UNIVERSITÀ degli STUDI di CATANIA
Dipartimento di Agricoltura, Alimentazione e Ambiente
Di3A

UNIVERSITÀ DEGLI STUDI DI CATANIA

Agricultural, Food and Environmental Science

XXXV Cycle

NEW GENOMIC TECHNIQUES APPLIED TO CITRUS FOR THE OBTAINMENT OF SEEDLESS FRUITS

Lara Poles

Advisor:
Prof. Stefano G. La Malfa

Co-advisor:
Dr. Concetta Licciardello

Coordinator:
Prof. Antonio Biondi

Ph. D. attended during 2020/2022

Acknowledgements

I would like to acknowledge all people that contributed and improved my research project but also give me suggestions and support during these three years, before and now.
I would like to express my gratitude to my supervisor Prof. Stefano La Malfa and to my co-supervisor Dr. Concetta Licciardello for providing me the opportunity to work on this project and for their support and guidance toward this dissertation.

Special thanks to Prof. Alessandra Gentile who guided and supported me with her important advices, thanks to you I apply for this PhD.
Many thanks to Prof. Gaetano Distefano and Dr. Stefania Bennici for teaching me a lot about citrus flowering, one of the main topics of this project, and for helping me to finalize this work.
I would like to thank my colleagues, in particular the rome group (Angelo, Fabrizio and Helena), the four room's group (Valeria, Giuliana and my desk mate Chiara), Sebastiano, Leonardo and Mario that collaborated in the analyses and helped me a lot to finalize this project.

I would like to thank Di3A (Paolo, Francesco, Giuseppina, Giulia, Fabio) and CREA (Carmen, Giusi, Damiano) coffee friends and all the professors, researchers and technicians from the Di3A at University of Catania and from the CREA of Acireale for their scientific help but also for letting me feel at home in a new city.

Finally, I would also like to thank both the PhD coordinators that I met during these three years, Prof. Alessandro Priolo and Prof. Antonio Biondi, it was nice to share with you this experience, PhD courses and activities were great opportunities to learn and grow together with my colleagues.

Index

Research highlights

- Conventional breeding strategies have many limitations in citrus, New Genomic Techniques can be a valid alternative
- Genotype, explant type, and other factors affect the regeneration potential and transformation efficiency of commercial citrus varieties
- Transgenesis and genome editing can be successfully applied for the improvement of citrus quality traits and the development of new tolerant or resistant varieties
- 'Monreal' *de novo* genome was assembled and its S-genotype was defined as S_7S_{11}, same as 'Comune'
- RNA-seq analysis of self-pollinated pistils from both 'Monreal' and 'Comune' revealed the lack of expression of *S_7-RNase* in the self-compatible genotype
- RNA-seq of self-pollinated pistils from both 'Monreal' and 'Comune' followed by gene ontology identified 2,965 DEGs, mostly showing oxidoreductase and transmembrane transport activity
- 'Monreal' and 'Comune' RNA-seq analysis compared to the reference genome enabled the identification of 7,781 genes characterized by the presence of one or more SNPs among the two genotypes, mostly located on scaffold 7
- Genome editing can be applied in citrus, most of the edited plants showed a high mutation rate of reads
- The use of dual sgRNA approach can be efficiently used to knockout *IKU1* gene in citrus, 16 edited plantlets have been obtained

Abstract

Seedlessness is a highly desirable trait for citrus fresh fruit and one of the factors that mainly contribute to its subsequent high market value. The presence of seed, often hard and having a bad taste are a big hindrance for consumers that prefer seedy fruits only when the seedless alternative is unavailable. In Citrus seedlessness is a complex trait and can be induced by different mechanisms, including the presence of triploidy, male or female sterility, parthenocarpy, if the fruit develops without ovules fertilisation, or stenospermocarpy, if the fruit contains partially formed seeds that have aborted after fertilization; another reason can be the presence of self-incompatibility (SI) reaction that prevents seed formation, especially if the variety is cultivated in isolated blocks in the absence of cross pollination. In order to elucidate and describe the genetic basis of SI mechanisms, an integrated approach based on both genomic and transcriptomic data was developed on clementine (*Citrus clementina* Hort. ex. Tan.), one of the most common varietal groups in the Mediterranean area. In particular, the genome of 'Monreal' clementine was *de novo* assembled and the S-genotype of 'Comune' clementine (self-incompatible) and its natural self-compatible mutant 'Monreal' was defined as S_7S_{11}; RNA-seq comparison of self-pollinated pistils from both genotypes identified 2,965 differentially expressed genes, most showing an oxidoreductase and transmembrane transport activity and revealed the lack of expression of *S_7-RNase* in 'Monreal'. These data compared to the reference genome enables the identification of 7,781 genes characterized by the presence of one or more SNPs among the two genotypes, mostly located on scaffold 7 containing the S locus suggesting their involvement in the SI regulation.

The obtainment of seedless citrus varieties has been pursued for a long time using different techniques; the application of new-generation biotechnologies, also known as New Genomic Techniques (NGTs) represents a valid alternative to traditional techniques, such as hybridization, mutagenesis and selection, as it allows the introduction

of precise modifications without altering the original genetic background of the variety considered. Among the NGTs, genome editing application seems powerful for the improvement of elite cultivars, especially for gene knockout or for the insertion of genes and mutations conferring novel features; in citrus it had been successful applied in editing genes involved in citrus canker susceptibility. Here, CRISPR/Cas9 system was successfully used to knock out *IKU1*, a gene involved in the regulation of seed size, since the loss of function mutations in *HAIKU* (*IKU*) pathway genes cause a decrease in the dimension of Arabidopsis mutant's seed. Therefore, we used a dual-single guide approach on the homologous of *IKU1* in citrus, transforming three seedy varieties, including two model species and one seedy sweet orange variety. Sixteen plants were analysed confirming that *IKU1* gene and the translated protein were interrupted. In particular, among the edited plantlets, 4 samples displayed a large deletion of 327 bp present between the two cut sites of sgRNA1 and sgRNA2, while 2 samples showed an inversion.
Phenotypic evaluations, that are still undergoing due to plant juvenility phase, will help to understand the role of *IKU1* gene in *HAIKU* pathway and its importance for the obtainment of seedless new cultivars.

Keywords: CRISPR/Cas9, self-incompatibility, clementine, genome editing, transformation, regeneration, seed

Riassunto

L'apirenia è una caratteristica molto ricercata negli agrumi e uno dei maggiori fattori che contribuisce al suo aumento di valore nel mercato. La presenza di semi nei frutti, spesso duri e di cattivo gusto, è un grande ostacolo per i consumatori che preferiscono i frutti con semi solo quando un'alternativa apirena non è disponibile. La mancanza di semi negli agrumi è un tratto complesso e può essere causato da diversi meccanismi quali ad esempio la presenza di sterilità maschile o femminile, la triploidia, la partenocarpia, quando la fecondazione dell'ovulo non avviene o la stenospermocarpia, quando invece lo sviluppo dei semi è interrotto a fecondazione avvenuta; in altri casi l'apirenia può essere causata da reazioni di auto-incompatibilità che impediscono la formazione del seme, specialmente se la varietà viene coltivata in blocchi isolati dove l'impollinazione incrociata è impedita. Per caratterizzare e descrivere le basi genetiche del meccanismo di auto-incompatibilità sono state studiate due varietà di clementina (*Citrus clementina* Hort. ex. Tan), uno dei gruppi varietali più diffusi nella regione Mediterranea. La cv. di clementine 'Comune' (auto-incompatibile) e la cv. 'Monreal', il suo mutante naturale auto-compatibile, sono state caratterizzate mediante un approccio integrativo che combina dati genomici e trascrittomici. In particolare è stato assemblato *de novo* il genoma di 'Monreal' ed è stato definito il genotipo del *locus S* delle due varietà, ('Comune' e 'Monreal') S_7S_{11}; il confronto delle reads ottenute tramite RNA-seq dei pistilli autoimpollinati di 'Comune' e 'Monreal' ha portato all'identificazione di 2965 geni differenzialmente espressi, molti dei quali sono responsabili di processi di ossidoriduzione e attività di trasporto trans-membrana e ha permesso di rilevare la mancanza di espressione dell'*RNasi* S_7 in 'Monreal'. Questi dati, allineati con il genoma di riferimento hanno permesso di identificare 7781 geni caratterizzati dalla presenza di uno o più SNP presenti nei due genotipi, per lo più localizzati nello scaffold 7, lo stesso che contiene il *locus S*,

suggerendo il suo coinvolgimento nella regolazione dei geni coinvolti nell'auto-incompatibiltà.
L'ottenimento di piante di agrumi apirene è stato perseguito a lungo utilizzando diverse tecniche; l'applicazione delle biotecnologie di seconda generazione, anche note come Nuove Tecniche Genomiche (NGTs) rappresenta una valida alternativa alle tecniche tradizionali, quali l'ibridazione, la mutagenesi e la selezione, in quanto consente di introdurre modifiche precise senza alterare il background genetico originale della varietà considerata. Tra questi, l'applicazione del genome editing in agrumi sembra molto promettente per il miglioramento di cultivar selezionate, specialmente per il silenziamento genico o per l'inserzione di geni e mutazioni che portano nuove caratteristiche; esso è stato applicato con successo per la modifica di geni coinvolti nella suscettibilità al cancro degli agrumi. In questa tesi, il sistema CRISPR/Cas9 è stato utilizzato con successo per silenziare il gene *IKU1*, coinvolto nella regolazione della dimensione del seme; la perdita di funzione delle mutazioni nei geni del pathway di *HAIKU* (*IKU*) causa una diminuzione della dimensione del seme nel mutante di Arabidopsis. Pertanto, è stato utilizzato un approccio a doppia guida per l'editing dell'omologo di *IKU1* negli agrumi; la trasformazione è stata realizzata in tre genotipi di agrumi con semi, due specie modello e una varietà di arancio dolce. Sono state ottenute e analizzate sedici piante, confermando l'interruzione di sequenza nel gene *IKU1* e l'assenza di alcuni amminoacidi nella proteina tradotta. In particolare, tra le piante editate, 4 campioni presentano una grande delezione di 327 bp tra le due guide sgRNA1 e sgRNA2, mentre 2 campioni presentano un'inversione. Le valutazioni fenotipiche, al momento non possibili a causa della lunga giovanilità, consentiranno di verificare e confermare il ruolo del gene *IKU1* e la sua importanza per l'ottenimento di nuove varietà senza semi.

Parole chiave: CRISPR/Cas9, autoincompatibilità, clementina genome editing, trasformazione, rigenerazione, semi

1. General introduction

1.1 Origin and economic importance of Citrus species in the world

The genus *Citrus* belongs to the *Rutaceae* family, subfamily *Aurantioideae*, and the main cultivated species are sweet orange [*Citrus sinensis* (L.) Osb.], mandarin [*C. reticulata* Hort. ex Tan. and *C. unshiu* (Mak.) Marc. mainly], grapefruit (*C. paradisi* Macf.), pummelo [*C. grandis* (L.) Osb. or *C. maxima*], lemon (*C. limon* L. Burm. f.) and limes [*C. aurantiifolia* (Christm.) Swing. and *C. latifolia*].

In 2020, the worldwide cultivation of citrus, according to FAO, accounted for more than 10 million hectares, giving more than 150.000 million tons. The most widely cultivated species are oranges (75 million tons, Figure 1.1), followed by clementines, mandarins, tangerines and satsumas (38 million tons), lemons and limes (21 million tons), and grapefruits and pummelos (9 million tons). More than 80% of citrus fruits are consumed as fresh while the remaining are employed for industrial transformation, especially for juice production (FAO 2021). China (38 million tons), Brazil (19 million tons), and the United States (15 million tons) are the main citrus producers with some distinctions on the type of productive system: in China production is mainly marketed for internal use. Brazil is instead characterized by much larger farms and fruits are mainly employed for juice processing together with small and medium-sized growers that produce fresh fruit for the domestic market. In USA, citrus production is mainly centred in Florida, California, Texas, and Arizona where there are a range of small, medium, and large farms with a great deal of government oversight (Spreen et al. 2020).
According to FAO, Italy produced 2,94 million tons of citrus in 2020 corresponding to an average yield of 17 tons/ha with a harvested area of 145,100 ha (FAO, 2021).

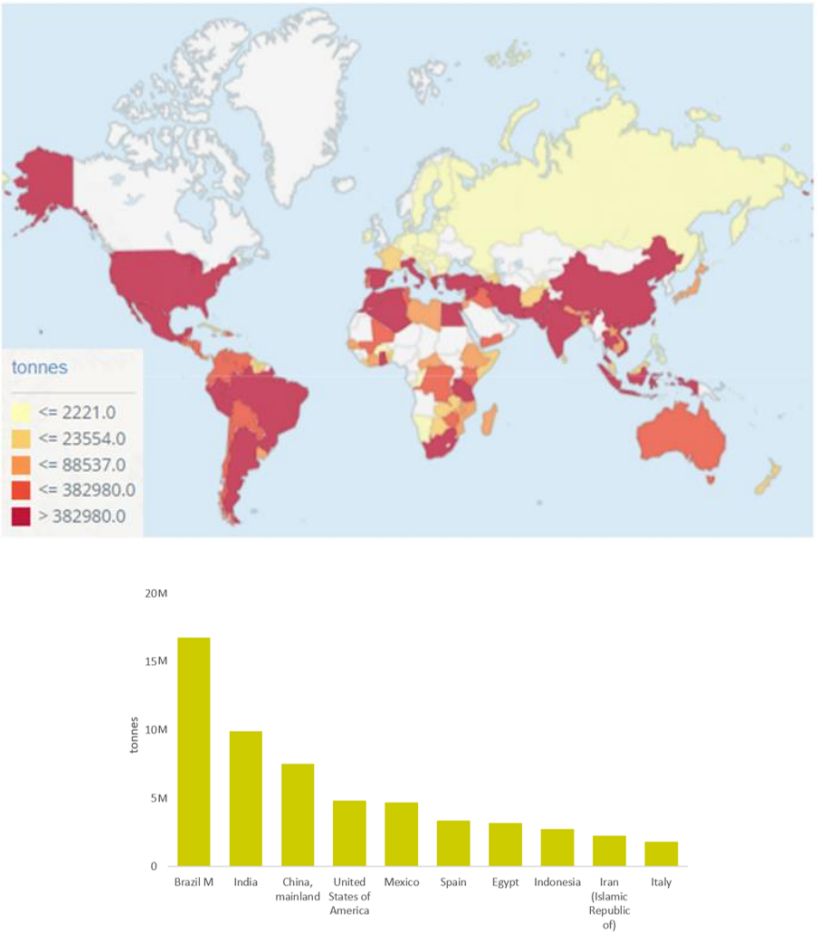


Figure 1.1 World orange production in 2020. Map with the production quantities of oranges by country and the top 10 orange producers in the world (FAO 2021).

Genomic, phylogenetic and biogeographic analyses suggest that citrus species originated during the late Miocene in the southeast of Asia, in a region that includes the eastern area of Assam, northern Myanmar and western Yunnan and that cultivated citrus arose from the natural interspecific hybridization between five ancestors: *C.*

medica L. (citron), *Fortunella japonica* (kumquat) *C. reticulata* Blanco (mandarin), *C. maxima* (L.) Osb. (pummelo) and *C. micrantha* Wester (a wild citrus belonging to Papeda subgenus) (Figure 1.2, Cuenca et al. 2018; Wu et al. 2018). Sexual hybridization between the ancestral species led to the development of modern varieties, such as lemons, sweet oranges and grapefruits, that growers kept, among their selection process, through clonal propagation (nucellar polyembryony or grafting). The phylogeny of the genus *Citrus* has been largely clarified thanks to the interrogation of the genomic variants among the *Citrus* species.

