweet orange (Liu et al. 2007; Pan et al. 2009; Yu et al. 2012), Flame and Rio Red grapefruit (Vanamala et al. 2005; Lester et al. 2007; Mendes et al. 2011; Costa et al. 2012; Chaudhary et al. 2017), and Moro blood oranges (Mondello et al. 2000; Rapisarda et al. 2003; Bernardi et al. 2010; Crifò et al. 2011; Butelli et al. 2012), to name a few (Table 11.1). Through comparison with their respective progenitors (wild type), these pigment-rich citrus mutants are advantageous to reveal differences in pigment composites, biosynthetic mechanisms, gene expression patterns, genomic structures, transcriptomes, proteomes, postharvest degradation process, health benefits, and other subjects of interest (Tao et al. 2005; Liu et al. 2007; Alquezar et al. 2008; Pan et al. 2009; Xu et al. 2009; Butelli et al. 2012; Costa et al. 2012; Yu et al. 2012; Wei et al. 2014a, 2018). In addition, these deeply colored cultivars also appear to have better market appeal and value largely due to the visual attraction and potentially extra dietetic/medicinal benefits of the rich pigments.

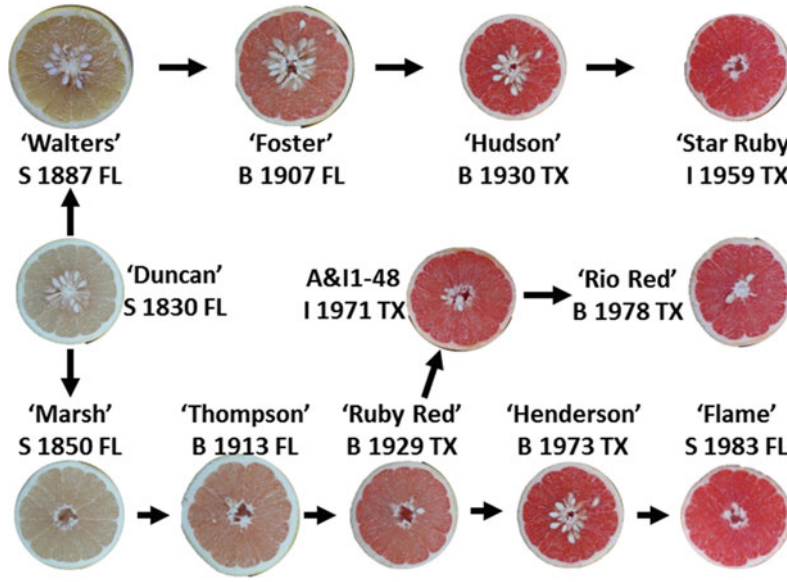

Fig. 11.2 Pigmentation change of main grapefruit (*C. paradisi*) mutant cultivars selected in different year in Florida (FL) and Texas (TX). The flesh colors changed from white to dark red and seed numbers from many to (nearly) zero through chance seedlings (S), bud sports (B), irradiated budwood or seed (I) (da Graça et al. 2004)

Table 11.1 Example of extensively studied red-fleshed citrus mutants/cultivars

Cultivars	Fruit types	Red pigments	Examples of references
Cara Cara	Navel orange	Lycopene	Carotenoid detection (Lee 2001); gene expression (Tao et al. 2007)
Hong Anliu	Sweet orange	Lycopene	Proteome comparison (Pan et al. 2009); transcriptome comparison (Yu et al. 2012)
Flame	Grapefruit	Lycopene	Gene expression (Mendes et al. 2011; Costa et al. 2012)
Rio Red	Grapefruit	Lycopene	Bioactive compounds (Vanamala et al. 2005; Lester et al. 2007)
Budd Blood	Blood orange	Anthocyanins	Anthocyanin detection (Lee and Castle 2001; Lee 2002)
Moro, Tarocco	Blood orange	Anthocyanins	Gene expression (Crifò et al. 2011); genomics mechanism (Butelli et al. 2012)

11.3 Pigment Compounds

Composition of citrus fruit pigments are determined by genotypes and affected by environmental factors (Gross 1987; Fanciullino et al. 2006; Nogata et al. 2006; Dhuique-Mayer et al. 2009; Chen et al. 2015). Carotenoids are the richest pigments in citrus fruit, with more than 115 carotenoid compounds that result in diverse characteristic colors (Pupin et al. 1999; Lee and Castle 2001; Kato et al. 2004; Fanciullino et al. 2006; Xu et al. 2006; Matsumoto et al. 2007; Melendez-Martinez et al. 2007; Dhuique-Mayer et al. 2009; Matsumoto et al. 2009). Carotenoid concentration, composition, and changing pattern during maturation vary greatly among citrus fruit types (Fig. 11.3) (Kato et al. 2004; Xu et al. 2006; Kato 2012) and cultivars within the same types (Table 11.2) (Pupin et al. 1999; Lee and Castle

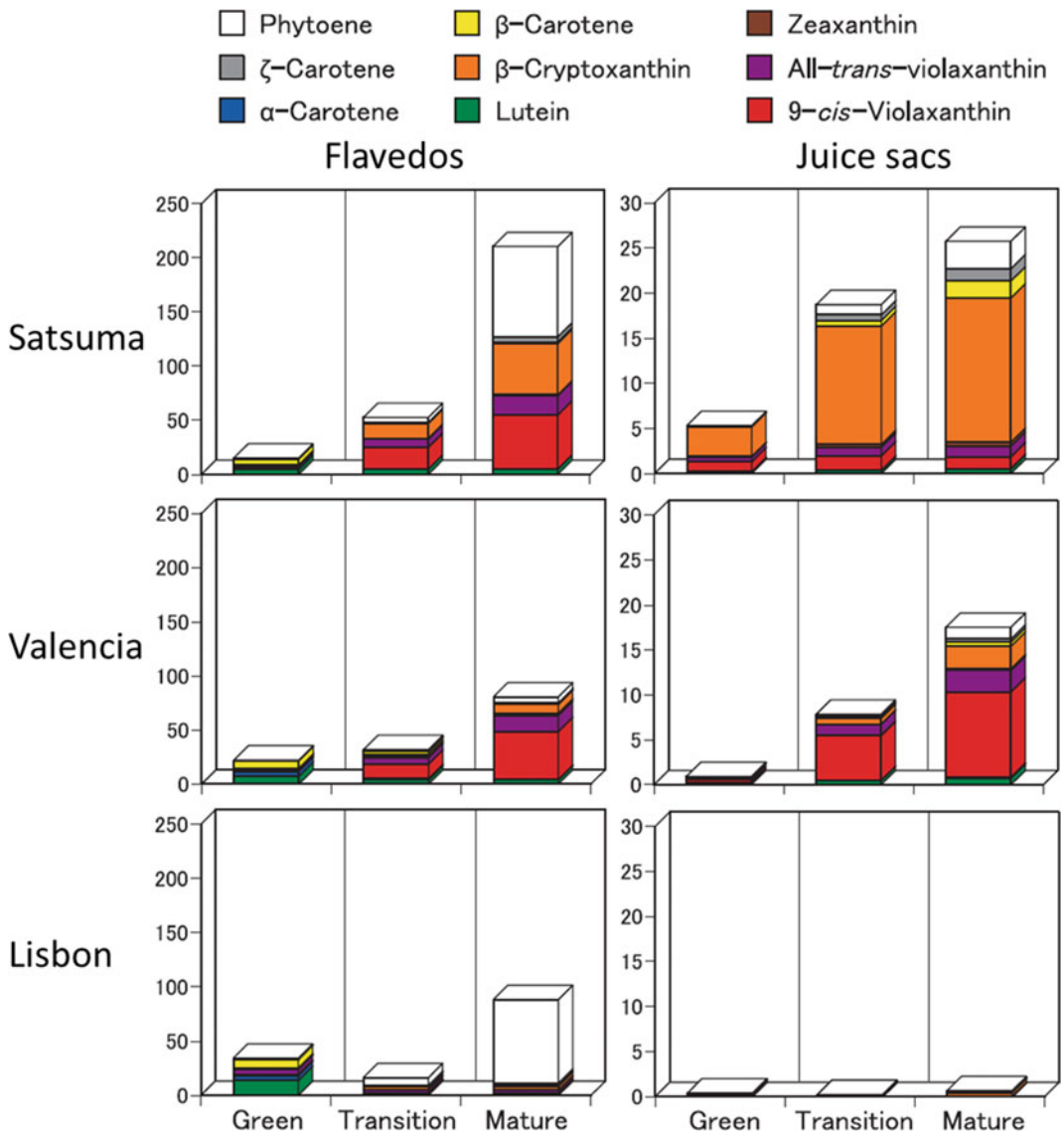

Fig. 11.3 Concentrations of nine carotenoids (milligram per gram fresh weight, μg/g) in flavedos and juice sacs of Satsuma mandarin (*C. unshiu*), Valencia orange (*C. sinensis*), and Lisbon lemon (*C. limon*) dramatically changed at the green, transition, and mature stages (Kato et al. 2004; Kato 2012)

2001; Wei et al. 2014a). For example, comparison of the same three sweet orange and two mandarin cultivars planted in three different geographic areas revealed variability in carotenoid content among the three areas, but close correlations were observed between β-cryptoxanthin and phytoene, and β-carotene and phytoene (Dhuique-Mayer et al. 2009). Such variability in carotenoid compositions was more interspecific than intraspecific, according to two independent reports, one using 24 citrus genotypes (Fanciullino et al. 2006) and the other using 42 citrus genotypes (Nogata et al. 2006). Among twenty-two Spanish orange genotypes measured in two seasons, most predominantly had xanthophylls (82.7–93.0%), except that Cara Cara had a higher proportion of carotenes (mainly lycopene) and Rohde Late and Ambersweet had β-cryptoxanthin (Stinco et al. 2016).

Table 11.2 Concentrations of five carotenoids (milligram per liter juice, mg/L) in hand-squeezed orange juice from seven cultivars (Pupin et al. 1999)

Cultivar	Sample no.	Lutein	Zeaxanthin	β-Cryptoxanthin	α-Carotene	β-Carotene	Total
Pera Rio	5	0.14–0.23	0.30–0.53	0.10–0.19	0.04–0.18	0.05–0.08	0.63–1.21
Natal	4	0.04–0.06	0.06–0.12	0.01–0.03	0.03–0.04	0.02–0.03	0.18–0.28
Valencia	3	0.06–0.07	0.04–0.10	0.02	0.03–0.07	0.02–0.05	0.17–0.31
Hamlin	3	0.03–0.08	0.06–0.27	0.02–0.06	ND–0.02	ND–0.02	0.11–0.45
Baia	1	0.05	0.04	0.08	ND	0.02	0.19
Lima	1	0.08	0.10	0.01	0.02	ND	0.21
Pera Coroa	1	0.08	0.15	0.05	0.05	0.04	0.37

ND = not detected

The predominant carotenoid in the Satsuma mandarin and Valencia orange juice sacs was β-cryptoxanthin and violaxanthin isomers, respectively, whereas the level of carotenoids was very low in Lisbon lemon juice sacs (Kato 2012). During the September to mid-January fruit maturation and color development period, the carotenoid concentration increased up to 4.9 times in Earlygold orange juice, compared to 3.9 times in Hamlin and 4.5 times in Budd Blood orange juices in the same period. Lutein and violaxanthin became the predominant pigments in Hamlin fruit at the early coloration stage, and β-cryptoxanthin became a major pigment in the late stage of maturation. Earlygold orange juice could reach grade A color number (i.e., 36) by late October to mid-November whereas Hamlin and Budd Blood juice did not reach the grade until January (Lee and Castle 2001).

Red-fleshed Cara Cara juice contained about 3.9 ppm of lycopene whereas ordinary navel orange juice had none. The red navel also had more β-carotene than its counterpart (Lee 2001). Another pleiotropic sweet orange mutant, Hong Anliu, also showed new fruit characteristics, such as substantial lycopene accumulation, high sucrose content, and low citric acid. Lycopene was primarily found in fruit albedo, segment membranes, and juice sacs, up to 1000-fold higher than that in these parts of its wild-type progenitor. The change of lycopene and other carotenoids was accompanied with the dramatic alteration of biosynthetic gene expression in albedo, segment membranes, and juice sacs but might be regulated differently in the three tissues. The differences suggested that lycopene might be synthesized in the juice sacs but transported accumulatively to the albedo and segment membranes. Interestingly, this mutation did not affect the carotenoid composition of leaves (Liu et al. 2007).

In Florida-grown Budd Blood blood orange, more than seven anthocyanin pigments were separated, revealing that cyanidin-3-(6″-malonylglucoside) was the primary anthocyanin (accounting for 44.8%), followed by cyanidin-3-glucoside (33.6%) and two other minor malonated anthocyanins (Lee 2002). Similar results were gained from quantification of anthocyanins from other blood orange juices; the major anthocyanins in the juice were also cyanidin-3-glucoside and cyanidin-3-(6″-malonylglucoside), plus six other minor anthocyanins: cyanidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-sophoroside, delphinidin-3-(6″-malonylglucoside), peonidin-3-(6”-malonylglucoside), and cyanidin-3-(6″-dioxalylglucoside). Four anthocyanin-derived pyranoanthocyanins (4-vinylphenol, 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol adducts of cyanidin-3-glucoside) were identified, which were formed through a direct reaction between anthocyanins and hydroxycinnamic acids during prolonged storage of the juice (Hillebrand et al. 2004).

11.4 Biosynthesis Genes

Genes in citrus fruit carotenoid and anthocyanin biosynthesis pathways have been identified, characterized, and reviewed (Kato et al. 2004; Lo Piero et al. 2005; Cotroneo et al. 2006; Andrade-Souza et al. 2011; Crifò et al. 2011; Kato 2012; Chen et al. 2015). The cDNA sequences of most genes in the carotenoid pathway shared high nucleotide identities among Satsuma mandarin, Valencia orange and Lisbon lemon, and other plant species as well. These genes included phytoene synthase (PSY), phytoene desaturase (PDS), zeta-carotene desaturase (ZDS), carotenoid isomerase (CRTISO), lycopene β-cyclase (LCYb), β-hydroxylase (HYb), zeaxanthin epoxidase (ZEP), and lycopene ε-cyclase (LCYe). With the transition of peel color from green to orange, in the flavedos of maturing Satsuma mandarin and Valencia orange fruit the change from α-carotenoid and lutein to β-carotenoid, β-cryptoxanthin, zeaxanthin, and violaxanthin was accumulated and accompanied by disappearance of LCYe transcripts and increase of LCYb transcripts. Massive accumulation of the three xanthophylls in both flavedos and juice sacs occurred with a simultaneously increasing expression of PSY, PDS, ZDS, LCYb, HYb, and ZEP, and decreasing expression of CRTISO (Kato et al. 2004).

In comparison of three isoprenoid and nine carotenoid genes, the expression of the isoprenoid genes and of carotenoid biosynthetic genes downstream PDS was higher in the pulp of Cara Cara than that of common navel orange, but not much different between the peel of both oranges. Increased levels of isoprenoid precursors in Cara Cara could be related to the accumulation of lycopene in the mutant (Alquezar et al. 2008). Besides, the amount of lycopene was negatively correlated with the expression of LCYe, but had little correlation with LCYb in the pulp in Cara Cara, suggesting that LCYe might prominently influence lycopene cyclization and downstream carotenoid biosynthesis (Wang et al. 2011).

Multiple alleles of some carotenoid genes expressed in citrus fruit may also play an additional regulatory mechanism of carotenoid biosynthesis (Alquezar et al. 2009; Chen et al. 2010; Costa et al. 2012; Wei et al. 2014b); and some of the alleles apparently were directly involved in the accumulation of lycopene in red-fleshed grapefruit (Alquezar et al. 2009; Costa et al. 2012). Light also affected peel pigmentation of red-fleshed grapefruit. The amount of lycopene in the peel of shaded grapefruit was 49-fold higher than that in the peel of light-exposed fruit while concentrations of downstream metabolites were remarkably reduced. This increment in carotenoids in light-shaded grapefruit was not accompanied by increasing mRNA levels of carotenogenic genes; however these genes were mostly up-regulated in light-exposed grapefruit. Light signals seemingly influenced both carotenoid biosynthetic capacity and sink strength at the molecular and structural level, and thus regulated carotenoid accumulation in the grapefruit (Lado et al. 2015).

A recent study revealed an R2R3-MYB transcriptional factor (CrMYB68) might directly regulate the transformation of α- and β-branch carotenoids (Zhu et al. 2017). Besides, a MADS transcription factor, CsMADS6, was coordinately expressed with fruit development and coloration. CsMADS6, acting as a nucleus-localized transcriptional activator, apparently directly bound the promoter of LCYb1 and activated its expression. Overexpression of CsMADS6 in citrus calli induced the expression of LCYb1 and other carotenogenic genes, including PSY, PDS, and carotenoid cleavage dioxygenase1 (CCD1), and increased carotenoid contents accordingly, suggesting CsMADS6 could regulate multiple gene targets in carotenoid metabolism (Lu et al. 2018).

Extensive studies were aimed at understanding anthocyanins biosynthesis, alteration, and genomics in blood oranges (Lo Piero et al. 2005, 2006a, b; Cotroneo et al. 2006; Bernardi et al. 2010; Crifò et al. 2011, 2012; Butelli et al. 2012). Anthocyanins were synthesized and accumulated

only in the flesh and rind of blood oranges, not in those of common (blonde) sweet oranges. Varied levels of anthocyanins were accumulated when blood orange fruit started ripening (Cotroneo et al. 2006; Rapisarda et al. 2008), but were affected by environmental conditions, particularly by temperatures during fruit coloration (Lo Piero et al. 2005; Crifò et al. 2011, 2012). Low temperatures could substantially enhance related gene expression and anthocyanins accumulation in blood oranges (Lo Piero et al. 2005; Crifò et al. 2011). Oroval Clementine and Moro orange and their pigmented hybrid (Omo-31) were analytically compared, indicating that juice yield, total soluble solids (TSS), total acidity (TA), and TSS/TA ratio of the hybrid were similar to those of Moro. At maturity stage the amount of anthocyanins, flavanones, and hydroxycinnamic acids in Omo-31 was found to be notably higher than those of the two parents, but vitamin had no differences among the three genotypes (Rapisarda et al. 2003).

Genes encoding chalcone synthase (CHS), anthocyanidin synthase (ANS), UDP-glucose-flavonoid 3-*O*-glucosyltransferase (UFGT), and others in the anthocyanin biosynthetic pathway in blood orange fruit, were characterized, in comparison with common sweet orange fruit. Results showed that the expression of CHS, ANS, and UFGT was up- and co-regulated in the blood oranges, but down-regulated in the common sweet oranges, suggesting that the absence of pigment in the common sweet orange cultivars was likely caused by lack of inducible or structurally functional genes (Lo Piero et al. 2005; Cotroneo et al. 2006).

Genomic analysis revealed blood oranges of Sicilian origin were mutated by insertion of a *Copia*-like retrotransposon adjacent to a gene encoding a MYB transcriptional activator of anthocyanin production. Expression of the gene was controlled by the retrotransposon and cold dependency was due to induction of the retrotransposon by stress. Intriguingly a blood orange of Chinese origin resulted from an independent insertion of a similar retrotransposon (Butelli et al. 2012). Dihydroquercetin and dihydromyricetin, two dihydroflavonols, are synthesized from dihydrokaempferol by hydroxylation reactions catalyzed by flavonoid 3′-hydroxylase (F3H) and flavonoid 3′,5′-hydroxylase, respectively (Doostdar et al. 1995). The gene encoding dihydroflavonol 4-reductase (DFR) was involved in the biosynthesis of anthocyanins. There are no differences in DFR coding and promoter regions between blood oranges and common sweet oranges, but DFR transcripts are normally detected only in blood oranges. Therefore, its expression should be under strict control of transcriptional factors (Lo Piero et al. 2006b), which included a homologous binding site for the transcriptional activation of DFR (Grotewold et al. 1994). Although acylated anthocyanins can account for more than 40% of the total anthocyanin content in some blood oranges, until now there has been no report about the exact genes required in blood orange anthocyanin acylation.

11.5 Postharvest Altercations

Postharvest changes of citrus fruit pigments were mostly studied during artificial degreening with ethylene and/or under different storage temperatures (Lo Piero et al. 2005; Rodrigo and Zacarias 2007; Matsumoto et al. 2009; Zhou et al. 2010; Crifò et al. 2011; Tao et al. 2012; Chaudhary et al. 2017). Similar to endogenous ethylene-induced fruit maturation and coloration with increasing carotenoid content in the flavedo of citrus fruit, exogenous ethylene treatment showed strong effects on the changes of carotenoid content and composition, and of carotenoid biosynthetic gene expression in the flavedo of Navelate navel orange. Ethylene stimulated an increase of phytoene, phytofluene, violaxanthin, and β-citraurin (an apocarotenoid), and a reduction of chloroplastic carotenoids. The changes were accompanied by up-regulation of PSY, ZDS, β-carotene hydroxylase (BCH), PDS, plastid terminal oxidase (PTO), LCYb and ZEP, and down-regulation of LCYe. In contrast, gibberellic acid delayed fruit degreening and reduced the ethylene-induced early carotenoid biosynthetic gene expression and the accumulation of phytoene, phytofluene and β-citraurin (Rodrigo and Zacarias 2007).

Comparing ethylene-degreened Star Ruby grapefruit to untreated controls after 35-day storage, β-carotene, lycopene, limonin, neohesperidin, didymin, 6,7-dihydroxybergamottin, 5-geranyloxy-7-methoxycoumarin, ascorbic acid, TSS, decay, fruit softening, taste, odor, and radical scavenging activity had little change; however, nomilin was significantly higher, flavonoids such as narirutin, naringin, and poncirin were significantly lower, and deacetyl nomilinic acid glucoside and bergamottin were somewhat reduced. The results suggested postharvest degreening with ethylene, while pigmenting the fruit, had minimal effect on nutritional quality (Chaudhary et al. 2012). Likewise, postharvest degreening of Ponkan fruit through ethylene fumigation or ethephon dipping pigmented the fruit with decreasing chlorophyll content and increasing total carotenoid content, during which orange-colored β-carotene and β-cryptoxanthin were accumulated whereas yellow-colored lutein, violaxanthin and 9-*cis*-violaxanthin were reduced. The changes in abundance of these carotenoids might be due to inhibited BCH expression in both treatments (Zhou et al. 2010).

Temperatures of postharvest storage and/or ethylene treatment also had complicated effects on carotenoid content and composition and biosynthesis gene expression, according to multiple studies on storage temperatures and ethylene treatment temperatures (Matsumoto et al. 2009; Carmona et al. 2012; Tao et al. 2012; Chaudhary et al. 2017). In general, the temperatures in this study had different effects on flavedo and juice sacs of Satsuma mandarin fruit; it appears to enhance a more rapid increase of carotenoid content and color change in the flavedo at 20 °C than at 5 °C (Matsumoto et al. 2009). After 12 weeks of storage, grapefruits showed no significant differences in lycopene, narirutin, poncirin, furocoumarins, and radical scavenging activity in the three low temperature-related treatments. Low temperature storage could help fruits to retain most compounds and fruit quality (Chaudhary et al. 2017). Likewise, the comparison of two storage temperatures on postharvest Cara Cara navel orange fruit drew a similar conclusion; storage temperature affected the carotenoid accumulation and gene expression in a tissue-dependent manner and the carotenoid biosynthesis was transcriptionally regulated (Tao et al. 2012). Navelina navel orange fruit were compared between two harvests (before and after the color breaker stage), and two storage temperatures (2 and 12 °C). Total carotenoid content and coloration in both flavedo and pulp of the fruit considerably increased at 12 °C, but remained almost unchanged at 2 °C, which were consistent with the increasing expression of PSY, PDS, ZDS, LCYb1, LCYb2, and CHX (β-carotene hydroxylase) at 12 °C and little change or slight decline at 2 °C. The increased carotenoids in peel color at 12 °C were mainly reddish apocarotenoid, citraurin, and slightly antheraxanthin; and those in the pulp were cryptoxanthin and xanthophyll, up to two and three times in the fruit harvested before and after the color breaker, respectively (Carmona et al. 2012).

Red grapefruit juice concentrates in plastic containers and metal cans during storage at −23 °C for 12 months could lose color, due to significant changes in major β-carotene and lycopene. In plastic containers, more than 20% of lycopene and about 7% of β-carotene were lost after the storage period. In metal cans, both pigment losses were slightly smaller. It was estimated that the shelf life for lycopene was 18 months in plastic and 26.1 months in metal; and that for β-carotene was more than doubled in both (Lee and Coates 2002). Irradiation also affected grapefruit bioactive compounds, including flavonoids (naringin and narirutin), lycopene, β-carotene, limonene, and myrcene (Vanamala et al. 2005). In comparison to organic grapefruit, conventional grapefruit apparently colored better and had higher lycopene; the juice was less tart and contained lower naringin (less bitter); and both showed higher acceptance by a consumer panel. But organic grapefruit had a commercially preferred thinner peel and the juice contained higher ascorbic acid and sugars and lower nitrate and furanocoumarins (Lester et al. 2007).

Moderately long storage of blood orange fruit at 4 °C caused the accumulation of anthocyanins and the expression changes of biosynthesis

genes, including phehylalanine ammonia lyase (PAL), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP-glucose flavonoid glucosyl transferase (UFGT). Anthocyanins in the juice vesicles of blood orange stored at 4 °C for 75 days were induced up to eight times higher than those stored at 25 °C; and correspondingly the expression of PAL, CHS, DFR, and UFGT was induced up to at least 40-fold more. The amount of anthocyanins in blood oranges stored at 4 °C for 45 days and then kept at 25 °C decreased concordantly with declining expression of CHS, DFR, and UFGT but remained significantly higher than that in the control kept at 25 °C. However, PAL transcripts almost disappeared immediately after the temperature change from 4 to 25 °C, implying biosynthesis of anthocyanins in blood orange might be regulated in different steps in the pathway (Lo Piero et al. 2005). Similar results were also observed in two other experiments. Anthocyanins and other polyphenols increased during cold storage of the fruit of Tacle and Clara, two triploid hybrids of Monreal Clementine x Tarocco blood orange, suggesting low-temperature storage enhanced some health compounds in Tacle and Clara fruit juice (Rapisarda et al. 2008). The amount of anthocyanins in blood orange fruit exposed to cold sharply increased after 6 days of storage, up to eight times higher than the measure on fruit before cold storage; the increase was accompanied by dramatic transcript increases of all quantified genes (CM1, PAL, CHS, DFR, ANS, UFGT, and GST). This work further confirmed that biosynthesis of anthocyanins in blood oranges is a cold-induced pathway. In addition, PAL, DFR, and UFGT were not responsive to cold in common sweet oranges, and an EST, encoding the transcription factor NAC domain protein and inducible by cold only in blood oranges might be a cold-responsive candidate gene that was able to trigger the biosynthesis of anthocyanins in blood oranges (Crifò et al. 2012).

11.6 Potential Health Benefits

The health benefits associated with the consumption of pigment-rich food, particularly of carotenoids and flavonoids, have been documented in reviews (Middleton et al. 2000; Jackson et al. 2008; Bowen et al. 2015; Parihar et al. 2015). In general, frequent consumption of pigment-rich fruits and vegetables, likely due to their antioxidant properties, is associated with a substantially lower risk of some cancers, cardiovascular diseases, and cell inflammations. Carotenoids reportedly could inhibit induced mutagenesis, reduce various neoplastic events, and enhance protection of cell immune systems for improved response to certain cancers (Bendich 1989; Garewal et al. 1993; Paiva and Russell 1999; Sharoni et al. 2012; Stinco et al. 2016). However, some benefits may be anecdotal or inconclusive. Epidemiologic studies have shown an inverse relationship between presence of various cancers and dietary carotenoids or blood carotenoid levels. It appears that carotenoids (including β-carotene) can promote health when taken at dietary levels, but may have adverse effects when taken in high dose by subjects who smoke or who have been exposed to asbestos. It will be the task of ongoing and future studies to define the populations that can benefit from carotenoids and to define the proper doses, lengths of treatment, and whether mixtures, rather than single carotenoids (e.g., β-carotene) are more advantageous (Paiva and Russell 1999; Stinco et al. 2016).

In addition, some carotenoids high in citrus fruit are essential precursors of vitamin A (also known as provitamin A carotenoids) in the human and animal diet, including α-carotene, β-carotene, and β-cryptoxanthin (Stewart 1977; Sanchez-Moreno et al. 2003). These provitamin A carotenoids can be metabolized intracellularly into vitamin A and act its many essential biological functions (Cazzonelli 2011; Walter and Strack 2011). However, others, such as lycopene,

lutein, and zeaxanthin, are not provitamin A carotenoids as they cannot be converted to vitamin A in humans and animals.