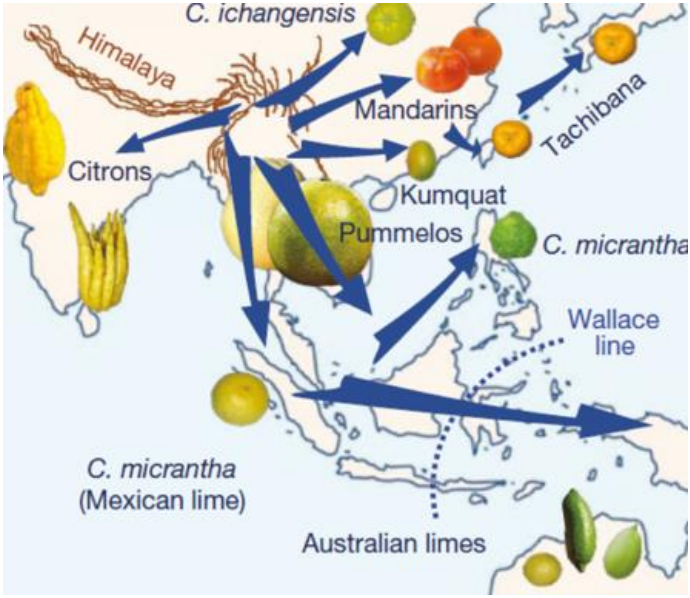
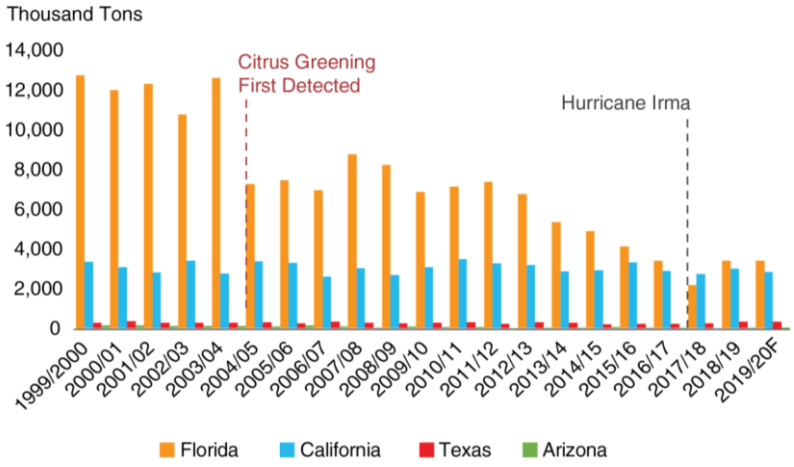


Figure 1.2 Proposed origin of citrus and ancient dispersal routes. The five progenitor citrus species (*C. medica*, *Fortunella*, *C. reticulata*, *C. micrantha*, and *C. maxima* have migrated from the centre of origin, the triangle formed by north eastern India, northern Myanmar and north western Yunnan (blue arrows). Modified from Wu *et al.*, 2018.

Citrus cultivation is vulnerable to numerous threats; nowadays the most serious disease at the global scale is the Huanglongbing

(HLB, also known as citrus greening), which was first identified in Florida in 2005. HLB is causing severe economic losses due to the fast progression of symptoms coupled with the rapid dispersal, the lack of resistant commercial citrus varieties and the absence of a durable control mechanism.

As an example, in recent years the USA production halved their production even though serval concauses contributed to such rapid decline (e.g.: the presence of citrus canker and some significant meteoric events such as storms and hurricanes), the appearance of HLB is certainly the main contributor to this decline together with main responsible factor. In particular, such a decrease in production was registered in Florida, in this state Citrus production registered a 300% decrease with a production passing from more than 12 million tons to 4 million tons (Figure 1.3).



Note: F = forecast. Citrus production includes production of grapefruit, oranges, and lemons; excludes production of tangerines, mandarins, and tangelos due to lack of data.

Source: USDA, Economic Research Service.

Figure 1.3 Citrus Production by the USA States. USA production declined by nearly 50% in recent years due to many reasons, firstly the spread of HLB found in Florida since 2005. (USDA, 2020)

1.2 Citrus genetic improvement

The progress made in citrus genetic and biotechnology research is changing the context of citrus breeding, potentially, accelerating the process and the release of new commercial varieties. Despite many limitations present in citrus for the application of sexual hybridization, clonal selection, induced mutagenesis and ploidy manipulation, these traditional methods are still used, enabling the selection of superior citrus cultivars; in the last decades the release of new genomes (Di Guardo et al. 2021; Wang et al. 2017, 2018; Wu et al. 2014) has opened a new possibility for many researchers to assess molecular mechanisms underlying agronomically-important traits, in some cases also characterizing genes responsible for a specific feature or involved in its regulation (Cuenca et al. 2018); this has important implications for the application of transgenesis and of new genomic techniques, (NGTs), including genome editing and CRISPR/Cas system. Due to the existence of some legal and ethical issues on the application of NGTs, at the moment the use of QTL discovery and the development of markers has become fundamental for the release of a new improved cultivar (Figure 1.4).

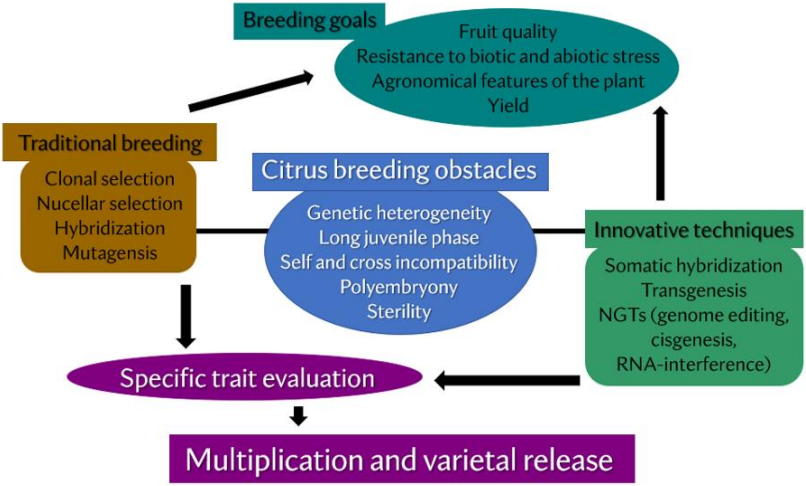


Figure 1.4 Citrus improvement trends. Overview of breeding goals, limitations, traditional and innovative approaches to overcome breeding challenges.

Citrus breeding has focused on many goals, essentially the development of new scions and new rootstocks. Sour orange (*C. aurantium*) has been the most used rootstock since 1800, however, in the last decade the outbreak of pest and disease (like citrus tristeza virus, CTV and HLB) had accelerated the development and the selection of new resistant rootstocks, among the other Troyer and Carrizo citranges [*C. sinensis* (L.) Osb. × *Poncirus trifoliata*], Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata*) and more recently Sunki mandarin (*C. sunki* Hort. ex Tan.) and trifoliate orange (*P. trifoliata*) hybrid rootstocks.

Among the most important breeding selection traits for fruit (Figure 1.4) there are peel and pulp colour, especially the presence and the accumulation of anthocyanins and carotenoids has been shown to have many beneficial effects on human health (Lila et al., 2004); a particular fruit flavour is very important, sweet or sour with a unique and rich

aroma (Raveh et al. 2020) while in grapefruit and grapefruit-like varieties a low furanocoumarins level is preferred (Fidel et al. 2016). Other breeding targets, very important for mandarin and mandarin-like groups, are fruit size and shape (from small and oblate to large and round fruits), seedlessness (fruit with less than 5 seeds) and ease of peeling, a trait determined by the degree of rind separation from the pulp (Raveh et al. 2020). Considering citrus production, yield and quality performance are also very important breeding goals, together with the harvesting and marketing period that should be as long as possible: the combination of different goals together, like the production of fruits with a long shelf-life obtained from cultivars that are disease resistant and stress tolerant is the biggest challenge for breeders, as it would ensure to growers the best profitability.

1.2.1 Traditional breeding

In citrus, as well as in all woody crops, the selection and development of novel varieties through traditional breeding are time-consuming and expensive. Breeding efforts are hampered by some peculiarities that are common to other woody plant species, like the high heterozygosity, the prolonged juvenile period (3-5 years) and the long generation time, making the time needed from the selection of promising new cultivars to the evaluation till their commercial release, of up to 25 years (Caruso et al., 2020).

For these reasons, the high diversity in terms of fruit quality and agronomical traits that are currently available for most citrus species is mainly due to the selection and asexual propagation of individuals originated by chance from bud sports (Peña et al., 2008). In addition, citrus are also characterized by a range of biological characteristics including non-climacteric fruit ripening and dormancy that makes the full utilization of the citrus variability difficult using conventional breeding techniques (Iglesias et al. 2007). Important limitations are also due to the difficulty to manage field evaluation for large progenies

and multigenerational breeding schemes of the fruit (Iglesias et al. 2007). In addition, the complex, and rather uncommon, characteristics of the reproductive system require the set-up of specific breeding plans for citrus that must take into account that:

- most of the important species show some degree of apomixis (thus adventitious embryos develop directly from nucellar cells limiting or precluding the development of zygotic embryos);
- many genotypes show parthenocarpy and/or self- or cross-incompatibility, and others show defective pollen and/or ovule sterility (Abouzari and Nezhad 2016; Pena et al. 2008; Raveh et al. 2020).

Despite enormous constraints and the long time needed, citrus breeders have selected new improved cultivars through the years using traditional methods such as hybridization, clonal selection and induced mutation.

Sexual hybridization has been applied all over the world both using diploid and triploid parents, especially to develop new mandarins, for example, the seedless mandarin 'Primosole' (Tribulato and la Rosa, 1993) and 'Mandared' (Russo et al. 2004) or for the development of new rootstocks like 'Carrizo' and 'Troyer'(Savage and Gardner 1965); the application of this method is particularly complicated in citrus due to many aspects, first, the long time needed for fruiting, second the high heterozygosity that causes a strong variability in the progeny of a controlled cross and finally the presence of a large number of apomictic varieties that cannot be used as female parents in cross-hybridization (Cuenca et al. 2018; Raveh et al. 2020).

Induced mutagenesis has been used widely in citrus, it ensures the obtainment of new varieties with a genetic background similar to the parent with some new features, like a different flesh colour (as 'Star Ruby' pink grapefruit originated from 'Duncan') or the lack of seeds (for example the seedless 'Tango' was originated from a

mutation of ‘Nadorcott’ mandarin, Cuenca et al. 2018), thanks to the exposition of the woody budsticks to specific mutagenesis agents, especially gamma rays; this method can be applied easily, it does not need the knowledge of how a gene is controlled, is relatively rapid and cost-effective but it can result in chimeric mutation and a large number of mutated plants need to be evaluated to discover desirable stable mutations (Cuenca et al. 2018). Also, spontaneous mutations are relatively frequent in citrus and has generated many known varieties like for example the navel and the ‘Tarocco’ oranges.

Despite the limitation described above, nowadays many breeding program around the world are still based on the expensive and time-consuming classical approaches; the selection of superior cultivars is done mainly through the generation of a large number of hybrid progenies screened in the field and/or in greenhouse with the help, if available, of markers for marker-assisted selection (MAS) (Caruso et al. 2020; Gill et al. 2022).

1.2.2 Novel breeding techniques

Since the 1990s, genetic transformation represented a promising and effective alternative for the genetic improvement of citrus. This is particularly true for transgenesis using *Agrobacterium tumefaciens* transformation and, at a less extent, polyethylene glycol (PEG) mediated DNA uptake process. In the last three decades, many traits were improved such as early flowering (Cervera, Navarro, and Peña 2009; Duan, Fan, and Guo 2010; Endo et al. 2005; Nishikawa et al. 2010; Peña et al. 2001; Pons et al. 2014; Velázquez et al. 2016), the tree architecture and the growth habitus (Distefano et al. 2013; Fagoaga et al. 2007a; Alessandra Gentile et al. 2004), the tolerance to abiotic stresses (Ali et al. 2012; Molinari et al. 2004; Orbović, Fields, and Syvertsen 2017), the improvement of fruit quality (Alquezar et al., 2008; Dutt et al., 2016; Hijaz et al., 2018; Koltunow et al., 2000; Li et al., 2002, 2003; Pons et al., 2014) and the resistance to biotic stresses

(CTV, Mal secco disease caused by *Plenodomus tracheiphilus*, citrus psorosis virus, citrus canker, *Alternaria alternata*, *Phytophtora*, HLB*)*, the most limiting factors for citriculture worldwide (Boscariol-Camargo, Takita, and Machado 2016; Chen et al. 2013; Cheng et al. 2017; Dutt et al. 2015; Fagoaga et al. 2006; Gentile et al. 2007; Miyamoto et al. 2008; Narayan et al. 2010; Qiu et al. 2020; Reyes et al. 2011; Robertson et al. 2018; Soler et al. 2012; Zhang et al. 2010).

An important contribution to the improvement of novel varieties is due to the development of the second-generation biotechnologies called new genomic techniques, NGTs (European Commission 2021) or new plant breeding techniques, NPBTs, (Eriksson et al. 2018a) that comprises genome editing and cisgenesis, techniques allowing the isolation of genes underlying the trait of interest and their precise modification or transfer into targeted varieties, without altering the unique characteristics of the original cultivar.

The first application of genome editing in citrus fruits has been achieved in 2014 targeting phytoene desaturase gene (Jia and Wang 2014a, 2014b) while other applications focused on the editing of genes involved in citrus canker susceptibility using *Agrobacterium*-mediated transformation of epicotyl tissues or *Xanthomonas citri* ssp. citri (Xcc)-facilitated agroinfiltration (Jia, et al. 2017; Jia et al. 2017; Jia and Wang 2020a; Peng et al. 2017; Zhu et al. 2019).

Other approaches aimed at accelerating the evaluation and the characterization of the transformed or edited plants (especially when the trait of interest is expressed in the flower or the fruit) were based on the reduction of the juvenility period. Successful attempts employed the overexpression of flowering meristem identity genes (Cervera et al. 2009; Peña et al. 2001) or citrus homolog of the flowering-time gene (Endo et al. 2005; Nishikawa et al. 2010; Pons et al. 2014) in juvenile tissues (Figure 1.5). Several authors focused on the use of mature tissue for transformation rather than the most widely

employed young tissue (Cervera et al., 1998, 2008; Dutt et al., 2018), or in the case of mandarins hybrids that are more difficult to transform with *A. tumefaciens*, protoplast or embryogenic calli can be a valid alternative (Dutt et al., 2018; Mahmoud et al., 2022).

Figure 1.5 Early flowering *CiFT* transformed plants. Carrizo citrange plants transformed with a vector containing *CiFT* gene displays premature flowering three months after the beginning of the experiment.

The use of biotechnology and the development of NGTs in citriculture is expected to potentially improve the efficiency of breeding programs. However, it must be considered that, at the moment, these plants cannot be cultivated for commercial purposes in the European Union (EU) by the current genetically modified plants (GMPs) legislation.

Currently, the EU regulation is based on a product-oriented approach while many researchers propose to surpass this approach for a technique-oriented paradigm (Eriksson et al., 2019, 2020). The

directive 2001/18/EC does not exclude NGTs that can mimic naturally occurring processes; in addition, the Directive's Annex 1B list exempts 'mutagenesis' from the definition, but it is unclear if 'mutagenesis' should be interpreted strictly, meaning a spontaneous mutation occurring naturally or it should also include the ones obtained through human manipulation, for example using gamma irradiation, and/or including precise mutagenesis techniques developed after 2001 (Eriksson et al. 2019).

Over the last 10 years, the European Commission requested the European Food Safety Authority (EFSA) to develop a scientific position on the plants obtained through NGTs. EFSA has published three opinions, one on site-directed nuclease (SDN)-1, SDN-2 and oligonucleotide directed mutagenesis (ODM, EFSA et al., 2020), one on cisgenesis and intragenesis (EFSA, 2022a) and one on the safety assessment of plants developed through SDN-3 (EFSA 2012). In 2022(b) EFSA better specify that the generation of new cisgenic and/or intragenic plants (and derived products) requires a case-by-case evaluation of the potential hazards; in addition, in a new statement (EFSA, 2022a) EFSA develop 6 criteria for the risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis (Figure 1.6).

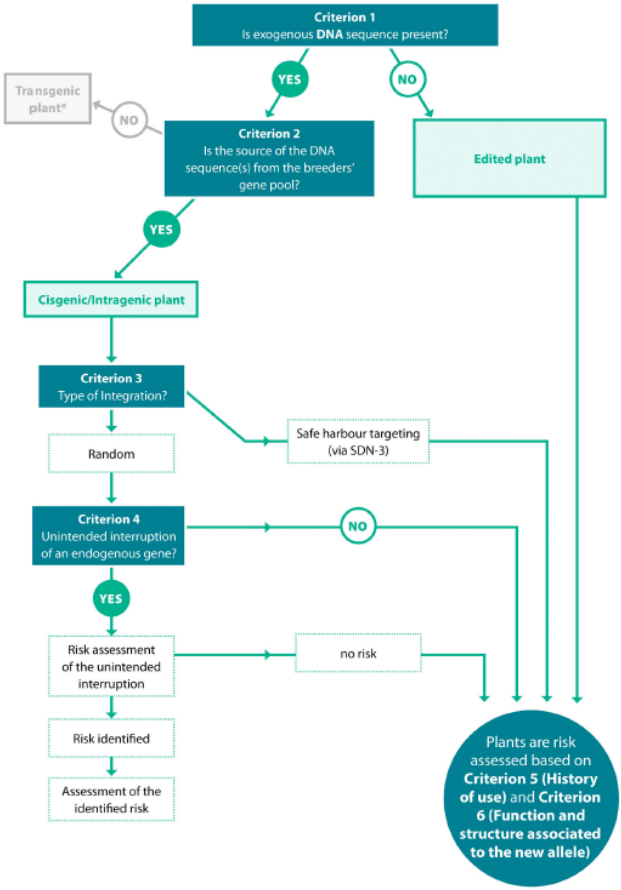


Figure 1.6 Criteria proposed by EFSA. The decision tree summarizes the 6 criteria for the risk assessment of plants developed through targeted mutagenesis, cisgenesis and intragenesis **(**EFSA, 2022a).

1.3 Case study: the obtainment of seedless citrus fruit

Seedlessness is a highly desirable trait for citrus fresh fruit and one of the factors that main contribute to its market value. Varieties are considered 'seedless' if the fruits have either no seed, traces of

aborted seeds or a much-reduced number of seeds; in particular citrus fruits with less than 5 seeds are considered to be seedless (Varoquaux et al., 2000; Gambetta et al. 2013). In citrus the presence of a large number of seeds greatly hinders consumer acceptability even if fruits are characterized by other positive organoleptic properties; seedless fruits are also more easily processed making the trait an important feature for varieties that are not intended for fresh consumption (Abouzari and Nezhad 2016; Vardi, et al. 2008).

Two main seedlessness mechanisms (Figure 1.7) have been described in plants, depending on the time at which the development of the seed is arrested: parthenocarpy, if the fruit develops without ovule fertilisation, stenospermocarpy, if the fruit contains partially formed seeds that have aborted after fertilization.

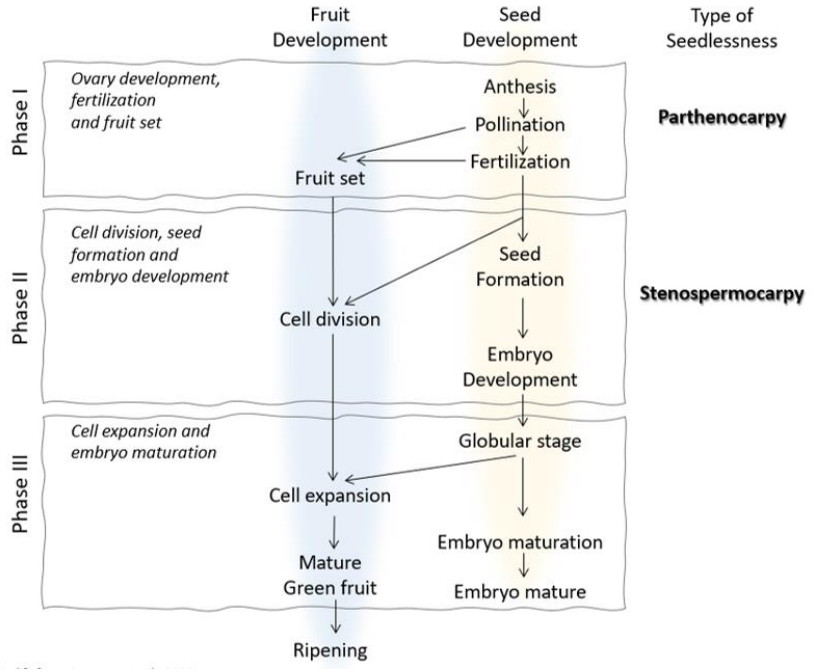


Figure 1.7 Seedlessness classification. Parthenocarpy and stenospermocarpy affect the seed development at different moment (modified from Varoquaux et al., 2000).

1.3.1 Parthenocarpy

The level of parthenocarpy can vary significantly among citrus species and it can require pollination (facultative parthenocarpy as for 'Star Ruby' grapefruit) or not (obligatory parthenocarpy as for 'Tahiti' lime) (Vardi et al. 2008; Varoquaux et al. 2000). Another distinction can be made between "stimulative parthenocarpy", if the pollination stimulus is required to set seedless fruits or, "vegetative parthenocarpy" (referred also as autonomous or autonomic) if fruit set occurs without pollination or any external stimulus (Montalt et al.

2021; Vardi et al. 2008). Parthenocarpy has been extensively studied and many researches highlighted a central role of endogenous phytohormones, especially auxins and gibberellic acid, coupled with epigenetic mechanisms that regulate fruit development; in addition, MADS-box transcription factors seem to play a role in the regulation of endosperm development and fruit set (Joldersma and Liu 2018; Vardi et al. 2008).

The lack of seeds can be due to different mechanisms: (i) the presence of female sterility that can comprise ovule sterility, failure of the pistil development up to the functional stage and the early stage arrest of the seed development; (ii) male sterility that is related to pollen development and fertility; (iii) the presence of self-incompatibility (SI) reactions that prevents seed formation in the absence of cross pollination (Montalt et al. 2021).

In past years traditional techniques have been widely used for the obtainment of seedless new commercial clones of citrus. Seedless mandarin generated through irradiation programs include 'Nero' and 'Nulessin' both originated from Clemenules', 'Orri', generated from 'Orah' hybrid ('Temple'×'Dancy'), and 'Tango', originated from 'Nadorcott' tangor (Cuenca et al. 2018; Pena et al. 2008). Selection of bud sport mutation has been used by Chinese breeders that through the years had identified 150 clones seedless or less seedy citrus plants, including 'Shatian' pummelo (Zhuang et al. 1994), 'Shatangju' (Zi-xing et al. 2006), 'Ponkan' and 'Xuegan' sweet orange (Biswas et al. 2020; Cheng et al. 1997).

Seedlessness has been obtained also by conventional hybridization; among the examples, there are the seedless mandarin 'Primosole' obtained from the cross of the 'Avana' mandarin with the 'Miyagawa' satsuma (Tribulato and la Rosa 1993), and the seedless mandarin 'Nectar' obtained by 'Wilking' self pollination (Vardi et al. 2008). Triploid breeding programs had produced sterile plants using sexual hybridization of tetraploid parents, protoplast fusion, embryo rescue,

and ploidy analysis by flow cytometry (Aleza et al. 2012); among the triploids released there are 'Mandared' (Russo et al. 2004), 'Safor' and 'Garbì'(Aleza et al. 2010).

In the last years, biotechnological approaches involving suicide genes expressed only in a specific tissue (i.e. the stigma or the tapetum) or at a specific time (for example during seedcoat development) could result in seedless fruits; Li et al., (2003) transformed 'Ponkan' mandarin inducing male sterility, using the *barnase* suicide gene, a cytotoxic ribonuclease that destroys tapetal cells and controls its expression using a tapetal-specific promoter.

Among the many high-quality seedless citrus varieties developed so far, 'Tahiti' lime [*C. latifolia* (Yu. Tanaka) Tanaka] and 'Oroblanco' (*C. grandis* x · *C. paradisi*) that have a triploid origin, 'Satsuma' mandarin (*C. unshiu* Marc.) and 'Washington navel' orange (*C. sinensis*) that display both female and male sterility and develop parthenocarpic fruit without pollination or fertilization (Iglesias et al., 2007) are widely cultivated and appreciated by consumers.

In self-incompatible varieties, seedless fruit occurs only if cross-pollination with compatible cultivars do not occur. SI is a mechanism that prevents self-fertilization and it is based on the discrimination between self- and non-self pollen. It has been classified into gametophytic or sporophytic depending on when (thus where in the style) the incompatible reaction occurs. In most citrus hybrids gametophytic SI is observed, this phenomenon is characterized by the arrest of the tube growth at 50% of the style, in other cases, the pollen germination is arrested earlier when the pollen is on the stigma (Newbigin, Anderson, and Clarke 1993).

Different horticultural techniques have been reported to reduce the number of seeds in the fruit. The cultivation of self-incompatible parthenocarpic cultivars in orchards that are isolated (often several kilometers) from cross-pollinators can avoid cross pollination, despite it is not compatible with intensive commercial production conditions

(Chao 2005; Vardi et al. 2008); pollen dispersal distance was studied using fluorescent AFLP markers and was about 500m for 'Clementina de Nules' pollen and 960m far for 'Nadorcott' (Chao, Fang, and Devanand 2005).

Another strategy is the use of anti-insect net coverage mostly in orchards where cross-pollination prevails, despite that air temperature and relative humidity can be altered under the net affecting the fruiting process both in terms of quality and yield (Otero and Rivas, 2017). Temperature has an important impact on citrus pollination and shifts from the optimal temperature affect the pollination performance and thus the production of seeds, inducing drastic modification to the male gametophytes (tapetal cells, microspores and pollen grains, Bennici et al. 2005; Distefano et al. 2012).

Exogenous application of gibberellic acid (GA) and copper sulphate ($CuSO_4$) can be used to reduce the number of seeds in citrus fruits.

Fruit development involves three phases: the first is the fruit setting, when the ovary can abort or proceed with wall cell divisions in order to set the fruit, the second is cell division followed by the third, cell expansion, in which the fruit reaches its final size by increasing its volume (Varoquaux et al. 2000). During the first phase, fruit set is promoted by fertilization that induces GA synthesis in the ovule re-activating cell division; pollinated ovaries of 'Pineapple' sweet orange shows an increase of GA level in pollinated ovaries at anthesis while in un-pollinated ovaries GA level was progressively reduced during and after the anthesis, leading to ovary abscission (Ben-Cheikh et al. 1997). In high parthenocarpic fruits, like 'Satsuma', fertilization doesn't occur and the autonomous activation of GA biosynthesis at anthesis in pericarp tissues activates ovary cell division determining a high proportion of fruits set. Facultative parthenocarpic fruits, like 'Clementine', lack the initial autonomous GA biosynthesis, thus failing the transition from ovary to fruit set, so the application of

exogenous GA during flower blossom increases fruit set but is non-effective in 'Satsuma' mandarin (Mesejo et al. 2008, 2016; Talon, et al., 1992).

In ‘Nadorcott’ mandarin (known commercially as Afourer®), GA applicated during anthesis reduced the percentage of seeded fruits and seeds number per fruit, but the most efficient treatment was obtained using 50 mg/L of GA coupled with 25 mg/L of $CuSO_4$ (Gambetta et al. 2013; Otero and Rivas 2017). In fact, it has already been proved that copper sulphate, applied as a foliar spray at full bloom, increases the percentage of seedless fruits and reduces seeds number per fruit under cross-pollination conditions; the presence of $CuSO_4 \cdot 5H_2O$ reduced in vivo pollen germination and/or interrupted pollen tube development, preventing pollen tubes from reaching the embryo sac (Mesejo et al. 2006).

1.3.2 Stenospermocarpy

Stenospermocarpy was only occasionally observed in ‘Valencia’ sweet orange (Koltunow et al. 1995) and described in ’Mukaku Kishu’ cherry orange fruit (*C. kinokuni* Hort. ex Tan.), a bud mutant of the seedy ’Kishu‘ (*C. kinokuni*) that is diploid, produces seedless fruits even if cross-pollinated and can transmits its seedless characteristics to its progeny (Yamasaki et al. 2007, 2009); the use of this cultivar in the breeding program had led to the development of other stenospermocarpic varieties such as ’Southern Yellow’ (Yamasaki et al. 2007).

‘Mukaku kishu’ and its seedless descendants are characterized by the presence of specific small seeds with an immature, soft, and edible seed coat (swollen seeds, classified as ‘type A’ in Figure 1.8) that differ completely from the seeds present in seedy cultivars, that had, in contrast, a mature, hard and dried seed coat (Figure 1.8). Histological investigation highlighted that, in the seedless varieties, the zygote of fertilized ovules divides into more cells but a few weeks

after pollination, it is inhibited mainly at zygote or proembryo stages. The arrest of seed development at an early stage after fertilization does not involve endosperm abortion (Yamasaki et al. 2007, 2009). The use of molecular markers and of segregating populations with full-sib families having the seedless *C. kinokuni* as a parent allowed the identification of the genomic region associated seedlessness and it seems that the process is controlled by a single dominant allele (Fs) with 'Mukaku kishu' having Fsfs genotype (Chavez and Chaparro 2011).

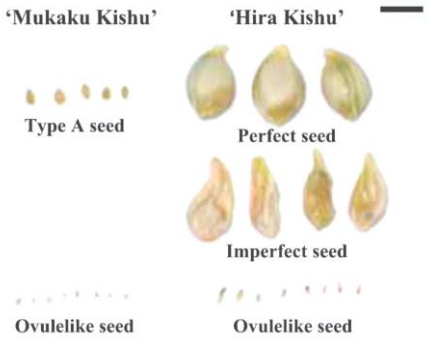


Figure 1.8 Comparison of seeds present in *C. kinokuni.* On the left, 'Mukaku kishu' seedless cultivar; type A swollen seeds and squashed ovule-like seeds; on the right, 'Hira kishu' seedy cultivar that shows normal developing seeds (perfect), poorly developing seeds (imperfect) and ovule-like seeds (squashed seeds). (bar = 0.5, Yamasaki et al., 2009)

Few studies have tried to induce stenospermocarpy in self-incompatible species grown under cross-pollination conditions: while Lewin and Monselise (1976) failed to produce seedless fruits spraying trees with naphthalene acetic acid, Mesejo and colleagues (2014) sprayed 'Afourer' tangor [*C. reticulata* Blanco × *C. sinensis* (L.) Osb.] with maleic hydrazide, a plant growth regulator that specifically interferes in the S phase of the cell cycle, and succeeds in inducing

fertilized ovule abortion applying it during the first and second week after anthesis.

1.4 Aim of the thesis and its outline

The dissertation project aims to provide novel insight on the obtainment of seedless citrus varieties through the use of NGTs. Firstly, this thesis focuses on the identification of candidate genes responsible for seedlessness in citrus fruits in order to use them to guide new seedless variety; among the many different mechanisms responsible for citrus lack of seeds, SI was chosen and deeply investigated through an integrative approach combining both genomic and transcriptomic analysis. In the meantime, regeneration and transformation potential of different citrus cultivars were investigated and optimized using different media combinations; NGTs were also studied and investigated to better plan the appropriate strategy to be used. CRISPR/Cas9 genome editing, one of the most famous NGTs was then applied to citrus using a gene chosen from literature involved in the control and the reduction of seed size in Arabidopsis.

Results will be presented and discussed as follow:

- Chapter II provides the state of art of NGTs in citrus and discusses the recent findings related to citrus transformation and regeneration protocols; limits and benefits of these techniques, together with factors affecting the successful application of transformation and genome editing in the manuscript 'Recent Advances of *In Vitro* Culture for the Application of New Breeding Techniques in Citrus' published the 24 July 2020 in the special issue of the journal Plants on the subject of 'Advances in Genetic Engineering Strategies for Fruit Crop Breeding'. https://doi.org/10.3390/plants9080938.

- Chapter III investigated the genetic bases of SI, one of the different mechanisms responsible for the lack of seed in citrus fruits; transcriptomic profiles of clementine (*Citrus clementina* Hort. ex. Tan) 'Comune' (self-incompatible) and its natural self-compatible mutant 'Monreal' were compared and many differentially expressed genes were identified, mostly showing a oxidoreductase and transmembrane transport activity. Genomic comparison revealed the S-genotype of these cultivar together with the presence of many SNPs, mostly located in the S-locus

- Chapter IV describes the successful application of CRISPR/Cas9 genome editing to reduce seed presence in citrus; the transformation was applied to three seedy genotypes, two model species and a cultivar of sweet orange, and the gene considered for the editing was of *IKU1*, the homologous of the one described in Arabidopsis HAIKU pathway, involved the regulation of seed size. Despite phenotypic evaluations of these edited plants are still not available, in 16 plants *IKU1* gene resulted modified and thus the deduced amino acid protein showed a different structure. The manuscript reporting these data is in preparation and it will be soon submitted.