Several studies have demonstrated that orange juice consumption can promote lower levels of oxidative stress and inflammation due to the antioxidant activity of citrus flavonoids and carotenoids, particularly of anthocyanins and lycopene (Dourado et al. 2015; Silveira et al. 2015; Balestra et al. 2016). In one experiment, clinical and biochemical assessments were performed at baseline and on the final day after volunteers consumed red-fleshed sweet orange juice daily for 8 weeks. No change was noticed in the abdominal obesity, but low-density lipoprotein cholesterol and C-reactive protein decreased with increasing antioxidant activity in serum after red orange juice consumption. In addition, insulin resistance and systolic blood pressure were reduced in normal-weight volunteers while diastolic blood pressure decreased in overweight volunteers. In this study, lycopene-rich orange juice also showed anti-inflammatory, antioxidant, and lipid-lowering properties that may prevent the development of metabolic syndrome (Silveira et al. 2015). Unlike other abundant carotenoids, such as α-carotene, β-carotene, lycopene, lutein, and zeaxanthin, β-cryptoxanthin is a xanthophyll found only in several fruits, including citrus, persimmon, and hot chili pepper. In order to determine whether β-cryptoxanthin had a protective effect on renal glomeruli, kidney inflammation and acute nephritis were induced in mice. In the β-cryptoxanthin-ingested mice, the pathological situations were decreased, as was migration of urinal proteins and inflammatory cells of the renal glomeruli, which were also correlated with the blood concentration of β-cryptoxanthin. The results suggested that acute nephritis was greatly improved by the ingestion of β-cryptoxanthin, which could be a new research subject for pharmacological therapy on human diseases (Hikita et al. 2016).

In comparison of the chemopreventive effect of common sweet orange juice, red-fleshed orange juice, and hesperidin on a T acute lymphoblastic leukemia (cancer) cell line, red-fleshed orange juice promoted cytotoxicity of the cells and decreased only the G0/G1 fraction; common sweet orange juice induced cell cycle arrest in the G0/G1 phase and decreased the cell accumulation in the G2/M phase; and hesperidin did not change the cell cycle. In addition, common sweet orange juice led to apoptosis in a manner different from red-fleshed orange juice and hesperidin had no effect on apoptosis. The results suggested potential chemopreventive effects of both orange juices on the cancer cells (Dourado et al. 2015). An anthocyanins-rich extract from blood orange could modulate the vascular response in recreational divers, including their hematocrit, body water distribution, and flow-mediated dilation. The extract also significantly reduced the potentially harmful endothelial effect of a recreational single dive. The underlying mechanism appeared independent of the putative antioxidant activities of anthocyanins (Balestra et al. 2016).

Comparative studies between consumption and non-consumption of 100% orange juice have yielded some interesting results, using large set of data acquired from children (n = 7250) of 2–18 years old and adults (n = 8,861) over 19 years old who participated in the National Health and Nutrition Examination Survey 2003–2006 (O'Neil et al. 2011, 2012). Among child consumers (2183 or 26.2% of the total participants), the usual intake was ∼300 ml orange juice per day. As a result, the energy intake for consumers was significantly higher than non-consumers. However, there was no difference in weight or body mass index, or in the risk of being overweight or obese between consumers and non-consumers. Compared with non-consumers, consumers had a higher percentage of the population meeting the Estimated Average Requirement (EAR) for vitamin A, vitamin C, folate, and magnesium. The Healthy Eating Index-2005 (HEI-2005) was significantly higher in consumers than that in non-consumer. Consumers also had higher intakes of total fruit, fruit juice, and whole fruit (O'Neil et al. 2011). The results from adult consumers showed similar trends. Among adult consumers (2,310 or 23.8% of the population), the usual intake of 100% orange juice was ∼210 ml per day. Compared

to non-consumers, consumers had a higher percentage of the population meeting the EAR for vitamin A, vitamin C, folate, and magnesium. Consumers were also more likely to be above the adequate intake for potassium. HEI-2005 was significantly higher in consumers than that in non-consumers. Consumers also had higher intakes of total fruit, fruit juice, whole fruit, and whole grain; and a lower mean body mass index, lower total cholesterol levels, and lower density lipoprotein-cholesterol levels. Finally, compared to non-consumers, consumers were 21% less likely to be obese and male consumers were 36% less likely to have metabolic syndrome (O'Neil et al. 2012). The results suggest that moderate consumption of 100% orange juice should be greatly encouraged as a healthy diet component to help individuals meet daily fruit, vitamin and micronutrient intake recommendations and thus gain healthy benefits to some extent.

11.7 Conclusion

Carotenoids and flavonoids are the two main types of pigment compounds coloring mature citrus fruit. Orange is the typical color in mature citrus fruit flesh and peel, but a wide spectrum of red, pink, orange, yellow, greenish, and whitish colors is also common. Easy visualization of deeper pigmentation in citrus fruit has led to selection of many better-colored mutants, including those with accumulation of lycopene or anthocyanins. With additional organoleptic attraction and marketing appeal, these valuable mutants also have been extensively used for various pigment studies. Biosynthetic and regulatory genes in the carotenoid and anthocyanin pathways in citrus fruit, including pigment alterations during postharvest degreening and storage, have been gradually uncovered through extensive comparison of those red-fleshed mutants with their progenitors. The knowledge of citrus fruit pigment biosynthesis genes and genetics helps promote the study of citrus fruit pigments of interest and efforts to increase these beneficial compounds through variety improvement. Results also showed potential nutritional and preventive medicinal benefits of some particular pigments (e.g., lycopene, β-cryptoxanthin, and anthocyanins) or citrus fruit/juice in general, including improved health biomarkers, lower risk of obesity and cardiovascular disease, and potential suppression of certain cancers. An extensive survey on 100% orange juice consumers, in comparison with non-consumers, suggested there were substantial benefits when 100% orange juice was regularly, moderately consumed. Orange juice and citrus fruit should be encouraged as a healthy daily diet component.

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Essential Oils in Citrus

12

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Abstract

Citrus essential oils are precious natural compounds characterized by a strong odor and formed as secondary metabolites. They are widely used in many fields all over the world and they have a large economic impact. Their pleasant flavors, bioactive capacity and nutritional value make them an integral part of pharmaceutical, agricultural, cosmetic and food industries. An insight into the chemical compositions of citrus essential oils and their presence in commercial citrus species is given. The chapter presents an overview on the different fields of application of citrus essential oils. The modern frontiers of genetics and biotechnologies open new opportunities for their use in human health, agriculture and environment; potentials, future prospects and challenges are also discussed.

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12.1 Introduction

Essential oils (EOs), also known as essences, volatile oils, etheric oils, or aetheroleum, are natural products formed by several volatile compounds (Sangwan et al. 2001; Baser and Demirci 2007). In nature, they play an important role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides. According to the International Standard Organization on Essential Oils (ISO 9235: 2013) and the European Pharmacopoeia (Council of Europe 2004) an essential oil is defined as the product obtained from plant raw material by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process (for *Citrus* fruits). The term 'oil' denotes the lipophilic and viscous nature of these substances, while the term 'essential' signifies their preciousness and typical fragrance of plants. They are used all over the world and their use is constantly increasing because of the strong demand for pure natural ingredients in many fields: cosmetics, flavors, fragrances, agriculture, food and health industries (with aromatherapy and phytomedicine). Essential oils of Citrus are the most popular natural essential oils and account for the largest proportion of commercial natural flavors and fragrances: in 2012, production of orange oil was around 55,000 tons and production of lemon oil was around 9,500 tons. Depending on the plant source, Citrus essential oils are extracted from pericarp, flower, fruit juice, crushed fruits, leaf and twigs with sometimes little green fruits.

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12.2 An Historical Overview

The origin of the essential oil industry began in ancient times in the Orient, especially in Egypt, Persia and India, where the process of distillation was first employed (Guenther 1950). From the scarce and extremely vague data obtained from Herodotus (484–435 B.C.), Pliny (23–79) and his contemporary Dioscorides, appear that essential oil of which the preparation was established was turpentine and camphor oil. The Muslim civilization strongly promoted the development of the spice trade and the distillation techniques, afterwards. During the Arab domination, between the end of the first and the beginning of the second millennium, the botanist Al-Beithar reported in his 'Dictionary of the Simple Remedies' (1200) the first technical description of essential oil extraction from citron fruits. The art of distillation was diffused in Europe by the catalan physician Arnald de Villanova (1235–1311). It was the Swiss medical reformer Bombastus Paracelsus von Hohenheim (1493–1541) that named the effective component of a drug '*Quinta essentia'*, so opening the way for research in the preparation of essential oils after his time. A noticeable progress in the knowledge of the nature of essential oil was made in the 16th century when the Neapolitan scientist Giovanni Battista Della Porta (1537–1615), in his 'De Destillatione libri IX' distinguish the nature of essential oils, describes their preparation, the ways of separating the volatile oils from water and the apparatus for this purpose. In this period, the industrial exploitation started in Sicily with the extraction of essential oils from orange, lemon and bergamot fruits. In the 17th and 18th centuries, chiefly the pharmacists improved methods of distillation and made valuable investigations into the nature of essential oils. Modern investigation starts with the 19 century when, thanks to French chemists disciple of Lavoisier (J.B. Dumas, M. Barthelot and others) a systematic study of essential oil allowed to understand their chemical structure. The work of O. Wallace (1847–1931), a German chemist, is considered a milestone in investigation and thoroughly comprehension of essential oils composition.

12.3 Chemical Composition

As mentioned, an essential oil is a natural matrix produced by steam distillation or hydrodistillation; the essential oils of Citrus are the unique obtained by a mechanical procedure (Rubiolo et al. 2010; Tranchida et al. 2012; Palazzolo et al. 2013). In other words, the Citrus essential oils are necessarily (forced) by-products of the Citrus fruits juices production, since the first step of any industrial procedure of the juice production, known as cold pressing process ('sfumatrice', 'pelatrice', in line F.MC. Food Machinery Corporation) is the removal of the essential oil to avoid their mixing with juice (Arce and Soto 2008). This is because in citrus fruits the essential oils are contained in glands localized in the epicarp or *flavedo,* more precisely in the region immediately below the epidermis of the fruit. These glands are between 0.4 and 0.6 mm in diameter and have no walls, but are enclosed instead by the remains of decayed cell matter, with no excretory outlets.

Citrus essential oils are complex mixtures of more than 200 compounds (Ruberto 2002, Tranchida et al. 2012; Palazzolo et al. 2013), whose content depends on several factors: genotype and chemotype of the plant, ripeness of fruits, vegetative stage of plants, agro-climatic conditions, extractive and analytical processes (Fanciullino et al. 2005; Hosni et al. 2010; 2013; Luro et al. 2012). Rootstocks may also affect volatiles contents and concentrations (Verzera et al. 2003; Benjamin et al. 2013).

Citrus essential oils are characterized by the predominance of terpenoidic compounds (in particular monoterpene hydrocarbons); the oxygenated components (mono and sesquiterpene) are in most of the species/cultivars at very lower levels. The Fig. 12.1 shows selected molecular formulas of the main volatile components of Citrus essential oils. In all citrus varieties, limonene is the main component with a percentage ranging between 60 and 95% of the total oil.

Unfortunately, the large amount of terpene hydrocarbons in Citrus essential oils represent a problem since these compounds scarcely contribute to the Citrus oil aroma and are rather

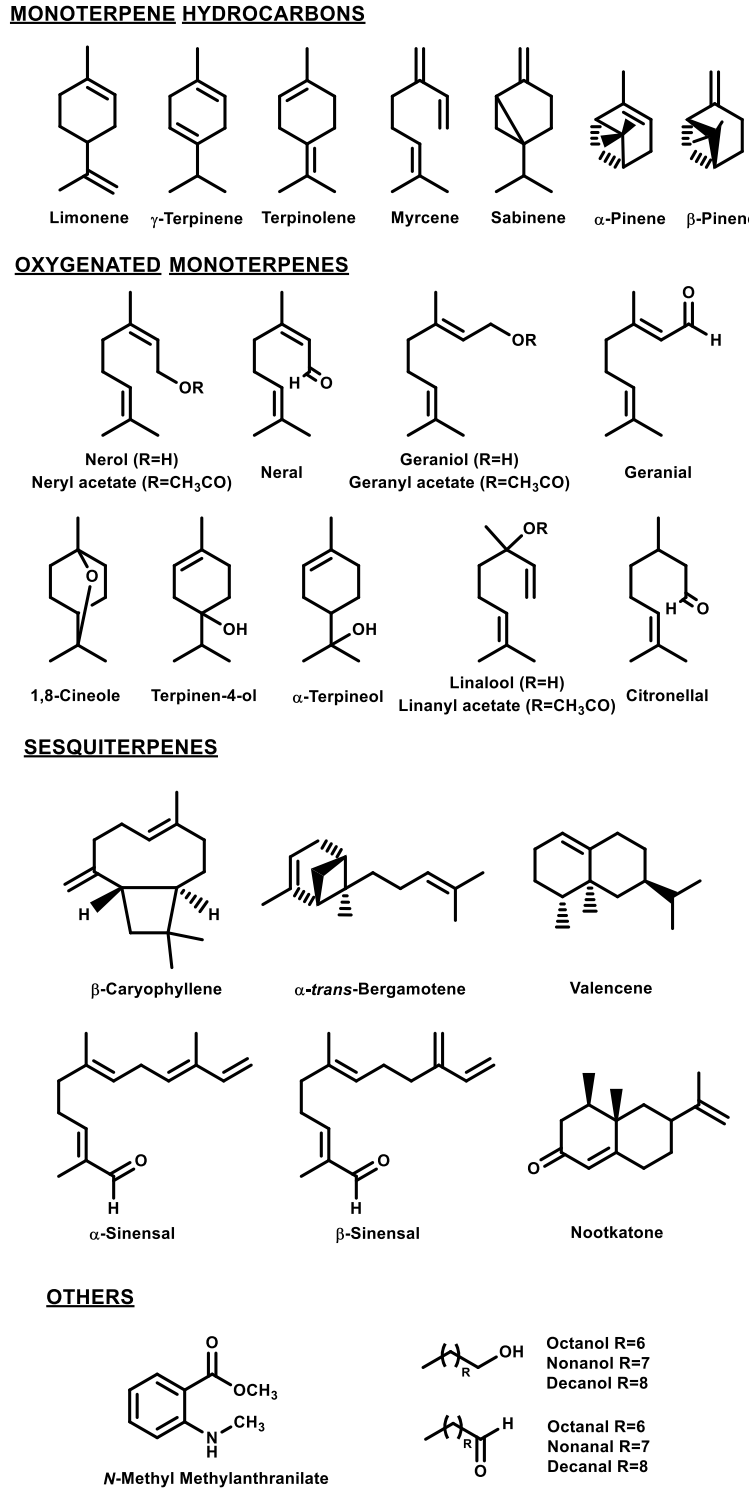

Fig. 12.1 Selection of the most representative volatile components of Citrus essential oils

unstable, being subjected to oxidation with formation of undesirable off-flavors. The removal of these components, with a procedure called 'deterpenation', produces oils with enhanced Citrus flavor, higher stability and increased solubility in water and alcohols, being of easier use in food and other employments. The deterpenation process can be carried out with a classic fractionated distillation or adopting new procedures, such as membrane separation, supercritical fluid extraction (SFE), extraction with ionic liquids (Ruberto 2002; Arce and Soto 2008).

A specific chemical feature characterizes the Citrus essential oil with respect to other oils. As consequence of the mechanical extraction, Citrus oils contain a variable amount of not volatile lipid components, which stratify together with the essential oils over the water layer used in the production process. These not volatile components are present in the external layer of the peel (flavedo): their concentration ranges from 1 to 15%, depending on the Citrus species, variety or cultivar and their composition is quite variable containing carotenoids, sterols and waxes, mixed with different groups of oxygen heterocyclic metabolites, such as polymethoxyflavones (PMF), coumarins and psoralens.

12.4 Main Citrus Essential Oils

Sweet orange (*Citrus sinensis* L. Osb) is the main and most cultivated fruit around the world. The increasing production of orange juice entails the recovering of thousands of tons of essential oils (around 75,000 tons/year, in 2013). In sweet orange essential oils are contained in fruit pericarp. All varieties/cultivars of sweet orange contain more than 90% of limonene. The large production of orange juice causes very low prices for the essential oil (it rarely reaches only 2 $/L) and the need to find new and alternative uses for it, as well as for limonene (Thomas and Bessière 1989; Ciriminna et al. 2014, 2018; Lubbe and Verpoorte 2011; Vieiria et al. 2018). The oxygenated components (the terpenoidic derivative linalool, neral, geranial and sinensal), together with some esters (neryl and geranyl acetate), and some not terpenoidic components (such as octanal and decanal aldehydes), strongly affect the sweet orange fragrance. The blood orange cvs Tarocco, Moro and Sanguinello, typical Sicilian products, have a higher content of oxygenated compounds than that of blond orange, cvs Washington navel, and Valencia. The composition of sour orange (*C. aurantium* L.) essential oil is very similar to that of sweet orange, with a slight difference due to a higher content of terpenoid esters that makes this oil particularly appreciated, mainly in the cosmetic sector.

Mandarin (*C. reticulata* Blanco) and clementine (*C. clementina* Hort. ex Tan.) represent the second group of Citrus fruits, after sweet orange. In mandarin, essential oils are synthesized from flowers, fruit pericarp, leaves and twigs with sometimes little green fruits. With respect to sweet orange oil, the oil of mandarin is characterized by a lower content of limonene (65–75% of total), followed by γ-terpinene (15–22%); in any case, the total amount of monoterpene hydrocarbons is similar to that observed for sweet orange. The oxygenated component is present at very low extent: the terpenes linalool, α-terpineol and sinensal, and the non terpene octanal and decanal, are the major compounds. However, the characteristic component of mandarin oil is the presence of an unusual nitrogen derivative, methyl-*N*-methyl anthranilate, which, notwithstanding its low amount, confers the typical mandarin fragrance. Clementine is a natural hybrid of sweet orange and mandarin and its essential oil is very similar to that of orange oil with over 95% of limonene, and very low amounts of the oxygenated components linalool, octanal and decanal.

Lemon (*C. limon* L. Burm. f) and lime (*C. aurantifolia* L.) represent the third group of Citrus fruit in order of importance. In lemon, essential oils are contained in flower, fruit pericarp, leaf and twigs with sometimes little green fruits, while in lime in pericarp, fruit juice or crushed fruits. The monoterpene hydrocarbon portion of these two species is very similar, being characterized by about 60% of limonene and 10–12% of the 2 monoterpene hydrocarbons, β-pinene and γ-terpinene. The difference between

the two species is due to the oxygenated portion: in lemon, two terpenoidic aldehydes, neral and geranial (usually defined together as citral), confer the typical lemon fragrance (other components concurring to the total aroma are the esters neryl and geranyl acetate), while in lime, the oxygenated portion is due to 1,8-cineole, terpinen-4-ol and α-terpineol. It is to underline that the lime oil is the only Citrus essential oil obtained by distillation of whole fruit.

Grapefruit (*C. paradisi* Macf.) is notified as a natural hybrid between sweet orange and pummelo (*C. maxima* (Burm.) Merr). In grapefruit, essential oils are obtained from the fruit pericarp. As well as in sweet orange, grapefruit oil is characterized by over 90% of limonene. Octanal, decanal and linalool are recorded as the major components of the aromatic portion. A specific component, the oxygenated sesquiterpene nootkatone, confers a typical aroma to grapefruit essential oils.

Bergamot (*C. bergamia* Risso) is produced in Calabria-Italy (95% of worldwide cultivation) and, to a small extent, in Ivory Coast, Guinea and Brazil. Not edible owing to its high bitterness, the fruit is cultivated mainly for the production of essential oils. The essential oil of bergamot is used mainly in cosmetic and perfumery industries (Forlot and Pevet 2012), where it has a great commercial value, but also in the food and confectionery industries as a flavoring for liqueurs, teas, toffees, candies, ice creams, and soft drinks. It has a peculiar composition: the content of the monoterpene hydrocarbon fraction rarely reaches 60% (limonene, γ-terpinene and β-pinene are the main components), whereas the oxygenated fraction is highly represented, unlike the other Citrus oils, being linalyl acetate about the 30% and linalool about 10% of the total oil composition. These two compounds define the flavor notes of the bergamot oil; for this reason, international buyers evaluates the quality of a bergamot oil according to the amount of oxygenated compounds and, in particular, of linalool and linalyl acetate.

Yuzu (*C. junos* Sieb. ex Tanaka) is well-known in far Eastern countries because of the pleasant aroma from the outer rind. Recently, yuzu essential oil has gained a great interest due to its unique properties industrially used in sweet production, beverages, cosmetics and perfumery, and also in aromatherapy (Sawamura 2005). Limonene is the most predominant compound of yuzu oil (63.1–68.1%).

Petitgrain oil is produced by the distillation of leaves and twigs of all Citrus species (orange, mandarin, lemon), even though the most appreciated one is that coming from sour orange, which is particularly rich in linalyl acetate (ca. 50%) and linalool (ca. 30%). Neroli oil is obtained by distillation of flowers of *C. aurantium*. Also in this case, the oxygenated terpenes linalool, linalyl acetate, nerolidol and geranyl acetate are the main components. Because of these particular features and the very low oil yield, both these oils are very expensive. They are used mainly in the preparation of exclusive perfumes.

12.5 Uses

The current use of citrus essential oil sweeps in a wide range of fields:

Pharmaceutical and Therapeutic. A vast number of studies demonstrate the pharmaceutical and therapeutic potential of essential oils and their individual constituents (Burt, 2004; Edris 2007; Bakkali et al. 2008). Their role and mode of action have been studied with regard to the prevention and treatment of cancer, cardiovascular diseases including atherosclerosis and thrombosis, as well as their bioactivity as antibacterial, antiviral, antioxidants, anti-inflammatory, analgesic and antidiabetic agent. The phenolic component present in essential oils has been recognized as the bioactive constituent with antimicrobial activity. The mechanism of action is not fully understood: essential oils components act involving several targets in the bacterial cells, rendering the microbial cell membrane permeable and leading to loss of homeostasis, leakage of cell contents and death. Essential oils and their individual components showed cancer suppressive activity when tested on a number of human cancer cell lines including

glioma, colon cancer, gastric cancer, human liver tumor, pulmonary tumors, breast cancer, leukemia and others (Bhalla et al. 2013). Recent studies performed with the Citrus essential oils established their potential anticancer effectiveness and assessed their efficiency in reducing local tumor volume or tumor cell proliferation by apoptotic and/or necrotic effects (Visalli et al. 2014). Syrian *C. limon* essential oil showed a cytotoxic effect on the human colorectal carcinoma cell line LIM1863 when studied *in vitro* (Jomaa et al. 2012). Celia et al. (2013) and Navarra et al. (2015) verified that bergamot essential oils exhibited anticancer activity in different *in vitro* assays against human neuroblastoma cells. More recently, a Chinese group showed the inhibitory effect on the proliferation of human lung cancer and prostate cell lines of the essential oil from a Navel orange peel (Yang et al. 2017). A review dealing with several therapeutic effects of limonene reported how this compound alone or in combination with other natural products exerted anticancer effects against human gastric and colon cancer cells (Vieria et al. 2018).

Aromatherapy is a complementary and alternative therapy that has gained a lot of attention in the last 15-20 years. It uses essential oils as the main therapeutic agents. Essential oils are administered through inhalation, massage or application on the skin surface, providing a feeling of well-being to the body and showing a curative potential on mind and spirit (Maeda et al. 2012). Compounds from essential oils enter the body (via the olfactory mucosa or the bloodstream by lung absorption) and may directly influence the brain's limbic region, affecting a person's emotional responses, heart rate, blood pressure and breathing. Recent clinical trials revealed the positive effect of lemon oil inhalation on nausea and vomiting of pregnancy, of citrus aurantium oil on anxiety, and bergamot oil on mood states, parasympathetic nervous system activity and salivary cortisol levels (Yavari Kia et al. 2014; Namazi et al. 2014; Watanabe et al. 2015).

Cosmetic. The pleasant odor and the distinctive taste make the essential oils one of the most important components in flavoring and perfume industries (Burt 2004; Sawamura 2011). Due to its intense fragrance and freshness and the ability to fix the aromatic bouquet of aromas, the essential oil of bergamot is used as one of the main basic constituents for the manufacture of perfumes.

Agricultural. The environmental problems caused by the massive application of pesticides (high toxicity, non-biodegradable properties, residual effects in soils, water resources and crops, selective resistance) in agriculture have been the matter of concern for the public opinion in the last years. In addition, the regulatory measures for pesticides use have become stricter. Current control focuses more on the use of alternative contact pesticides and other innovative greener phytosanitary methods. Natural products are excellent alternatives to synthetic pesticides. The properties that make them suitable for use in insect management include multiple modes-of-action and sites-of-action in the insect nervous system and elsewhere; these may account for the wide range of pesticidal actions (viz., contact, knockdown, fumigant toxicity) and sublethal behavioural actions (viz., deterrence, repellence). Owing to their volatility, the oils and their constituents are environmentally non-persistent. Toxicological tests indicate that most essential oil chemicals are relatively non-toxic to mammals and fish, and meet the criteria for 'reduced risk' pesticides. The insecticide activity of orange oils and the repellent capacity of lemon oils has been proved (Koul et al. 2008; Raina et al. 2007; Jaenson et al. 2006).

Food industries. Control of food spoilage and pathogenic bacteria is mainly achieved by chemical control, but the use of synthetic chemicals is often associated to undesirable aspects, such as carcinogenicity, acute toxicity, teratogenicity and slow degradation periods. Moreover, the emergence of bacterial antibiotic resistance in the food chain is a further concern. Demand of consumers for food without synthetic and harmful chemicals is therefore increasing. Consequently, interest in natural, non-synthesized food additives as potential alternatives to conventional antimicrobials to extend

shelf life, combat food pathogens, improve the quality of stored food products and protect the environment, has heightened. Among natural products, EOs are gaining interest as potential food additives and are widely accepted by consumers because of their relatively high volatility, ephemeral and biodegradable nature (Burt 2004; Holley and Patel 2005; Hyldgard et al. 2012; Rivera Calo et al. 2015). Among the great variety of essential oils, citrus fruit EOs and their major components have received attention in the food industry since they have been recognized as safe (GRAS) by the Food and Drug Administration (2005) and many foods tolerate their presence (Fisher and Phillips 2008). The antimycotoxigenic activity of Citrus EOs in food system has been proved by Phillips et al. (2012) that reported a strong inhibition of mycelial growth for the phytopathogenic fungi *Penicillum chrysogenum, Aspergillus niger* and *Alternaria alternata* on grain, following the application of vapour of citrus EO. Essential oils from *C. reticulata*, *C. maxima*, *C. sinensis* and *C. aurantifolia* displayed broad fungitoxic spectrum and antiaflatoxigenic activity against different food contaminating moulds (Razzaghi-Abyaneh et al. 2009; Singh et al. 2010a; 2010b; Velazquez-Nunez et al. 2013; Jing et al. 2014; Trabelsi et al. 2016). Moreover, there would be no chance of alteration in the organoleptic properties of food commodities when citrus EOs or their components are used as preservatives because the monoterpenes present in these oils are widely used as natural ingredients in many food products, soaps, soft drinks, cosmetics and perfumes for their lemon-like flavour and odor (Shukla et al. 2009).

Essential oils exert also potent and broad-spectrum antimicrobial activity *in vitro*, and to a smaller degree in foods, against common food-borne pathogens (Oussalah et al. 2007; Callaway et al. 2011; Muthaiyan et al. 2012). Limonene, the major chemical component of citrus EOs, and orange terpenes, alone or in combination, showed lethal effects against 11 different strains of *Salmonella* on a disc diffusion assay (O'Bryan et al. 2008), against *Campylobacter* spp and *Cinnamonum coli* (Nannapaneni et al. 2009), against *Escherichia coli* and *Salmonella* onto beef at the chilling stage of processing (Pittman et al. 2011). The antimicrobial activity, however, seems to be strictly oil-dependent and it is very hard to know which constituents or mixtures of them are responsible for the bacteriostatic or bactericide effect (Mandalari et al. 2007; Espina et al. 2011); in general, Gram-positive organisms seem to be much more susceptible to EOs than Gram-negative organisms (Rivera Calo et al. 2015). The substitution of synthetic additives with EOs with antimicrobial effect is still premature due to high cost, food matrix composition and possible sensory changes of food characteristics as a function of the EOs dose. The application of citrus EOs might be recommended to reduce the use of chemical additives, to maximize the use of existing resources and to minimize adverse effect of by-products in the environment.

Recent research has focused on the development of edible/biodegradable packaging for food product as substitute of conventional plastic materials. In this contest, the incorporation through emulsification of citrus essential oils into edible films positively impact the most relevant properties of edible films and coatings, namely microstructural, physical, antioxidant and antimicrobial (Sanchez-Gonzales et al. 2010; Tongnuanchan et al. 2012; Atarés and Chiralt, 2016).

Nanoformulations can solve problems related to EO application on large scale as volatility, hydrophobicity and tendency to oxidize (Campolo et al. 2017). In order to enhance the antimicrobial activity of essential oils in food, protect the essential oil from oxidation or evaporation and minimize the impact on the quality attributes of the final product, a nanoencapsulation delivery system has been positively tested (Donsì et al. 2011). Nanometric delivery system, due to the subcellular size, may increase the passive cellular absorption mechanisms, reducing mass transfer resistances and increasing antimicrobial activity (Donsì et al. 2011). Several examples of application of nanoformulated citrus essential oils have been reported as biocides for pest control (Campolo et al. 2017) and food preservatives (Ribeiro-Santos et al. 2017) and

additive for develop new active food packaging materials (Vilela Dias et al. 2013).