2. Recent Advances of *In Vitro* Culture for the Application of New Breeding Techniques in Citrus

This chapter was published as:
Poles, L., Licciardello, C., Distefano, G., Nicolosi, E., Gentile, A., & La Malfa, S. (2020). Recent advances of *in vitro* culture for the application of new breeding techniques in citrus. Plants, 9(8), 938. https://doi.org/10.3390/plants9080938.

2.1 Abstract

Citrus is one of the most important fruit crops in the world. This review will discuss the recent findings related to citrus transformation and regeneration protocols of juvenile and adult explants. Despite the many advances that have been made in the last years (including the use of inducible promoters and site-specific recombination systems), transformation efficiency, and regeneration potential still represent a bottleneck in the application of the new breeding techniques in commercial citrus varieties. The influence of genotype, explant type, and other factors affecting the regeneration and transformation of the most used citrus varieties will be described, as well as some examples of how these processes can be applied to improve fruit quality and resistance to various pathogens and pests, including the potential of using genome editing in citrus. The availability of efficient regeneration and transformation protocols, together with the availability of the source of resistance, is made even more important in light of the fast diffusion of emerging diseases, such as Huanglongbing (HLB), which is seriously challenging citriculture worldwide.

Keywords: regeneration, transformation, genome editing, genotype, agroinfiltration, promoter, selectable-marker genes, disease resistance

2.2 Introduction

Citrus is one of the most important fruit crops in the world. In 2018, the surface devoted to citrus production totalled 11.1 million hectares, with a huge production of oranges (75 million tons), followed by clementines, mandarins, tangerines and satsumas (34 million tons), lemons and limes (19 million tons), and grapefruits and pummelos (9 million tons) (FAOSTAT, 2019).

However, the global citrus industry relies substantially on large-scale monoculture, and it is threatened by several diseases with a great economic impact in the main production areas, such as China, Brazil, Mexico, United States, and some Mediterranean countries.

The development of novel varieties with improved resistance to various pests and pathogens is one of the main aims of citrus breeding programs; conventional breeding strategy in citrus has demonstrated numerous limitations due to biological characteristics common to woody plants, such as long juvenile period, large size, long generation time, and also the lack of knowledge on how the most important horticultural traits are inherited. In addition, citrus display other limitations, such as nucellar polyembryony, self-incompatibility, and high heterozygosity, that genetic engineering and New Plant Breeding Techniques (NPBTs) (Eriksson et al. 2018b; Limera et al. 2017) can overcome, leading to the development of novel varieties with the incorporation of selected traits, while retaining the unique characteristics of the original cultivar.

NPBTs include different biotechnological tools that are used to induce DNA modification, such as insertion, deletion, gene replacement, or stable gene silencing. Genome editing, or sequence-specific nuclease technology, involves the production of a permanent and inheritable mutation in a specific DNA sequence that can be inaccurately repaired by the plants' own repair mechanism (leading to gene knock-out), or that can be accurately repaired using a DNA-repair template (leading to target mutation or gene replacement)

(Bortesi and Fischer 2015; Jinek et al. 2012; X. Liu et al. 2017).

Cisgenesis or intragenesis approaches are based on transformation with genetic material from closely related species capable of sexual hybridization, in contrast to transgenesis, where genetic material can be mixed between species; in particular, while cisgenesis involves the use of a copy of a complete natural gene, intragenesis allows *in vitro* recombination of different gene elements (Lusser et al. 2012; Schouten et al. 2006).

Other important techniques include trans-grafting, a method where a non-genetically modified (GM) scion is grafted on a GM rootstock leading to better performance of the top and to the production of GM-free fruits (Limera et al. 2017; Lusser et al. 2012), and RNA interference (RNAi), a mechanism activated by the presence of target double-stranded DNA molecules that results in the inhibition or suppression of gene expression (Chhetri et al. 2019; Martínez de Alba et al. 2013).

Compared to other fruit tree species, some citrus varieties are really amenable to tissue culture (Grosser et al. 2000), and micropropagation and transformation have been widely used for many agronomically important varieties using different types of explants, such as epicotyls, shoot segments, protoplasts, and embryogenic cells.

Among the others, *in vitro* juvenile tissues are the most used due to their high morphogenic ability and the polyembryonic nature of many cultivars that enables the production of true-to-type plants by seed germination; however, this strategy cannot be adopted in seedless varieties, such as ‘Navel’ oranges and ‘Satsuma’ mandarins or monoembryonic species like ‘Clementine’, where only the zygotic embryo develops from the seed (Dutt et al. 2018). To overcome this problem, other tissues with morphogenic potential, such as mature tissues or cell suspensions derived from embryogenic callus, need to be used for these cultivars (Dutt et al. 2010, 2018; Li et al. 2002).

This review aims to summarize the progress achieved in citrus

genetic engineering, with particular focus on the transformation of juvenile and mature tissues, factors affecting the regeneration and selection of the transgenic shoots, and their main applications; new advances in citrus biotechnology, such as the use of selectable marker genes, inducible promoters, and genome editing will also be described. The availability of an optimized organogenesis protocol associated with an efficient *Agrobacterium*-mediated gene transfer system will contribute to a successful application of NPBTs and the development of novel varieties with improved quality features or resistance traits.

2.3 Regeneration of Citrus for Genetic Transformation

Citrus tissues are recalcitrant to regeneration and transformation; common systems use nucellar seedling internodes due to the polyembryonic nature of most citrus cultivars, but this cannot be applied to seedless genotypes or to species that are difficult to regenerate via organogenesis, such as mandarins. The use of cell suspensions or protoplasts obtained from embryogenic callus can represent a valid alternative for genetic transformation, to obtain plant recovery through somatic embryogenesis rather than the induction of adventitious shoots (Dutt et al. 2018; Omar et al. 2016).

2.3.1 Genotype Influence

The availability of an organogenesis protocol based on the culture of juvenile explants allowed the production of transgenic plants for many citrus species, with variable degrees of success in terms of transformation efficiency (TE) (Orbović and Grosser 2015; Pena et al. 2004); genotype is one of the main factors influencing the effectiveness of the protocol, as some genotypes are considered easy to transform (e.g., citranges (*Citrus sinensis* L. Osbeck. × *Poncirus trifoliata* L. Raf.) (Dutt and Grosser 2009; Peña et al. 1995), ‘Duncan’

grapefruit (*C. paradisi* Macf.) (Dutt and Grosser 2009; Orbović and Grosser 2015), while others are regarded as recalcitrant (e.g., 'Clementine' (*C. clementina* Hort. ex Tan.) (Cervera et al. 2008) and sour orange (*C. aurantium* L.) (Ghorbel et al. 2000)).

Despite the narrow genetic diversity present in citrus (Wu et al. 2014), the differences that exist between cultivars of the same species are sufficient to affect their organogenic response and the frequency of transformed explants after *Agrobacterium* infection (Table 2.1).

Table 2.1 – Regeneration (RE) and transformation (TE) efficiencies of different citrus species. Explant types considered are epicotyl segment (ES), mature internode segment (MIS), and mature nodal segment (MNS) with buds removed.

Species	Cultivar	Explant type	RE (%)	TE (%)	Reference
Citrus sinensis L. Osb. × *Poncirus trifoliata* L. Raf.	'Carrizo' citrange	ES	37.5	20.6	##
Citrus sinensis L. Osb.	'Valencia'	ES	28.8	23.8	##
Citrus sinensis L. Osb.	'Valencia'	MIS	9.12	0.88	##
Citrus sinensis L. Osb.	'Tarocco'	MNS	74.7	9.1	##
Citrus sinensis L. Osb.	'Pineapple'	MIS	23	6.1	##
Citrus sinensis L. Osb.	'Jincheng'	ES	28.3	4.7	##
Citrus sinensis L. Osb.	'Jindan'	ES	13	3.6	##
C. reticulata 'Sunki' × *Poncirus trifoliata* 'Flying Dragon'	'US-942'	MIS	29.42	3.96	##
Citrus clementina	'Clemenules'	MIS	1.28	0.3-3	##
Citrus paradisi Macf.	'Ruby Red'	MIS	10.70	1.05	##
Citrus medica L.	'Etrog'	MIS	9.49	1.49	##
Poncirus trifoliata L. Raf.	'Precocious trifoliate orange'	ES	66.1	57.4	##

Among sweet orange cultivars, despite the fact that epicotyl explants of 'Valencia' and 'Jincheng' had similar regeneration potential (28.8% and 28.3%, respectively), they showed different percentages of TE, with the second cultivar being almost five-fold lower. The highest percentage is reached by *P. trifoliata*, a citrus related genotype with a short juvenile period particularly useful for functional genomics studies, and by 'Carrizo' citrange, one of the most responsive species to transformation among citrus; in all cases, the

transformation process reduces the percentage of regenerated shoots in all the examples shown (Table 2.1).

2.3.2 Source of Explant Type

Citrus epicotyls show a good *in vitro* morphogenic response, and therefore have been mostly used for the standardization of regeneration protocols (Singh and Rajam 2010); considering mature shoots, internodes of 1 cm have been used for the regeneration and the transformation of adult tissues in citrus (Almeida et al. 2003; Cervera et al. 2005, 2008).

The use of thin sections of mature stems has been explored in sweet orange and has resulted in higher percentages of regenerated and transformed shoots (35% TE) (Kobayashi et al. 2003, 2017) with respect to longer internodes.

Another alternative is the use of leaf discs, especially material from propagated or greenhouse-grown plants, which would assure abundant supply and a low risk of contamination. In sweet orange, only a few reports have been successful; no bud induction was obtained on leaf discs of mature 'Hamlin' (Almeida et al. 2003) or 'Thompson' navel (Esmaeilnia and Dehestani 2015), while a regeneration rate of 60% was reached using 'Valencia' (Khan et al. 2009), and the TE of its leaves was 23.33% (Khan et al. 2012).

Regeneration is possible through somatic embryogenesis that can be induced using appropriate culture media and starting from ovules of immature fruits (Omar et al. 2016); the embryogenic cell suspension obtained can be maintained and transformed directly, via *Agrobacterium* infection, or indirectly, isolating protoplasts (Dutt and Grosser 2010; Grosser and Gmitter 2011). Citrus protoplasts obtained from leaves are not totipotent and do not develop into somatic embryos, while the ones obtained from embryogenic cell cultures have the best potential for proliferation and embryo regeneration (Omar et al. 2016). Protoplasts are usually transformed using a polyethylene

glycol (PEG)-mediated DNA uptake process or via electroporation (Guo et al. 2005; Omar et al. 2007).

2.3.3 Basal Media and Other Factors Influencing Organogenic Response

Basal culture media influence the morphogenesis performance, and the most stimulating are MS (Murashige and Skoog 1962) and MT (Murashige and Tucker 1969), irrespective of the cultivar analyzed (Boscariol et al. 2006; Cardoso et al. 2010; M. Cervera et al. 1998; Dutt and Grosser 2009; Esmaeilnia and Dehestani 2015; Ghorbel et al. 2000; de Oliveira et al. 2010); Woody plant medium (WPM) (Lyoyd and McCown 1980) is mainly used for the elongation of adventitious shoots to ensure a larger dimension to facilitate micrografting *in vitro* (Almeida et al. 2003; Cervera et al. 2008; Kobayashi et al. 2003).

The efficacy of the medium in the regeneration process, in terms of hormone concentration, has been investigated among different cultivars. Many reports have shown a promotive effect in citrus shoot regeneration using low cytokinin concentration (1–3 mg/L), depending on the cultivar (Cervera et al. 2005; Rodríguez et al. 2008).

The addition of cytokinin 6-Benzylaminopurine (BAP) was sufficient to induce organogenesis from mature and juvenile explants of many sweet orange genotypes, except for the ‘Navelina’ cultivar; to increase the regeneration efficiency of this genotype, an auxine, 1-Naphthaleneacetic acid (NAA), was added to the regeneration medium containing BAP, increasing the percentages of callus growth, bud formation, and also TE (from 0 to 3%). The same treatment resulted in an opposite effect when applied to ‘Pineapple’ genotype, and a reduction of TE from 6 to 0% was observed (Rodríguez et al. 2008). The same combination (BAP and NAA) in the regeneration medium gave good results for mature sweet oranges (‘Pera’, ‘Valencia’, ‘Natal’, and ‘Hamlin’ (Almeida et al. 2003)), rangpur lime

(de Oliveira et al. 2010), and sour orange (Ghorbel et al. 2000); however, auxin supplementation did not improve the regeneration of 'Carrizo' citrange, 'Mexican lime' (*C. aurantifolia* Swingle), lemon, rough lemon, 'Cleopatra' mandarin, *P. trifoliata*, *C. macrophylla*, and clementine (Pena et al. 2004; Rodríguez et al. 2008).

When plant cells are subjected to stress, such as wounding or cutting, ethylene biosynthesis increases, affecting plant regeneration (Navarro-García et al. 2016). Different ethylene inhibitors have been evaluated in citrus tissue cultures. Among others, silver ions (Ag+) can interfere with ethylene receptors, improving cell regeneration; for example, the addition of $AgNO_3$ had a weak effect on 'US-942' rootstock regeneration compared to the influence of other phytohormones (Marutani-Hert et al. 2012).

In addition, the use of antioxidants can improve regeneration and TE; for example, lipoic acid improved the transformation of epicotyl segments of 'Mexican lime' by five-fold compared to control explants (Dutt et al. 2011).

Among the environmental conditions, photosynthetic radiation and incubation temperature are factors affecting the performance of *in vitro* tissue culture; in particular, it was reported that temperature of approximately 27°C was adequate for the development of adventitious buds in sweet orange seedlings (Duran-Vila et al. 1992), and a period of incubation in darkness promotes an organogenesis response. Organogenesis from mature internodes of 'Pera', 'Valencia', 'Natal', and 'Hamlin' oranges occurred directly from the explants without intermediate callus formation with a continuous 16-h photoperiod, and indirectly in darkness culture. Histological sections showed structural changes in the cambium with an intense cell proliferation at both cut ends after 15 days of culture (callus proliferation), and several meristematic regions differentiated from the callus tissues, leading to the formation of adventitious buds after 30 days (Almeida et al. 2003); similar observations were reported in sweet orange regeneration and

grapefruit (Marutani-Hert et al. 2012).

2.4 Citrus Transformation Protocols

Since the first attempt in citrus transformation in 1989 (Kobayashi and Uchimiya 1989) that used a PEG-mediated strategy on protoplasts, *Agrobacterium*-mediated transformation has been shown to be the most widely used method, and approximately 90% of the transgenic plants were produced using this methodology (Gong and Liu 2013).

The most used protocol for juvenile tissue explants transformation starts with the preparation of epicotyl segments; after *Agrobacterium* infection, explants are blotted dry and placed on cocultivation medium for 3 days at low light intensity. Subsequently, explants are transferred on regeneration and selection medium for 2 weeks in the dark until the formation of a white callus and then in a 16-h photoperiod with light; among the different protocols published for genetic transformation of citrus seedlings, few differences in the medium composition are reported (Dutt and Grosser 2009; Orbovic et al. 2015; Sendin and Filippone 2019). Difficulties in the low rooting efficiency of regenerated shoots (Duran-Vila N. 1989; Peña et al. 1995) were circumvented by the use of *in vitro* shoot micrografting (Peña et al. 1995) and minigrafting (Marques et al. 2011) onto decapitated seedlings of citrange germinated *in vitro*.

Besides many factors affecting the transformation, preincubation of explants in a hormone-rich medium prior to bacterial infection have been shown to increase the genetic transformation rates (Birch 1997; Dutt and Grosser 2009; Orbović and Grosser 2015; Peng et al. 2019), activating cells at the cut end of explants and stimulating their divisions and de-differentiation. In juvenile tissues, 3 h of incubation in a MS medium supplemented with 13.2 μM BAP, 0.5 μM NAA and 4.5 μM 2,4-D was sufficient to increase the morphogenic

competency in ‘Carrizo’ citrange, ‘Duncan’ grapefruit, ‘Hamlin’ orange and ‘Mexican lime’ (Dutt and Grosser 2009), while for sour orange, a pre-culture of 1 day in MS medium containing 1 mg/L BAP and either 0.3 mg/L NAA or 0.3 mg/L 2,4-D, resulted in a stress response (Ghorbel et al. 2000); also in the transformation of stem segments of adult ‘Tarocco’ oranges, the preincubation period of 6 h was sufficient to increase TE, while a prolonged period resulted in explant necrosis (Peng et al. 2019).

In mature tissues, a cocultivation phase after *Agrobacterium* infection in medium rich in auxin and in darkness conditions promotes hormone enrichment in the infected cells and stimulates callus formation (Cervera et al. 2005, 2008; Magdalena Cervera et al. 1998; Rodríguez et al. 2008).