Other uses

Citrus essential oils have been also evaluated in the field of conservation of cultural properties and for their effects in the control of biodeterioration of documentary heritage. *C. sinensis* oils in the vapor phase showed significant inhibitory activity against fungal and bacterial strains isolated from different documentary supports and indoor environments of repositories, without negative environmental and human impacts (Borrego et al. 2012).

12.6 Future Perspective and Strategies

Consumption of citrus essential oil is constantly enhancing year after year because of the strong demand for pure natural ingredients in many fields. Thus, increasing the production of citrus essential oil has become a crucial objective for several breeders. The target, however, presents severe difficulties. Conventional breeding methods effective in creating useful variability and improve essential oil yield and uniformity are hampered in Citrus because of several factors: apomixis, diffuse pollen and ovule sterility, sexual incompatibilities, long juvenile period, are the most relevant. Moreover, the poor knowledge of the metabolic pathways by which essential oils are biosynthesized, makes the challenge even more complicated. The ever-increasing demand for citrus oils together with their high cost and, for some of them, their scarcity, encourages the flavour industry to consider biotechnology as an appropriate tool for improvement of citrus oil yield and quality. The application of biotechnological approaches to citrus essential oil improvement start from a quite low baseline, however, a range of biotechnological tools, such as somatic hybridization and molecular genetics, can help to circumvent some of the barriers associated with the reproductive biology of citrus. Somatic hybridization via protoplast fusion is an additive process capable to capture the genetic diversity of the gene pools by combining (fusing) the nuclear, chloroplast and mitochondrial genomes of desired parental protoplasts in novel arrangements, therefore creating unobtainable homokaryon or heterokaryon biotypes (Davey et al. 2005; Eeckhaut et al. 2013; Grosser and Gmitter 1990; Johnson and Veilleux 2001). The potential heterozygosity is extremely large depending on cumulative allelic differences between the contributing parents (Grosser and Gmitter 2011). The large extent of genomic arrangement and recombination following ploidy manipulation may have a deep impact on the chemical composition of somatic hybrid fruits, aiming to a presence of distinctive and original traits in phytochemical characters (Gancel et al. 2002, 2003; 2005a; 2005b; Tusa et al. 2007; Abbate et al. 2012; Fatta Del Bosco et al. 2013; 2017; Napoli et al. 2016). Gene expression differences between citrus allotetraploid somatic hybrids and their parents have been analyzed through quantitative RT-PCR assay. It revealed that the genes controlling the biosynthetic pathways of aromatic compounds (of the peel oil) are not inherited in an additive fashion in the allotetraploid hybrids but may be subject to dosage effects, likely over-dominance, co-dominance and other complex interactions in gene expression regulation (Gancel et al. 2003; Bassene et al. 2009a, 2009b).

In the last two decades, omics approaches (genomics, transcriptomics, proteomics, metabolomics, hormonomics, ionomics or phenomics), has been increasingly employed to gain insight into the biology of yield in plants (Swanson-Wagner et al. 2009; Syrenne et al. 2012; Thao and Tran 2016). Omics technologies have enhanced the knowledge on cellular processes, including gene and protein regulations, and metabolic pathways for economically important traits (Galland et al. 2012; Hong et al. 2016).

Development of high-throughput sequencing technologies, such as Illumina (https://www.illumina.com/techniques/sequencing.html), PacBio (https://www.pacb.com/), Optical Mapping (http://rtlgenomics.com/bionano/), have made easy the whole sequencing and assembly of complex genomes of plants. Nowadays genomes

of several crop are sequenced (http://www.genome.jp/kegg/catalog/org_list.html) and available, such as grapevine (Jaillon et al. 2007), apple (Velasco et al. 2010), tomato (Sato et al. 2012), Valencia orange (Xu et al. 2012), asparagus (Harkess et al. 2017). The genome comparison of sequences and re-sequencing of different cultivars are an effective approach to identify genes involved in specific traits, including regulation and production of bio-compound.

Different omic approaches focused on citrus genus. Transcriptome and proteome analyses of late-ripening sweet orange mutant were carried out (Wu et al. 2014; Zhang et al. 2014), highlighting the presence of multiple ripening events in citrus which suggested the key role of abscissic acid (ABA), sucrose and jasmonic acid (JA) in citrus ripening. Recently, Voo and Lange (2014) reported a protocol for the isolation of essential oil gland cells of citrus fruit peel through single cell omics. Katz et al. (2010) identified by proteomic approach 1,500 proteins in citrus fruit juice sac cells, quantifying their amounts at three developmental stages and developing a protein database with a comprehensive sequence database of citrus genes, ESTs and proteins, named iCitrus. Metabolome profiles of different citrus species were associated to sensitivities against greening (HLB) disease. Higher levels of the amino acids, organic acids and galactose were observed in HLB sensitive sweet orange varieties (Cevallos-Cevallos et al. 2012). In addition, differences in the phenylalanine, histidine, limonin and synephrine were observed in asymptomatic and symptomatic fruits (Chin et al. 2014), suggesting as metabolomics can generate biomarkers for important traits in citrus.

Omics were also used to investigate the regulation of oil biosynthesis in seed with the aim to increase their yields in several crops (Hajduch et al. 2011; Gupta et al. 2017). Since, in citrus, the essential oils composition provides valuable information related to organoleptic properties linked to product quality, the comprehensive untargeted analysis of biochemical constituents is the major objective of metabolomic studies. Due to their high added value, careful attention was paid to ensure the oils' genuineness and authenticity. In this way, Mehl et al. (2015) developed a multiblock data modelling to integrate heterogeneous signals collected from GC-FID, H-NMR, UHPLC-TOF/MS- and UHPLC-TOF/MS + platforms to obtain a complete characterisation of cold pressed lemon oil (CPLO), identifying relevant biomarkers.

12.7 Conclusions

The increasing demand in citrus natural extracts from the manufacturers of foods, cosmetics and pharmaceuticals and the possibility of linking the chemical contents with particular functional properties call for further and strong efforts in developing new studies on essential oils of citrus species and varieties.

In the future, genomic approaches such as genome resequencing, allele mining and genomic selection, will integrate the techniques for genotype obtaining, characterization, and selection, allowing the recovery and build-up of desirable crop phenotypes in novel and targeted citrus hybrid species.

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Abiotic Stress Resistance

13

Angela Roberta Lo Piero

Abstract

Citrus, one of the most important fruit crops in the world, is sensitive to many environmental stresses often leading to poor tree growth and reductions in fruit yield and quality. Citrus is most often grown in warm climates with well-drained soils, therefore acceptable growth conditions depend upon the quality and quantity of irrigation water and the risk of cold temperatures. Citrus species do not develop a powerful root system and in well-drained soils of subtropical semiarid zones might be subjected to water deficit especially during hot dry summers. Then, these areas often require supplemental irrigation that may prompt the use of low quality water thereby increasing soil's salt concentration. Even when irrigation water is of good quality, the use of fertilizers and other agro-chemicals raises the likelihood of salts to rise in the soil causing salinity stress, especially high chloride, which in turn is rather detrimental to citrus growth and fruit quality and yield. Negative soil characteristics such as excess calcium, high pH and mineral imbalances also affect citrus fruiting. In calcareous soils, for example, the high pH causes Fe-immobilization in unavailable forms for plant absorption thus causing iron deficiency. In addition, in areas characterized by scarcely drained soil, flooding can affect the soil structure depleting O_2 for the radical tissues and provoking a reduction in iron solubility. Moreover, these damaging stresses do not come alone but in combination, in some cases acting according to an additive effect thus leading to a more restricted plant development. In this chapter, citrus plant behavior under the main abiotic stress conditions, such as drought, salinity and low temperatures will be deeply described, taking into account that important differences among genotypes have been described in their response. The effect of heat, flooding and heavy metal stresses will be also considered although the reader can refer to the existing literature to examine in depth these abiotic stresses. In conclusion, the consequences of the combined effect of more than one stress type at once, occurrence that normally mimics the natural environmental conditions, will be also reviewed.

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13.1 Drought Stress

Being a perennial tree, citrus is frequently exposed to soil and atmospheric drought. Water deficit periods negatively affect citrus plant productivity in many aspects, including reduction in growth and metabolism, which lead to a decrease in fruit yield and quality (Pérez-Pérez et al. 2008). In order to cope with water shortage, plant's first response is the decrease of stomatal conductance (Kramer and Boyer 1995). The consequence in these cases is the loss of photosynthesis due to stomatal reduction in CO_2 uptake and the shift of resources into root growth, in order to maximize water uptake, at expense of photosynthetic and reproductive tissues. Plants subjected to drought are seriously affected by secondary damages caused by oxidative stress due to the down regulation of the photosynthetic process that impairs the photosynthetic electron transport chain that, being over-reduced, results in the generation of reactive oxygen species (ROS) (Apel and Hirt 2004). In plant cells, the excessive production of ROS is potentially harmful to lipids, proteins and nucleic acids, whose oxidation may in turn leads to detrimental effects such as enzyme inhibition, chlorophyll degradation, disruption of membrane integrity, loss of organelle functions and reduction in metabolic efficiency and carbon fixation (Scandalios 2005). In these latter cases, several enzymatic antioxidant systems or antioxidant molecules might counteract the aforementioned deleterious effects by a ROS '*scavenging*' activity (Garcia-Sanchez et al. 2010). Additionally, plants avoid cell dehydration by preventing water loss via either cell wall hardening or promoting water influx, because of active solute accumulation that decreases the osmotic potential (Zhang et al. 1999). The water soluble low molecular weight compounds known as osmolytes are one of the strategies to face the stress situation. The most important solutes produced by plants include proline, glycine betaine, sugars, polyols and polyamines (Parida and Das 2005). Among the above mentioned osmolytes, proline is most extensively studied amino acid which accumulates under drought, salinity and oxidative stress (Szabados and Savouré 2010). In this respect, de Campos et al. (2011) report that transgenic Swingle citrumelo over-accumulating proline, due to the constitutive expression of the *Vigna aconitifolia* key-enzyme for proline synthesis, copes with water deficit better than control plants, This is due not only to the well-known osmoprotective function of proline but also because it contributes to ameliorate the secondary oxidative stress induced by drought (Zaher-Ara et al. 2016; Şahin-Çevik et al. 2017). In addition, soluble sugars have been shown to be important osmoprotectants that play a major role in cellular osmotic adjustment by protecting cellular structures exposed to environmental stress. In this respect, *Ptr*A/NINV, an alkaline/neutral invertase gene of *Poncirus trifoliata* is an important gene implicated in sucrose decomposition, and plays a positive role in dehydration tolerance by promoting osmotic adjustment, ROS detoxification and photosynthesis efficiency (Dahro et al. 2016). A number of studies have demonstrated that polyamines function in stress tolerance largely by modulating the homeostasis of ROS due to their direct, or indirect, roles in regulating antioxidant systems or suppressing ROS production (Liu et al. 2015). The polyamine accumulation predominantly results from de novo biosynthesis, which involves a series of reactions catalyzed by five enzymes, among which arginine decarboxylase (ADC) has been shown to act as a rate-limiting enzyme for their accumulation under abiotic stress (Liu et al. 2006). In citrus, changes in free polyamine content and transcription of genes participating in polyamine metabolism are significantly induced under drought (Fu et al. 2016). In addition, the overexpression of *Fc*WRKY protein of *Fortunella crassifolia* in lemon confers drought tolerance by promoting production of putrescine via regulating ADC expression (Gong et al. 2015). As citrus culture occurs mainly in dryland, breeding citrus programs focused in the selection and use of scion-rootstock combinations with better responses to drought conditions. Many years of field observations showed that Rangpur lime is one of the most drought-tolerant rootstocks (Davies and Albrigo 1994) whereas some of the widely used commercial rootstocks, such as trifoliate orange

(*P. trifoliata*) and sour orange (*C. aurantium*) are not tolerant to drought stress. Romero et al. (2006) report that Cleopatra mandarin trees (*C. reshni*) are able to tolerate moderate water stress, being more efficient in soil water use than Carrizo citrange [(*C. sinensis* (L.) Osb. X *P. trifoliata* (L.) Raf.], in deficit irrigation conditions. Long-term exposure of the grafted trees to a gradually increasing water deficit and subsequent recovery revealed distinct strategies of drought acclimation that are induced by the different rootstocks. Trees grafted onto the drought-tolerant rootstock Cravo Rangpur lime (*C. limonia*) Santa Cruz selection exhibit less water conservation but an increased cell wall elasticity that contributes to turgor maintenance and its related processes of growth and photosynthesis. On the contrary, the drought-tolerant Sunki Tropical mandarin (*C. sunki*). Tropical selection and the drought sensitive trifoliate orange Flying dragon rootstocks induce a water conservation strategy by increasing tissue rigidity. However, Sunki Tropical is also able to induce osmotic adjustment, conferring thereby a more efficient water conservation strategy than Flying dragon by attenuating cell dehydration and shrinkage (Gonçalves et al. 2016). The influence of recurrent water deficit on the physiological, molecular and hormonal changes in Valencia orange (VO) grafted on two rootstocks with different soil water extraction capacities: Rangpur Lime (RL) and Sunki Maravilha (SM) was also investigated (Neves et al. 2017). The results indicate that epigenetic alterations involving DNA methylation are implicated in drought tolerance in *Citrus* genus. Upon successive drought events, the VO/SM scion/rootstock combination presents acclimatization characteristics that enable higher tolerance to water deficit, which in turn can facilitate the whole plant survival. Accordingly, frequencies of methylated polymorphic fragments identified by the MSAP technique are markedly different between VO/RL and VO/SM plants suggesting that recurrent drought triggers memory to stress more clearly in VO plants grafted onto SM than in VO plants grafted onto RL. Moreover, as epigenetic marks in plants can be transmitted by meiosis and mitosis, the genetic and physiological changes observed in VO/SM plants exposed to recurrent drought could be maintained during the subsequent development of these organisms (Neves et al. 2017). Another factor influencing the behavior of plants in relation to drought is the polyploidy (Allario et al. 2013, 2011; Hussain et al. 2012). In citrus, it has been shown that tetraploid plants (4x) cultivated in greenhouse and subjected to drought present higher drought tolerance than the respective diploid plants (2x) (Allario et al. 2013, 2011; Hussain et al. 2012). Such behavior could be associated to morpho-physiological differences more favorable in the 4x plants, such as lower stomata density, as well as, the existence of genes differentially expressed in roots and associated to abscisic acid production (Allario et al. 2013). More recently, based on an interactomic approach, the response to drought of two different scion/rootstock combinations presenting different polyploidy (the diploid and autotetraploid Rangpur lime rootstocks grafted with 2x Valencia Delta Sweet orange scions), named V/2xRL and V/4xRL, was analyzed in the attempt to identify proteins involved in response to drought. The V/2xRL plants implement some tolerance mechanisms and the global plant response to drought is rapid resulting in a general tendency to dehydration avoidance, which, however, does not allow the plant survival in long terms. Differently, the V/4xRL plant response strongly affects plant development but presents many advantages in case of prolonged drought (de Souza et al. 2017). As it has been mentioned before, some of the widely used commercial rootstocks are not tolerant to drought stress. Consequently, a lot of work has been done to isolate genes involved in drought tolerance with the further aim of genetically engineering rootstocks having this new trait. The candidate genes belong to the regulatory genes such as transcription factors that act as significant coordinators to transduce stress signals and to regulate the expression of a second gene category, the structural genes, that also include enzyme encoding genes. Several regulatory genes have been identified in a subtractive cDNA library constructed from drought-stressed and non-stressed Rangpur lime leaves (Şahin-Çevik et al. 2017), and

consisting of different types of NAC transcription factors, RING zinc finger proteins and bZIP proteins. WRKY genes induced in response of drought stress have been also identified in the *Citrus* relative *P. trifoliata* (Şahin-Çevik et al. 2013; Şahin-Çevik and Moore 2013). A genome-wide analysis of *Citrus* R2R3MYB genes identified a group of R2R3MYB genes that responded to at least one abiotic stressful treatment and some of them responded to multiple treatments, representing a promise for improving citrus adaptation since plants often undergo multiple stresses concurrently (Xie et al. 2014). Under drought stress (Rodrigo et al. 2006), abscisic acid (ABA) de novo synthesis might be promoted, leading to accumulation of higher levels of ABA. The ABA signaling components have been increasingly unraveled, allowing the establishment of a relatively clear ABA signaling pathway associated with stress adaptation (Pizzio et al. 2013). The reaction catalyzed by 9 cis epoxycarotenoid dioxygenase (NCED) is regarded as the rate-limiting step in the regulation of ABA biosynthesis, and, the ABA levels under the stresses are closely correlated with the transcript abundance of NCED genes. The ectopic expression in tobacco of NCED from *C. reshni* confers enhanced tolerance to drought suggesting its potential use for genetic engineering to develop drought resistant crops (Xian et al. 2014). Finally, aquaporins are membrane proteins that act as channels for water and other small-uncharged molecules of great physiological significance. In a genome wide study, De Paula Santos Martins et al. (2015) characterized the complete set of *Citrus* species acquaporins, and, among them they identified a TIP isoform, named *Cs*TIP2;1, which improves the leaf water and oxidative status, photosynthetic capacity, transpiration rate and water use efficiency of plants subjected to a progressive soil drying (De Paula Santos Martins et al. 2017). The glutathione transferases (GSTs) are members of a superfamily of enzymes with pivotal role in the detoxification of both xenobiotic and endogenous compounds. Transgenic tobacco plant over-expressing *C. sinensis* GSTs exhibited both drought and salinity stress tolerance (Lo Piero et al. 2006, 2009, 2010, 2011; Lo Cicero et al. 2015). Transgenic tobacco plants overexpressing *Cs*GSTs also showed tolerance against fluorodifen and alachlor herbicides (Lo Cicero et al. 2017) thus representing promising candidate genes that can be helpful for phytoremediation of residual xenobiotics in the environment and overall to develop genetically modified crops with high resistance a range of abiotic stresses.

It has been shown that a short-term period of water depletion is essential for inducing dormancy requested for citrus flowering, especially in tropical growing regions, in which winter cold is not sufficient to stimulate flowering (Chica and Albrigo 2013). A recent report reveals an interaction between water deficit and hormones in the activation of the florigen related genes, with the process requiring the transcriptional regulator GIGANTEA (GI) and the hormones abscisic acid (ABA), gibberellic acids (GAs), and indole 3-acetic acid (IAA). At the beginning of water deficit treatment, GAs and IAA are decreased and ABA is rapidly increased in the buds. GI protein directly up-regulates the expression of the FT protein, a mobile florigen signal, that along with nutrients are gradually transported from leaves to the bud, where FT protein interacts with several others regulators leading to the up-regulation of floral organ gene (Li et al. 2017).

13.2 Salinity

Relative to many crop plants, *Citrus* has been classified as a salt-sensitive crop (Maas 1993) because of saline irrigation water reduces tree growth and fruit yield relatively more than in many other crops (Grieve et al. 2007). Salinity affects all plant physiological responses and production in three ways: nutritional imbalances, osmotic stress and toxic ion stress. In *Citrus*, salinity can causes leaf nutrient deficiencies resulting in high ratios of Na^+/Ca^{2+}, Na^+/K^+, Na^+/Mg^{2+}, Cl^-/NO_3^- and $Cl^-/H_2PO_4^-$ (Grattan and Grieve 1992), which can cause reductions in growth. Dissolved salts in the nutrient solution exert an osmotic effect that reduces the

availability of free water through physical processes that impair water extraction from soil by roots. Growth and yield of all plants are reduced by decreased leaf water potential (Maas 1986). The effect of osmotic stress is different when stress increases gradually avoiding salt shock and allowing the plant to adjust, compared to the situation when osmotic stress in the soil solution increases abruptly (Levy and Syvertsen 2004). When salinity stress is gradual, the osmotic effect is practically negligible as the downward osmotic adjustment in citrus leaves is very effective at maintaining turgor (Garcia-Sanchez and Syvertsen 2006). In contrast, osmotic stress can occur from an abrupt increase in salinity of the soil solution that can result from high saline irrigation water, excessive fertilization or when a light rain leaches accumulated salts into the root zone (Levy and Syvertsen 2004). The effect of salinity on plant growth is not only related to osmotic effects, but growth reductions in citrus also can be related to a gradual accumulation of toxic levels of Cl^-, Na^+ or boron (B) in leaves (Levy and Syvertsen 2004). Exactly how the salts exert their toxicity remains unknown. Salts may build up in the apoplast and dehydrate the cell, they may build up in the cytoplasm and in the chloroplast inhibiting enzymes involved in carbohydrate metabolism and photosynthetic process (Munns and Tester 2008). Nevertheless, it is well known that Cl^- and Na^+ toxicity reduces the CO_2 assimilation in citrus trees determining a marked reduction of the photosynthesis process and provoking a possible impairment of the electron transport chain. In Carrizo citrange, salt-induced oxidative stress response can be alleviated by pre-treatment with ammonia. Upon salinity, citrus seedlings grown with NH_4^+ show a reduction of H_2O_2 levels in parallel to an increase of catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) activities compared with the control plants. Moreover, N-NH_4^+ plants are able to keep high levels of reduced glutathione (GSH) upon salinity and are able to induce glutathione-S-transferase (GST) and phospholipid hydroperoxide glutathione peroxidise (PHGPx) mRNA accumulation. Based on this evidence, sublethal concentrations of NH_4^+ might act as a mild oxidative stressor, which triggers antioxidant cellular machinery that can provide resistance to subsequent salt stress (Fernández-Crespo et al. 2014). In addition, it has been demonstrated that pre-exposure to H_2O_2 or NO can ensure sour orange plant survival in a high salt environment, and this result supports the notion that interaction between H_2O_2 and NO production may be locally and systematically sensed in citrus plants (Tanou et al. 2012). Cl^- is an essential micronutrient for higher plants, involved in the regulation of important cellular functions like enzyme activity, membrane potentials, pH gradients and electrical excitability (Marschner 1995). In addition, Cl^- is a major osmotically active solute in the vacuole involved in both turgor and osmoregulation processes (Marschner 1995; Colmenero-Flores et al. 2007). Under salt stress conditions, woody perennial plants frequently exhibit toxicity symptoms to chloride (Cl^-), rather than to sodium (Na^+) accumulation (Munns and Tester 2008). In citrus, the evidence for the detrimental effect of chloride is mostly supported by the association between genetic differences in the rate of Cl^- accumulation in leaves and the plant's salinity tolerance (Moya et al. 2002). Thus, Bañuls and Primo-Millo (1992) showed that NaCl and KCl stress have similar toxicities, while $NaNO_3$ resulted less harmful. Using different rootstock/scion combinations (Bañuls and Primo-Millo 1995) and different chloride salts (Brumós et al. 2009), higher correlations between shoot Cl^- accumulation than between Na^+ accumulation and defoliation were found. This difference may arise because Na^+ is withheld effectively in the woody roots and stems whereas Cl^- continues passing to the leaves and becomes the more significant toxic component of the saline solution (Munns and Tester 2008). This statement does not imply that Cl^- is more toxic than Na^+, rather that citrus under NaCl stress conditions are better excluding Na^+ from the leaf than Cl^- (Brumós et al. 2009) when a Cl^--sensitive rootstock is used. Therefore, it is believed that the physiological basis for citrus tolerance to salt stress is mostly related to the Cl^- exclusion capacity, or to the plant ability

to restrict Cl^- uptake and transport from root to shoot, a mechanism whose efficiency is particularly dependent upon rootstock performance. Carrizo citrange and rough lemon (*Citrus jambhiri* Lush.) are defined as Cl^- includers because their limited ability to exclude the anion, which correlates with their sensitivity to salt stress (Bañuls and Primo-Millo 1995; Levy and Syvertsen 2004; Alvarez-Gerding et al. 2015). Cleopatra mandarin and Rangpur lime have been classified as chloride excluder rootstocks, and hence are salt-tolerant plants. Sour orange is considered a good Na^+ and Cl^- excluder (Gimeno et al. 2009) and is commonly used in many citrus-producing regions in the world, especially in areas with high pH and calcareous soils. Nevertheless, it is highly susceptible to *Citrus tristeza virus* (CTV) (Moreno et al. 2008). Other trifoliate rootstocks such as trifoliate hybrids, C22 and C146 (*Citrus sunki* Hort. Ex Tan. × *Poncirus trifoliata* L. Raf Swingle) have been proven tolerant to CTV and, probably due to the ability of trifoliate orange to sequester and limit toxic ion translocation throughout the plant (Walker 1986), they are more tolerant to saline irrigation water than the sour orange rootstocks. Tolerant rootstocks apparently possess more efficient root mechanism for chloride exclusion than sensitive ones (Moya et al. 2002; Fernández-Ballester et al. 2003). It has been shown that these mechanisms rely on plasma membrane (PM) transporters that regulate root Cl^- uptake. In sweet orange, a genome-wide survey of the aquaporins or major intrinsic protein (MIP) gene family identified a total of 34 open reading frames (ORFs) encoding MIP proteins characterized by tissue-specific gene expression, and different expression profiles up on abiotic and biotic stresses (de Paula Santos Martins et al. 2015). In addition, the expression of two MIP genes, PIP1 and PIP2, has been investigated in roots of Cleopatra mandarin and Carrizo citrange, subjected to salt treatment (Rodríguez-Gamir et al. 2012). PIP1 and PIP2 gene expression differences among citrus genotypes appear to affect Cl^- exclusion from leaves, thus determining their aforementioned different tolerance to salinity. Different physiological parameters have been investigated from a wide range of varieties and species belonging to the *Citrus* genus subjected to salt stress with the aim to seek new sources of tolerance that might be specie-specific (Hussain et al. 2015). Large differences were observed within *Citrus* genus indicating that Cleopatra mandarin and pummelo (*C. maxima*) presented good tolerance to salt stress while citron (*C. medica*) is very sensitive. In citrus, rootstocks are propagated by seedlings of polyembryonic seeds. Some citrus produce in their seeds somatic embryos originated from nucellar cells, a maternal tissue, and therefore the regenerated plants reproduce the maternal characters. Citron and pummelo are two species that are not able to produce polyembryonic seeds. This is the reason for their uselessness as rootstock in modern agriculture where homogeneity and reproducibility of plant phenotypes are the conditions of plant commercialization.

It has been previously reported that all species in the *Citrus* genus are diploid, with 2n = 2x = 18 chromosomes (Rose et al. 1998) and they are easily crossed producing fertile hybrids (Barrett 1992). Several studies have revealed that chromosome doubling in citrus, might possibly arise during seed formation and tetraploid seedlings arise spontaneously in nuclear cells (Cameron and Soost 1968; Saleh et al. 2008). The occurrence of tetraploidy in some citrus species has led to smaller, more desirable trees with larger fruits (Barrett 1992). Ruiz et al. (2016) attempted to compare diploid (2x) *C. macrophylla* genotype to the respective double diploid (4x) and their results show that genome duplication improves the tolerance to saline toxicity, because of the lower Cl^- accumulation in leaves delays the damage, this effect may be linked to the reduced transpiration rate of the 4x genotype. Many efforts have been made to improve tolerance of the rootstock by transferring genes related to salt or drought tolerance, especially those functioning in osmotic adjustment or membrane stabilization (Gong and Liu 2013). Accumulation of proline has been shown to be correlated with stress tolerance. 1-Pyrroline-5-carboxylatesynthetase (P5CS) is the key gene involved in proline synthesis, being feedback inhibited by proline itself. Molinari et al. (2004) delivered a site-directed mutagenesis P5CS mutant gene (p5cs) into the citrus rootstock

Carrizo citrange, which retained similar kinetic characteristics to the wild type P5CS except for the eliminated feedback inhibition. Thus, the transgenic plants present superior osmotic adjustment and significantly higher photosynthetic rate than the control plants. More recently, Fu et al. (2011) reported that overexpression of a betaine aldehyde dehydrogenase gene cloned from *Atriplex hortensis* in trifoliate orange leads to enhanced salt stress tolerance, which may be correlated with the low levels of lipid peroxidation, protection of the photosynthetic machinery, and increase in K^+ uptake. The glyoxalase pathway involving glyoxalase I (gly I) and glyoxalase II (gly II) enzymes is required for glutathione-based detoxification of methylglyoxal and has been indicated as probable candidate genes in conferring salinity tolerance. The transformation of Carrizo citrange rootstock with these genes coding for the glyoxalase system (*Bj*GlyI and *Pg*GlyII) enhances their salt stress tolerance (Alvarez-Gerding et al. 2015). Very recently, next-generation RNA-seq technology was applied to analyze the gene expression profiling of citrus roots over a 24-h period of salt treatment (Xie et al. 2018). A total of 1831 differentially expressed genes (DEGs) were identified, and, based on functional annotation, the salt overly sensitive (SOS) and reactive oxygen species (ROS) signaling pathways were found to be involved (Xie et al. 2018). Meanwhile, genes involved in hormone metabolism and signaling play important roles in salt stress. In addition, a multitude of transcription factors (TFs) including WRKY, NAC, MYB, AP2/ERF, bZIP, GATA, bHLH, ZFP, SPL, CBF, and CAMTA were identified. The genes related to cell wall loosening and stiffening (xyloglucan endotransglucosylase/hydrolases, peroxidases) are also involved in salt stress response. Based on these data and previous research achievements, a putative model has been proposed, which might be helpful in understanding the regulatory network of salt stress response in citrus roots (Xie et al. 2018). The putative model showed that salt stress firstly acts on the plasma membrane, making its lipid bilayer more fluid, consequently induces Ca^{2+} influx in the cytoplasm and orderly amplifies Ca^{2+} signaling cascades (e.g., CMLs, CAMs, and CBLs) and MAPK (mitogen activated protein kinase) cascades, and further regulates the expression of a series of salt stress-responsive genes to confer salt tolerance to citrus (Xie et al. 2018) (Fig. 13.1). A similar mechanism has been proposed for the signal transduction cascade induced by low temperature and heat stress, as described below.