The majority of Citrus species are recalcitrant to *Agrobacterium*-mediated transformation; in fact, this genus is not a natural host for *A. tumefaciens*, and so their mutual interaction has not evolved at the optimum level, as for other species (Singh and Rajam 2010). To increase the rate of success, the disarmed hypervirulent *A. tumefaciens* strain EHA105, a derivative of the most virulent strain, A281 (Magdalena Cervera et al. 1998; Ghorbel et al. 2001; Pena et al. 1998), or AGL-1 (Orbovic et al. 2015) were used, and the insertion of additional copies of *vir* genes from *A. tumefaciens* enhanced the transformation efficiency (Cervera et al. 2008; Ghorbel et al. 2001). Acetosyringone, a phenolic compound secreted by wounded plant tissues, can stimulate *vir* gene activation, and its addition increased the TE in juvenile explants of ‘Carrizo’ citrange (Cervera et al. 1998) and sweet orange (Dutt and Grosser 2009), but had no effect on ‘Duncan’ grapefruit and ‘Mexican lime’ (Dutt and Grosser 2009).

For citrus species that are difficult to regenerate via organogenesis, such as mandarins, the use of cell suspensions or protoplasts obtained from embryogenic callus can represent a valid alternative for genetic transformation (Dutt et al. 2018).

Agrobacterium-mediated transformation strategies using cell suspension cultures, seed-derived epicotyl segments, mature stem segments and PEG-mediated transformation using protoplast strategies were compared in the transformation of recalcitrant 'W Murcott' (*C. reticulata* Blanco × *C. sinensis* L. Osbeck). Epicotyl segments and mature explants resulted in high regeneration efficiency (68% and 34%, respectively) and low TE (1.23% and 0.33%, respectively); juvenile cell suspensions and protoplasts showed higher TE, with values of 29% and 11%, respectively, with a large number of cells that were potentially amenable to transformation. Despite the fact that suspension cells offer the possibility to avoid chimeras due to the single-cell origin of regenerated somatic embryos, these techniques require a long time for regeneration and plant recovery compared to other strategies, and the regenerated plants are still juvenile, requiring years for production (Dutt et al. 2018).

In addition, biolistic methods, recently applied for the transformation of epicotyl explants of 'Carrizo' citrange with low TE (0.3–1.9 transgenic shoots per paired shot), can be optimized and become a valid alternative to *Agrobacterium*-mediated transformation (Wu et al. 2016, 2019).

Cultivation of plant cells and tissues with subsequent regeneration of the entire plants can be avoided using *in planta* transformation methods; this strategy was applied to 'Shatian' pummelo (*C. maxima*), 'Jincheng' and 'Xinhui' oranges, leading to TEs of 20.41% (Y. yan Zhang et al. 2017), 46.3%, and 39.5%, respectively (Hong et al. 2000); it is performed under non-sterile conditions and is faster than conventional tissue culture techniques; in fact, plants obtained using this method could be graft-propagated in 3 months post-transformation. Briefly, the apical meristem and primary leaves of pummelo seedlings were removed, and the decapitated epicotyls were winded by Parafilm to form funnels for *Agrobacterium* inoculation; then, funnels were removed, and wounds were wrapped

with Parafilm and maintained in the dark. Following three days of co-culture, Parafilm wrap was removed, and cotton balls saturated with selection agent were used to soak the wounds of putative transformed seedlings three times. Seedlings were then wrapped again with Parafilm, kept in the dark for two weeks and then transferred to a greenhouse with natural lighting.

Citrus leaves can also be infiltrated with *Agrobacterium* for transient expression assays, useful for the characterization of gene function and the evaluation of candidate genes, e.g., 'Duncan' grapefruit (Figueiredo et al. 2011), 'Eureka Frost Nuclear' (Sendín et al. 2012), 'Eureka Frost', and 'Lisboa Frost' lemons, and 'Troyer' citrange (Enrique et al. 2011).

Agroinfiltration procedure was implemented in 'Mexican lime' using intermediate-aged leaves and setting *Agrobacterium* concentrations and buffer composition (Li et al. 2017). In addition, a pre-treatment with *Xanthomonas citri* (*Xcc*) before *Agrobacterium* infection significantly enhanced transient protein expression in different citrus species ('Duncan' grapefruit, 'Valencia' orange, 'Key' lime, 'Carrizo' citrange, sour orange, and 'Meiwa' kumquat), eliciting cell divisions (Jia and Wang 2014a, 2014b). *Xcc*-facilitated agroinfiltration was used to hasten transgene function assays in *Cre*/lox (Jia and Wang 2014b) and Cas9/sgRNA systems (Jia et al. 2016; Jia, Zhang, et al. 2017; Jia, Orbovi, and Wang 2019; Jia and Wang 2014b).

2.4.1 Selectable Marker Strategy

In most transformation systems, identification and selection of transgenic shoots are performed using genes that confer resistance to selective chemical agents, such as antibiotics or herbicides that are usually co-transformed with a gene of interest (Orbovic et al. 2015; Orbović and Grosser 2015); in citrus *nptII* (neomycin

phosphotransferase II from *E. coli*), confers resistance to the antibiotic kanamycin, which is commonly used (Pena et al. 2008), but once the transformation has taken place, the marker gene is not useful anymore, and it represents an undesirable obstacle for biosafety issues and public concerns (Ballester, Cervera, and Peña 2008, 2010; Zou et al. 2013).

The *Citrus* genus is highly heterozygous, and its long generation cycles make the segregation and removal of marker transgenes in the progeny difficult (Ballester et al. 2007; 2010).

Under non-selective conditions, transformed and non-transformed segments compete in the same space for shoot development, and non-transgenic events would be more competent to regenerate and prevailed over the transformed segments (Ballester et al. 2010; Rodríguez et al. 2008); moreover, the selection of transgenic plants directly by molecular analyses could result in gene silencing (Domínguez et al. 2002; 2004) and in laborious, expensive, and time-consuming screenings. To avoid this risk, reporter markers, such as *β-glucoronidase* (*uidA* or *GUS*), which needs the extractive assay to be detected, and green fluorescent protein (GFP, Figure 2.1), a viable reporter gene, can be used to rapidly screen and select transformed shoots (Cervera et al. 2008; A. Domínguez et al. 2002; Ghorbel et al. 1999).

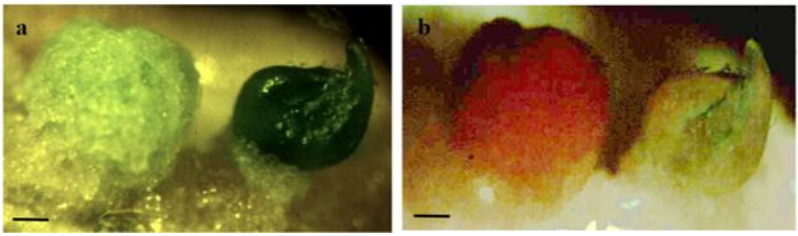


Figure 2.1 - Callus and shoot in 'Troyer' citrange internode observed under a stereomicroscope with white light (a) and 480 nm-excited blue light (b); in the latter case, it is possible to discriminate the 'escape' callus (red) and the fluorescent transgenic shoot (green). Bar = 1 mm.

Type and concentration of the antibiotics influence the regeneration process, irrespective of the cultivar. De Oliveira *et al.* (2010) evaluated the regeneration of 3 cultivars of oranges, 'Bahia', 'Valencia', and 'Pera', from adult tissues, testing different concentrations and types of antibiotics (timentine, cefotaxime, meropenem, and augmentin), and the best responses were obtained with 500 mg/L of cefotaxime. In addition, it was pointed out that, after transformation, in the selection and regeneration processes, different kanamycin concentrations had the smallest effects on the regeneration and TE compared to cytokinin type and concentration, and 50 mg/L kanamycin was sufficient to balance the growth of transgenic and non-transgenic cells (Peng et al. 2019).

Over the years, many efforts have been made to find alternative methods to replace the nptII selection system. One option is the phosphomannose isomerase (PMI)/mannose conditional positive selection system (*manA* gene, Joersbo et al. 1998; Miles and Guest 1984), which promotes the growth of transformed cells capable of synthesizing PMI enzyme on a medium that has mannose as a carbon source. It was first used by Boscariol *et al.* (2003) in the transformation of sweet oranges, with TEs of 3–23% depending on the cultivar ('Valencia' 23.8%, 'Natal' 12%, 'Pera' 7.6%, and 'Hamlin' 3%), and Ballestrer *et al.* (2008) concluded that it was an excellent candidate for citrus transformation, yielding TEs of 30% for citrange epicotyls and 13% for sweet orange mature internodes. Recently, PMI selection has been applied for the biolistic transformation of Carrizo citrange to increase the TE obtained with kanamycin selection, 0.7%, to 1.9% transgenic shoots per shot, avoiding the introduction of antibiotic resistance in plants (Wu et al. 2016, 2019).

An ideal strategy for overcoming the biosafety problems associated with selectable marker genes is the direct production of transgenic plants containing only the gene of interest. Site-specific recombination systems enable the removal of the marker gene after

the selection phase through the use of the multi-auto-transformation (MAT) vector system. This method combines two elements, first a positive selection using the isopentenyl transferase gene (*ipt*, which catalyzes the production of a precursor of several cytokinins, Ebinuma et al. 1997), and then a site specific recombination system R/RS from *Zygosaccharomyces rouxii* (Sugita et al. 1999), in which the R recombinase removes the DNA fragment placed between two recognition RS sites from the transgenic cells after transformation.

The MAT vector system was used in citrus transformation first by Ballester *et al.* (2007), but the excision of the RS fragment was not always efficient and precise due to the constitutive expression of the R recombinase gene, and the *ipt* marker was clearly distinguishable in sweet orange (Zou et al. 2013), but not in citrange (Ballester et al. 2007). In 2008, Ballester *et al.* improved the MAT vector system using an inducible R/RS-specific recombination system with transgenic-shoot selection through expression of the *ipt* gene and the indoleacetamide hydrolase/tryptophan monooxygenase (*iaaM*/H) marker gene, which causes the development of shoots exhibiting a characteristic shooty phenotype (Endo et al. 2002). In this case, R recombinase gene expression was controlled by the inducible GST-II-27 promoter from maize (Endo et al. 2002; Lyznik et al. 2003), and the *uidA* reporter gene was included in the T-DNA but outside the RS fragment to facilitate the screening of regenerated shoots. The TEs obtained with this system were 7.2% for citrange and 6.7% in Pineapple orange, which were significantly lower if compared with kanamycin selection, which resulted in TEs of 40% and 15%, respectively; however, with this method regeneration of non-transformed escape shoots was not precluded for any genotype (Ballester et al. 2008).

Other site-specific recombination methods are based on the bacteriophage P1 *Cre*/loxP and on the yeast Flp/FRT (Lyznik et al. 2003). In the *Cre*/loxP-mediated site-specific DNA recombination

system, Cre recombinase specifically recognizes loxP sequences and performs a precise autoexcision of the DNA segment between the two sites (Dale and Ow 1991). This technology has been used by Zou *et al*. (2013) in the genetic transformation of 'Jincheng' orange. The vector includes an *ipt* gene and *Cre* recombinase inserted between the two loxP recognition sites, while the GFP reporter gene was located outside to monitor the transformation; both *Nosp* and Cauliflower mosaic virus (*CaMV35S*) promoters were evaluated in driving *Cre* recombinase expression, and the first was more suitable (100% deletion efficiency compared to 81.8% of *CaMV35S*).

Problems of chimerism and inefficient deletions can be avoided by limiting the expression of the *Cre* gene with the use of tissue-specific (Boszorádová et al. 2014; van Ex et al. 2009; Hamzeh et al. 2015; Kopertekh et al. 2010; Moravčíková et al. 2008) or inducible promoters, for example activated by heat shock (Chong-Pérez et al. 2012; Cuellar et al. 2006; Dalla Costa et al. 2016).

2.4.2 Role of the Promoter

An important component to choose for the development of transgenic crops is the promoter element, which has an essential role in gene regulation at the transcriptional level; the characterization of gene regulatory sequences and their associated binding proteins provides valuable tools for plant genetic engineering.

A wide range of promoters derived from plants, viruses or bacteria has been used in plant genetic transformation. In *Citrus*, the most used is the CaMV35S promoter (Odell, Nagy, and Chua 1985), which targets gene expression throughout the plant (Boscariol et al. 2006; Cardoso et al. 2010; Cervera et al. 1998; Dutt and Grosser 2009; Ghorbel et al. 2000).

The availability of promoters and gene regulatory sequences derived from citrus is particularly important in the generation of

intragenic or cisgenic plants, which use genetic material derived from the same species or from closely related ones. In addition, the availability of different constitutive promoters is important to avoid the risk of homology-dependent gene silencing caused by the use of the same constitutive promoters to express multiple transgenes (Meyer and Saedler 1996); Erpen *et al.* (2018) identified the regulatory sequences from the *cyclophilin* (*CsCYP*), *glyceraldehyde-3-phosphate dehydrogenase C2* (*CsGAPC2*), and *elongation factor 1-alpha* (*CsEF1*) citrus constitutive genes, which exhibited constitutive gene expression in the vegetative tissues of transgenic 'Hamlin' orange.

Additional studies on the regulatory elements of these promoters will enable the use of compact transformation vectors containing only the regulatory components instead of the entire plant promoter, considerably larger than the commonly used viral promoters (Erpen Dalla Corte et al. 2020).

In addition, in genetic engineering, a constitutive expression of the gene of interest is not always needed, and in many cases, gene expression could be limited to a particular developmental stage or particular organ or tissue. Promoters controlling spatio-temporal gene expression were evaluated in citrus. For example, the fruit-specific promoters that have been isolated thus far include the "type-3 metallothionein-like gene", which confers preferential expression in juice sacs (Endo et al. 2007), and the Cl111 promoter gene isolated from acid 'Eureka' lemon and acidless lime (*C. limettioides* Tan.), which is pulp and flower organ-specific (Sorkina et al. 2011). For putative seed-specific expression, the CuMFT1 promoter has been isolated from 'Satsuma' mandarin (*C. unshiu* Marc., Nishikawa et al. 2008).

Promoters that drive transgene expression preferentially to vascular systems were developed especially to target defence-related protein and to reduce or minimize expression in other parts of the plant. Among them, the citrus *phenylalanine ammonia-lyase* (*PAL*)

promoter (*CsPP*), which drives gene expression preferentially to xylem vessels, was useful against *Xylella fastidiosa* (de Azevedo et al. 2006), while phloem-specific promoters could be useful for Huanglongbing disease, associated with a phloem-limited Gram-negative bacterium. Dutt et al. (2012) evaluated the activity of four phloem-specific promoters in citrus transforming 'Mexican lime', and histochemical GUS analysis revealed vascular-specific expression of the gene at different levels, depending on the promoter. *Rice tungro bacilliform virus promoter* (RTBV, (Bhattacharyya-Pakrasi et al. 1993) was the most efficient, followed by *rolC* from *Agrobacterium rhizogenes* (Schmulling, Schell, and Spena 1989), then *Arabidopsis thaliana sucrose-H+ symporter* (*AtSUC2,* Sauer and Stolz 1994) and *Oryza sativa sucrose synthase l* (*RSs1,* Miyata et al. 2012; Shi et al. 1994).

Specific phloem gene expression was also studied in Hamlin and Valencia oranges using the promoters *C. sinensis phloem protein 2* (*CsPP2*), *A. thaliana phloem protein 2* (*AtPP2*), and *AtSUC2*; although the TE was low (from 0.2% to 4.5% among the two cultivars), the *attA* gene was preferentially expressed in the phloem (Tavano et al. 2019).

Another possibility is the use of inducible promoters, especially pathogen-inducible promoters, to engineer plant lines with durable disease resistance and to avoid the presence and accumulation of antibacterial proteins in fruits.

The *A. thaliana heat shock protein 70B* promoter was used in an *Xcc*-facilitated agroinfiltration experiment for the temporal control of transgene expression in 'Duncan' grapefruit; leaves subjected to agroinfiltration and incubation for 4 h at 42°C showed GUS staining, confirming the action of the inducible promoter in modulating GUS transient expression (Jia and Wang 2014b).