13.3 Low Temperature

Low temperature is one of the most common environmental stresses and it can potentially causes severe losses to major economically important plants especially to those growing in subtropical and tropical regions such as *Citrus* varieties, which are therefore considered susceptible to cold. In particular, cold stress, which includes chilling (<20 °C) and freezing (<0 °C) temperatures, adversely affects the full genetic potential of plants owing to both the direct inhibition of the metabolic reactions and, indirectly, through cold-induced osmotic, oxidative and other stress types. Although the temperature sensor has not been identified unambiguously in plants (Penfield 2008), it seems that cold stress can mediate the alteration in the cell membrane fluidity this likely being the primary event of stress perception and injury (Chinnusamy et al. 2007) (Fig. 13.1). After stress recognition, the signal is communicated within cells and throughout the plant and culminates in an appropriate physiological response (Chinnusamy et al. 2007). This sensory machine leads to increased Ca^{2+} levels in the cytoplasm, which act as second messenger for signal transduction to the stress inducible genes. The elevated Ca^{2+} levels in the cell leads to activation of ICE transcription factors (inducer of CBF expression) which binds to cis acting element called as inducer of CBF expression region (ICEr) which in turn leads to the expression of CBF transcription factors. The CBF genes (C-repeat-binding factor) control major share of the low temperature induced genes so that many biochemical and physiological changes occur that can contribute to enhance cold tolerance

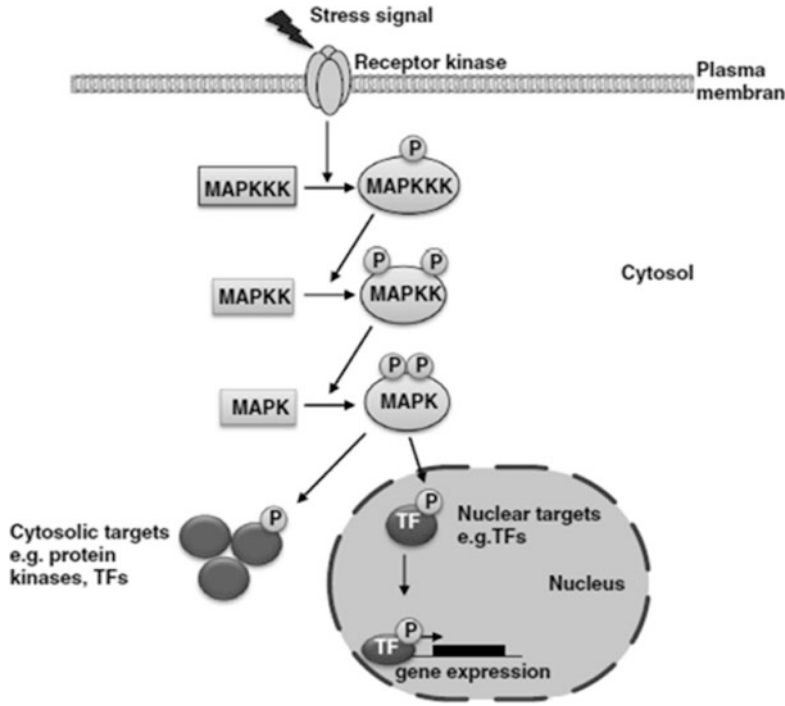

Fig. 13.1 A typical MAPK cascade pathway under drought, cold and heat stress. It consists of least three protein kinases namely MAPKKK, MAPKK and MAPK, whose activation takes place in a sequential manner through protein phosphorylation. The activated MAPK phosphorylates specific transcription factor, phospholipases, and cytoskeletal proteins leading to expression of genes in response to environmental stimuli (Danquah et al. 2013)

(Gilmour et al. 1998). These differently regulated genes are involved in a variety of cellular functions including metabolism, transcription, protein fate, transport facilitation, biogenesis, communications and signal transduction, cell rescue and defense, and cell death and aging (Şahin-Çevik and Moore 2006; Şahin-Çevik 2013; Lang et al. 2005). In many plants, including citrus, the maximum freezing tolerance is not constitutive but it can be induced in response to non-freezing temperatures below 10 °C according to a process called cold acclimation (Thomashow 1998). Periods of warm weather before a freeze event will make citrus plants less cold-acclimated and thus more susceptible to the freezing conditions (Davies and Albrigo 1994). Gene expression differences during acclimation have been associated with freeze tolerance in *Citrus* (He et al. 2012; Lang et al. 2005; Zhang et al. 2005a, b). There is significant variation among citrus species and relatives for cold tolerance (Inch et al. 2014): within *Citrus* genus, cold-hardiness ranges from cold-tolerant to cold-sensitive (Soost and Roose 1996). The most cold-tolerant commercial *Citrus* variety is considered *C. unshiu* (Satsuma mandarin), whereas Mexican lime lemon, and citron are considered the most susceptible to freeze damage (Davies and Albrigo 1994). *Poncirus* and *Fortunella* are considered the most cold-tolerant genera that are cross-compatible with *Citrus*. *Poncirus* is an unique genus because, in contrast to most citrus species, it has deciduous leaves and undergoes winter dormancy. These marked differences are probably adapted traits acquired during the evolutionary process in Northeast Asia when the trees were subjected to cold conditions. During hard winters, since no transpiration occurs, they can withstand temperatures as low as −20 °C with proper acclimation (Yelenosky 1985; Lang et al. 2005). Once *P. trifoliata* was recognized as cold-hardy it was used in breeding programs mainly in Florida (Soost and Cameron 1975) and other countries such as Japan and Russia (Gmitter 1994) in efforts to produce cold-tolerant commercial citrus varieties. Breeding within the *Citrus* genepool is complicated by many factors including prolonged juvenility, self- and cross-incompatibility, polyembryony (apomixis), heterozygosity, and inbreeding depression

(Grosser and Gmitter 1990; Soost and Cameron 1975). In addition, *Poncirus* is used as a cold-tolerant rootstock for some commercial citrus production (Ebel et al. 2008). However, when grafted, the cultivars are no longer deciduous, their cold tolerance is much more limited, and the production of scion varieties with good fruit quality, acceptable tree characteristics, and cold hardiness has been unsuccessful mainly because the fruit of the hybrids tends to contain high levels of poncirin, which gives it a disagreeably bitter taste. Although the use of trifoliate orange rootstock has been considered anyway one of the most effective ways to boost cold tolerance, the use of genomics and molecular biology techniques such as gene cloning, genetic transformation and the application of genome editing techniques to transfer cold tolerance traits can overcome problems associated with conventional breeding and provide new approaches for understanding and improving cold tolerance in *Citrus*. This requires the availability of genes that can be used in genetic transformation studies. Genes for cold tolerance have been mainly identified in *P. trifoliata*, (Şahin-Çevik and Moore 2006; Peng et al. 2014; Şahin-Çevik 2013) and in *C. unshiu* (Lang et al. 2005). Among them, transcription factors are important regulatory proteins that function to control the expression of target genes through the binding to specific cis-acting elements within the promoters (Şahin-Çevik et al. 2013; Şahin-Çevik and Moore 2013; Geng and Liu 2018). In particular, an enormous progress in deciphering significant components implicated in the cold signaling network has been done. One of the most important finding has been the identification of C-repeat binding factor (CBF) genes belonging to APETALA2/ETHYLENE RESPONSE FACTOR, that play crucial roles in regulating a large spectrum of cold-regulated (COR) genes (He et al. 2012). Another important breakthrough has been the characterization of Inducer of CBF Expression (ICE1) of *P. trifoliata* encoding a MYC-type basic helix–loop–helix (Huang et al. 2015) and governing the expression of CBFs, which in turn regulate the downstream target genes. To confirm its role, ectopic expression of *Pt*ICE1 in lemon confers cold tolerance by modulation polyamines levels regulating antioxidant systems or suppressing ROS production (Huang et al. 2015). As regards the hormonal control of the cold stress response, it has been shown that genes involved in ABA biosynthesis and the ABA signaling pathways are induced by cold in *Poncirus* (Şahin-Çevik and Moore 2006). Nine-cis-epoxycaretonoid dioxygenase (NCED), a key enzyme in ABA biosynthesis that catalyzes the conversion of 9-cis-epoxycarotenoids to xanthoxin as well as a bZIP transcription factor have been identified as cold-responsive (Xian et al. 2014). Since some bZIP transcription factors induce gene expression through cis-elements that include the ABA response element (ABRE) in *Arabidopsis* (Jakoby et al. 2002), increased expression of such a gene may indicate that an ABA-dependent pathway is activated during cold acclimation in *Poncirus*. Ethylene is also increased when plants are exposed to cold as consequence of the increased expression of 1-aminocyclopropene- 1-carboxylate (ACC) oxidase, which is responsible for converting ACC to ethylene (Şahin-Çevik and Moore 2006). Three groups of genes encoding functional protein have been detected as related to cold temperature responses (Zhang et al. 2005a, b). The first group include up-regulated proline/betaine transporter, nitrate transporter and water channel protein known to be associated with osmotic adjustment of plant cells under adverse environment. The second group includes up-regulation of early light inducible protein and aldo–keto reductase. Proteins encoded by these genes were reported to alleviate the damages caused by photo-oxidative stress, which simultaneously exists in combination with other environmental stresses. The third group includes down-regulation of chlorophyll a/b binding proteins, photosystem II OEC23 and carbonic anhydrase. The proteins encoded by these genes are related to adjustment of plant photosynthesis under stress condition (Zhang et al. 2005a, b). Down regulation of these genes can lower photosynthesis efficiency, and probably decreases generation of excess energy, which can produce ROS that are toxic to plants. Unlike *P. trifoliata*, in *C. unshiu*, as mentioned before

one of the most cold hardy commercial *Citrus* species, genes involved in osmotic adjustment, photo-oxidative protection and photosynthesis repression are not detected under cold stress. This might be due to the differences in cold acclimation between these two species (Lang et al. 2005). More recently, the early response of *Poncirus* to cold has been explored in detail, and the results demonstrated that although a few genes are commonly induced in response to both short and long-term cold acclimation, majority of early cold-responsive genes are different from previously identified late cold-responsive genes (Şahin-Çevik and Moore 2006; Şahin-Çevik 2013). Although several cDNA clones showing homology with heat shock proteins (HSPs), responsible for the functional conformations of the protein, and late embryogenesis abundant proteins (LEA), involved in membrane and protein stabilization, were identified in short cold period, their expression is not increased significantly enough to be classified as cold induced genes. This result suggests that HSPs and LEA proteins are possibly involved in relatively late (Şahin-Çevik and Moore 2006) response rather than in the early response to cold stress (Şahin-Çevik 2013). In addition, in order to respond to the required availability of a wide range of cold responsive genes to be employed in the genetic transformation studies, a huge amount of investigations have been focused on the identification of cold responsive genes during fruit postharvest storage at low temperatures (Sanchez-Ballesta et al. 2003; Lo Piero et al. 2005, 2014; Maul et al. 2008; Zhu et al. 2011; Crifò et al. 2011, 2012; Lo Piero 2015; Lafuente et al. 2017; Pannitteri et al. 2017). This also because data clearly show that cold responsive genes are differentially expressed among tissues pointing out the importance of analyzing the transcriptional changes of specific organs (Ganeshan et al. 2008). Although the topic is interesting, it probably lies outside of the aim of this book chapter, then, for completeness of information, referring to the published literatures is needed. However, considering their peculiarity, I report some of the results obtained after the post-storage exposure of blood oranges cv Tarocco Sciara to low temperature, during that the change in gene expression was evaluated. Overall, it was observed the enhancement of transcripts involved in the defense mechanisms against oxidative damage, osmoregulation processes, lipid desaturation as well as many ESTs implicated in the primary and secondary metabolisms. In particular, the results showed that cold stress induces transcriptomic modifications directed towards the increase of flavonoid biosynthesis, including those reactions involved in anthocyanin biosynthesis, thus indicating that anthocyanins have a crucial role as protective antioxidant molecules during cold stress in pigmented fruits (Crifò et al. 2011, 2012). Few studies evaluated the response of orange fruits to freezing stress *in planta* in order to understand the biochemical and molecular basis of the changes that later derive in internal and external damage symptoms (Perotti et al. 2015). Using two-dimensional differential gel electrophoresis to analyze exposed and non-exposed fruits of Valencia Late sweet orange, proteins and compounds involved in regulatory functions, iron metabolism, oxidative damage and carbohydrate metabolism are found to be the most affected. Interestingly, three glycolytic enzymes are induced by cold, and there is an increase in fermentation products (volatiles); all of that suggests that more energy generation might be required from glycolysis to counter the cold stress. Moreover, a notable increase in sugar levels is observed after frost, but it is not at the expense of organic acids utilization. Consequently, these results suggest a probable redistribution of photo-assimilates in the frost-exposed plants, tending to restore the homeostasis altered by this severe type of stress (Perotti et al. 2015). Several studies have reported that tetraploidization is an effective way to improve stress tolerance in *Citrus* (Allario et al. 2013; Tan et al. 2015; Ruiz et al. 2016). Recently, Oustric et al. (2018) demonstrated that genome doubling of the allotetraploid somatic hybrid FlhorAG1 (FL-4x) can confer greater tolerance to cold stress than the diploid parents, the Willowleaf mandarin (WLM-2x) and the *Poncirus* Pomeroy (POP-2x) and their respective doubled-diploids (WLM-4x and POP-4x). When

subjected to cold, FL-4x shows less accumulation of oxidative markers than diploid and doubled-diploid WLM and POP genotypes, and a sharp increase in some antioxidant activities (superoxide dismutase SOD, ascorbate peroxidase APX, and glutathione reductase GR) suggesting that greater antioxidant capability in FL-4x should make this allotetraploid hybrid more tolerant to low temperatures than the two WLM genotypes. Low temperatures are one of the most important environmental cues to induce flowering (Davenport 1990; Krajewski and Rabe 1995). Species belonging to the genus *Citrus* are evergreen trees and have interesting and distinct flowering behaviors. After several years in the juvenile phase, adult citrus trees show seasonal periodicity of flowering. In subtropical climates, floral induction occurs from autumn to early winter. After the floral inductive period, citrus trees start the morphological development of floral buds in winter and then bloom in spring without a long rest period. Genetic analyses showed that gene FLOWERING LOCUS T (FT) is involved in the low temperature induction of flowering in Satsuma mandarin (*Citrus unshiu* Marc.) (Nishikawa et al. 2007). In adult trees, the expression of total CiFT in stem and leaf tissues is enhanced by the low-temperature (15 °C) treatment, and accompanies an increase in the flowering potential. More recently, flower bud development, pollen performance both in vitro and in vivo during the progamic phase, and early fruit development have been characterized in a self-incompatible clementine variety under different temperature treatments (15, 20, 25 or 30 °C) simulating the range from cool to hot weather (Distefano et al. 2018). At 15 °C (the lowest temperature) flower buds grow very slowly and reach their final size after 14 days. Compared with this, growth is significantly accelerated at higher temperatures, from 15 to 30 °C, leading to much more rapid flower bud development and much earlier anthesis. Overall, the aforementioned results suggest that, in citrus, low temperatures are essential to trigger flowering in adult trees but once the flower bud is formed warmer temperatures induce a quicker development of the flower buds.

13.4 Heat and Flooding

Climate change includes changing precipitation patterns along with different episodes of extreme heat, probably making the use of traditional rootstocks becomes compromised. Cleopatra mandarin, frequently used as a rootstock due to its tolerance to high salinity levels and viruses, is a sensitive genotype to heat stress, partially as a result of a high incidence of oxidative stress (Zandalinas et al. 2016a). This was likely associated to a poor efficiency of the antioxidant machinery of this citrus genotype (Arbona et al. 2008), especially under conditions of drought and heat stress acting simultaneously (Zandalinas et al. 2017a). Furthermore, the oxidative damage observed in Cleopatra leaves under these stress conditions could be associated to the effect on photochemistry (Sharma et al. 2012). In this sense, PSII was identified as the most heat-sensitive component of the photosynthetic system (Sharma et al. 2012). Salycilic acid is a phytohormone that could regulate thermotolerance mechanisms such as maintenance of membrane integrity (Clarke et al. 2004) and protection of PSII complex (Wang et al. 2014). Therefore, the remarkable salycilic acid accumulation and increased signaling during heat stress could be related to the strong sensitivity of this citrus genotype to high temperatures and aimed to reduce the damage to PSII and protect against the effect of the oxidative damage. In addition to salycilic acid, ABA represents a key phytohormone which is repressed by heat stress probably avoiding stomatal closure, and keeping high transpiration rates to cool leaf surface. Heat stress does not vary either ABA levels or *Cs*NCED1 expression in leaves of Cleopatra, however, an induction of ABA catabolism is observed (Zandalinas et al. 2016b). Carrizo plants are found to be more tolerant to high temperatures than Cleopatra mandarin as PSII performance are affected only by the stress combination. Furthermore, higher transpiration and photosynthetic rates along with reduced oxidative damage are also identified as additional traits behind this increased tolerance (Zandalinas et al. 2016b).

Poor soil drainage combined with excessive rainfall or irrigation can produce soil and submerged tissues waterlogging referred as flooding. One major constraint deriving from excess water is the progressive reduction in the soil O_2 concentration and a decline of aerobic root respiration, which impairs ATP synthesis disturbing the whole plant metabolism. Although the response is variable among species and cultivars (García-Sánchez et al. 2007), *Citrus* is considered a flooding-sensitive crop that responds to waterlogging by restricting stomatal conductance. Nutritional imbalance changes in nutrient partitioning may appear in waterlogged plants, depending on plant species and soil type (Pezeshki 2001); in Carrizo citrange, flooding reduces nitrogen concentration as a consequence of impaired uptake and transport, and the observed changes in carbohydrate distribution suggest that translocation from leaves to roots is reduced, leading to significant starch accumulation in leaves and further decreases in roots (Martínez-Alcántara et al. 2015). In addition, flooding inhibits both Fe-uptake as result of the synergistic inactivation of H^+-ATPase and ferric chelate reductase that represents the preferential regulator of the Fe acquisition system under flooding conditions (Martínez-Cuenca et al. 2015).

13.5 Heavy Metals

Heavy metals are one of the most important chemical pollutants in the world (Chehregani and Malayeri 2007). Although they occur naturally in the soil, heavy metals also enter in the environment through chemical wastes, chimney gases, industrial products, pesticides and chemical fertilisers (Cope 2004). Plants can take-up heavy metals from the soil and water via their roots and from the atmosphere via the leaves (Wedding et al. 1975; Cataldo and Wildung 1978). Heavy metals such as Fe, Mn, Zn and Cu are microelements for plant growth and development and become very toxic in high concentrations (Haynes and Swift 1985). Instead, Cd, Hg and Pb have no biological function in plants and are toxic even at very low concentrations (Doganlar and Yurekli 2009). Heavy metals induce morphological and physiological changes in plants including chlorosis and inhibition of seed germination and root-shoot development (Ouzounidou 1994; Aksoy et al. 2000; Kiran and Sahin 2005; Doganlar and Atmaca 2011). In addition, these elements cause biochemical changes such as formation of ROS which results in altered levels of enzymatic and non-enzymatic antioxidants and proteins, decreases in pigment content and lipid peroxidation (Peralta-Videa et al. 2004; Kiran and Sahin 2005). Plant responses to high concentrations of heavy metals generally imply activation of the sulphur assimilation pathway to provide an enhanced supply of GSH for the biosynthesis of phytochelatins (PCs), which play a major role in metal sequestration (Cai et al. 2011). With increasing of pollutions such as the extensive usage of Cu- and Mn-containing bactericides in citrus production, heavy metals toxicity has become a threat for citrus genotypes. Cleopatra mandarin and Carrizo citrange rootstocks are relatively tolerant to Cd^{2+} and do not show leaf damage after prolonged period of being watered with 300 μM Cd^{2+}. Citrus roots efficiently retain Cd^{2+} avoiding its translocation to the shoots and Cleopatra mandarin translocates less Cd^{2+} than Carrizo. With increasing Cd^{2+} concentrations all gas exchange parameters decrease more in Carrizo than in Cleopatra mandarin (López-Climent et al. 2011). In contrast, the same genotypes watered with higher Cd^{2+} concentration (3 mM) show damage within a few days, significantly decreasing the photosynthetic rate, transpiration and stomatal conductance. *Citrus* roots under the aforementioned different concentrations of Cd^{2+} either do not modify or reduce their levels of abscisic, salicylic and jasmonic acids thus indicating that there is no specific hormonal response to the metal in these citrus genotypes. Moreover, the data indicate that, although Cd^{2+} exclusion is a powerful mechanism to avoid heavy metal raising into photosynthetic organs, the capacity to maintain optimum GSH levels to feed phytochelatin biosynthesis could also be an important factor in stress tolerance (López-Climent et al. 2014).

Various other protein families have been shown to play the key roles in avoiding heavy metal toxicity as plants have developed a series of strategies that include the tight regulation of metal uptake, efflux, chelation, trafficking, and intracellular sequestration (Clemens et al. 2002; Hall 2002). Cation diffusion facilitator (CDF) family proteins, usually called metal tolerance proteins (MTPs), are one class of important metal transporters that are involved in metal efflux from the cytoplasm, either to the extracellular space or into subcellular compartments. Sequencing and assembly of the sweet orange genome (Xu et al. 2013) provided the opportunity to perform a systematic analysis of this gene family by a genome-wide identification and to analyze their expression patterns in roots and leaves under Zn, Mn, Cu, and Cd toxicity (Fu et al. 2017). A total of 12 MTP genes have been identified in sweet orange and expression analysis indicated that most CitMTP transcripts are upregulated to various extents under heavy metal stress in different plant tissues. These findings provide systematic information on each of these citrus MTP genes may be utilized in future studies once individuated their specific roles in heavy metal detoxification (Fu et al. 2017). Finally, *Citrus* dehydrin, which accumulates mainly in response to cold stress (Crifò et al. 2011), shows metal binding capacity (Hara et al. 2005). Among the bound metals, the highest affinity was detected for Cu^{2+}- binding using a specific sequence containing His thus reducing metal toxicity in plant cells (Hara et al. 2005).

13.6 Combined Abiotic Stress

In the field, all plants undergo exposure to several abiotic stresses occurring simultaneously and interacting each other leading to a highly complex response that might be different or of different extent from that obtainable from the single stress type. Drought and high temperatures are two major abiotic stress factors that often occur simultaneously in nature, constituting a unique stress factor (Zandalinas et al. 2016a, b, 2017a, b, 2018). In two citrus genotypes, Carrizo citrange and Cleopatra mandarin, combined stress causes more injuries than each of the adverse environmental factors acting in isolation. Carrizo was found to be more tolerant than Cleopatra to high temperatures, applied individually or in combination with drought. This contrasting ability of Carrizo e Cleopatra plants to tolerate combined stress treatments seems to be a consequence of their different semi-polar metabolite profile (Zandalinas et al. 2017a). In particular, the activation of secondary metabolism is associated to combined stress sensitivity. Therefore, the increased basal tyrosine levels registered in Cleopatra plants correlates with its higher flavonoid concentration and could also account for the greater induction of these metabolites in this genotype. Due to its high sensitivity, Cleopatra requires a deep alteration of its primary and secondary metabolism in order to cope with stress-induced physiological and biochemical imbalances (Zandalinas et al. 2017a) whereas Carrizo does not. In addition, some responses to combined stress conditions such as ABA accumulation and stomatal conductance changes vary depending on the intensity of water stress (Zandalinas et al. 2018). During combined conditions of moderate drought and heat, stomatal conductance decreases despite the reduction of ABA levels supporting the idea that other mechanisms could be involved in regulating stomatal responses during a combination of drought and heat stress. In this sense, previous reports suggested that H_2O_2 and jasmonic acid (JA) could signal stomatal closure in plants subjected to combined drought and heat independently of ABA signaling by enhancing nitric oxide levels and triggering Ca^{2+} and SLAC1 function, a multispanning membrane protein expressed predominantly in guard cells that plays a role in regulating cellular ion homeostasis and S-type anion currents (Zandalinas et al. 2016a). Regarding the interaction of salt stress with other abiotic environmental factors, it has been shown that it often occurs along with poor soil drainage, flooding, high temperature and evaporative demand, drought, boron toxicity, and/or nutrient deficiencies and imbalances (Syvertsen and Garcia-Sanchez 2014). In addition, the

anticipated increase of CO_2 concentration in the atmosphere (IPCC 2014) will affect crop responses to all stresses including salinity. Boron can also be toxic to citrus plants since they are very sensitive to high B in irrigation water (Zandalinas et al. 2018).

13.7 Concluding Remarks

The content of this chapter highlights that in recent decades, citrus physiology and biochemistry under abiotic stresses have been extensively investigated. Field studies have revealed wide genotypic variability and disclosed several of the processes involved in *Citrus* tolerance and resistance. Many studies identified stress-regulated genes in *Citrus* and in *Citrus* relatives, such as *Poncirus*, and indicated the conservation of similar response pathways between these woody plants and *Arabidopsis*, an herbaceous plant. Moreover, in some cases, such as drought and cold stress, a common mechanism of stress perception and signal transduction has been proved thus suggesting a cross talk between different defense patterns. This investigation of the relationships among responses to various environmental stresses is highly required to cope with the announced changing climate, which will lead to rising temperatures, increase in precipitation abnormalities and freezing temperatures, and salinization that will expose *Citrus* trees to concurrent environmental constraints, probably also altering their ability to cope with biotic stress. Consequently, major efforts should be focused on the elucidation of the effects of combined stress and identification of tolerant genotypes, this knowledge being lacking at this moment or restricted to few stress types. The recently released genome and transcriptome analyses yielded new insights into the origin of sweet orange and provide a rich resource of genetic information for citrus breeding and genetic improvement. However, *Citrus* breeding programs for producing stress tolerant citrus varieties following conventional methods is strongly impeded by biological problems. The use of genomics and molecular biology techniques such as the novel genome editing techniques can take advantage of the existing knowledge of a huge amount of candidate genes for stress coping and can provide alternative approaches for generation of novel germoplasm with improved stress tolerance in *Citrus*.

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Biotechnological Approaches for the Resistance to Citrus Diseases

14

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Abstract

Citrus is one of the top fruit crops worldwide. Citrus production faces many challenges such as diseases, insects, and abiotic stresses. Genetic improvement of citrus using conventional breeding is a lengthy, costly, and time-consuming process. Biotechnological approaches such as *Agrobacterium*-mediated transgenic expression, Citrus tristeza virus (CTV)-mediated transient expression and CRISPR-based genome editing have shown tremendous potential to improve citrus against different diseases. Here, we summarize the progress in generating disease-resistant/tolerant citrus plants via biotechnological approaches.

Citrus is an economically important fruit crop grown in tropical and subtropical regions of the world. In recent years, citrus industry has been under immense pressure to develop new germplasm to overcome barriers to production from diseases, insects, and abiotic stresses. Especially, citrus Huanglongbing caused by *Candidatus* Liberibacter presents an unprecedented challenge to citrus production worldwide (Wang et al. 2017). Genetic improvement of citrus using conventional breeding is a lengthy and challenging process due to the complex reproductive biology of citrus including sexual incompatibility, highly heterozygous nature, nucellar seedlings, male or female sterility, and the long juvenile phase (Omura and Shimada 2016). Biotechnological approaches provide a promising alternative to engineer citrus plants that can resist the many abiotic and biotic stresses. The biotechnological approaches include the commonly used *Agrobacterium*-mediated transgenic expression, citrus tristeza virus (CTV)-mediated transient expression, and the forthcoming CRISPR-based genome editing. Here, we summarize the progress in generating disease-resistant/tolerant citrus plants via biotechnological approaches.

14.1 *Agrobacterium*-Mediated Transgenic Expression

The advent of genetic transformation technologies has facilitated the rapid germplasm improvement of citrus. *Agrobacterium*-mediated transformation, protoplast transformation, and particle bombardment methods have been successfully applied in various crop plants (Hansen

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and Wright 1999). These techniques allow us to introduce the gene(s) of interest into the genome of cultivars, modify, or silence selected genes of a cultivar.

Citrus tissues can be transformed by several methods. The *Agrobacterium*-mediated transformation process is the most commonly used technique. This process utilizes different explants as source tissues for transformation by the *Agrobacterium*. Among them, juvenile in vitro epicotyl segments (Dutt and Grosser 2009; Moore et al. 1992; Luth and Moore 1999) and many others, mature internode segments obtained from greenhouse-grown plants (Cervera et al. 1998; Almeida et al. 2003), or embryogenic callus obtained from unfertilized ovules (Dutt and Grosser 2010; Li et al. 2002) are the commonly used source tissues. Direct incorporation of DNA into protoplasts using electroporation (Niedz et al. 2003), biolistics (Wu et al. 2016), or PEG mediated (Olivares-Fuster et al. 2002; Guo et al. 2005; Omar et al. 2007; Fleming et al. 2000) have also been utilized.

Enhanced Biotic and Abiotic Stress Management Using a Transgenic Approach

Huanglongbing (HLB) caused by the phloem limited *Candidatus* Liberibacter asiaticus (*C*Las) (Jagoueix et al. 1996) has become a major issue globally, especially in citrus growing regions of the United States, China, and Brazil (da Graça et al. 2016). Several transgenic solutions have been devised to combat this disease. Mirkov and Gonzalez-Ramos (2013) claimed that constitutive overexpression of a spinach defensin gene resulted in enhanced HLB tolerance. Both constitutive overexpression and phloem targeted expression of the *Arabidopsis* NPR1 in the sweet orange cultivars Hamlin and Valencia (Dutt et al. 2015) resulted in the production of HLB tolerant transgenic sweet oranges. This was the first scientific report on transgene mediated resistance to HLB and its overexpression resulted in significantly lower HLB incidence when compared to non-transformed plants. Antimicrobial peptides have demonstrated promise against combating HLB. Dutt et al. (2008) overexpressed several antimicrobial peptides in the sweet oranges Hamlin and Valencia. Stover et al. (2013) screened several antimicrobial peptides in vitro for use in developing transgenic citrus resistant to HLB. A modified thionin peptide gene was observed to reduce the Liberibacter asiaticus (Las) titer in roots and scion of transgenic Carrizo rootstock, 12 months after graft inoculation (Hao et al. 2016). Similarly, phloem targeted expression of the *cecropin* B gene resulted in decreased susceptibility to HLB in sweet orange (Zou et al. 2017).

Antimicrobial peptides have been more extensively evaluated for citrus canker tolerance. Citrus canker caused by *Xanthomonas citri* ssp. *citri* is also a global problem resulting in leaf-spotting and fruit rind blemishing and can result in fruit drop and unmarketable fresh fruit (Brunings and Gabriel 2003). Expression of a dermaseptin gene in sweet orange plants reduced citrus canker symptoms (Furman et al. 2013) while the sarcotoxin IA gene reduced canker symptoms in transgenic sweet orange (Kobayashi et al. 2017). Introduction of the Attacin A (*attA*) gene into sweet orange cultivars Hamlin and Valencia reduced the canker disease symptoms (Cardoso et al. 2010; Boscariol et al. 2006). Transgenic plants regenerated via *Agrobacterium* transformation of mature axillary buds with antibacterial peptide genes Shiva A and *Cecropin* B showed enhanced resistance to canker (He et al. 2011). More recently, transgenic Carrizo plants expressing D2A21 peptide were developed which showed significant resistance to canker but not HLB (Hao et al. 2017). In addition to antimicrobial peptides, pathogen-related genes responsible for systemic acquired resistance such as *hrpN* or the *AtNPR1* were used with *Agrobacterium* transformation experiments to develop canker-resistant plants (Barbosa-Mendes et al. 2009; Zhang et al. 2010). Also, the rice derived *Xa21* gene was introduced into citrus via *Agrobacterium* (Mendes et al. 2010) and protoplast transformation systems (Omar et al. 2007) for citrus canker resistance. Recently, canker

tolerance was also demonstrated in transgenic W. Murcott plants overexpressing Xa21 under greenhouse conditions (Omar et al. 2018). Reduced susceptibility to citrus canker was also observed in transgenic sweet orange plants overexpressing the *MdSPDS1* gene responsible for polyamine biosynthesis (Fu et al. 2011a).

In addition to HLB and citrus canker resistance, transgenic strategies have also been utilized for resistance against CTV (Febres et al. 2003; Dominguez et al. 2000; Ghorbel et al. 2000; Gutierrez et al. 1997), C*itrus psorosis virus* (Zanek et al. 2008), *Phytophthora* spp (Fagoaga et al. 2001; Azevedo et al. 2006) and other biotic stresses. A commercially important Rio Red grapefruit was transformed with CTV untranslatable coat protein gene (*uncp*) and plant-derived insecticidal *Galanthus nivalis* agglutinin (*gna*) gene with an aim to protect the plants from CTV and aphids that transmit this virus (Yang et al. 2000). In addition to coat protein, transgenic plants developed by introduction of antisense constructs of CTV *RdRp* gene into epicotyl segments of grapefruit also exhibited enhanced resistance to CTV infection (Cevik et al. 2006). Fruit aroma chemistry has also been modified to improve resistance to pathogens and insect pests by introducing antisense constructs of genes responsible for terpene biosynthesis that down-regulate the production of terpenes in fruit peels (Rodriguez et al. 2011).

Apart from biotic stresses, transgenic approaches have been utilized for reducing chilling injury in Carrizo citrange and *Poncirus trifoliata* by suppressing the ethylene production with the introduction of ACC synthase antisense transgene *CS-ACS1* (Wong et al. 2001). Drought tolerance and osmotic adjustment were enhanced in rootstock Carrizo citrange by incorporating proline synthesis *p5cs* gene (Molinari et al. 2004). Being a commercially important rootstock, halotolerance gene *HAL2* extracted from *Saccharomyces* yeast was introduced into Carrizo citrange via *Agrobacterium* method for improving the performance of this rootstock in saline conditions (Cervera et al. 2000). Salt tolerance was also enhanced in trifoliate orange rootstock with the incorporation of betaine aldehyde dehydrogenase (*BADH*) gene which leads to synthesis of the osmoprotectant glycine betaine (GB) (Fu et al. 2011b).

14.2 CTV-Mediated Expression

The effect of HLB on the citrus industry is devastating and a quick solution is necessary to maintain it in Florida. As the causal bacterium is not cultured yet, direct in vitro screening is not possible. CTV and Las co-localize to the phloem tissue of their citrus host where the Asian citrus psyllid (ACP) feeds. Thus, CTV-based expression and/or RNAi vectors are being used as a screening tool to identify potential therapeutic products. Furthermore, due to its unusual stability and because it can be deployed rapidly, it is being considered as an interim control measure until transgenic or CRISPR/Cas9 plants can become available.

CTV was first reported in Argentina causing quick decline on the sour orange rootstock (Bar-Joseph et al. 2010). CTV is endemic to Florida. Some isolates of CTV in other parts of the world are extremely virulent and prevent profitable production of citrus. Most Florida isolates cause mild symptoms in most citrus genotypes. One exception is T36 that causes decline and death of trees on the sour orange rootstock. The decline has been avoided in Florida simply by using other rootstocks. On other rootstocks T36 isolates cause even milder symptoms than other isolates. Currently, a T36-based CTV vector is being used to overexpress genes or induce RNA interference (RNAi).

CTV, a positive-sense 19.3 kb single-stranded RNA virus, is a member of the closteroviridae. The RNA genome is organized into 12 open reading frames (ORFs), which are expressed via three different strategies (Karasev et al. 1995). The first strategy is a poly-protein strategy with post-translational processing. The second strategy is a +1 ribosomal frameshift that allows continued translation beyond a stop codon. Both strategies are used to express ORF1a and 1b,

which are involved in RNA replication. The 3' half of the genome is organized into 10 ORFs. Hsp70 homologue, p61, CPm, CP, and p20 ORFs are involved in virion formation (Peremyslov et al. 2004; Satyanarayana et al. 2000). CP, p20, and p23 are silencing suppressors with different modes of action (Lu et al. 2004). P6 is a potential movement protein. All these genes are essential for the systemic infection of all citrus plants. P33, p18, and p13 are host range determinants not necessary for the infection of some citrus hosts (Tatineni et al. 2008). The 10 ORFs are expressed via a nested set of 3'coterminal sub-genomic RNAs (Hilf et al. 1995). The transcription of each ORF is driven by a subgenomic controller element upstream of the coding sequence (Gowda et al. 2001). The 3' half of the genome was explored for expression of foreign sequences via different strategies to develop CTV-based expression/RNAi vectors (Folimonov et al. 2007; El Mohtar 2011; EL Mohtar and Dawson 2014; Hajeri et al. 2014).

CTV as an Expression/RNAi Vector

The first CTV vector had an extra gene inserted between the minor and major coat protein ORFs (Folimonov et al. 2007). Further studies identified three new locations within CTV that can differentially express genes of different lengths (El Mohtar 2011; EL Mohtar and Dawson 2014). The first two positions had the extra gene added between p13 and p20 or between p23 and 3' nontranslated region (NTR). In addition, the substitution of the p13 ORF and its controller element was successful. The different positions could be combined to express multiple foreign genes from the same vector. Using green fluorescent protein (GFP) as a reporter gene, CTV vectors have been shown to be exceptionally stable. There are trees infected with CTV vectors that are still expressing GFP almost 15 years after inoculation. In 2012, the vector was used to efficiently induce silencing of the phytoene desaturase (PDS) citrus gene via RNAi (Hajeri et al. 2014). The plants are still showing the bleaching phenotype characteristic of PDS silencing 6 years after inoculation.

CTV-Mediated Expression of Antimicrobial Peptides and Proteins

CTV is being used to transiently express small antimicrobial peptides (AMPs, 10-70 amino acids) to either directly target Las or to help the plant tolerate the infection. Targeting the bacteria directly is based on expressing small antimicrobial peptides that kill or reduce Las titer in the plant. More than 100 AMPs with different modes of action have been or are being screened for activity against Las. Furthermore, potential PAMPs peptides of Las are being expressed through CTV-based expression vectors to trigger the citrus plant defenses. CTV vectors are also being used to express lytic phage proteins that are directed against the bacterial cell wall causing its disintegration and killing the bacteria. Two proteins from the Las prophage have been selected for CTV-mediated expression.

Use of CTV RNAi Vectors Against Psyllids

A major goal is the deployment of CTV RNAi vectors against the psyllid. The idea is to introduce truncated sequence of psyllid genes into the CTV vector. The plant will load these genes into its silencing machinery producing abundant amounts of siRNA in phloem cells. Upon feeding on the citrus phloem, the ACP insect acquires the siRNA, which silences the psyllid gene and prevents reproduction of the next generation of psyllids. More than 20 ACP genes are being targeted for silencing. For example, it has been used to transiently express truncated abnormal wing disc (*Awd*) gene of *Diaphorina citri*, the insect vector of Las. Consequently, feeding *D. citri* nymphs led to altered *Awd* expression and malformed-wing phenotype in adults and increased adult mortality (Hajeri et al. 2014).

Prescreening for CRISPR-Cas9 Genome Editing Genes

Many researchers believe that CRISPR-Cas9 could be used to successfully engineer resistance/tolerance to HLB in citrus by editing either susceptibility genes or negative regulators of plant defense. However, CRISPR-Cas9 is a difficult technique to employ in citrus on a list of potential target genes. Thus, CTV is being used to

induce RNAi against citrus genes to prescreen potential genes for targetting using the CRISPR/Cas9 technique. Around 40 plant genes are being targeted for silencing by CTV RNAi vectors. The major advantage of using the CTV vector to silence genes is speed. Although nothing is fast in citrus, silencing a potential gene using the CTV vector is much quicker and easier than directly using CRISPR/Cas9 modification. Using CTV also has additional advantages. One is that several different vectors with potential targets can be examined in parallel. Most of the potential targets will be examined in the first screening run. Also, more than one target gene sequences can be inserted into the same vector to silence more than one plant gene. Thus, if more than one gene was predicted to be modified by CRISPR to provide tolerance, these genes could be silenced simultaneously by the CTV vector. Perhaps more importantly, the CTV vector can be graft transmitted to mature plants, allowing the determination of the effect of the silenced gene on mature characteristics such as fruit development and juice flavor. Finally, if silencing a target gene using the CTV vector results in HLB resistance or tolerance, this in itself could be used as a short-term management possibility for HLB in the field.

14.3 CRISPR Technology in Citrus Disease Management

The CRISPR/Cas modules are adaptive immune systems of prokaryotes against invading phages and plasmids by cleaving the foreign DNA, or, in some cases, RNA, in a sequence-dependent manner (Jinek et al. 2012; Barrangou et al. 2007). Approximately 84% of archaea and 48% of bacteria genomes contain CRISPR-Cas systems (Marraffini 2013). A CRISPR locus consists of a CRISPR array and diverse *cas* genes. The CRISPR array comprises short direct repeats interspaced by variable DNA spacer sequences which are acquired from virus and plasmid genes. The spacers enable the recognition and cleavage of the invasive viruses and plasmids (Barrangou et al. 2007). CRISPR/Cas-mediated adaptive immunity consists of three stages: adaptation, expression, and interference (van der Oost et al. 2009; Wiedenheft et al. 2012; Barrangou and Marraffini 2014; Marraffini 2015). During the adaptation stage, short pieces of foreign DNA (called protospacers) from invading viruses or plasmids are processed and incorporated into the CRISPR loci (Barrangou et al. 2007; Garneau et al. 2010). In the expression stage, the CRISPR array is transcribed, which is further processed into mature CRISPR RNAs (crRNAs). The pre-crRNA binds to either Cas9 or to a multisubunit complex, forming the crRNA-effector complex after further processing involving bacterial RNase III and transactivating CRISPR RNA (tracrRNA) (Deltcheva et al. 2011) or by an endonuclease subunit of the multisubunit effector complex. The interference stage involves crRNA-directed cleavage of invading cognate virus or plasmid nucleic acids by Cas nucleases.

The continuous arms race between prokaryotes and invading viruses and plasmids have driven rapid evolution of highly diverse CRISPR-Cas systems (Takeuchi et al. 2012; Koonin and Wolf 2015). Based on the repertoire of *cas* genes, the sequence similarity between Cas proteins and the locus architecture, the CRISPR-Cas systems have been classified into two classes that are subdivided into six types (Makarova and Koonin 2015; Shmakov et al. 2015). The Class 1 systems are present in bacteria and archaea and include the most common and diversified type I, type III that is mainly presented in archaea, as well as the rare type IV (Koonin et al. 2017). Class 1 systems encompass effector complexes composed of four to seven Cas protein subunits. The Class 2 systems (types II, V, and VI) are less common and are mostly restricted to bacteria. Class 2 effector complex consists of a single multidomain protein represented by Cas9 and Cpf1 (Makarova et al. 2015). The ability to easily program sequence-specific DNA targeting and cleavage by CRISPR-Cas components render them a very useful tool for genetic engineering in a wide range of eukaryotes including various plant species and prokaryotes (Mohanraju et al. 2016).

CRISPR-Cas9 mediated genome editing of citrus has been successfully conducted previously (Jia and Wang 2014a; Jia et al. 2016b; LeBlanc et al. 2017; Peng et al. 2017; Zhang et al. 2017; Jia et al. 2017b).

Major Tasks in the Application of CRISPR Technology for Genome Editing of Citrus

There are multiple tasks or challenges facing the application of CRISPR technology for gene editing of crops especially citrus: identification of critical traits for targeting, foreign DNA free in modified plants, off-target issue, expanding the toolbox of genome editing, and optimizing the procedure and improving the efficacy.

Critical traits for targeting. Genome editing can be used to improve many different aspects of citrus such as color, nutrition, metabolic engineering, quality, yield, seedlessness, and stress resistance (both biotic and abiotic stress). CRISPR-mediated genome editing has been successfully used to generate disease-resistant citrus varieties against bacterial canker disease caused by *Xanthomonas citri* (Jia and Wang 2014a; Jia et al. 2017b; Peng et al. 2017). Specifically, Cas9/sgRNA has been utilized to modify the PthA4 effector binding elements (EBEs) in the promoter region as well as the coding region of the *CsLOB1* (*Citrus sinensis* Lateral Organ Boundaries) gene (Jia et al. 2016a, b). *CsLOB1* is a susceptibility gene for citrus canker, which is induced by the pathogenicity factor PthA4 via its binding of the EBE_{PthA4}-CsLOBP (Hu et al. 2014). Genome editing of the coding region of the disease susceptibility gene *CsLOB1* in citrus leads to the development of canker resistant plants (Jia et al. 2016a, b). Deletion of the entire EBE_{PthA4} sequence from both *CsLOB1* alleles confers a high degree of resistance to citrus canker (Peng et al. 2017).

Foreign DNA free in genome-modified plants. To avoid all the headaches of deregulations related to transgenic and GMO (genome-modified organisms) plants (Hartung and Schiemann 2014), it is critical that the genome-modified plants do not contain foreign DNAs originating from pathogens or other organisms that are not naturally associated with plant chromosomes during evolution, traditional crossing, conventional mutagenesis, or sexually compatible species. Plants modified by the CRISPR technology have potentials to be free of foreign DNAs and to be indistinguishable from plants generated by conventional breeding or mutagenesis. Plants stably transformed with CRISPR/Cas may contain unwanted insertions of plasmid DNA at both on-target and off-target sites (Woo et al. 2015). Even though the foreign DNA may in principle be removed by genetic segregation, this is not feasible in plants that reproduce asexually. Specifically, the crossing approach for citrus is laborious and time-consuming, particularly considering the long juvenile period for citrus. Backcrossing of citrus will lead to loss of traits of the parental cultivars. Additionally, constant expression of Cas9/sgRNA in transgenic plants may lead to accumulation of off-target effects. Transient expression of either Cas9-sgRNA ribonucleoproteins, Cas9/sgRNA DNA or RNA has been used successfully to generate non-transgenic genome-modified plants (Zhang et al. 2016; Liang et al. 2017; Svitashev et al. 2016; Woo et al. 2015). Recently, Cas9/sgRNA DNA and Cas9-sgRNA ribonucleoproteins have been used successfully to edit the genes of protoplast cells of citrus in the Wang lab.

Expanding the toolbox of genome editing. The specificity of CRISPR/Cas9 mediated gene editing is determined by both the sgRNA and PAM, which, on the other hand, also limits the repertoire of sequences that it can target. For Cas9/sgRNA based on *Streptococcus pyogenes*, the PAM sequence of 5'-NGG-3' is required (Cong et al. 2013). Multiple Cas9 orthologs from type II CRISPR-Cas systems, which recognize different PAMs, have been characterized and engineered for genome editing. For example, SaCas9 of *Staphylococcus aureus* recognizes NNGRRT or NNGRR(N) (Kleinstiver et al. 2015a; Ran et al. 2015), the PAM sequence for StCas9 of *Streptococcus thermophiles* is NNAGAAW (Deveau et al. 2008), and the PAM for NmCas9 of *Neisseria meningitides* is NNNNGATT (Hou et al. 2013). Interestingly, Cpf1 is the effector protein for type V CRISPR-

Cas system which recognizes a PAM sequence of 5'-TTN-3' (Zetsche et al. 2015). The different PAM sequences recognized by Cas9 orthologs have significantly increased the repertoire of sequences that are suitable for site-directed mutagenesis. In addition, modification of the PAM-binding domain of Cas9 can change the PAM specificities (Kleinstiver et al. 2015b), which further expanded the use of genome editing. Both SpCas9/sgRNA and SaCas9/sgRNA have been successfully used to conduct genome editing of citrus (Jia and Wang 2014a; Jia et al. 2017a).

Optimization of the CRISPR-Cas9 mediated genome editing of citrus. Optimization of CRISPR-Cas9 mediated genome editing of citrus includes optimization of delivery of genome engineering reagents and improving the design of Cas9-sgRNA. Here, we summarize the current progress in the relevant areas in citrus biotechnology.

Delivery of genome editing reagents into citrus cells is the major barrier for successful genome modification. The delivery methods include plasmid transformation by biological organisms such as Agrobacterium and viruses (e.g., CTV) as well as reagents delivery via protoplast transfection and microprojectile bombardment. Previously, it has been difficult to conduct agroinfiltration-mediated transient expression in citrus leaves. Pretreatment of citrus leaves with *Xanthomonas citri* significantly enhanced transient protein expression in citrus leaves and delivery of Cas9/sgRNA (Jia and Wang 2014a, b). This has been suggested to be due to the excessive cell division caused by *X. citri* infection, which mimics the fast dividing epicotyl segment suitable for *Agrobacterium*-mediated transformation.

Optimization of expression of Cas9-sgRNA has been used to improve the efficacy of genome editing in plants. Cas9 has been traditionally driven by 35S promoter whereas sgRNA has been driven by U3 or U6 promoter. sgRNA is a small non-coding RNA and requires an accurate 5'-end to keep its target-specific sequence. Transcripts from U3 and U6 promoters start with the nucleotides "A" and "G", respectively, thus restricting the targeting range and potentially the efficiency of Cas9. RNA processing systems have been engineered for sgRNA processing: tRNA processing system (Xie et al. 2015), self-cleaving ribozyme (Gao and Zhao 2014), and the ribonuclease Csy4 (Nissim et al. 2014). Both 35S and U3/U6 have been used to drive expression of sgRNA in citrus (Jia and Wang 2014a; Jia et al. 2017b; Peng et al. 2017; Zhang et al. 2017). 35S promoter is an RNA polymerase II promoter which synthesizes precursors of mRNAs and most snRNA, whereas U6 or U3 are polymerase III promoters, which synthesize tRNAs, rRNA 5S, and other small RNAs. In most studies, sgRNAs are driven by U6 or U3 promoters (Kim et al. 2016; Wei et al. 2017). It seems 35S, U6, and U3 promoters all work in promoting the transcription of sgRNA (Kim et al. 2016; Jia and Wang 2014a). However, U6 or U3 might be more efficient than 35s for driving the transcription of sgRNA since Pol II created RNA will be capped and poly-A-tailed, so the half-life of the RNA in the nucleus will be shorter than that synthesized by RNA polymerase III.

The 35S promoter is the most commonly used to drive the expression of Cas9. However, Cas9/sgRNA gene editing efficacy has been improved by driving the expression of Cas9 using different promoters including the dividing cell-specific INCURVATA2 promoter (Hyun et al. 2015); the cell division-specific YAO promoter (Yan et al. 2015), and the germ-line-specific SPOROCYTELESS promoter (Mao et al. 2016) in Arabidopsis. Besides 35S promoter, the YAO promoter has been successfully used to drive the expression of Cas9 in citrus (Zhang et al. 2017).

Codon-optimization of Cas9 has also been used to maximize Cas9 activity in plants (Bortesi and Fischer 2015). Plant codon-optimized SpCas9 gene has been used in citrus previously (Peng et al. 2017). Codon-optimization of other Cas9 orthologs is also recommended.

In addition, heat stress has also been shown to increase the efficacy of gene editing by CRISPR/Cas9. LeBlanc et al. (2018) demonstrated that Arabidopsis and citrus plants subjected to temperature at 37 °C showed

significantly higher frequencies of CRISPR-mediated mutations compared to plants grown at 22 °C. This seems to have resulted from that SpCas9 is more active in creating double-strand DNA breaks at 37 °C than at 22 °C.

Off-target Issue

Besides their target sites, Cas9 protein and orthologs can also create unwanted cleavages at off-target sites with high sequence similarity to target sequence, thus causing off-targeting mutations. For example, SpCas9 not only recognizes 5'-NGG-3' as the PAM sequence, but also can cleave sites with a 5'-NGA-3' or 5'-NAG-3' PAM sequence at lower efficacy (Hsu et al. 2013). In addition, mismatches in the PAM-distal sequence at the 5' terminus are tolerated, whereas mismatches in the seed region, the 10-12 nucleotides right upstream of PAM are not tolerated. Off-targets can cause negative effect which must be monitored carefully and avoided as much as possible (Koo et al. 2015).

Multiple approaches have been reported to reduce off-target issue associated with Cas9-sgRNA mediated genome editing. First, optimization of sgRNA by selecting unique target sequences which differ from other sequences by at least 2 or 3 nucleotides reduces off-target effects (Cho et al. 2014). Second, application of D10 mutant nickase version of Cas9 pairing with two sgRNAs that each cleaves only one strand decreases off-target effect (Ran et al. 2013). This approach extends the target sequence from 23 bp to 2×23 bp. Third, fusing dead SpCas9 (dCas9, which results from mutations of both cleavage domains of SpCas9, i.e., D10A for RuvC and H840A for HNH) with the FokI nuclease domain at the N-terminus also reduces off-target problem (FokI-dCas9) (Tsai et al. 2014). In addition, nontransgenic genome editing approaches as described above have the potential to reduce off-targets.

Overall, *Agrobacterium*-mediated transgenic expression, CTV-mediated expression, and CRISPR-based genome editing have shown tremendous potential to improve citrus against different diseases. However, their applications remain at the early stage. The scientific community needs to further optimize the tools, rigorously test the end products to avoid negative effects, and appropriately address the public concerns regarding crops containing those elements.

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Genetic Basis of Resistance to Citrus Canker Disease

15

Ziniu Deng and Xianfeng Ma

Abstract

Citrus canker, caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*), is a destructive quarantine disease worldwide. As no commercial cultivars are resistant to the disease and its control is difficult, selection of resistant genotypes becomes the essential solution. A large quantity of citrus genotypes were screened for their behaviors to the pathogen infection during the past decades, unfortunately almost all the tested genotypes are susceptible when they are artificially inoculated with *Xcc*. The pathogen infects citrus host through attachment on the tissue surface, and then penetration into the tissue for colonization. The successful infection relies on the formation of biofilm, which is affected by different factors including extracellular polymeric substances (EPS) containing mainly extracellular polysaccharides, quorum sensing, etc. The canker disease development depends on the virulent effector PthA4 secreted into citrus cells through the Type III protein secretion system (T3SS). The plant has two layers of defence responses to pathogen attack, i.e., the basal defence realized by pattern recognition receptors (PRRs) to detect microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) to trigger PAMP-triggered immunity (PTI) and the effector-triggered immunity (ETI) based on the highly specific interaction between products from pathogen avirulence genes (*Avr*) and products from host resistance genes (*R*). The XacFhaB, Lipopolysaccharides (LPSs) and flg22 are PAMPs identified in *Xcc*. The PRR *FLS2* was identified in kumquat and mandarin genotypes. The rhizobacteria strains were found to effectively activate plant defence and significantly reduce symptom development in leaves challenged with *Xcc*. A few resistance genes, like *Citrus NPR1 homolog 1* and *Avr9/Cf-9* genes, were cloned. Breeding for citrus genotypes resistant to *Xcc* has continuously carried on for long time. The majority is achieved by genetic transformation, and among the reports, anti-bacterial peptide genes have been widely used, followed by the transferring resistant genes from other plants in citrus. The results, however, indicated only different levels of reduction of susceptibility to Xcc were gained. Further investigation of resistant mechanism and identification of resistant genes are indispensable for breeding of citrus genotypes really resistant to canker disease.

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15.1 Introduction

Citrus bacterial canker disease (CBC) is a destructive quarantine disease worldwide. It was first reported in India and Java in the mid of nineteenth century, however its original center is still unknown (Li et al. 2007). And now the disease occurs in the citrus producing areas all the world except Europe (Fig. 15.1; CABI 2018).

15.1.1 The Disease Groups

According to the pathogenicity and the host range, the CBC disease is located into five groups of strains, i.e., group A strains (Asian canker strains or Oriental canker strains) which is the most invasive pathogen and infects almost all the citrus cultivars; group B strains causing cancrosis B mainly to lemon [*Citrus limon* (L.) Burm. f.] and lightly to Mexican lime [*C. aurantifolia* (Christm.) Swing.], sour orange (*C. aurantium* L.) and pummelo [*C. grandis* (L.) Osb.]; group C strains causing cancrosis solely to Mexican lime; group D strains causing Mexican lime bacteriosis reported only in Mexico, and group E strains causing citrus bacterial spot which was only reported in nurseries in Florida (USA) and observed in the rootstock Swingle citrumelo [*Poncirus trifoliata* (L.) Raf. X *C. paradisi* Macf.]. The groups B and C were described in South America and were gradually supplanted by CBC-A (Civerolo 1985; Das 2003). Two variants of group A strains were identified. One, found in Southeast Asia, causes typical group A symptoms only to Mexican lime and designated as A^* (Vernière et al. 1998) and another, detected in Florida, USA, affects Mexican lime and Alemow (*C. macrophylla* Westr.) and named A^w (Sun et al. 2004).