Zou et al. (2014) evaluated the functionality of the pathogen-inducible promoters *PPP1* (Peng et al. 2004) and *hsr203J* (Pontier et

al. 1994) from tobacco and glutathione S-transferase (*gst1*) from potato (Malnoy et al. 2006) to drive expression of the GUS gene in response to the *Xanthomonas axonopodis* pv. *citri* (*Xac*) pathogen; the *PPP1* promoter was the most efficient promoter induced by *Xac* and wounding in transgenic 'Jincheng' orange. The promoter gst1 was used in 2009 by Barbosa-Mendes et al. (2009) to drive expression of the *hrpN* gene (from *Erwinia amylovora* (Burr.)) in 'Hamlin' transgenic plants and by Sendin et al. (2017) to control the expression of the Bs2 gene in 'Pineapple' orange, both resulting in a reduced susceptibility to citrus canker.

The pathogenesis-related *PR5* gene promoter, which is rapidly induced after *X. citri* infiltration upon wounding (Cernadas et al. 2008), was used for driving the citrus *MAPK* (*CsMAPK1*) gene in 'Troyer' citrange transgenic plants, reducing citrus canker symptoms (de Oliveira et al. 2013).

Targeted expression is one of the most important aspects for the future development of value-added crops and for the application of NPBTs; public concerns about the use of pathogen-derived constitutive promoters have led to the isolation of plant-derived promoters that are more likely to be accepted and to the development of spatiotemporal gene expression that limits the presence of transgenes in the transformed cultivars (Dutt et al. 2014).

2.5 Attempts to Reduce the Long Juvenility

Like other woody plant species, citrus has a long juvenile phase that prolongs the time for agronomic evaluation, delaying the release of new varieties; this characteristic becomes even more severe when the genetic improvement concerns rootstocks in which the level of polyembryony and the evaluation of the effect on scions require a very long time to be considered. For these reasons, the search for mature material to be used as explant source is of paramount importance,

whereas most citrus genetic transformation systems utilize explants derived from juvenile tissues.

In citrus, the reproductive stage starts after 5 to 10 years; this period can be shortened by several biotechnological strategies, like the use of genes involved in flower initiation, the use of mature plant tissues or the use of genotypes with short juvenile periods, particularly useful for citrus functional genomics (Duan et al. 2010; Yang et al. 2007).

In the latter case, seedling stem segments of precocious trifoliate orange, an extremely early flowering mutant from *P. trifoliata* that has a juvenile period of 1–2 years, were used by Tong et al. (Tong et al. 2009) and by Tan et al. (Tan et al. 2009); TEs of 57.4% and 20.7%, respectively, were recorded. In addition, kumquat (*F. crassifolia* Swingle), a species close to *Citrus* that has a juvenile phase of only 2–3 years and bears fruit several times per year (Yang et al. 2007, 2016), and 'Mini-Citrus Hongkong' kumquat (*F. hindsii*), which shows a very short juvenile period of approximately 8 months (Zhu et al. 2019), were used.

The over-expression of flowering meristem identity genes in juvenile tissues leads to a shorter generation time and was first used in citrus by Pena et al. (2001), transforming Carrizo citrange seedlings with the *Arabidopsis LEAFY* (*LFY*) or *APETALA1* (*AP1*) genes and reducing the juvenility phase of transformed plants from 7 years to 12–20 months. In the transgenic plants obtained, flowering remained under endogenous and environmental controls, and the new feature was inherited by the offspring; in particular, AP1-transgenic citranges were fully normal and behaved as rapid-cycling trees, showing a generation time of approximately one year from seed to seed, allowing faster propagation and genetic transformation studies, making possible the rapid evaluation of flower or fruit traits (Cervera et al. 2009). The strategy was also applied to 'Meiwa' kumquat, which showed a TE of 4.08% using epicotyl segments (Duan et al. 2010).

Generation time was also reduced by the constitutive expression of the CiFT gene in trifoliate orange, the citrus homolog of the flowering-time (FT) gene in *Arabidopsis*; transformants flowered 12 weeks after being transferred to the greenhouse (Endo et al. 2005; Nishikawa et al. 2010). 'Pineapple' orange was transformed to increase their β-carotene content with the simultaneous overexpression of the FLOWERING LOCUS T from sweet orange (*CsFT*); early fruiting phenotype (approximately 1 year after being grafted in the greenhouse) was observed, with two fruiting cycles per year displayed by transgenic plants, which enabled a rapid characterization of fruit quality traits (Pons et al. 2014).

Another strategy is the use of virus vector based on citrus leaf spot virus (CLBV) expressing the *CiFT* gene, which promotes precocious flowering within 4 to 6 months in juvenile plants of several citrus species (Velázquez et al. 2016).

Finally, the genetic transformation from mature tissues represents a valid strategy to bypass the long juvenile phases and to decrease the time and cost for the obtainment of new varieties for which fruit characteristics must be evaluated for years. For these reasons, quick and easy protocols for transformation of mature tissues are required to accelerate functional genomics studies, including a better understanding of genes underlying quality traits (Rodríguez et al. 2008).

The use of adult tissues in fruit crops for *in vitro* culture is hampered by the high level of contamination and the reduction or loss of morphogenetic abilities (Almeida et al. 2003), in fact, the transition between juvenile and adult stages results in a progressive loss of competence for organogenesis and embryogenesis (von Aderkas and Bonga 2000). However regeneration from adult somatic tissues is highly recommended for clonally vegetatively propagated fruit tree crops, in order to maintain genetic uniformity of the cloned plants, especially for the highly heterozygotic species, such as citrus.

Transformation of mature tissue of citrus was first described by Cervera et al. (1998) and has proven to be successful in the transformation of 'Pineapple' orange (Cervera et al. 1998), where it has led to the production of transgenic plants (6.1% TE) flowering and bearing fruits in 14 months after being transferred to the greenhouse; this system is also a valid alternative for the transformation of citrus seedless and monoembryonic varieties and was patented in Europe and the United States procedure (Pena et al. 1998). The protocol included three steps, starting with the *ex vitro* invigoration of source plant material by grafting adult buds onto juvenile vigorous rootstock, such as *C. volkameriana*. A second step consists of the optimization of tissue culture conditions to shift explant citrus cells to a competent state for *Agrobacterium*-mediated transformation and regeneration; explants are usually sterilized, co-cultivated with the engineered *A. tumefaciens* for 15 min, blotted dry on sterile paper and placed horizontally on co-cultivation medium rich in auxins for three days, with cocultivation at low light intensity. Internodes are then transferred to regeneration medium containing opportune hormones and antibiotics and are maintained in the dark for 2–4 weeks. Finally, in the third step, regenerated shoots are checked for their transgenic nature, micrografted onto decapitated seedlings of Troyer citrange germinated *in vitro* and acclimatized.

This method was also optimized for the transformation of the more recalcitrant clementine increasing transgenic plant regeneration efficiency of this genotype from 0.3 to 3% (Cervera et al. 2008), although this genotype showed lack of bud uniformity in sprouting and morphology. In particular, the concentration of 2,4-D used in the co-cultivation medium was doubled from 2 to 4 mg/L (increasing transformation frequency by 1.7- to 2.3-fold), the co-cultivation period was reduced from three to two days, and the dark period after co-cultivation was extended from 2–4 to 5–6 weeks. As clementine was more recalcitrant to *A. tumefaciens* infection, additional copies of

virG and *virE* were introduced into the plasmid used in the transformation, which led to a consistent enhancement of transformed plants obtained from 1.5- to 2.3-fold. In clementine, only regeneration under non-selective conditions was adequate to recover a sufficiently large number of transgenic shoots, distinguished by the *GUS* test or *GFP* visualization; they also lowered the kanamycin concentration to 25 mg/L. However, while an increase in callus induction was observed, shoot regeneration remained low. The same authors report that using WPM medium instead of MS, longer shoots, easier to be micrografted, were obtained (4 mm instead of 2 mm length, Cervera et al. 2008).

He et al. (2011a) used a novel *Agrobacterium*-mediated transformation system for mature auxillary buds leading to TEs of 7.5% for Jincheng and 8.3% for Newhall, both commercial orange cultivars. This method involved the use of mature shoots and did not contemplate the use of either hormones, antibiotics selection and solid medium, because all passages were carried out on MS liquid medium and a filter paper bridge.

Transgenic plants obtained start to blossom and bear fruits in the second year after the last grafting. The high-frequency transformation was attributed to the use of rootstock that enhanced nutrition for shoot development, to the absence of kanamycin selection and to the regeneration ability of the auxillary meristem in micrografted shoot.

An optimized protocol for mature tissue transformation was published in 2015 by Orbovic et al. (2015), with a TE of 12.8% using ‘Hamlin’ orange; compared to the previous protocols, this included a stronger sterilization process and the addition of another antibiotic selection (10 mg/L of Meropenem during the first 2 weeks of selection).

Adult tissues (stem segments) of ‘Tarocco’ orange were transformed with a TE of 11.7% (Peng et al. 2019) using a protocol

that included a pre-incubation step, commonly used for the transformation of juvenile material but never employed in mature explant transformation experiments. The highest percentage for the transformation of mature tissue was reached with 'Pera' orange, with 35% of transgenic plantlets (Kobayashi et al. 2017) using thin transversal segments (1–2 mm) of newly elongated shoots from greenhouse plants instead of internodal segments.

2.6 Success in Transgenesis Applied to Citrus

Genetic engineering has been strongly considered for the development of novel citrus varieties, offering a wide range of tools and strategies that enable the insertion or the editing of desirable traits into elite commercial cultivars. The applications of transgenesis are wide and include resistance to biotic and abiotic stresses and the control of fruit quality traits.

Several traits have been considered for genetic transformation, including early flowering (See Section 1.4, 'Attempts to reduce the long juvenility'), tree architecture and growth habitus (Distefano et al. 2013; Fagoaga et al. 2007b; Gentile et al. 2004), tolerance to abiotic stresses (Cervera et al. 2000; Molinari et al. 2004; Orbović et al. 2015, 2017), improvement of fruit quality (Dutt et al. 2016; Guo et al. 2005; Hijaz et al. 2018), in particular carotenoid content (Alquezar et al. 2008; Pons et al. 2014), and seedlessness (Koltunow et al. 2000; Li et al. 2002; Li, et al., 2003b). Thus far, the main aspects rely on biotic stresses, as these are the most limiting factors for citriculture worldwide. In the last years, great interest has been devoted to the development of novel varieties showing resistance to citrus greening (Huanglongbing, HLB).

HLB is considered the most devastating citrus disease worldwide (FAO2015); for example, the citrus utilized production in the United States in the 2017–2018 season (6.13 million tons) was

decreased by 20% from the 2016–2017 season and by 66% with respect to the record high production of the 1997–1998 season (17.8 million tons); moreover, Florida's on-tree value of the 2017–2018 citrus crop ($551 million) was the lowest since the 1976–1977 season ($530 million) (United States Department of Agriculture 2019). Greening is associated with 3 species of phloem-restricted Gram-negative bacteria*: Candidatus Liberibacter asiaticus* (*CLas*) and *C. Liberibacter americanus*, which are transmitted by the Asian citrus psyllid *Diaphorina citri*, and *C. Liberibacter africanus*, which is transmitted by the insect *Trioza erytreae* (Bové 2006; Coletta-filho et al. 2013; Gottwald et al., 2007). No curative methods are available for the disease; to ensure citrus survival in Europe, preventive measures are currently being developed within an European project (www.prehlb.eu).

Different strategies can be adopted to confer disease resistance to citrus cultivars. Genetic transformation with the constitutive expression of antimicrobial peptides (AMPs), a set of peptides of the innate immunity with antimicrobial activity (Boman 2003), has been used to control bacterial diseases, such as HLB and citrus canker (Schaad et al. 2006). In citrus the most used AMPs are insect-derived *attacin A* (Boscariol et al. 2006; Cardoso et al. 2010; Soriano et al. 2019; Tavano et al. 2019), *creopin B* and *Shiva A* (He et al. 2011b; Zou et al. 2017), *sarcotoxin IA* (Kobayashi et al. 2017), *a thionin* (Hao, Stover, and Gupta 2016) and *dermaseptin* (Furman et al. 2013).

In addition, the introduction in plants of resistance genes (R-genes) coding for proteins that recognize pathogen avirulence gene products (avr-gene, (Flor 1971)) can confer race-specific resistance, e.g., the pepper R-gene Bs2 used against citrus canker (Sendín et al. 2012; 2017).

Another possibility is the use of heterologous expression of receptors, which identify conserved molecules in the pathogen and trigger the plant's immune response to a wide range of

microorganisms; to enhance citrus canker resistance, the genes that have been considered are *Xa21*, a receptor kinase-like protein from rice (Mendes et al. 2010; Omar et al. 2018), and the *Flagellin Sensitive 2* (*FLS2*) receptor gene from *Nicotiana benthamiana* (Hao, et al. 2016).

Alternative approaches against pathogen diseases have aimed to enhance the systemic acquired resistance (SAR), the plant's inducible defence mechanism that increases innate resistance to further infection by pathogens (Kuc 1982). The SAR response is induced by salicylic acid and is associated with the production of pathogenesis-related (PR) proteins that confer long-lasting broad-spectrum resistance; in citrus, this strategy was used against citrus canker (Boscariol-Camargo et al. 2016; Chen et al. 2013; Zhang et al. 2010) and HLB using *NPR1* (Dutt et al. 2015; Qiu et al. 2020; Robertson et al. 2018).

To improve plant defence against fungi, the overexpression of genes encoding products with *in vitro* antifungal activity has been used, e.g., the *chit42* gene from *Trichoderma harzianum*, leading to an increased resistance of transgenic lemons to different fungi (such as *Phoma tracheiphila* and *Botrytis cinerea* (Distefano et al. 2008; Gentile et al. 2007) and conferring resistance to some post-harvest pathogens (Muccilli et al. 2020).

Pathogen-derived resistance was used against Citrus Tristeza Virus (CTV), which replicates in phloem vessels and is transmitted by *Toxoptera citricida*, an aphid vector; the *p25* coat protein from CTV was used to transform 'Mexican lime' (Domínguez et al. 2002), and it was demonstrated that plants exhibiting post-transcriptional gene silencing (PTGS) also showed resistance to CTV due to the accumulation of p23-specific small interfering RNAs (siRNAs) (Fagoaga et al. 2006). RNAi, the approach that involves the knockdown of gene expression mediated by siRNAs using specific double-stranded RNA molecules, was applied to control CTV (Cheng et al. 2017; Soler et al. 2012), citrus psorosis virus (Reyes et al. 2011),

citrus canker (Enrique et al. 2011) and fungal pathogens, such as *Alternaria alternata* (Miyamoto et al. 2008) and *Phytophtora* spp. (Narayan et al. 2010).

Commercialization of disease-resistant citrus cultivars will presumably take many years, but the development of resistant or tolerant new genotypes that will replace susceptible varieties is one of the most realistic long-term solutions to many devastating diseases, such as HLB; until that time, it is important to incentivize cooperation in pest and disease management to guarantee vector control and tree monitoring (Paiva et al. 2020; Singerman and Rogers 2020).

2.7 Genome Editing

One of the most important NPBTs is genome editing, a technology based on programmable nucleases that produce site-specific DNA double-strand breaks (DSBs), which trigger endogenous DNA repair systems, resulting in targeted modification. The first tool used was zinc-finger nuclease (ZFN) followed by transcription activator-like effector nucleases (TALENs) in 2011. Since 2013, clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) nucleases have become the most popular method for plant genome editing (Kim and Kim 2014).

In the CRISPR-Cas system, an adaptive immune system of prokaryotes (Barrangou et al. 2007), Cas nuclease is directed by a single guide RNA (sgRNA) that recognizes a target DNA sequence flanked by a protospacer adjacent motif (PAM) and generates specific DSBs. Nuclease-induced DSBs can be repaired by the non-homologous end-joining (NHEJ) pathway, which leads to the introduction of insertion/deletion mutations (*indels*) of various lengths, or by homology-directed repair (HDR), which is useful to introduce specific point mutations or to insert desired sequences through recombination of the target locus using DNA ‘donor

templates' present at the moment of DSB formation (Sander and Joung 2014).

Since the first application of genome editing in plants, much progress has been made in the development of CRISPR-based editing tools; numerous Cas variants and orthologs with specific PAMs have been discovered together with precise genome editing by base editors, expression systems for multiplexing, transcription regulation and epigenome editing (Zhang et al. 2019).

In citrus, Jia and Wang (2014a) reported the first genome editing using the Cas9/sgRNA system and *Xcc*-facilitated agroinfiltration on Valencia orange. The delivery of Cas9 and sgRNA were accomplished with a particular agroinfiltration that consists of an initial inoculation of *Xcc* followed by an *Agrobacterium* infiltration on 'Valencia' leaves; the target gene was the endogenous *Citrus* phytoene desaturase (*CsPDS*) gene, an enzyme required for the biosynthesis of carotenoid pigments that results in a white-colored (albino) phenotype when it is silenced or mutated (Agüero et al. 2014). The mutation rate was approximately 3.2–3.9%, with no off-target mutagenesis detected. Jia and Wang (2014b) applied the same strategy on 'Duncan' grapefruit and, being a grapefruit hybrid between pummelo and sweet orange (Xu et al. 2013), they were able to apply the Cas9/sgRNA system to specifically modify one of the two CsPDS alleles of the variety. Subsequent application of genome editing has focused on editing genes involved in citrus disease resistance, especially in citrus canker. Most of the studies were performed to target the CsLOB1 gene (*C. sinensis Lateral Organ Boundaries 1*), a disease-susceptibility gene upregulated by *PthA4*, a transcription activator-like effector of *Xcc* (Hu et al. 2014; Yang et al. 2011), in particular to target the effector binding elements (EBEs) of PthA4, which are located in the promoter of the *CsLOB1* gene (EBEPthA4-CsLOBP), and should confer resistance to the disease without losing *CsLOB1* function.

Peng et al. (2017) edited 'Wanjincheng' orange using 5

different constructs to modify different regions along *EBEPthA4-CsLOBP*; through the transformation of epicotyl segments, they obtained 16 lines (42% TE) with *EBEPthA4* modifications and 4 mutation lines that showed enhanced resistance to citrus canker.

'Duncan' grapefruit epicotyl transformation was achieved by Jia et al. (2016) and resulted in 4 lines with targeted modification of only *EBEPthA4 CsLOBP Type I* with a mutation rate of 15.63–81.25%; the transgenic plants were susceptible to *Xcc* infection. In 2017, Jia et al. (2017) succeeded in disrupting the coding regions of both alleles of *CsLOB1*, and no canker symptoms were observed in the lines DLOB9 (mutation rate of 89.36%), DLOB10 (88.79%), DLOB11 (46.91%), and DLOB12 (51.12%) after *Xcc* inoculation. In both studies, no off-target mutation was detected, but only a few among the possible off-targets were subjected to analysis; an alternative strategy to reduce off-target mutations is the use of a different type of nuclease, such as CRISPR derived from *Prevotella* and *Francisella* (CRISPR-Cpf1), a new class II CRISPR-Cas system (Zaidi, Mahfouz, and Mansoor 2017; Zetsche et al. 2015) that has been used to edit tobacco, rice and soybean (Endo et al. 2016; Hu et al. 2017; Xu et al. 2017; Yin et al. 2017). In comparison with Cas9, Cpf1 exhibits little to no off-target activities in plant cells (Tang et al. 2017), has a different protospacer adjacent motif (T-rich PAM instead of G-rich one, NGG), generates cohesive ends with four or five nucleotide overhangs (compared with SpCas9, which produces blunt ends), promoting an HDR mechanism, and among the other features, Cpf1 requires shorter CRISPR RNAs (crRNAs 43 nucleotides instead of 100 of Cas9), making this system more suitable for multiplexed genome editing (Zaidi et al. 2017; Zetsche et al. 2015). *Lachnospiraceae bacterium ND2006* Cas12 (LbCas12a) was used to edit 'Duncan' grapefruit EBEPthA4-CsLOBP; epicotyls were transformed via *Agrobacterium*, and the biallelic mutation efficiency obtained was 5%, with no off-targets observed (Jia, Orbovi, et al.

2019). Recently, Jia et al. (2019) published a protocol for the application of the CRISPR/Cas system via *Agrobacterium*-mediated transformation of epicotyl tissues in citrus, and the CRISPR/Cas9 system has been applied to 'Mini citrus Hongkong' kumquat (Zhu et al. 2019). Despite the low TE of *Agrobacterium*-mediated transformation (0.2–4%), it was possible to apply CRISPR/Cas9 and achieve a T1 generation in approximately 15 months; the modifications of target genes in the CRISPR-modified *F. hindsii* were predominantly 1-bp insertions or small deletions, and all T1 seedlings showed a mutation rate of 100% at the sgRNA1 targeting site.

Another approach was used by Wang et al. (2019), editing the transcription factor CsWRKY22 that was negatively correlated with citrus canker resistance. Epicotyls of 'Wanjincheng' orange were transformed, and the transgenic plants W1-1, W2-2, and W2-3 showed 85.7%, 79.2%, and 68.2% mutation rates, respectively, with off-target frequencies of 3.0-16.0%; resistance evaluation indicated that transgenic plants delayed the development of canker symptoms.

Although all these studies demonstrate how CRISPR/Cas9 technology can be exploited for citrus genome editing, accelerating the breeding process and combining multiple favourable traits, there is a need for more precise biotechnology tools than those that are currently available.

One of the problems is the efficiency of the editing obtained; despite the fact that several computational tools are now available for designing guide RNAs targeting a specific gene, the editing efficiencies might be different due to the existence of variant alleles not included in online citrus genome databases (Xu et al. 2013); for this reason, the investigation of the sequence of the gene of interest (Jia et al. 2016; Jia, Zhang, et al. 2017; Peng et al. 2017), the functionality evaluation of many sgRNAs using *Xcc*-facilitated agroinfiltration (Jia et al. 2016; 2017; 2019; Jia and Wang 2014a), and the *in vitro* cleavage analysis of the construct before citrus

transformation (Wang et al. 2019) represent fundamental steps to increase editing efficiency.

The low frequencies of mutations induced by the CRISPR/Cas9 system used in citrus were improved by Zhang et al. (2017), who used a different promoter to drive Cas9 expression, replacing the CaMV35S promoter with the *A. thaliana YAO* sequence (Yan et al. 2015) and increasing the frequency of mutational events from 3.2–3.9% (Zhang et al. 2017) to 75% using the same sgRNA. Le Blanc et al. (2018) also demonstrated that temperature has an effect on mutation rate achieved by the CRISPR/Cas9 system; Carrizo citrange transgenic plants containing *pYAO:SpCas9* and sgRNA targeting *CsPDS* genes that were exposed to several heat stress treatments (24 h at 37°C and 24 h at 24°C repeated seven times) showed an increase in targeted mutagenesis (100% *CsPDS* alleles mutated) with respect to those continuously grown at 24°C (approximately half of the *CsPDS* alleles mutated). This result suggests that all CRISPR/Cas9 systems require higher temperatures to achieve optimal editing efficiency, regardless of the promoter used to regulate Cas9 expression (LeBlanc et al. 2018), and that many aspects of the functioning of this technology are still to be explored.

Jia and Wang (2020a) generated homozygous and biallelic canker-resistant pummelo in the T0 generation via the CRISPR-Cas9 system with a 100% mutation rate in the *EBE* region of the *LOB1* promoter. Zhang et al. (2017) also developed a bifunctional selectable and visible marker for citrus (*eGFP-NPTII*) that improved the recovery of transgenic events expressing high levels of Cas9, reducing the number of promoters present in the vector. In citrus, special efforts to control CRISPR/Cas9-mediated chimeric mutation are required, and the optimization of regeneration protocols will offer a great opportunity to select transgenic events and reduce the formation of chimeric mutations (Wang et al. 2019).

Other options include the use of embryogenic calli

transformation that rarely produce transgenic chimeras (Dutt et al. 2018; Li et al. 2002) and a transient approach using purified CRISPR/Cas9 ribonucleoproteins to edit plant protoplasts, which has been tested in wheat (Liang et al. 2017) and applied to grape and apple (Malnoy et al. 2016).

Other concerns are related to the findings of new target genes for editing and to genetically modified organisms legislation; knowledge of plant pathogen interactions and mechanisms is critical to the development of new varieties with improved quality or resistance to disease via the CRISPR/Cas system (Caserta et al. 2020). The legislation of genome-edited plants is still a debated issue at international scientific and political forums, and many countries are in the process of drafting the regulatory frameworks for their use (Kim and Kim 2019).

2.8 Conclusions

The development of novel citrus varieties with improved quality and resistance to biotic and abiotic stresses is one of the main purposes of breeding programs. Thus far, the use of conventional breeding techniques in citrus has been shown to be time consuming and default due to the many limitations of typical of tree crops, such as the long juvenility and high heterozygosity.

The application of NPBTs could overcome these problems, offering new tools that combine site-specific and targeted editing with a reduction in the time for plant breeding, thus leading to lower production costs. Many aspects need to be considered to apply transgenesis to citrus, among them: (i) the organogenic response is largely genotype-dependent, and (ii) the regeneration efficiency for many commercial varieties is still low. Other aspects of great relevance rely on the establishment of appropriate strategies to limit the expression of the transgenic gene in a particular organ and on

techniques to efficiently remove selectable marker gene(s).

Despite the numerous papers published over the last several years, the availability of new sequencing data has greatly advanced the knowledge on genes underlying pathways of interest. This aspect will certainly over new opportunities for the establishment of targeted breeding programs. The availability of germplasm collections encompassing a high fraction of the allelic variability characterizing Citrus heirloom varieties and/or landraces represents a valuable genetic reservoir that can be readily transferred into other varieties through NPBTs for the definition of novel varieties characterized by superior agronomical traits.

3. Integrated approaches to investigate the genetic basis of *Citrus clementina* self-incompatibility

3.1 Abstract

Self-incompatibility (SI) is a physiological mechanism used by flowering plants to prevent self-fertilization and promote outbreeding. Among citrus species, several pummelo, mandarin, and mandarin-like accessions show SI behavior. In these species, SI is coupled with a variable degree of parthenocarpy ensuring the production of seedless fruits, a trait that is highly appreciated by consumers, especially in orange and mandarin. In citrus, cross-pollination studies showed the presence of a gametophytic SI system based on *S* ribonucleases (S-RNases) and *S-locus F-boxes* (SLFs) interrupting the self-pollen tube growth in the upper/middle part of the style. Several *S-RNase* and *SLF* homologs genes have been identified in citrus accessions so far, but there are evidences that other genes can be involved in the SI response as well. In the present study we define the *S*-genotype of the self-compatible 'Monreal' clementine (*Citrus clementina* Hort. ex Tan.), a natural mutant of the self-incompatible 'Comune' clementine, using primers designed on the basis of the available *S-Rnase* sequences and of the 'Monreal' *de novo* genome assembly. The analysis clarified that both 'Comune' and 'Monreal' clementines are characterized by the presence of a S_7S_{11} genotype. RNA-seq analysis of unpollinated pistils at mature stage from both clementine genotypes revealed the lack of expression of S_7*-RNase* in 'Monreal'. RNA-seq analysis followed by gene ontology studies enabled the identification of 2.965 differentially expressed genes (DEGs), most of which involved in oxidoreductase and transmembrane transport activity. Furthermore, the alignment of the RNA-seq reads against the reference genome of 'Comune' clementine led to the identification of 7.781 genes characterized by the presence of at least one polymorphism between the two genotypes. Most of the identified mutations were located on scaffold 7 containing the *S*-locus suggesting their involvement in the regulation of specific pathways such as SI. The present work shed light on the genetic mechanism causing the loss of SI in 'Comune', a trait that has a strong

economic impact and will help in the setup of future breeding programs for seedless citrus varieties.

Keywords: genome assembly, RNA-seq, SNPs, genetic improvement, Seedlessness

3.2 Introduction

Self-incompatibility (SI) is an important mechanism that has been evolved to prevent self-fertilization and inbreeding in plants. This reproductive strategy is controlled by a single genomic region, the *S*-locus, that contains two tightly linked genes, the pollen and pistil determinants, and is characterized by a number of alleles determining self and inter- compatibility or incompatibility in the varieties of a target species. SI can be classified into 2 types, sporophytic (SSI) and gametophytic (GSI). The latter is the most widespread among plants, it is found in *Solanaceae, Rosaceae* and *Plantaginaceae;* incompatible pollen tubes growth is arrested in the style and not at the surface of the stigma (Newbigin et al. 1993) as the case of SSI. *Citrus* is also characterized by GSI: the female *S* determinant, encoded by a class of III *S* ribonuclease (S-RNase) isolated in the pistil, can inhibit the growth of pollen tubes by degrading RNAs; the male *S* determinant comprises multiple *S*-locus F-boxes (SLFs) that are the component of a SKp1-Culling-F-box (SCF) complex that promotes the growth of compatible pollen by ubiquitinating and degrading nonself *S-RNases* in a 26A proteasome-dependent manner (Hu et al. 2021).

Recently, Liang and colleagues (2020) identified fourteen *S-RNase* genes and multiple *SLFs* through the *in silico* analysis of the available citrus genomes. One year later, three more *S-RNase* sequences (S_{15}, S_{16}, and S_{17}) were identified (Honsho et al. 2021). Structural analysis confirmed that citrus *S-RNases* are characterized by the same general structure as those found in *Plantaginaceae,*

Solanaceae, and *Rosaceae* that include five conserved domains (C1–C5) and 5 hypervariable domains (HV1–HV5 (Honsho et al. 2021; Liang et al. 2020).

Furthermore, it was demonstrated the existence of another *S-RNase* (*S_m-RNase*) isolated from *C. sinensis* and identified in *C. reticulata* that seems to be responsible for the loss of functional SI in SC species: the coding sequence of this gene is shorter due to a deletion at position 443 of an adenine resulting in a frameshift mutation causing the occurrence of a premature stop codon; in addition, it was found that the *S_m-RNase* was expressed in the style at a much lower level than other *S-RNases* (Liang et al. 2020).

SI in *Citrus* has been mainly described in pummelo, even though this mechanism is more important in mandarin-like varieties like clementine; in these varieties the presence of some degree of parthenocarpy enables the obtainment of seedless fruits when the plants are cultivated in solid blocks, preventing cross-pollination (Ollitrault et al. 2021). Despite the importance of understanding the molecular basis and the mechanism of regulation of SI, most of these informations are still not available and poorly understood.

In this chapter, two clementine (*C. clementina* Hort. ex Tan.) varieties, SI 'Comune' (abbreviated as ComSI) and its natural SC (self-compatible) mutant 'Monreal' (abbreviated as MonSC) are analysed to characterize the genetic bases of SI through whole-genome sequencing and transcriptomic analysis.

In previous studies, Distefano et al. (2009) demonstrated that the mutation leading to self-compatibility in MonSC affected pistil functions. In particular the histological analyses demonstrated as in MonSC both self- and cross-pollinated pollen tubes reach the ovary, while in self- and cross-pollinated ComSI, pollen tubes arrested their growth in the upper or middle style (recognizing the pollen of the SC mutant as self-pollen).

This work aims to elucidate the genetic basis controlling SI in

mandarin combing genomic and transcriptomic data, in order to identify candidate genes implicated in pollen-pistil interaction.

3.3 Material and methods

3.3.1 Plant material

ComSI and MonSC clementine plant tissues were collected from 10-years-old trees grown at the experimental field of the University of Catania. Young leaves were collected for genomic DNA analysis. Virgin styles were collected 24h after anthesis, frozen in liquid nitrogen, homogenized and then stored at −80 °C until analysis.

3.3.2 PCR amplification and Sanger sequencing

Genomic DNA was extracted from 100 mg of fresh young leaves of ComSI and MonSC using ISOLATE II Plant DNA Kits (Bioline, Meridian Life Science, Memphis, TN, USA) according to the manufacturer's instructions; the extracted DNA was quality checked using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis. Specific primer pairs were used designed on the different *S-RNases* (Table 3.1). PCR reactions were performed in a total volume of 20 µL containing 100 ng genomic DNA, 1x PCR buffer II, 2 mM magnesium chloride, 0.2 mM dNTPs, 0.2 µM of each primer (Table 3.1) and 1 U of MyTaq DNA polymerase (Bioline). Amplifications were conducted in thermal cyclers GeneAmp 9700 and 2700 (Applied Biosystem) using an initial denaturation step at 94°C for 10 min, followed by 35 cycles at 94 °C for 30 sec, 62-57 °C (depending on the primer pair used, see Table 3.1) for 45 sec and 72 °C for 2 min with a final elongation at 72 °C for 10 min. Amplicons were separated by electrophoresis on 1.5% agarose gel and amplificated bands were extracted and purified with ISOLATE II PCR and Gel Kit (Bioline) following manufacturer's

instructions; purified bands were then sequenced (Eurofins Scientific, Luxembourg) and aligned against the already available *C. clementina* and purpose-built MonSC genomes.