15.1.2 The Pathogen

The causing pathogen of citrus bacterial canker disease is *Xanthomonas citri* (Gabriel et al. 1989). The species and related pathtypes were modified several times. *X. campestris* pv. *citri* (Dye 1978) and *X. axonopodis* pv. *citri* (Hasse) Dawson (Vauterin et al. 1995) were commonly used previously. And now *X. citri* subsp. *citri*

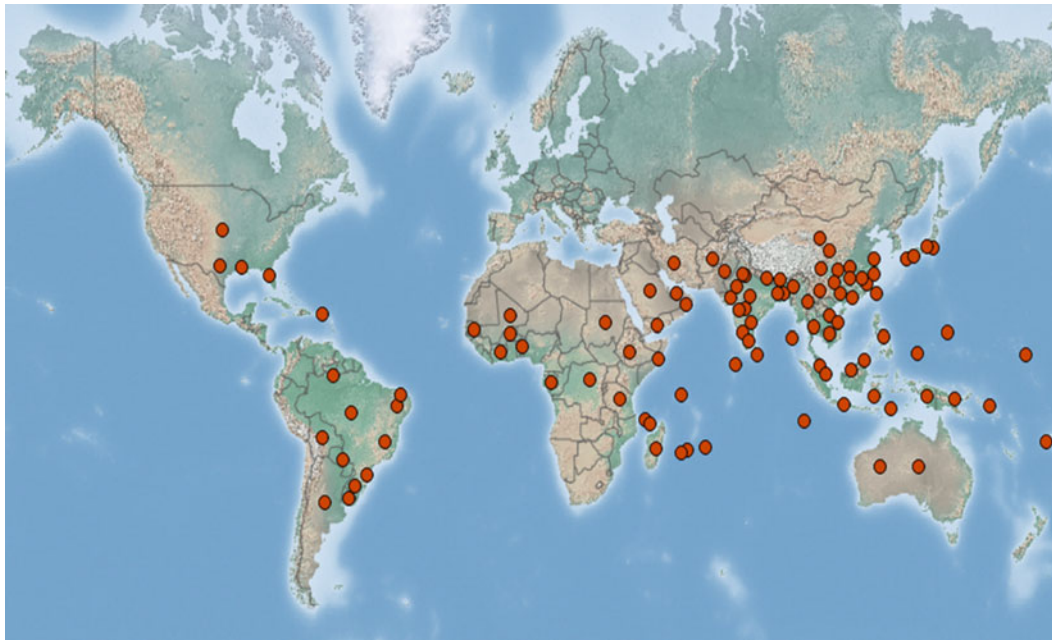

Fig. 15.1 Distribution map of citrus canker in the world (CABI 2018)

(Schaad et al. 2006) has been widely accepted. In accordance to the five groups of disease, there were corresponding pathogen strains. Group A and its variants A^* and A^w are located in pathtype A; Group B/C/D was combined in *X. campestris* pv. *aurantifolii*, and group E strains as *X. campestris* pv. *citrumelo* (Gabriel et al. 1989), which were later replaced by *X. axonopodis* pv. *aurantifolii* and pv. *citrumelo* (Vauterin et al. 1995). In reality, the diseases caused by the group C no longer exist in nature. The disease caused by group B may also no longer exist in nature because the pathogen strain has weak competitiveness with the group A strains which were introduced into the areas previously occupied by the group B strains (CABI 2018). The group D strains are now called 'mancha foliar de los citricos' and its pathogen is *Alternaria limicola* which is not a CBC disease (Palm and Civerolo 1994). Therefore, the pathogen causing citrus bacterial canker disease is restricted to *X. citri* subsp. *citri* (*Xcc*) in the present chapter if it is not specifically indicated.

15.1.3 The Host Range and the Disease Symptoms

The pathogen causes necrotic lesions on leaves, shoots and fruits. As the disease develops, defoliation, badly blemished fruits, premature fruit drop, twig dieback and general tree decline will appear. The typical lesions are erumpent, callus-like, with water-soaked, oily, yellowish-tan colored margins that become brown with age (Fig. 15.2). Although the canker disease is hardly to kill the tree, it indeed causes tree weakening, reducing production and the worst is that the fruit with canker symptoms is of low economic value. The diseased fruits may have no commercial value as they are prohibited in the market in areas where citrus is commercially cultivated without the canker disease.

Our observation indicated that most commercial citrus cultivars are susceptible to the disease, but they have different sensibility levels. In natural conditions, sweet orange (*C. sinensis* L. Osb.),

Fig. 15.2 The typical erumpent symptom on leaf and fruit of citrus bacterial canker disease

sour orange, pummelo, grapefruit, lemon and lime are susceptible showing typical symptoms in the orchard; while mandarins are considered as tolerant because no canker symptoms will appear on the trees when they are cultivated in an isolated orchard far away from other susceptible cultivars. If the mandarin trees are grown inside or near susceptible cultivars, like sweet orange, lemon, they will show typical symptoms, too. In the family *Rutaceae*, *Poncirus trifoliata* and its hybrids citrange (*P. trifoliata* x *C. sinensis*), citrumelo and many *Citrus* hybrids are quite sensible to canker disease; Kumquat (*Fortunella* spp.) scarcely shows canker symptoms.

15.1.4 The Disease Cycle

The pathogen initiates the infection process on young leaves, shoots and fruits. According to our continuous observation on fixed trees and fully investigation in the chosen orchards for over 5 years, the disease development is tightly related to leaf and fruit development stages and climate conditions. The infection only can start on the young leaf from bud burst to the fully expanded leaf yellowish green in color. When the young leaf turns into dark green and becomes functional leaf, the infection is hardly observed. For the fruit, the infection begins after the first period of fruitlet drop (usually in May in the subtropical regions) and continues till the color break. The disease development is regulated by the interaction between temperature, rainfall and wind speed. In the spring shoot growth period when the air temperature is between 15–25 °C, it needs 40–50 days from bud burst to the first symptom appearance, and in the summer and fall shoots growth period with the air temperature stably over 25 °C, the symptom becomes visible just 15–20 days after bud burst. For the fruits, visible lesions can be seen 15 days after anthesis. When air temperature is ≤ 20 °C, the canker lesion does not occur, even if there is rainfall and wind. When the temperature raises to ≥ 20 °C, the symptom will appear in one week. Then the rainfall is important for wetting the bacteria lesions on the infected leaves and twigs, and to spread onto the surface of the young growing leaves, shoots and fruits by wind. At the dry climate wind will not spread the bacteria or the rain will not make the bacterial spreading without wind. Thus means that wind together rainfall is essential for the pathogen spreading, and air temperature is the key factor for disease development.

The pathogen is usually introduced to the new cultivation areas through the movement of infected citrus fruits, budsticks and nursery plants, and inadvertent re-introduction is highly likely mainly through pass-by person, tools and vehicles. Despite the quarantine restrictions that are effective in many countries, and the eradication programs performed or that are on-going in some places, this pathogen remains a threat to all citrus-growing regions in the entire world (Gottwaldst al. 2002; CABI 2018).

15.1.5 The Control of the Disease

The pathogen can remain infective in the soil for very long time, as it was observed that citrus trees were re-infected by *Xcc* when they are replanted in the infected soil. The fundamental way to control the canker disease is to use disease-free (healthy) nursery plants. The certified healthy plants should be propagated with tested disease-free rootstock seeds and budsticks in the registered nurseries isolated from any citrus and trifoliate orange plantations. The equally important way is to use good sanitation and hygiene practices to prevent the orchard from the introduction of the pathogen. People (workers and visitors), vehicles, equipment and tools can spread diseases, therefore, it is important to perform disinfection of people and vehicles going into orchard and keep all the tools and orchards clean.

When the disease is present, all the efforts should be laid on the reduction of the damages to the minimum. Eradiation of the infected and the around trees in the epidemic center when the disease just appeared in a short time was effectuated in different countries. In Florida, USA the eradication of canker disease was

performed three times. The disease first appeared around 1912 and was declared eradicated from Florida and the adjacent states in 1933. Citrus canker was occurred again in Florida in 1986 and was declared eradicated by 1994. However, 3 years later the disease re-emerged in the same area where the canker disease had occurred 1980s. In the meantime, a new and separate infestation of citrus canker was discovered in urban Miami in 1995 (Gottwald et al. 2002). At this time, a cooperative state/federal citrus canker eradication program (CCEP) was established. All the trees within a distance of 579 m as a radius around the infected trees were removed. Despite extensive eradication efforts, which resulted in the removal or cutting back of over 1.56 million commercial trees and nearly 600,000 infected and exposed dooryard citrus trees statewide, the infected area has increased to 1701 km^2 till 2002. The eradication program was demised in January 2006 (Lowe 2009; Dewdney and Graham 2016). Eradication programs were established also in New Zealand and Australia. An outbreak in Queensland, Australia, in 2004 was declared eradicated (IPPC 2009). *Xcc* has then been detected in Northern Territory and Western Australia and is under eradication (IPPC 2018).

The re-emergence of the canker disease in the eradicated areas implies that the pathogen is consistently present in fields as latent infections, but at an undetectable level. The absence of the disease from the field (even all the citrus trees have been removed) for 10 years is not sufficient to declare that the disease has been eradicated.

In the canker infected orchards, copper sprays are the common methods for the control of canker disease. The control is usually not so effective, and heavy sprays can cause food security problems. Another problem of this approach is the high cost and laborious practice which increase production cost and reduce the benefit.

Generally, citrus canker disease is difficult to be eradicated and controlled, and most commercial cultivars are susceptible. Breeding for resistant cultivar may be an essential way to completely control the disease.

15.2 Evaluation of Citrus Genotypes for Resistance to Canker Disease

Resistant/tolerant genotypes are considered the fundamental way to control citrus canker disease, thus a large quantity of citrus genotypes were screened for their behavior towards the pathogen infection during the past decades. Gottwald et al. (1993) evaluated 53 genotypes for their behavior to canker disease in the field by pinprick inoculation of *Xcc* and the results revealed that all the citrus genotypes showed similar typical symptoms without significant differences. In a greenhouse assay, 582 accessions including 319 varieties of sweet orange were screened for resistance. After being inoculated by spraying with *Xcc* (10^8 cfu/ml), the tested genotypes showed a wide range of reaction with approximately 13% of the tested accessions resistant to citrus canker, 42% moderately resistant, 20% susceptible, and 25% highly susceptible (Amaral et al. 2010). In this experiment, however, it is questionable that some accessions within one species (like sweet orange, mandarin) were observed to react differently to the disease.

A 6-year study was conducted to compare the susceptibility of 186 genotypes of citrus including sweet orange, mandarin (clementine, satsuma, etc.), sour orange, lemon, lime, grapefruit, and four mandarin hybrids under natural inoculation in field. The number of lesions per leaf was assessed for 18 times during the experimental period (up to 4 times per year). Based on the observed results, satsuma and lemon had the lowest number of lesions (mean lesions per leaf = 4.32 and 4.26, respectively) and were considered as the most resistant genotypes, while grapefruit and sweet lime had the highest number of lesions (14.84 and 10.96 lesions per leaf, respectively) and were classified as the most susceptible genotypes. Mandarins, sour oranges and sweet oranges ranged in the intermediate severity group (5.48–9.56 lesions per leaf). The mandarin hybrids also showed a range of *Xcc* severity but all were in the more resistant groups (5.26–7.35 lesions per leaf). No genotype was observed to be immune to *Xcc* (de Carvalho et al. 2015).

Seedling progenies of 94 genotypes were evaluated for the resistance to citrus canker disease. The results indicated that the progenies of 14 genotypes did not exhibit canker symptoms during the 2 years' observation. *C. nobilis* and *C. sunki* were the only species in *Citrus* that had a low severity in some observation years, while in other years the severity was up to 26% to 38% when no chemical control for canker disease was applied in the citrus groves adjoining to the experimental field site. In general, *C. reticulata* and related mandarin-like genotypes had lower incidence and severity of symptom than *C. grandis*, *C. limon* and related species. *Microcitrus, Eremocitrus*, kumquat hybrid and *C. halimii* did not show symptoms, while the progeny of *Poncirus* and its hybrids were among the most severely diseased at all assessment dates (Stover et al. 2014).

Sweet orange is very susceptible to citrus canker disease, and limited evaluations have been made on sweet orange selections. In total 25 Pera sweet orange selections were evaluated for the resistance to *Xcc* by wound inoculation under greenhouse conditions. Canker disease severity was also assessed on the tested 25 selections at three locations in the field, relying on natural inoculum and conditions to cause disease. In the greenhouse experiments, five selections (EEL, Bianchi/CC, Ipiguá, Olimpia, IAC 2000/1) and Ovale Siracusa consistently had the smallest diameter lesions, although differences in lesion diameter were small. Results from the field experiments indicated that EEL and Ovale Siracusa were less affected by canker disease (Gonçalves-Zuliani et al. 2016).

Somatic hybrids were evaluated for their behaviors to the infection of *Xcc*. The genotypes included two autotetraploids, nine triploid hybrids of Lakeland limequat (*C. aurantifolia* × *Fortunella japonica*) and their progenitors (Lakeland limequat, the autotetraploids Femminello lemon and Giant Key lime, and the somatic hybrids Key [also known as Mexican] lime + Valencia orange and Hamlin orange + Femminello lemon). Meiwa kumquat (*F. crassifolia*) and Pineapple sweet orange were used as known resistant and susceptible standards, respectively. The bacterial pathogen solution of 10^3 and 10^4 cfu/ml were inoculated by injection infiltration through stomata on the abaxial surface of immature leaves under greenhouse conditions. Lesion number per inoculation site and bacterial population per lesion were recorded 15–19 days after inoculation. Susceptible and resistant genotypes were separated based on lesion number per inoculation site and bacterial population per lesion. 'Lakeland' limequat is a promising seed parent for breeding acid citrus fruit that is resistant to ACC. Lesion number per inoculation site is sufficient for assessment of resistance of citrus genotypes to canker disease without the necessity of conducting bacterial population assays (Viloria et al. 2004).

In order to find resistant genotypes within *Citrus*, an evaluation was performed to screen the species that usually do not have symptom in the field where the canker disease is present. The evaluated genotypes were 14 accessions, among which 8 from *C. ichangensis*, 5 from *C. junos* and one from *C. medica* (named Citron C-05). Immature leaves were inoculated with 2.5×10^7 cfu/ml of the pathogen by puncture inoculation, and it was found that 3 accessions of *C. ichangenesis* (Huaihua Sanye and 'No. 25–86') and 2 of *C. junos* (Shenju and Zhencheng) had fewer disease spots on pin holes with delayed symptom appearance; while Citron C-05 did not show any lesion (Table 15.1). These six genotypes were further tested in an epidemic field by puncture and spray inoculation for 2 years. The results (Table 15.2) revealed that only citron showed complete and active resistance to canker disease, with tissue necrosis on the pin holes; the other 5 accessions were all infected with different disease incidences. Therefore, the citron accession C-05 is the only resistant genotype within the genus *Citrus* (Deng et al. 2010).

Looking through all the experiments to evaluate the citrus genotypes for their resistance to canker disease, it is easy to note that the inoculation and susceptibility/resistance evaluation methods are inconsistent and nonstandard. The inoculation methods used are spraying, puncture (wound) inoculation, injection infiltration and natural infection. These methods may detect different types of resistance and then give

Table 15.1 Disease incidence observed in the primary assay by puncture inoculation

Year	Tested plants	*C. ichangensis*			*C. junos*		Citron C-05	'Bingtang' sweet orange
		Huaihua	Sanye	No. 25–86	Shenju	Zhengcheng		
2007	Plants in field	36	36	39	61	26	30	20
	Inoculated plants	17	13	9	16	11	12	12
	Inoculated pin-holes	510	390	270	480	330	360	360
	Lesions	69	75	42	70	42	0	177
	Disease incidence (%)	13.5	19.2	15.5	14.6	12.7	0	49.2
2008	Plants in field	4	4	5	5	5	10	6
	Inoculated plants	4	4	5	5	5	10	6
	Inoculated pin-holes	120	120	150	150	150	300	180
	Lesions	10	12	9	6	5	0	90
	Disease incidence (%)	8.3	10.0	6.0	4.0	3.3	0	50.0

Table 15.2 Disease incidence in the in vivo assay by spraying inoculum in 2007

	C. ichangensis			*C. junos*		Citron C-05	'Bingtang' sweet orange
	Huaihua	Sanye	No. 25–86	Shenju	Zhengcheng		
No. of inoculated leaf	17	13	9	16	11	14	12
No. of infected leaf	2	1	1	2	1	0	4
Disease incidence (%)	11.8	7.7	11.1	12.5	9.1	0	33.3

different even opposite results to the same genotype. Spray inoculation is similar to natural infection which can detect the possible physical barrier to prevent from the attachment and the penetration of the pathogen as well as the resistance inside the host cells. The injection infiltration avoids the physical barrier and tests only the resistance inside tissue. The puncture (wound) inoculation is the mixed way composing of the function of both injection infiltration and spray inoculation. It is important to set up the standard inoculation method and use both spray and injection infiltration to get complete picture of the resistance. It is not suggested to use the puncture (wound) inoculation as it easily gives ambiguous results. The inoculation concentrations are quite different ranging from 10^3 to 10^8 cfu/ml. The concentration of *Xcc* inoculum depends on the inoculation method with 10^8 cfu/ml for spray inoculation and 10^3–10^6 cfu/ml for injection infiltration.

15.3 Pathogenesis of *Xcc*

In the infection, the bacterial pathogen *X. citri* subsp. *citri* should attach the surface of young leaves, shoots and fruits and then penetrate into inside the tissues where it will cause symptoms. The citrus genotypes show quite different behaviors to the pathogen infection. It is necessary to understand the interaction between the pathogen and the host.

15.3.1 The Pathogen Genome

X. citrus subsp. *citri* (strain 306) has one circular chromosome comprising 5,175,554 base pairs (bp), and two plasmids: pXAC33 (33,699 bp) and pXAC64 (64,920 bp) (da Silva et al. 2002). The variant of the Asiatic strain (*X. citri* subsp. *citri*) A^w (strain AW12879) was also sequenced and it contains one chromosome (5.32 Mb and 64.71% G-C content) and two circular plasmids, pXcaw19 (18,869 bp and 63.07% G-C content) and pXcaw58 (58,317 bp and 61.85% G-C content). The genome consists of 4,760 annotated CDS, of which 3,457 could be assigned to a COG functional category (Jalan et al. 2013).

15.3.2 *Xcc* Infection Process

The *Xcc* bacteria are brought in the orchard by infected nursery plants or other infected plant materials, transportation vehicles, tools and people walking through, and spread by rainfall and wind. The bacterial pathogen is watered by humidity and blown to nearby trees. The infection process was fully reviewed by Das (2003).

The *Xcc* bacteria in canker lesions are the main source for dispersing in the orchard. They are embedded in a dense matrix of extracellular polysaccharides, which is produced in host tissues by the bacterium itself. Together with extracellular polysaccharides, the pathogen is spread by rainy wind. It was reported that the extracellular polysaccharides has protective effects against water dilution effect and air desiccation, providing benefits for the bacterial ecology (Goto and Hyodo 1985). It was observed that the bacteria were adhered to host tissues via the protection of the EPS of *Xcc* and citrus agglutinin interaction and that the process might involve in the initial step for establishing the host–parasite relationship in citrus canker (Takahashi and Doke 1984).

Biofilm is considered as the protector for bacteria from environmental stresses, host defence mechanisms and antimicrobial compounds (Karatan and Watnick 2009). The biofilm is supported by extracellular polymeric substances (EPS), thus the EPS was considered as 'house of the biofilm cells'. The EPS contains mainly extracellular polysaccharides and other substances such as proteins, nucleic acids (eDNA) and lipids (Flemming et al. 2007). These substances provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells, and they each have certain function (Flemming and Wingender 2010). In the case of *Xcc*, the biofilm formation on host surface is essential in the epiphytic survival of this pathogen prior to the development of canker disease. Before entering into plant tissues, *Xcc* should grow on leaf surfaces in the biofilms (Rigano et al. 2007).

The biofilm development is regulated by numerous factors and can be broadly grouped into four different phases including contact/attachment on the surface, sessile growth phase governed by intercellular interaction, i.e., quorum sensing (QS) factors, biofilm maturation induced exopolymeric substances (EPS) matrix synthesis and finally detachment (Saxena et al. 2019).

Numerous studies have been carried out on the factors affecting the formation of *Xcc* biofilm. The following elements are found to be necessary in the biofilm establishment: Xanthan production (Rigano et al. 2007), quorum sensing (Siciliano et al. 2006), a filamentous haemagglutinin-like adhesin (Gottig et al. 2009), flagellum synthesis (Malamud et al. 2011), a UTP-glucose-1-phosphate uridylyltransferase

(Guo et al. 2010), LPS biosynthesis (Li and Wang 2011; Yan et al. 2012) and glucan biosynthesis (Malamud et al. 2012), a two-component signal transduction system encoded by colS/colR (Yan and Wang 2011) and a LOV protein (Kraiselburd et al. 2012). Later on, new genes were revealed from 23 mutants related to biofilm formation, and the importance of exopolysaccharide production, motility and cell surface structures was reinforced in the biofilm formation process (Malamud et al. 2013). Outer membrane proteins and receptor or transport proteins (Zimaro et al. 2013; Ficarra et al. 2016) and the type III protein secretion system contributes (Zimaro et al. 2014) were reported to contribute to the *Xcc* biofilm formation.

Xanthan is the main EPS in *Xanthomonas* genus playing an important role in biofilm formation (Rigano et al. 2007), but it does not play an essential role in citrus canker at the initial stages of infection or in the incompatible interactions between *Xcc* and non-host plants, but facilitates the maintenance of bacteria on the host plant, possibly improving the efficiency of colonization of distant tissue (Dunger et al. 2007).

Quorum sensing (QS) plays essential roles in biofilm development. Pathogenic bacteria in biofilms utilize QS mechanisms to activate virulence and develop antibiotic resistance. Free-living bacterial cells, through QS mechanisms, establish cell-to-cell communication by secretion of small signaling molecules, which lead to a wide range of effects on bacterial genetics and physiology. Different QS signaling molecules have been identified in bacteria, and in Gram-negative bacteria, it was found that N-acyl homoserine lactones (AHLs) cross cell membranes and bind to regulatory proteins in recipient cells (Braeken et al. 2008; Kalia 2013; Saxena et al. 2019). Further study revealed that the RpfC-RpfG two-component system was a major and conserved signal perception and transduction system for DSF (Diffusible Signal Factor) family signal-mediated QS in *Xcc*. The unique genes controlled by RpfF alone indicate the complexity of the QS pathway and the involvement of additional sensory mechanisms in *Xcc*. The unique genes controlled by RpfC and RpfG, respectively, support the possibility that RpfC and RpfG play broader roles in gene regulation other than transduction of DSF signals (Guo et al. 2012).

It is commonly believed that the bacteria, after successfully attached on the surface of citrus tissues, penetrate inside the tissue through natural openings, like the stomatal pores or wounds (Gottwald et al. 2002). Nevertheless, the differences among cultivars in stomata structure and density on leaves expanded by two third or fully expanded were not related to their susceptibility to citrus canker disease (Graham et al. 1992). We also observed that the resistant genotype citron C-05 had the stomata structure and density similar to the susceptible sweet orange cultivars (Table 15.3). When the pathogen bacteria were sprayed on the leaf surface of sweet orange or citron C-05, they grew randomly on the whole leaf surface without preference to gather around the stomata (Fig. 15.3).

15.3.3 *Xcc* Pathogenicity

The Type III protein secretion system (T3SS) is conserved in many Gram-negative pathogenic bacteria (Zimaro et al. 2014). The T3SS of *Xcc* is necessary in pathogenicity. As the T3SS mutants are unable to grow in host plants indicating that *Xcc* T3SS is responsible for the secretion of effector proteins, and hypersensitive response and pathogenicity (*hrp*) cluster composes of the genes encoding the T3SS (Dunger et al. 2005). Most *Xanthomonas* genomes sequenced to date have the *hrp/hrc* gene cluster, which render these cells capable of assembling a fully functional T3SS (Ryan et al. 2011). All citrus canker-causing strains have similar T3SS gene clusters (Jalan et al. 2013).

Through the analysis of genomic sequence of *Xanthomonas* (da Silva et al. 2002; Jalan et al. 2013), different effectors were found. One of the most important T3SS-delivered effectors found among the citrus canker strains is PthA4 and its homologues. This protein is a T3SS effector delivered inside the plant cell and is a key effector responsible for canker development. Its

Table 15.3 Leaf stoma density and size of different citrus genotypes

Genotypes	Stoma density (No./mm^2)	Stomatal apparatus		
		Major axis (μm)	Minor axis (μm)	Major axis/Minor axis
'Bingtang' sweet orange	551.28b	23.34a	20.43ab	1.14
'Newhall' navel orange	530.40b	23.40a	20.31ab	1.15
'Succari' sweet orange	527.83c	23.03a	19.41ab	1.19
'Yamamoto' satsuma	515.13b	22.53a	19.78ab	1.14
Citron C-05	533.33b	21.42a	18.67a	1.15
kumquat	353.84a	21.67a	21.13b	1.03

Means in the same column followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05)

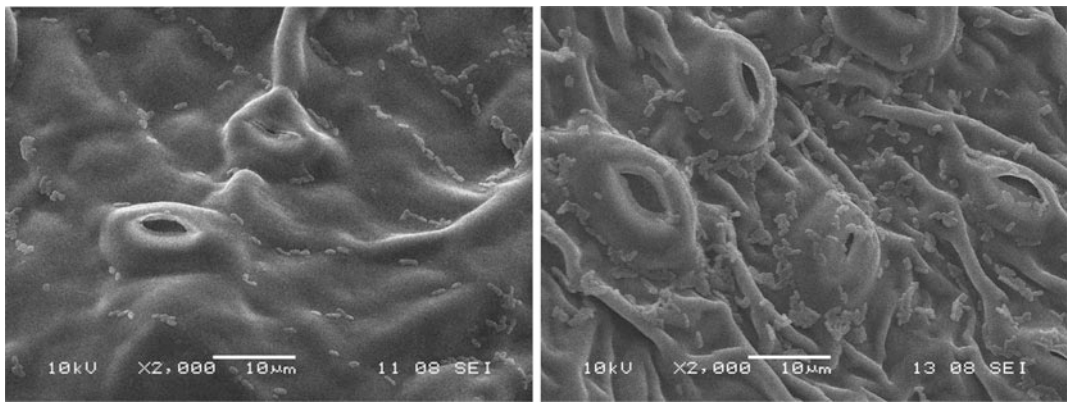

Fig. 15.3 SEM observation of *Xcc* on the leaf surface of 'Bingtang' sweet orange. The bacterial suspension of 10^8 cfu/ml DL509 isolate was sprayed on leaf surface and the SEM observation was performed 3 (left) and 7 (right) days post inoculation

presence alone is capable of inducing canker formation, while its absence suppresses the appearance of cankers (Al-Saadi et al. 2007).

Although the precise mechanism by which the *Xcc* pathogen induces canker is unknown, the transcriptional activator-like (TAL) effector *pthA* exhibits pleiotropic pathogenicity and avirulence functions, and it plays a central role in the activation of host genes implicated in cell division and growth (Al-Saadi et al. 2007; Duan et al. 1999; Hu et al. 2014; Lee et al. 2008; Lee and Schneewind 2001; Pereira et al. 2014; Soprano et al. 2013; Swarup et al. 1991, 1992; Yan and Wang 2012). It was also found that the PthA expression level was positively correlated with the *Xcc* pathogenicity (Li et al. 2014a).

The TAL effectors have conserved (almost identical) repeats that include a repeat variable di-residue (RVD). Each of the RVDs can recognize one nucleotide and the RVDs target a given DNA sequence so as to target specific DNA sequences (Pereira et al. 2014). The TAL effectors are proteins that can control gene expression of the host cell in which they are delivered, where they can enter the cell nucleus and act as transcriptional regulators favoring pathogen development.

All the *Xanthomonas* TAL effectors have similar structure. The N-terminal region contains the type III translocation signal; the central part is repetitive, consisting of nearly identical 102 bp-direct repeats and each repeat encodes 34 amino

Table 15.4 Amino-acid sequence identity values (%) among PthA homologs from all known host range groups of Xanthomonas citri strains

Effectors	PthA	PthA4	Apl1	PthAW	PthA[a]	PthA*2	PthA1	PthA2	PthA3	Apl2	Apl3	PthB	PthC
	17.5a	17.5	17.5	17.5	17.5	15.5	16.5	15.5	15.5	15.5	23.5	17.5	17.5
PthA	100	100	100	99	98	92	95	93	92	94	84	87	87
PthA4		100	100	99	98	92	95	93	92	94	84	87	87
Apl1			100	99	98	92	95	93	92	94	84	87	87
PthAW				100	97	92	95	93	92	93	84	87	87
PthA*					100	92	95	93	92	93	84	87	87
PthA*2						100	95	96	97	97	79	82	83
PthA1							100	95	95	95	81	84	85
PthA2								100	98	99	79	82	82
PthA3									100	98	79	82	82
Apl2										100	80	82	82
Apl3											100	75	75
PthB												100	98
PthC													100

[a]Number of repeats
Al-Saadi et al. (2007) MPMI 20(8):934–943

acids (The number of repeats varies between 1.5 and 33.5). The tandem repeats are very similar, however, the number, order, and length of repeats varied, and the hypervariable aa position occurs at 12 and 13, which determine the DNA base-pair recognition specificity of each repeat. The C-terminal contains nuclear localization signal (NLS) and activation domain (AD). NLS mediates import into the plant cell nucleus and AD is necessary for activating plant gene expression (Boch and Bonas 2010).

Different *pthA* homologs were gradually identified. From the sequencing of *Xcc* genome, 4 TAL-effector *pthA*s were found and named *pthA1*, *pthA2*, *pthA3* and *pthA4* (da Silva et al. 2002), and by screening a library, three homologs named *apl1*, *apl2*, *apl3* were identified (Kanamori and Tsuyumu 1998). Through analyses of four pathogen groups (A*, Aw, B, C), four more homologs were found and they were named *pthA**, *pthA*2*, *pthAW*, *pthC* (Al-Saadi et al. 2007) and *pthB* (El-Yacoubi et al. 2007). All the *pthA* homologs have the structure similar to other *Xanthomonas* TAL effectors. Differences among the *pthA* homologs exist mainly in the number of the tandem repeats varying from 15.5 to 23.5 (Table 15.4). The functional tests of the *pthA* homologs revealed that only those homologs carrying 17.5 repeats appeared to encode a pathogenicity gene that was required for elicitation of citrus canker disease such as *pthA*, *pthA**, *pthAW*, *pthB* and *pthC* genes (Al-Saadi et al. 2007).

15.3.4 Citrus Disease Susceptibility Genes

In comparison with researches on the pathogenicity of the bacteria, the mechanisms on the susceptibility of the host have been investigated much less. Until 2014, one citrus disease susceptibility (S) gene, *CsLob1*, was reported contemporaneously by two research groups (Hu et al. 2014; Li et al. 2014b). The promoter of *CsLob1*has PthA effector binding element (EBE) and the expression of the S gene is driven by PthAs (Hu et al. 2014).

In addition to *Lob1*, various citrus genes were identified as potential direct targets of PthAs, which included *Lob2*, *Lob3* and *DIOX* (citrus dioxygenase) with different expression levels (Pereira et al. 2014; Abe and Benedetti 2016).

Although PthA4 is indeed the TAL effector needed for canker formation, the other PthA copies seemed to have an important role in canker development, especially in some citrus varieties (Abe and Benedetti 2016). PthA1 and PthA3 contribute to canker development in Pera sweet orange, but not in Tahiti lemon. This phenomenon, however, did not correlate with the activation of the *Lob1* gene, but with the induction of other PthA targets, including *Lob2* and *DIOX*. The results imply that the activation of multiple S genes, such as *Lob1* and *DIOX*, may be necessary for full canker development (Abe and Benedetti 2016).

15.4 Resistance Mechanism

15.4.1 Overview of Plant Resistance to Bacterial Pathogen

The classic 'gene-for-gene' model indicated that the resistance needs interaction of complementary pairs of dominant genes, one in the host and another in the pathogen. A loss or alteration to either the plant resistance (R) gene or the pathogen avirulence (*Avr*) gene leads to disease (Flor 1971). The *Xanthomonas avrBs3*, as mentioned above, is necessary to deliver the *avr* gene product into plant cells via T3SS, while the R gene encodes proteins that can recognize Avr-gene-dependent ligands. Following pathogen recognition, the R protein is presumed to initiate the defence signaling to impair pathogen ingress (Hammond-Kosack and Jones 1997).

One of the best characterized *avr* genes in bacterial pathogens is the *avrBs3* gene and its homologues. Genes homologous to *avrBs3* are present in different pathogenic *Xanthomonads* and share at least 95% identity with most variability residing in the repeat region. Some *avrBs3* family members have a dual function. For example, the *pthA* gene from *Xcc* is required for pathogenicity (citrus canker symptoms) on citrus and functions as an avirulence gene on non-host plants. The *avr* function requires bacterial *hrp* (hypersensitive reaction and pathogenicity) genes that encode a type III protein secretion system. Plant recognize the pathogen *avr* gene by single R genes (Bonas and Van den Ackerveken 1999).

Plants resistance to bacterial disease involve several different defence mechanisms to prevent bacterial infection. The first line of protection from an initial pathogen infection is achieved through passive defence mechanisms such as physical and chemical barriers, which may prevent the bacterial pathogen penetrate into plant tissue (Ade and Innes 2007). Then plants have the basal defence against bacterial pathogens and a second layer of the plant immune system that will be described in detail in the following part.

15.4.2 Brief Introduction to the Plant Innate Immune System

Plants have evolved effective innate immune systems to protect themselves from the attack of most potential pathogens. The plant immunity consists of two mechanistically connected and evolutionarily interrelated branches, i.e., the basal defence realized by pattern recognition receptors (PRRs) to detect microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) to trigger PAMP-triggered immunity (PTI), and the effector-triggered immunity (ETI) based on the highly specific interaction between products from pathogen avirulence genes (*Avr*) and products from host resistance genes (R), according to the above mentioned 'gene-for-gene' hypothesis (Dodds and Rathjen 2010; Jones and Dangl 2006; Muthamilarasan and Prasad 2013).

15.4.2.1 PAMP-Triggered Immunity (PTI)

MAMPs or PAMPs are highly conserved molecules within a class of pathogens or microbes and they usually have essential functions in fitness or survival of the pathogens or microbes. PRRs are receptor-like kinases (RLKs) or receptor-like proteins (RLPs) containing ligand-binding domains that recognize PAMPs/MAMPs at the cell surface and activate immune responses (Monaghan and Zipfel 2012). According to the

identified a few PAMP–PRR pairs, the RLKs have a single pass transmembrane (TM) domain for anchorage, a variable N-terminal extracellular domain (ECD) for ligand binding and a C-terminal intracellular kinase domain (KD) that relays downstream signaling. Some RLKs have the leucine-rich repeats as extracellular domain (LRR–RLKs) such as Flagellin Sensing 2 (FLS2) and EF-Tu Receptor (EFR) that activate PTI responses by sensing elicitor epitopes from bacterial flagellin (flg22) and elongation factor Tu (elf18) (Dalio et al. 2017; Zipfel et al. 2004); and there are RLKs containing LysM motifs in the ECD (LysM–RLKs) that play dual roles in peptidoglycan (PGN) and chitin perception in plant innate immunity, such as LYM1, LYM3, CERK1 in Arabidopsis and LYP4 and LYP6 in Rice (Liu et al. 2012; Willmann et al. 2011). As LYM1 and LYM3 lack cytoplasmic domains, transmembrane signaling likely requires an additional protein(s). Thus, it must complex with KD proteins to transduce the signals in the cytoplasm after PAMP recognition by the ECD, and it was found that CERK1 was involved in both PGN sensitivity and immunity to bacterial infection (Willmann et al. 2011). Similarly, the rice orthologues LYP4 and LYP6 recognize peptidoglycans as PAMPs but complexes with LysM RLKs are necessary to trigger immunity responses (Zipfel 2014).

15.4.2.2 Effector-Triggered Immunity (ETI)

Effectors are pathogen molecules that can modify host cell structures and manipulate function, facilitating infection and/or triggering defence responses. They evolve fast and are structurally divers (Dalio et al. 2017). When the effectors are secreted into the host cells, they reach their cellular target either at the intercellular interface of the host and pathogen cells (apoplastic effectors) or inside the host cells (cytoplasmic effectors) (Dalio et al. 2017).

R genes usually have different structures, among them, the majority has the LRRs (Leucine rich repeats) domains having an important role for recognition specificity. The main group of R gene has a central nucleotide binding site (NBS) domain, a C-terminal LRR region and an N-terminal variable domain mainly identified as TIR (Toll/Interleucina-1) or CC (Coiled coil) (Gururani et al. 2012). Besides TIR-NBS-LRR and CC-NBS-LRR, other R-genes include the RLKs (containing an extracellular LRR, a transmembrane domain and a cytoplasmic kinase domain), RLPs (which are similar to the RLKs but lack the kinase domain) and cytoplasmic enzymatic R-genes that contain neither LRR nor NBS groups (Dalio et al. 2017; Gururani et al. 2012).

ETI is the host defence triggered by recognition of pathogen effectors by plant immune receptors (R proteins). This effector (Avr)-R protein interaction can be direct or indirect. In the direct model the R protein directly detect the effector, in the indirect model (guard hypothesis), the R protein interacts, or guards, a host protein known as the guardee which is the target of the effector (Avr) protein. When the R protein detects changes of the guardee protein, it activates defence signaling leading to resistance. Based on this model, R proteins detect the virulence activity rather than physical presence of effectors, and subsequently activate defence (Dangl and Jones 2001).

15.4.3 Citrus-*Xcc* Interaction

15.4.3.1 PAMPs Derived from the Factors Related to Biofilm Formation

The *Xcc* should attach the host tissue surface by forming biofilm to initiate the infection process. Strategies that combat biofilm-associated diseases, such as anti-biofilm substances, quorum quenching molecules, bio-surfactants and competitive inhibitors (Saxena et al. 2019) may be the possible PAMPs.

The *Xcc* possesses a non-fimbrial adhesin protein, XacFhaB, which is required for bacterial attachment and is an important virulence factor for the development of citrus canker (Gotting et al. 2009). Later it was found that the adhesin XacFhaB had the function to trigger plant defence responses. The XacFhaB was divided

into three regions and each adhesin regions were investigated for the possibility to play PAMP roles inducing basal immune response in host and non-host plants. All the adhesion regions demonstrated the PAMP function to induce basal defence reaction to certain levels. When citrus leaves were pre-infiltrated with XacFhaB regions, growth of *Xcc* was inhibited, thus confirmed the induction of defence responses and the possible role in control of citrus canker (Garavaglia et al. 2016).

Lipopolysaccharides (LPSs) are essential and distinctive structures of Gram-negative bacteria being a major component of the bacterial cell surface. LPS from *Xcc* is essential in biofilm formation and considered as a virulence factor. *Xcc* LPSs were also recognized as PAMPs showing the role in the activation of basal defences in both host (*C. sinensis* Valencia late) and non-host (*Nicotiana tabacum* Petit Havana) plants (Petrocelli et al. 2012).

15.4.3.2 Citrus PRR FLS2—*Xcc* PAMP Flagellin Interaction

The *Xcc* flg22 was observed to introduce higher ROS production in the resistant genotypes Nagami kumquat (*Fortunella margarita*) and Sun Chu Sha mandarin (*C. reticulata*) but not in the susceptible citrus genotypes Duncan grapefruit (*C. paradisi*) and navel orange (*C. sinensis*). It was also found that several defence genes (*GST1*, *EDS1*, *NDR1*, *PBS1*, *RAR1*, *SGT1*, *PAL1*, *NPR2* and *NPR3*) were significantly induced by *Xcc* flg22 in Nagami kumquat, but not in susceptible Duncan grapefruit (Shi et al. 2015, 2016); thus the *Xcc* flg22 could be considered as a PAMP that might trigger basal defence response to *Xcc* attack mandarin and kumquat genotypes.

FLS2 is the bacterial flagellin/flg22 receptor, two *FLS2* genes (*FLS2-1* and *FLS2-2*) were identified and sequenced from the susceptible Duncan grapefruit (*CpFLS2-1* and *CpFLS2-2*) and the moderately tolerant Sun Chu Sha mandarin (*CrFLS2-1* and *CrFLS2-2*). Only one *FLS2*, however, was identified from Nagami kumquat (*FmFLS2-1*), and the *FLS2-2* gene was probably deleted from the kumquat genome. The expression level of *FLS2-2* was higher than *FLS2-1* in response to *Xcc* flg22. And the induced *FLS2-2* expression level was higher in canker-resistant mandarin than in the susceptible grapefruit. In Nagami kumquat *FLS2-1* showed the highest steady-state expression levels, which was not induced by Xflg22. Further *Agrobacterium*-mediated transient expression assays indicated that FmFLS2-1 and CrFLS2-2 from canker-resistant genotypes conferred stronger Xflg22 responses and reduced leaf canker symptoms in the susceptible grapefruit genotype (Shi et al. 2015, 2016).

15.4.3.3 Systemic Defence Response Against *Xcc* Induced by Rhizobacteria

Three rhizobacteria strains (*Burkholderia territorii* strain A63, *B. metallica* strain A53 and *Pseudomonas geniculata* strain 95) were found to effectively activate plant defence and significantly reduce symptom development in leaves challenged with *Xcc*. The expression of SA-signaling pathway marker genes (*PR1*, *PR2*, *PR5* and *SAM-SACM*) was enhanced by root application of *P. geniculate*, and expression of *PAL* and *SAM-SACM*, two genes involved in the phenylpropanoid pathway as well as the biosynthesis of SA and MeSA were induced following *Xcc* inoculation, respectively. Moreover, *P. geniculata* root-treated plants contained higher levels of reactive oxygen species (ROS) in aerial tissues than control. The results suggested that rhizobacteria can modulate citrus immunity resulting in a systemic defence response against *Xcc* under greenhouse conditions (Riera et al. 2018).

15.4.4 Resistance Genes

The ortholog of Arabidopsis *NPR1* gene, *Citrus NPR1 homolog 1* (*CtNH1*) was identified and cloned. When the *CtNH1* was transferred into the susceptible cultivar Duncan grapefruit plants, three transgenic lines had high levels of *CtNH1*

transcripts. These three lines were inoculated by leaf infiltration and showed less severity of canker symptoms than the wild-type Duncan grapefruit (Chen et al. 2013).

The *Avr9/Cf-9 rapidly elicited protein (ACRE)* (a R gene) was isolated and its expression was induced in kumquat inoculated with *Xcc*. *ACRE* genes code for the regulatory proteins with diverse functions important in Cf-9 (a R gene)-mediated resistance, HR and basal defence. The kumquat-*Xcc* interaction analysis revealed that the resistance was the product of an active rather than a passive response and that ROS, HR and general defence-associated genes were induced during this response (Febres et al. 2009).

15.5 Breeding for Resistant Citrus Genotypes

As no commercial cultivars are resistant to canker disease, breeding for resistance by conventional hybridization has made limited progress. Genetic transformation has widely utilized to generated transgenic lines with certain less susceptibility to the disease.

15.5.1 Transfer of Antimicrobial Peptides in Citrus Genome to Enhance Resistance to *Xcc*

The antimicrobial peptides genes such as *attacin*, *cecropin* and *sarcotoxin*, have been introduced in different citrus genotypes. Natal, Pera and Valencia sweet orange cultivars were transformed with the insect-derived *attacin A* (*attA*) gene. Four Valencia and one Natal transgenic lines showed a significant reduction in disease severity caused by *Xcc* with the reduction ranging from 45.2% to 77.8% in comparison with the wild types (Cardoso et al. 2009). Pineapple sweet orange plants were transformed with *dermaseptin* gene, and transgenic plants showed symptom levels of up to 50% less than the wild-type plant (Furmana et al. 2013).

Cecropin B and *Shiva A* genes were also transferred into Jincheng orange and Newhall navel orange genomes to enhance resistance to *Xcc*. Among the 40 transgenic lines obtained, 11 demonstrated enhanced resistance to citrus canker disease and one did not show any symptom in both the greenhouse and open field assays (He et al. 2011). An anti-bacterial peptide gene *AATCB* was introduced into blood orange genome, and 11 out of 19 transgenic lines had less severity canker symptoms compared with the wild type (Peng et al. 2015).

Thionins were reported having the antimicrobial activity against plant pathogens. The modified thionin (*Mthionin*) was transferred into Carrizo citrange genome, and 9 out of 11 transgenic lines did not show canker development at the *Xcc* inoculation concentrations of 10^4–10^5 cfu/ml. Compared to the wild-type plants most of the transgenic plants expressing Mthionin reduced the disease severity (Hao et al. 2016).

The *sarcotoxin IA*, an antimicrobial peptide isolated from the flesh fly (*Sarcophaga peregrina*) was used to transform Pera sweet orange. The transgenic plants expressed sarcotoxin IA peptide fused to the PR1a signal peptide from *N. tabacum* for secretion in the intercellular space. Compared to non-transgenic controls, the transgenic plants had lower *Xcc* population and incidence of canker lesions (Kobayashi et al. 2017).

15.5.2 Genetic Transformation of Exotic Genes in Citrus Genome to Enhance Resistance to *Xcc*

Up to now, no resistance gene has been identified and cloned in citrus and its relatives, therefore, genes from other plants are used to transform citrus in order to gain enforced resistance to *Xcc*.

An apple spermidine synthase gene (*MdSPDS1*) was introduced into Anliucheng sweet orange via *Agrobacterium*-mediated transformation of embryogenic calli. Two transgenic lines (TG4 and TG9) were less susceptible to *Xcc* than the wild type plants. It was observed

that, following *Xcc* attack, TG9 had significantly higher free spermine (Spm) and polyamine oxidase (PAO) activity showing an apparent hypersensitive (HP) response and the accumulation of H_2O_2 compared with the wild type (Fu et al. 2011).

A rice PRR gene *Xa21* was introduced into Anliucheng sweet orange via *Agrobacterium*-mediated transformation of embryogenic callus. The tolerance to citrus canker disease of the three recovered transgenic lines T2, T4 and T6 was assessed by in vitro pin-puncture inoculation. The results showed that all the three transgenic lines conferred improved resistance to citrus canker bacterium infection and the T4 transgenic line displayed the highest resistance (Li et al. 2014c). The same *Xa21* gene was transferred into W. Murcott mandarin by direct DNA uptake using a protoplast transformation system, and the transgenic plants challenged with the citrus canker pathogen showed a reduction in lesion number and bacterial populations within lesions compared to the wild type plants (Omar et al. 2018).

The pepper *Bs2*, a resistant gene, under the control of a pathogen-inducible promoter from glutathione S-transferase gene of potato, was transferred in Pineapple sweet orange and seven transgenic lines were obtained. The disease symptoms reduced up to 70% in transgenic lines with respect to non-transformed wild-type plants. This reduction did not happen when a mutant strain of *Xcc* with a disruption in *avrBs2* gene was used for inoculations. Additionally, a canker symptom reduction was correlated with levels of the *Bs2* expression in transgenic plants (Sendín et al. 2017).

The Harpin protein, produced by Gram-negative bacteria and encoded by the *hrp* gene, elicits the hypersensitive (HP) response and systemic acquired resistance (SAR) in plants. The *hrpN*, controlled by a pathogen-inducible promoter *gst1* and together with a signal peptide for protein secretion to the apoplast, was introduced in Hamlin sweet orange. Six *hrpN* transgenic lines were evaluated for the susceptibility to *Xcc*. Some of the transgenic lines showed reduction in susceptibility to citrus canker in comparison with the wild-type plants. One transgenic line exhibited normal vegetative development and displayed very high resistance to the pathogen, estimated as up to 79% reduction in disease severity (Barbosa-Mendes et al. 2009).

The *Xcc pthA-nls* was transferred into sweet orange via *Agrobacterium*-mediated transformation and 12 sense-nls (nls +) and 9 antisense-nls (nls−) transgenic lines were obtained. The nls + transgenic lines showed no typical lesion development, while the wild types and the nls− transgenic lines had typical symptoms in the in vitro assays. In vivo assay results indicated that the nls + transgenic lines showed resistance to the disease, in comparison with the wild types and the nls− transgenic clones. When 10^4–10^7 cfu/ml of pathogen was spray inoculated, the nls + transgenic clones did not show any symptom, while the wild types and the nls− transgenic lines had 100% disease development with whatever concentration of inoculum. Two transgenic clones were confirmed to be resistant to citrus canker disease in the repeated inoculation (Yang et al. 2011).

15.5.3 Regeneration of Resistant Genotypes by Modifying Susceptible Gene *CsLob1*

CsLOB1 (*Citrus sinensis* Lateral Organ Boundaries) is a susceptibility gene for citrus canker and is induced by the pathogenicity factor PthA4, which binds to the EBEPthA4-CsLOBP to induce CsLOB1 gene expression. The PthA4 effector binding elements (EBEs) in the CsLOB1 Promoter (EBEPthA4-CsLOBP) of the *CsLOB1* gene was modified in Duncan grapefruit. Four transgenic Duncan grapefruit plants with targeted modification of EBEPthA4-T1 CsLOBP were obtained; however, the canker symptoms in the transgenic lines were similar to those of wild type after being inoculated with wild-type *Xcc*. It was supposed that activation of a single allele of susceptibility gene *CsLOB1* by PthA4 is sufficient to induce citrus canker disease, and mutation in the promoters of both alleles of *CsLOB1*

is probably required to generate citrus canker-resistant plants (Jia et al. 2016).

CRISPR/Cas9/sgRNA technology was utilized to modify the canker susceptibility gene *CsLOB1* in Duncan grapefruit. Among the six transgenic lines, four had no canker symptoms at 4 days post inoculation (DPI) with *Xcc*. Pustules caused by *Xcc* were observed in later stages, which were much reduced compared to that on wild-type grapefruit. The pustules on DLOB9 and DLOB10 did not develop into typical canker symptoms (Jia et al. 2017).

Modification of *CsLOB1* promoter was also performed in Wanjincheng sweet orange. Sixteen lines that harboured EBEPthA4 modifications were identified, and four mutation lines (S2–5, S2–6, S2–12 and S5–13), in which promoter editing disrupted induction in response to *Xcc* infection, showed enhanced resistance to citrus canker compared with the wild type. No canker symptoms were observed in the S2–6 and S5–13 lines (Peng et al. 2017).

15.5.4 Breeding for Genotypes Resistant to *Xcc* by Cell Culture

In vitro mutation of Bingtang sweet orange calluses by exposure to ethyl methane sulphonate (EMS) was performed and somaclones tolerant to citrus canker disease were selected. Sweet orange leaves inoculated by *Xcc*-crude extract showed the symptoms similar to those inoculated by *Xcc* bacterial inoculum. The young shoots of susceptible genotypes cultured in 10% of *Xcc*-crude extract solution become brown and died in 1 week, while the resistant citron C-05 grew normally under the same conditions. After two steps (cell suspension and plants) of selection by *Xcc*-crude extract solution, the survived plants were tested by in vitro and in vivo inoculation with *Xcc*. One somaclone, named DG-2, was identified to be resistant to the canker disease (Ge et al. 2015).

Cybridization, a somatic hybridization approach that combines the organelle and nuclear genomes from different species, was used to create cybrids between citrus canker resistant Meiwa kumquat (*Fortunella crassifolia*) and susceptible grapefruit cultivars. The obtained cybrids with grapefruit nucleus contained either kumquat mitochondria and kumquat chloroplasts or kumquat mitochondria and grapefruit chloroplasts. All the cybrids with kumquat chloroplasts had a significantly lower canker disease severity than the grapefruit controls, the cybrids with grapefruit chloroplasts had a significantly higher number of lesions than those with kumquat chloroplasts (Murata et al. 2019).

15.6 Conclusion

The citrus bacterial canker disease has appeared for over 100 years, and the citrus-*Xcc* interaction has been extensively and intensively studied; the results, however, have biased the pathogen researches. The pathogenesis and pathogenicity of *Xcc* have been well investigated and many factors related to the pathogen infection and canker disease development have been identified through systematic studies. On the contrary, the resistance mechanism has not been understood and no resistant genes have been characterized so far. This is mainly due to the lack of resistant genotypes in *Citrus*, especially in the commercial cultivars. We found a resistant citron genotype in *Citrus*, named Citron C-05, by a long time screening of local germplasm (Deng et al. 2010). Systematic evaluation of the resistance for Citron C-05 has been completed and its resistance has been confirmed. Now three populations have been established, including the segregating hybrid population between the susceptible Shatian pummelo and Citron C-05, and two self-crossed progeny populations of Citron C-05 and of Eureka lemon. These segregating populations are under investigation for better understanding the genetic basis and molecular mechanism of the resistance to citrus canker disease.

Numerous researchers have made efforts to breed resistant genotypes in their genetic improvement programs, nevertheless, almost all the obtained citrus genotypes have demonstrated only reduced susceptibility to certain extent,

which is far away from real resistance. And except for a few works by cellular biotechnological approaches, all the genotypes are generated via genetic transformation, thus their release in the field is strictly prohibited or at least extremely difficult. Identification of resistant genes and comprehensively understanding of the resistance mechanism are indispensable for breeding citrus cultivars resistant to canker disease and commercially utilizable.

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Molecular Mechanisms for Resistance to Biotic Stresses

16

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Abstract

Diverse pathogens including viruses, viroids, fungi, and bacteria are responsible of diseases on Citrus. Some of them in addition represent a treath to Citrus industry in some specific areas, others are either worldwide spread or have a restricted distribution. Breeding program searching for resistance to a given pathogen must take into consideration the nature of the interaction being studied. In addition a large number of data generated by sequencing projects will contribute to the identification of individual genes or groups of genes potentially associated with resistance to biotic and abiotic factors. This chapter introduce the molecular basis of plant resistance to innate immune response elicited by non-specific elicitors and how successful pathogens have evolved to evade them or trigger them later in the infection so that they become infective. The other paragraphs are dedicated to illustrating three important disease model studies caused by a fungus (Alternaria brown rot), an oomycete (Phythophthora root rot) and a virus (Citrus Tristeza).

16.1 Plant Diseases that Pose a Threat to Citrus Industry

Cultivated citrus species are susceptible to many diverse pathogens including viruses, viroids, fungi, and bacteria. Some of them are responsible of diseases considered the most limiting factors for the development of citrus industry in some specific areas, others are either worldwide spread or have a restricted distribution. Historically, *Phytophthora* and 'tristeza' diseases, are known since long time and have changed the citrus production systems to budding on rootstocks by the beginning of the nineteen-century (Moreno et al. 2008). Since then many diseases and pests exclusively associated with the canopy (scion) or the root system (rootstock) have developed, along with those resulting from the interaction between them.

The progressive specialization of cultivation on a regional base and the use of monoclonal types of citrus were favorable conditions for the

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pathogens in different areas, and their spread increased by the huge increase of transportation and globalization. Nowadays climate changes are contributing in a great measure to further expand the world list of economically important and destructive citrus diseases. Amongst the most threaten are: Citrus variegated chlorosis (CVC); Citrus leprosis (CiL); Huanglonbing (HLB, previously known as greening); Citrus sudden death (CSD); Citrus bacterial canker (CBC); Citrus black spot and Alternaria brown spot (Timmer et al. 2000).

The narrow genetic base used by the citrus industry, the high genetic plasticity of citrus that allow them to adapt to different conditions, and vegetative propagation by buds and use of rootstocks from nucellar embryos are among the factors associated to severity of pests and diseases (Machado et al. 2011). Therefore, any breeding program searching for resistance to a given pathogen must take into consideration the nature of the interaction being studied. Several of them are of relevance due to the productivity losses they cause and the high cost of their control. The work on breeding for resistance and genetic mapping in different countries has been focused on different target according to regional relevance of citrus and specific problems. The largest interest has been devoted to destructive pest and pathogens, frequently included in the quarantine list of the Regional Plant Protection Services worldwide. The large number of data generated by sequencing projects has stimulated the use genotyping arrays to study the expression of thousands of genes, contributing to the identification of individual genes or groups of genes potentially associated with several metabolic pathways, resistance biotic and abiotic factors as well expression of QTL (Quantitative Trait Loci) at different environmental conditions. The integration of the database with the construction of arrays for gene expression studies is helpful to understand host-pathogen interactions and other traits of interest (Machado et al. 2011; Gmitter et al. 2012).

This chapter first introduces the molecular basis of plant resistance to innate immune response elicited by non-specific elicitors and how successful pathogens have evolved to evade them or trigger them later in the infection so that they become infective. The other paragraphs are dedicated to illustrating three important disease model studies caused by a fungus (Alternaria brown rot), an oomycete (Phythophthora root rot) and a virus (Citrus Tristeza). An extensive literature review on the pathogen- citrus interaction was recently published by Dalio et al. (2017). Two important disease model studies caused by bacteria, namely Huanglongbing and CBC, will be deepen in separate chapters (Chaps. 14 and 15).

16.2 Plant Resistance

16.2.1 Plant Innate Immunity—PRRs and PTI

The perception of environmental signals and the ability to respond accordingly are essential for all organisms in nature to survive. The innate immune system provides such abilities and protects organisms like plants and animals. Mammals have evolved a sophisticated adaptive immune system that relies on creation and selection of somatic diversity for recognition of pathogen-derived molecules. In contrast, plants rely on cell-autonomous innate immunity, activated upon pathogen detection by either cell surface or intracellular receptors. Plasma membrane located Pattern Recognition Receptors (PRRs) have evolved to detect specific molecular patterns that are interpreted by the plant cell as danger signals. These danger signals can be either infectious non-self determinants, such as microbe- or Pathogen Associated Molecular Patterns (PAMPs/MAMPs), or self-molecules, Damage Associated Molecular Patterns (DAMPs) that are released upon pathogen perception or pathogen-induced cell damage, and activate PAMP Triggered Immunity (PTI) (Macho and Zipfel 2014; Choi and Klessig 2016; Duxbury et al. 2016). PTI regulates a wide array of responses, including regulation at a cell to organism level, aimed at hampering pathogen growth and disease progression. Early PTI events include the rapid generation of Reactive Oxygen

Species (ROS), the activation of Mitogen-Activated Protein Kinases (MAPKs), and the expression of immune-related genes (Macho and Zipfel 2014). While plants defective in PTI signaling appear to be more susceptible to adapted but also non-adapted pathogens. This indicates that PTI is sufficient against the majority of the plant pathogenic microbes, while the best demonstration regarding its biological relevance is the necessity for adapted successful pathogens to interfere by the active suppression of this first layer of plant defence in order to cause disease (Macho and Zipfel 2014). These are the so called Successful pathogens.

16.2.2 Plant Innate Immunity—NLRs and ETI

The successful pathogens have developed pathogenicity components (largely known as effector proteins) to block PTI. Plants from their side, carry a repertoire of intracellular Nucleotide Binding-Leucine Rich Repeat (NB-LRR or NBS-LRR or NLRs) receptor proteins, which structurally and functionally resemble mammalian Nod-like Receptors (NLRs) to recognise pathogen effectors (Jones and Dangl 2006). Plant genes that code NLR receptors are generally known as disease resistance (*R*) genes and their products directly or indirectly intracellularly detect pathogen effector proteins (Jones and Dangl 2006; Duxbury et al. 2016; Jones et al. 2016). The importance of NLRs to plant defence is illustrated by the expanded complement of NLRs in plants compared to NLRs in most animals. For example, *Arabidopsis thaliana* carries ~120 full length NLRs, rice carries ~595 NLRs and wheat (*Triticum aestivum*) ~1224 NLRs, while most mammals have ~20 NLRs (Jacob et al. 2013; Sarris et al. 2016). NLR-mediated defence is termed Effector-Triggered Immunity (ETI) and often concludes to the induction of the hypersensitive response (HR), a form of programmed cell death that restricts pathogen's spreading to neighbouring host tissues and biotrophic pathogen growth. For historical reasons, recognized effectors that trigger ETI are often referred to as avirulence (Avr) proteins. The main difference between plant NLRs and mammalian NLRs is that plant NLRs detect pathogen effector proteins, while mammalian NLRs recognize DAMPs or relatively conserved pathogen molecules such as flagellin or peptidoglycan (Eitas and Dangl 2010; Maekawa et al. 2012; Duxbury et al. 2016; Mermigka et al. 2020; Mermigka and Sarris, 2019). The activation of plant and animal NLRs relies on a signal transduction ATPase with numerous domains (STAND) domain function, which is under strict control to prevent auto-activation (Leipe et al. 2004; Duxbury et al. 2016). The NB domain of plant NLRs belongs to nucleotide-binding, Apaf-1, R-protein and CED-4 (NB-ARC) class (Williams et al. 2014), and in the auto-inhibited or 'inactive' state, is proposed to be ADP-bound, while the exchange of ADP to ATP allows the NB-ARC domain to adopt an activated or 'active' state (Williams et al. 2014). A strict regulation mechanism, which usually includes intra-molecular interaction with the LRR domain and other domains, keeps the NB-ARC domain in the 'inactive' state in the absence of microbial ligands, preventing auto-immunity (Maekawa et al. 2012; Duxbury et al. 2016).

In plants there have been characterized two main groups of NLRs based on the N-terminal part of the protein. There are NLRs that either carry a Toll/Interleukin-1 receptor/Resistance protein domain (TIR) or a coiled-coil (CC) protein domain in their amino-terminal end. In mammalian NLRs the amino-terminal domain usually harbours a caspase-activation and recruitment domain (CARD), a pyrin domain (Pyr) or baculovirus inhibitor-of-apoptosis repeats (BIRs) (Maekawa et al. 2012; Duxbury et al. 2016). Both groups of plant NLRs are involved in pathogen recognition, but the two subfamilies have distinct genetic requirements and cluster separately in phylogenetic comparisons of their NB-ARC domains (Jones and Dangl 2006; McHale et al. 2006; Andolfo et al. 2014; Sarris et al. 2016). It is noteworthy that TIR-NLR-triggered immunity is often attenuated or abolished at or above 28 °C (Dinesh-Kumar et al. 1995; Zhu et al. 2010; Heidrich et al. 2013).

16.2.3 Plant Innate Immunity—Effectors Recognition 'Ligand and Guard/Decoy Models'

Although many NLR/effector protein pairs have been studied, the molecular mechanisms of effector perception by plant NLRs, such as precise knowledge of the interactions of protein domains, and mechanisms linking NLR/effector interaction with the activation of downstream resistance signalling pathways, remain unclear. In some cases, plant and animal NLRs function in pairs to mediate immune recognition (Narusaka et al. 2009; Eitas and Dangl 2010; Williams et al. 2014; Sarris et al. 2015; Saucet et al. 2015). Activation of ETI can occur via direct physical interaction of an effector with a plant NLR receptor, ('the ligand-receptor model'), followed by defence activation, resulting in transcriptional re-programing (Keen 1990; Ravensdale et al. 2012). Few cases of direct NLR/effector interactions have been reported so far (Jia et al. 2000; Dodds et al. 2006; Krasileva et al. 2010; Catanzariti et al. 2010; Cesari et al. 2013). The 'guard' and 'decoy' models provide plausible explanations for recognition of multiple effectors. According to those models, some plant NLRs monitor the integrity of host proteins with which they associate. These NLRs 'guard' host proteins that are targets of effectors, or host proteins that resemble those targets (Duxbury et al. 2016; Ntoukakis et al. 2014).

16.2.4 Plant Innate Immunity—Effectors Recognition 'the Integrated Decoy Model'

Interestingly, several plant NLRs reveal fusions with unusual protein domains (e.g. the WRKY domain of RRS1 in Arabidopsis, the heavy-metal associated domain of RGA5 in rice, mitogen-activation protein kinase domain of AT4G12020 and Lim domain of CHS3 in Arabidopsis, and many more), while the roles of these domains in defence activation remain largely unknown (Meyers et al. 2003; Nishimura and Dangl 2014; Sarris et al. 2016). The rice CC-NLR pair RGA4/RGA5 carries out recognition of two microbial effectors, AVR-Pia and AVR1-CO39, by direct interaction with RGA5 through a small unusual protein domain located at the C-terminal region, related to heavy metal–associated domains (Cesari et al. 2013). However, the exact mechanism of perception and defence activation remains unknown. Notably, some of these extraneous protein domains have evolved by duplication of pathogen effector target proteins and have been incorporated into plant NLR immune receptors (Sarris et al. 2016). Such an NLR is the Arabidopsis RRS1 that contains a WRKY domain fused to its C-terminus (Narusaka et al. 2009; Sarris et al. 2015; Saucet et al. 2015). An early hypothesis relating to RRS1 is that it represents a protein that has the capability to recognize, signal and activate defense through the binding of its WRKY domain to DNA (Narusaka et al. 2009). PopP2, an acetyltransferase effector from *Ralstonia solanacearum*, interacts with this C-terminal domain (Deslandes et al. 2003). *RRS1* is encoded in the genome in a head-to-head orientation with *RPS4*, which encodes another phylogenetically distinct TIR-NLR required for RRS1-dependent effectors' recognition. This raises the question of whether *RRS1* acquired the WRKY domain exclusively as a decoy against interference with other WRKY transcription factors that are involved in defence (Eulgem and Somssich 2007). The gene pair *RPS4/RRS1* also confers recognition of the *Pseudomonas* effector AvrRps4 and an unknown factor from *Colletotrichum higginsianum* (Birker et al. 2009). This is consistent with the theory that limited number of host components is differentially targeted by different pathogens (Sarris et al. 2016).

Two breakthrough studies concerning NLRs with integrated domains have led to new conjecture regarding the role of these domains (Sarris et al. 2015; Le Roux et al. 2015). These works together show that PopP2 can bind and acetylate the WRKY domain of RRS1, reducing its DNA binding activity and triggering HR.

Furthermore, Sarris et al. (2015) show that the *P. syringae* effector AvrRps4 can also bind to RRS1's WRKY domain and trigger HR. These studies also show how these effector proteins can associate with multiple WRKY transcription factors. A remaining puzzle in the field of plant NLRs with integrated domains (NLR-IDs) is whether or not the IDs are true decoys or participates as functional domains (e.g. real WRKY transcription factor in RRS1) in the plant physiology that occurs subsequent to activation. However, there are scientific opinions suggesting that this must be considered the endgame of the guard model whereby the guard and guardee become linked genetically to ensure adaptive co-evolution and eventually fuse to become one unit.

16.2.5 Plant Innate Immunity—Citrus spp NLRsomes

Citrus species are amongst the most important fruit trees and have been cultivated for more than 4000 years (Barrett and Rhodes 1976; Scora 1975; Wang et al. 2015). Molecular markers analysis and phylogeny showed that cultivated *Citrus* species (sweet orange, grapefruit, and lemon) are derived from three original cultivated *Citrus* species: *C. medica* (citron), *C. reticulata* (mandarin) and *C. maxima* (pummelo) (Wang et al. 2015). A recent work deals with the identification and comparison of the NB-ARC domain-containing genes from three *Citrus* genomes: *C. clementina*, *C. sinensis* from USA and *C. sinensis* from China. The authors describe the identification of similar numbers of NBS domain-containing genes amongst these three genomes. The authors describe the identification of 618, 650 and 508 NLR genes from *C. clementina*, *C. sinensis* China and *C. sinensis* USA genomes respectively (Wang et al. 2015). While, a recent and yet unpublished genome analysis performed by this chapter's author (Sarris P. F. and Pavlidis P. et al. unpublished data) based on the NCBI deposited *Citrus* genomes, reveal the presence of 623 NLRs in *Citrus clementina* cv Clemenules (NCBI BioProjects: PRJNA232045, PRJNA223006); 813 NLRs in Valencia sweet orange (NCBI BioProjects: PRJNA225998, PRJNA86123) and 491 NLRs in Miyagawa wase satsuma (NCBI BioProject: PRJDB5882). Further studies are on-going regarding the identification and the phylogenetic analysis of *Citrus* NLRs that carry integrated domains (Citrus NLR-IDs).

16.3 Disease Case Studies

16.3.1 Phythophthora Diseases

Phythophthora spp. are responsible of serious soil borne diseases of citrus, including damping off in the seedbed, root and crown rot in nurseries, foot rot and brown rot of fruits, causing significant economic losses to citrus industry. After the severe outbreak of the nineteen-century citrus propagation system moved to the use of the resistant rootstock sour orange. The most prevalent species include: *P. boehmeriae*, *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. drechsleri*, *P. hibernalis*, *P. megasperma*, *P. palmivora*, *P. nicotianae* (Panabieres et al. 2016).

P. nicotianae and *P. citrophthora* cause severe damage in citrus nurseries and orchards worldwide. *P. citrophthora* is mostly associated to trunk gummosis, whereas *P. nicotianae* is associated to root rot (Panabieres et al. 2016). Root rot and crown rot are critical for the grove survival (Graham and Menge 2000). Citrus rootstocks show a different level of sensitivity to *Phytophthora* spp. Almost all varieties are susceptible to gummosis, from highly susceptible (*C. sunki*) to resistant (*P. trifoliata*). Some hybrids of *C. sinensis* x *P. trifoliata* are resistant to *P. citrophthora* and less resistant to *P. nicotianae*.

Several studies investigated the hemibiotroph behavior of *P. nicotianae* that establishes itself in host tissues as a biotroph and once inside switch to a necrotrophic phase of growth. Recently reviewed by Dalio et al. (2018) there are old and new evidences of the mechanisms that are involved in the resistance to *Phytophthora*

spp. In different species of citrus rootstocks resistance appears related to phenolic compounds concentration. In both stem gummosis and root rot infections, total phenol content is higher in the leaves of resistant varieties than in those susceptible.

A correlation was found between phytoalexin production by the pathogen and the ability of *P. trifoliata* and Swingle citrumelo rootstocks to regenerate roots from the tip of the infected roots themselves. The phytoalexins escoparone and esculin have been detected in the bark and roots of citrus in response to infection by *P. citrophthora* and *P. nicotianae* associated to the inhibition of the pathogens. Escoparone accumulation was faster in the resistant cultivars.

Studies of *P. nicotianae* transcriptome in order to understand the basis of citrus gummosis, have identified genes that encode cell wall degrading proteins, such as phospholipases, glucanases and endopolygalacturonases, elicitins (ELI), effectors that induce necrosis in plants, such as crinkling and necrosis-inducers (CRN) and necrosis inducing proteins (NIP) (Rosa et al. 2007). Ten different elicitin classes of parasiticein, differently expressed, have been identified in vitro in *P. parasitica* (Panabières et al. 2005). In *P. parasitica*—citrus interaction in susceptible cultivars elicitins are up-regulated at the later stages of infection, associated to tissue necrosis (Boava et al. 2011a, b).

An increase in expression of effectors was detected in the late stage of infection in the susceptible *C. sunki* rootstock (susceptible) (Dalio et al. 2018). *Phytophthora* species are able to secrete two types of effectors related to their localization in plant tissues: the apoplastic or extracellular effectors, such as elicitins and NPP-like effectors; and cytoplasmic effectors, such RxRL and Crinkler effectors (CRNs), which possess special amino acid motifs in their structure enabling their entry inside cells independent of the presence of the pathogen (Hogenhout et al. 2009; Kamoun 2009).

Researches involving the model *P. trifoliata* (resistant) and *C. sunki* (susceptible) rootstocks, showed that defense genes, such as pathogenicity-related genes with anti-oomycete properties, and genes that act in plant water conductivity, are activated in the host in response to infection by the pathogen through signal transduction chains. These genes are also regulated by plant hormones, including salicylic acid (SA) and abscisic acid (ABA) (Boava et al. 2011a, b). In deep, the studies have reported the changes in global gene expression profiles and have shown differentially expressed genes involved in several processes, such as cell defense, photosynthesis and carbohydrate metabolism (Boava et al. 2011a, b). An increase in expression of above-mentioned effectors was observed in late stages of infection by *Phytophthora* spp., when pathogens enter the necrotrophic stage and promote hypersensitive response (HR) and necrosis in tissues of susceptible plant varieties, including citrus (Boava et al. 2011a; Oßwald et al. 2014) and more specifically *C. sunki* rootstock (susceptible) (Dalio et al. 2018).

Pathogenesis-related proteins (PRs) PR1, PR2 and PR5, which are responsive to salicylic acid (SA), have anti-oomycete properties, contributing directly to the defense against *Phytophthora* spp. (Dalio et al. 2017). The evaluation of the response to *P. nicotianae* infection in the susceptible (*C. sunki*) and resistant (*P. trifoliata*) genotypes of citrus showed that PR genes such as PR1, PR2, PR3 and PR5 were more up-regulated in *P. trifoliata* than in Sunki tangerine. This result suggests the involvement of these transcripts in mechanisms of resistance to citrus gummosis (Boava et al. 2011b). In addition, Boava et al. (2011b) showed that the expression of POX genes, others related to PR, and lipoxygenase (LOX), a gene that has been widely associated with plant defense against pathogens and both were over expressed in the resistant rootstock at later stages of infection, compared to the susceptible ones. ESTs from citrus species such as *C. sinensis*, *C. clementina* and *P. trifoliata* under various conditions, including biotic stresses in the CitEST project (Guidetti-Gonzalez and Carrer 2007) helped to identify a gene of the type TIRNBS-LRR, named RPS protein 4 (RPS4), and another gene named late embryogenesis abundant 5 (LEA5), both responsive to abscisic acid (ABA), had an increase in gene expression in resistant hybrids of *P. trifoliata*

(resistant parent) and *C. sunki* (susceptible parent) after infection by the hemibiotrophic pathogen *P. nicotianae* and that are differentially expressed between the resistant and susceptible parent (Boava et al. 2011a).

16.3.2 Alternaria Brown Spot of Tangerines

Two different pathotypes of fungus *Alternaria alternata*, the 'tangerine pathotype' and the 'rough lemon pathotype' are causal agents of Alternaria Brown Spot (ABS) an important disease of tangerines and their hybrids and of a similar disease that affects only rough lemon and Rangpur lime, respectively (Timmer et al. 2000, 2003). ABS affecting leaves, twigs and fruit have a large economic impact on tangerines and hybrids cultivation. Symptoms on young leaves and young shoots appear as brown to black spots and could be surrounded in the leaves by a yellow halo; affected leaves often abscise. Brown to black lesions tiny to large crater-like lesions develop on fruits.

The disease cycle is simple since there is no teleomorph known for *A. alternata* (Timmer et al. 2003). Conidia are produced primarily on the surface of lesions on mature or senescent leaves and on blighted twigs when lightly moistened or at high humidity and also wind dispersed.

The host specificity of the tangerine and rough lemon pathotypes of *A. alternata* depends upon the production of host-specific toxins (HSTs) that are also responsible for necrotrophic colonization and that possess the same selectivity as the pathogens (reviewed by Tsuge et al. 2013). Most HSTs are considered to be pathogenicity factors, required to invade tissue and induce disease and determines the host range of toxin-producing pathogens. *Alternaria* pathotypes produce HSTs which are diverse ranged chemical compounds, from low molecular weighted peptides to cyclic peptides. The toxin from the rough lemon pathotype was named ACR or ACRL-toxin, and that from the tangerine pathotype was named ACT-toxin (Miyamomto et al. 2010). The toxin is released during the germination of conidia, and rapidly affects the plasma membrane integrity of susceptible host cells. ACT-toxin causes veinal necrosis and electrolyte leakage from susceptible leaves but not on resistant leaves (Kohmoto et al. 1993). The mode of action of ACT toxin is still uncertain, but a rapid loss of electrolytes from leaf tissues and ultrastructural changes of cells treated with the toxin indicated that the primary action site of ACT-toxin was likely the plasma-membrane.

Although HSTs are responsible for pathogenicity a number of studies have investigated the role of other potential virulence factors. A mutant of Citrus Alternaria depleted in endopolygalacturonase (endoPG) production, important for fungal penetration, endopolygalacturonase showed reduced ABS symptoms (Isshiki et al. 2001). Fruits of Fortune mandarin, *Citrus limon* and *Citrus paradisi*, inoculated with *A. alternata* showed a degradative metabolism of flavonoids (flavanones, flavones and polymethoxyflavones) and *de novo* synthesis of the phytoalexin caused by an extracellular fungus laccase. Study of the substrate specificity of this enzyme revealed that flavonoids are substrates of *A. alternata* laccase suggesting its role in pathogenesis.

Citrus genotypes have been tested in order to evaluate the resistance to the fungus. In the overall more studies defined that clementine, Willowleaf and satsuma mandarins are resistant whereas genotypes Dancy and Fortune mandarins, Orlando, Minneola and Nova tangelos and the Murcott tangor are susceptible. Diploid progeny analysis suggested that inheritance of ABS resistance in citrus is controlled by a single recessive allele. Therefore, resistant cultivars are considered to be recessive homozygous for this locus, whereas susceptible cultivars could be heterozygous or homozygous dominant. Since the single locus inheritance of resistance, segregation is expected therefore markers for assisted selection were investigated.

In a recent study, a region containing the so-called ABSr locus, near the centromere on chromosome III using bulked-segregant and half-tetrad analyses from triploid populations was

located (Cuenca et al. 2013). This region was flanked by a Simple Sequence Repeat (SSR) marker (TTC8) and a Single Nucleotide Polymorphism (SNP) marker (CiC3248-06), found at 3.77 and 1.71 cM from the ABSr locus, respectively, delimiting a 3.3 Mb genome region. Moreover, no recombination was observed between another SSR marker (AT21) and the ABSr locus. This locus seemed to be included in a genomic region very rich in disease resistance homologous genes.

In a further study Cuenca et al. (2016) fine mapped the ABSr locus on LG III of the clementine's genetic map, using a 268-diploid progeny arising from a heterozygous susceptible × resistant hybridization, and identified candidate genes for resistance. This study also allowed to develop SNP molecular markers for efficient Marker Assisted Selection in citrus breeding programs.

Cuenca et al. (2016) limited the candidate region containing the ABSr locus to 1.5 cM flanked by two SNP markers at 1.1 and 0.4 cM, corresponding to 366 kb in the clementine reference genome (between positions 25.496.094 and 25.862.085 in the chromosome III). This region contained eight genes harboring NBS-LRR repeats and, candidate as genes for ABS resistance. Among the identified resistance genes, Ciclev10018637 and Ciclev10023511 encode for a Leucine-Rich Repeat (LRR) receptor-like protein with serine/threonine kinase domain. LRR receptor-like kinases (LRR-RLK) have a central role in signaling during pathogen recognition and activation of plant defense mechanisms, and developmental control. Both genes were mapped very close to the most significant SNP related to ABS resistance (SNP08). Another strong candidate for ABS resistance found within the region of interest and close to the SNP08 was the gene Ciclev10024361, encoding for an S-adenosyl-L-methionine dependent methyltransferases superfamily protein, with thiopurine S-methyltransferase superfamily protein. This gene is a good target for achieving resistance against necrotrophic pathogens, and therefore, for resistance to ABS.

Numerous genes involved in resistance to necrotrophs studied in other pathosystems were blasted against the citrus genome identifying many putative hortologues many of which located in chromosome III, although outside the region of interest, which deserve further attention (Cuenca et al. 2016). However, the study of the strongest candidate genes for ABS resistance in Citrus, Ciclev10018637, Ciclev10023511, and Ciclev10024361 could allow a good starting point to determine whether these genes are really involved in the Alternaria-citrus interaction.

In the same study it was determined that a single SNP marker, SNP08 flanking the ABSr locus, could discriminate the susceptible from the resistant genotypes. The SNP marker linked with the dominant susceptible allele of the ABSr locus is *G*.

16.3.3 Citrus Tristeza

Citrus tristeza is caused by the Citrus Tristeza Virus (CTV, Closteroviridae): a pathogen that changed the course of the citrus industry (Moreno et al. 2008). CTV can cause any of four distinct syndromes in citrus plants, depending on the virus isolate and the scion/rootstock combination. Decline (D-CTV) is a bud union disease that develops only in susceptible scion/rootstock combinations, when grafted on sour orange rootstock. The observed decline can be extremely rapid ('quick decline'), with wilting and death of trees occurring within a few days or weeks, or it can be a slower process, occurring over months or even years. Stem pitting (SP-CTV) is induced by an aberrant cambium development, resulting in pits in the wood, which reduces the plant growth, the size of the fruit and productivity, irrespective of the rootstock, and can affect both rootstock and grafted varieties. Seedling yellows (CTV-SY), characterized by stunting and leaf chlorosis, affects only sour orange, lemon and grapefruit. A fourth form of disease is associated to a complete lack of symptoms on most hosts, except for vein clearing and stem pitting, when the virus multiplies to high titers (Dawson et al. 2013). CTV decline is kept under control by

using resistant rootstocks, while stem pitting is mitigated by cross protection using weak strains of the virus.

CTV is a positive-sense single-stranded RNA (ssRNA) virus, member of the genus Closterovirus, with a complex genome (19.3 kb RNA divided into 12 ORFs and two UTRs), causing serious economic losses to citrus industry worldwide. CTV is a phloem-associated virus. It replicates in the cytoplasm of companion or phloem parenchyma cells of its hosts. It is graft-transmitted through the vegetative multiplication of infected host plants and by aphids in a semi-persistent manner (Moreno et al. 2008). Following the most recent review of current knowledge on CTV, the large number of isolates reported have been grouped into strains (Harper 2013). Their characterization is a prerequisite for a reliable control, breeding and surveillance program. Sequencing and biological assays demonstrates that CTV isolates assigned to a strain can differ remarkably in their phenotypes and infected plants may contain a pool of sequence variants from a single strain or several strains, resulting in isolates with mixed virus populations (Harper 2013). As stated by EFSA Opinion on CTV (EFSA PLH Panel 2017) 'a combination of biological, molecular and, possibly, serological data are needed for a conclusive characterization of the genetic and pathogenic features of a CTV isolate'.

Natural resistance

Host interference in CTV infection mechanisms is dependent on the citrus genotype. The virus systemically infects its hosts using only the long-distance movement from source-to-sink, while cell-to-cell movement is absent or limited to only small clusters of adjacent cells (Folimonova et al. 2008), likely related to the interaction of virus gene products with specific hosts (Dawson et al. 2013). Citrus species counteract the attack of CTV by RNA silencing, a central host defense reducing viral degradation to contain replication and restrict the virus to phloem cells. The p20 and CP proteins overwhelm intercellular silencing while p20 and p23 suppress intracellular silencing (Lu et al. 2004).

The constitutive expression of p23 appears to be responsible of the CTV titer in sour orange, allowing the virus to escape from the phloem in sour orange and sweet orange (Fagoaga et al. 2011). Nevertheless, it has been observed that viral replication and infection are not completely blocked, and the virus and its hosts reach a balance so that the virus remains in the host without causing severe symptoms or plant death. Other non-conserved genes-p33, p18 and p13-are necessary in different combinations for movement and overcoming host resistance (Dawson et al. 2013). The balance between expression of the effectors induces substantial changes in the severity of symptoms (Tatineni and Dawson 2012), or in the accumulation of miRNAs (Ruiz-Ruiz et al. 2011) and alters the plant small RNA regulatory pathway, resulting in symptom expression.

In a broad sense most Citrus species as seedlings are tolerant to the disease, despite some low symptoms. A variable degree of natural CTV resistance had been found in some citrus and related genotypes, including *C. maxima*, *C. aurantium*, *Atlantia ceylanica*, *Fortunella crassifolia*, *Poncirus trifoliata*, *Severinia buxifolia*, and *Swinglea glutinosa* (Garnsey et al. 1987, 1997; Ghosh et al. 2014; Bernet et al. 2004, 2008; Yoshida et al. 1983; Mestre et al. 1997). However, their resistance is not absolute, and depend on the CTV isolates tested (Garnsey et al. 1997; Dawson and Mooney 2000). *P. trifoliata* is resistant to most CTV isolates, and used as rootstock to effectively control the damage caused by seeding-yellow and decline-inducing CTV strains, but is susceptible to stem-pitting and resistant breaking isolates that can overcome this resistance, and are able to replicate and systemically invade resistant plants (Harper 2013). In citrus production area with *P. trifoliata* as rootstock, it is necessary to be on guard against occurrence of stem-pitting strain.

Being only *P. trifoliata* sexually compatible with citrus, many *Citrus*× *Poncirus* hybrids have been developed by conventional breeding. Some hybrids showing resistance to CTV have been widely used as rootstocks for citrus tree, such as Swingle citrumelo (*C. paradisi* × *P. trifoliata*)

(Castle et al. 1993), Carrizo citrange (*C. sinensis* × *P. trifoliata*) (Castle and Tucker 1998), US-812 (*C. reticulata* × *P. trifoliata*) (Bowman and Rouse 2006). While have no way to be used as scion cultivar because the coincident introgression of some undesirable traits from *P. trifoliata*. Moreover, conventional breeding of citrus has many problems including inbreeding depression, polyembryony, and long juvenility period, indicating its application in scion improvement for CTV resistance seems practically impossible.

The upsurge of genetic transformation of resistant gene has raised hope to overcome the shortcomings of conventional breeding in scion improvement. Since the resistance of *P. trifoliata* was conferred by a single dominant Mendelian gene designated Ctv (Gmitter et al. 1996; Fang et al. 1998), which induces broad-spectrum resistance to most CTV isolates. Apparently, it works by confining the movement of the virus to the root cells. Sequence analysis of the Ctv genomic region show CTV resistance is in a 121-kb region comprising ten genes (Rai 2006) and another different linkage group (Mestre et al. 1997). Grapefruit plants transformed with some of these ten candidate Ctv-R genes showed different levels of resistance: lack of infection, slow spread or initial infection followed by its abortion (Rai 2006). Despite a CC-NB-LRR R protein has been characterized the corresponding Avr CTV gene is still unknown (Rai 2006) and some of the viral proteins recognized by NB-LRR are not VSRs (de Ronde et al. 2014). Recently, the researches of Sahin-Cevik et al. (2014) and Gomez-Munoz et al. (2017) revealed WRKY transcription factors, the RNA silencing and salicylic acid defense pathways may play role in CTV resistance of citrus, which provide reference for identification of the key resistance gene in future.

Virus-driven resistance

Citrus plants can obtain virus resistance by the infection of a mild strain of the same virus in advance. This virus-derived resistance is called cross protection, which has been applied in CTV control. Several citrus producer countries have successfully isolated mild strains protective against severe SP-CTV isolates, including Brazil, South Africa, Australia, Japan and China (Costa and Muller 1980; Van Vuuren et al. 1993; Broadbent et al. 1991; Zhou et al. 2008). In Brazil, the protective CTV isolates developed by Muller and Costa have proven highly effective for over 40 years, and have been established in more than 80 million Pera trees as pre-immunization (Zanutto et al. 2013). Zhou et al. (2002) found CTV cross protection is caused by post-transcriptional gene silencing (PTGS), and its efficiency depend on the similarity between mild and severe virus strains in genetic background. A super infection exclusion mechanism has been shown in homologous genotypes inside the strain T36 (Folimonova 2013). It is possible that cross protection could be influenced and even broken by the change of host and field environment (Powell et al. 1992; Scott et al. 2013), which limit the development of this control strategy.

Another virus-driven resistance just like a simplified cross protection, is conferred by viral genes or sequences which are transformed into plant genome by genetic engineering. Some transgenic citrus lines with CTV major coat protein gene (p25), exhibited protection against CTV, but some others were susceptible (Dominguez et al. 2002). Similar results were also observed in transgenic citrus lines with other CTV genes. This resistance difference among transgenic lines have relationship with the accumulation of transgene-derived siRNA (Fagoaga et al. 2006; Lopez et al. 2010), indicating the second virus-derived resistance is also caused by PTGS. To directly utilize PTGS as means to impart CTV resistance into citrus plants, transformed vectors with stem-loop structure were designed to attenuate or block virus gene expression. Soler et al. (2012) found the stem-loop structure targeting simultaneously the three viral silencing suppressors (p20, p25 and p23) may achieve full resistance to CTV in citrus.

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