Table 3.1 List of primer designed from *S-RNase* conserved and hypervariable domains.

Target	Accession (Species)	Primer name	Sequence (5'-3')	Product size
S_1-RNase	MN652897.1 (*C. maxima*)	S1FW	CTACTCTCTGCTACGCAATCA	334
		S1RV	CACTTCCTTCAGCAGATAACC	
S_2-RNase	MN652898.1 (*C. maxima*)	S2FW	CGCTGGGGGAAAAACATTGGAA	429
		S2RV	TTGTTTGCTTGGACACCTACGC	
S_3-RNase	MN652899.1 (*C. maxima*)	S3FW	GGGATTCTTGCATCGCTGGAAC	451
		S3RV	GACTCGGAGCAGGGAACTTGAT	
S_4-RNase	MN652900.1 (*C. maxima*)	S4FW	CAGTTCTGGTTTTGACCACTT	277
		S4RV	ATGTTCCCAGAAGCCTATATG	
S_5-RNase	MN652901.1 (*C. maxima*)	S5FW	GATTCTTCTCTTGTTGCCGAC	264
		S5RV	ACTGTCACTACCGGTTACAGC	
S_6-RNase	MN652902.1 (*C. maxima*)	S6FW	ATTGAAGAAGTACTGGCCAAG	272
		S6RV	TTAGCAGATAACGGTTAACGC	
S_7-RNase	MN652903.1 (*C. maxima*)	S7FW	TGGCCGAGTCTCATTTCGAAG	368
		S7RV	TCCCCACAGTTCTCGGTTTTG	
S_8-RNase	MN652904.1 (*C. maxima*)	S8FW	AGGCAATTCGTCTTTAAGAGG	392
		S8RV	TAATTCTCTTACGGCAAGTGG	
S_9-RNase	MN652905.1 (*C. maxima*)	S9FW	CTCAAAATTCTTCGGGATTCC	379
		S9RV	AATGTGTTTAAGAGGTCCGTG	
S_{10}-RNase	MN652906.1 (*C. reticulata*)	S10FW	GTCTCTCCACTTGGGACAAGG	358
		S10RV	CGGCAGCTCTCTCCATTAATC	
S_{11}-RNase	MN652907.1 (*C. reticulata*)	S11FW	CGCTGTTCAGAGTAAAGCTGGC	243
		S11RV	AGTGGATCTTTGTCGCGGGTTA	
		S21FW	CTCTAATGGGCAAACACTGAGC	(for qRT-PCR)
		S22RV	TGCCAGCTTTACTCTGAACAGC	
S_{12}-RNase	MN652908.1 (*Atalantia buxifolia*)	S12FW	GACAAATCTCTTTGGAACAAT	197
		S12RV	GCCCTATTTAATGTGTCTAAG	
S_{13}-RNase	MN652909.1 (C. cavaleriei)	S13FW	AGTCTCCTCCGTCGTAACACT	224
		S13RV	TTGTCACTACTGGTTACAGCC	
S_{14}-RNase	MN652910.1 (C. medica)	S14FW	AAATGGTTCCGGCCCAGGTAA	443
		S14RV	CACAGTTATCTGCGCGCAAGT	
S_{15}-RNase	LC575202.1 (C. tamurana)	S15FW	CAGGTACAGGCAAAACAGGCAG	305
		S15RV	TGTTAGATCGACAGCCCTTCGG	
S_{16}-RNase	LC575207.1 (C. maxima)	S16FW	CTGGCCAGTAAACAGTACCGGA	310
		S16RV	ATCCACCTCCTTGTACGGTTGG	
S_{17}-RNase	LC575209.1 (C. hassaku)	S17FW	TCTCTTTCCCTTGGCTCTGCTC	221
		S17RV	CCGATGAAAGAATGGTGCGGTC	

3.3.3 *'Monreal' sequencing and genome assembly*

The *de novo* assembly of the MonSC genome was carried out combining both short and long reads technologies. Short reads sequencing was performed with Illumina next-generation technology (PE-150 reads) with an average reads depth of 100X, while long reads sequencing was carried out with Oxford Nanopore Technology (ONT) with an average read depth of 30X. ONT reads were assembled using the Flye aligner (Kolmogorov et al. 2019), then the quality of the draft assembly was improved with Pilon (Walker et al. 2014). The quality of the *de novo* assembly was tested with the Benchmarking Universal Single-Copy Orthologs (BUSCO v 5.4.3; Manni et al. 2021; Simão et al. 2015) by using the embryophyta_odb10 dataset (-l parameter) featuring 1.614 target genes.

3.3.4 *Total RNA extraction*

Total RNA was extracted from frozen styles using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, USA) and treated with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, Saint Louis, USA) following the protocol described by Distefano et al. (2013). Extracted RNA was quantified using a NanoDrop-2000 (Thermo Scientific, USA) spectrophotometer and total RNA integrity was assayed by 1% agarose gel electrophoresis and then stored at −80 °C for further analysis.

3.3.5 *Quantitative real-time PCR (qRT-PCR) analysis*

The cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) assays were run on the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) in 20

µL total reaction volume containing 1 × PCR buffer II, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of forward and reverse primer (Eurofins Genomics), 1.5 µM SYTO9 (Life Technologies, UK), 1 µL of the synthesized cDNA and 1 U of MyTaq DNA polymerase (Bioline, UK). The *S_{11}-RNase* and *S_7-RNase* genes were amplified using S21FW-S22RV and S7FW-S7RV primers, respectively (Table 3.1). The citrus Elongation Factor 1-alpha gene (*EF-1α*, accession AY498567) was used as a housekeeping reference gene (Distefano et al. 2009). Thermal cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 5 s, 59 °C for 20 s, and 72 °C for 2 min. The expression level of *S_{11}-RNase* and *S_7-RNase* genes relative to the EF-1α transcript was calculated following the mathematical model described by (Livak and Schmittgen 2001). The values reported are the mean ± SD of at least three independent assays. Statistical analyses were performed using ANOVA (LSD test, $p < 0.01$).

3.3.6 RNA-seq analysis

Total RNA extracted from ComSI and MonSC pistils was prepared and submitted to Novogene for library preparation and sequencing. Three biological replicates per each accession were employed for RNA-seq analysis (pair ends 150 reads). An average of 20 million reads per sample was analysed. Raw reads were aligned against the MonSC genome employing the Spliced Transcripts Alignment to a Reference (STAR) RNA-seq aligner (Dobin et al. 2013); then reads were counted using the FeatureCounts software (Liao, et al., 2014), while differentially expressed genes (DEGs) were identified using the DESeq2 R package (Ignatiadis et al. 2016; Love, et al., 2014). The significant DEGs were determined using a threshold of FoldChange ≥ 2 with an adjusted *P*-value ≤ 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

databases enrichment analysis of DEGs were implemented by ShinyGO bioinformatic tool (Ge et al. 2020).

3.3.7 SNP detection and annotation

The availability of the aligned RNA-seq allowed the identification of the single nucleotide polymorphisms (SNPs) occurring between Monreal and Comune mandarin using the SNPeff software (Cingolani et al., 2012). The analysis permitted the identification of SNPs located within sequencing regions (e.g. genes, regulatory regions). SNPs distribution was displayed employing the R package named.

3.4 Results

3.4.1 'Monreal' genome assembly

The assembly of the MonSC genome resulted in a genome size of 374.511 Mb, with a N50 of 140 Kb and a mean genome coverage of 57X. The quality of the *de novo* assembly was assessed using BUSCO and results are displayed in Table 3.2. In particular, the low number of fragments (21, 1.3%) and undetected (9, 0.6%) genes indicates a consistent genome assembly in line with the other reference genomes of *Citrus* (Table 3.2).

Table 3.2 Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis and descriptive statistics. Size of the genome, Number of scaffold and N50 of the MonSC genome compared with the related genome of ComSI and *Citrus reticulata*. In all genomes 1.614 target genes of the embryophyta_odb10 dataset were considered for the BUSCO analysis.

Species name	'Monreal' clementine	*Citrus* x *clementina*	*Citrus reticulata*
Version	V1.0	V1.0	V1.0

Complete BUSCOs	Number	1584	1366	1345
	%	98.1%	94.90%	93.40%
Complete and single-copy BUSCOs	Number	1461	1329	1309
	%	90.5%	92.30%	90.90%
Complete and duplicated BUSCOs	Number	123	37	36
	%	7.6%	2.60%	2.50%
Fragmented BUSCOs	Number	21	36	49
	%	1.3%	2.50%	3.40%
Missing BUSCOs	Number	9	38	46
	%	0.6%	2.60%	3.20%
*Total BUSCO groups searched		1614**	1440	1440
Total size of assembly (bp)		374163419	3E+08	3,47E+08
No. of scaffolds/contigs		22436	1398	90139
N50 (bp)		139 KB	3,1E+07	1585532

3.4.2 S-RNase identification in MonSC and ComSI

Primers listed in Table 3.1 were designed on the specific *S-RNase* to identify the alleles characterizing MonSC and ComSI (Figure 3.2a). PCR amplification led to the identification of one band for each sample (Figure 3.1) that was sequenced and compared with the already available sequences: the ones obtained from the amplification with S7FW and S7RV had 98% of similarity for ComSI and for MonSC with S_7*-RNase* (MN652903.1), while those amplified employing S11FW and S11RV had the 99% and the 98% of similarity

with the S_{11}-*RNase* of *C. reticulata* (MN652907.1) for ComSI and MonSC, respectively.

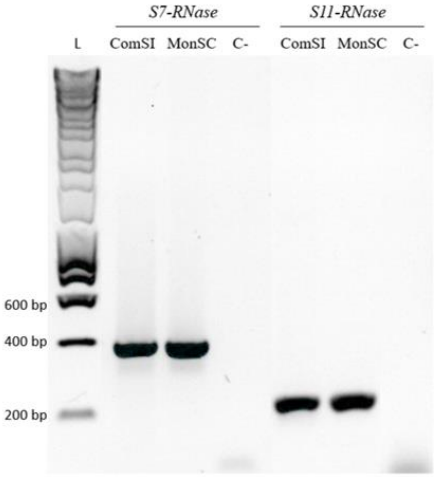


Figure 3.1 Visualization on agarose gel of amplified PCR product using S7FW, S7RV, S11FW and S11RV primers; C- = negative controls; L = ladder.

To confirm these data, the sequences of the two amplicons were aligned against the MonSC genome. Both S_7-*RNase* and S_{11}-*RNase* were identified, confirming that both MonSC and ComSI share a S_7S_{11} genotype at the *S*-locus. The complete sequences of both S_7-*RNase* and S_{11}-*RNase* were then retrieved using the MonSC reference genome, with S_7-*RNase* located in contig 25338, while S_{11}-*RNase* in contig 4474. S_7-*RNase* sequence was compared with that of the already available S_7-allele (MN652903.1 from *C. maxima*) and it showed an insertion of 93 bp in the region between C2 and C3 domains, probably in the HV1 region. The analysis of the S_7-*RNase* sequence gene on JBrowse genome viewer confirms that the insertion matches with an intron, that was predicted to be located inside the HV1 region and it is the only intron sited in the conserved *S*-*RNase* gene (Figure 3.2a)

(Liang et al. 2020).

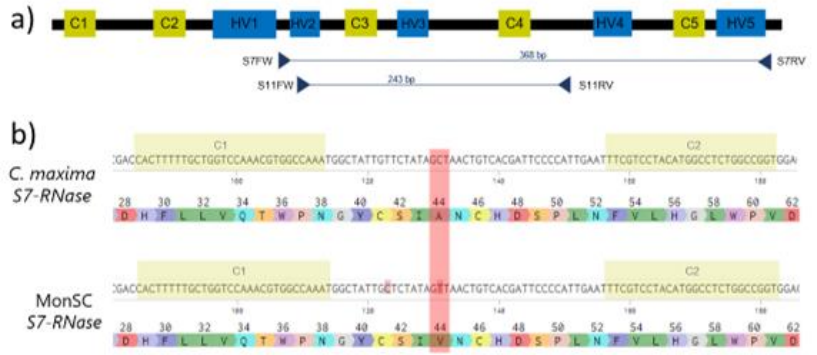


Figure 3.2 Schematic representation of the *S-RNase* structure, and *C. maxima* and MonSC sequence alignment of *S_7-RNase*. (a) *S-RNase* shows 5 conserved (C1-C5, in green) and hypervariable domains (HV1-HV5, in blue). Blue arrows at the bottom indicate PCR primers used to amplify S_7 and S_{11} alleles and the respective size of PCR amplicons. (b) The comparison between the two sequences enables the detection of a SNP present at the 131° base, that leads to the presence of valine instead of adenine in the first hypervariable region at 44° amino acids.

In addition, the *S_7-RNase* coding sequence of MonSC shows a 'T' instead of 'C' at 131° base, leading to the presence of a different amino acid (valine instead of alanine) at the 44° position. Both amino acids highlight an hydrophobic side chain; the change is located between C1 and C2, in the first hypervariable region (Figure 3.2b).

3.4.3 qRT-PCR and RNA-seq analysis

To confirm the expression of the *S-RNases*, a qRT-PCR analysis on pistil tissue sampled 24 hours after anthesis of MonSC and ComSI has been carried out. Data confirmed the effective expression of *S_{11}-RNase* and *S_7-RNase* in ComSI, while in MonSC the *S_7-RNase* was not expressed (Figure 3.3).

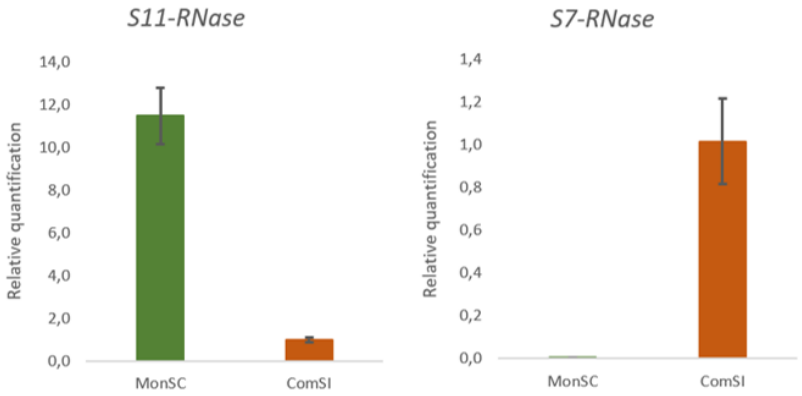


Figure 3.3 qRT-PCR analysis of *S_7-RNase* and *S_{11}-RNase* in styles from MonSC and ComSI collected 24 h after anthesis. *S_7-RNase* gene is much less expressed in the styles of MonSC than in those of ComSI.

An RNA-seq analysis was then performed on the same tissue to investigate the expression of genes putatively involved in down-regulation of the S_7-RNase in MonSC.

First, to better elucidate the expression of the *S-RNases*, *S_7-RNase* and *S_{11}-RNase*, transcriptomic profiles were visualized (Figure 3.4). No significant differences were observed in the *S_{11}-RNase* transcripts (as shown in Figure 3.4a); on the other side, major differences were observed in *S_7-RNase* expression with MonSC showing no expression, while ComSI is characterized by expression levels compared to those observed for *S_{11}-RNase* (Figure 3.4b), in agreement with qRT-PCR results.

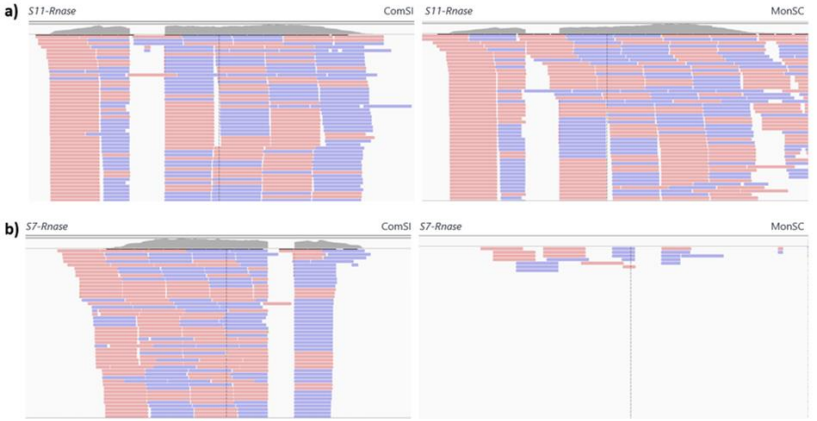
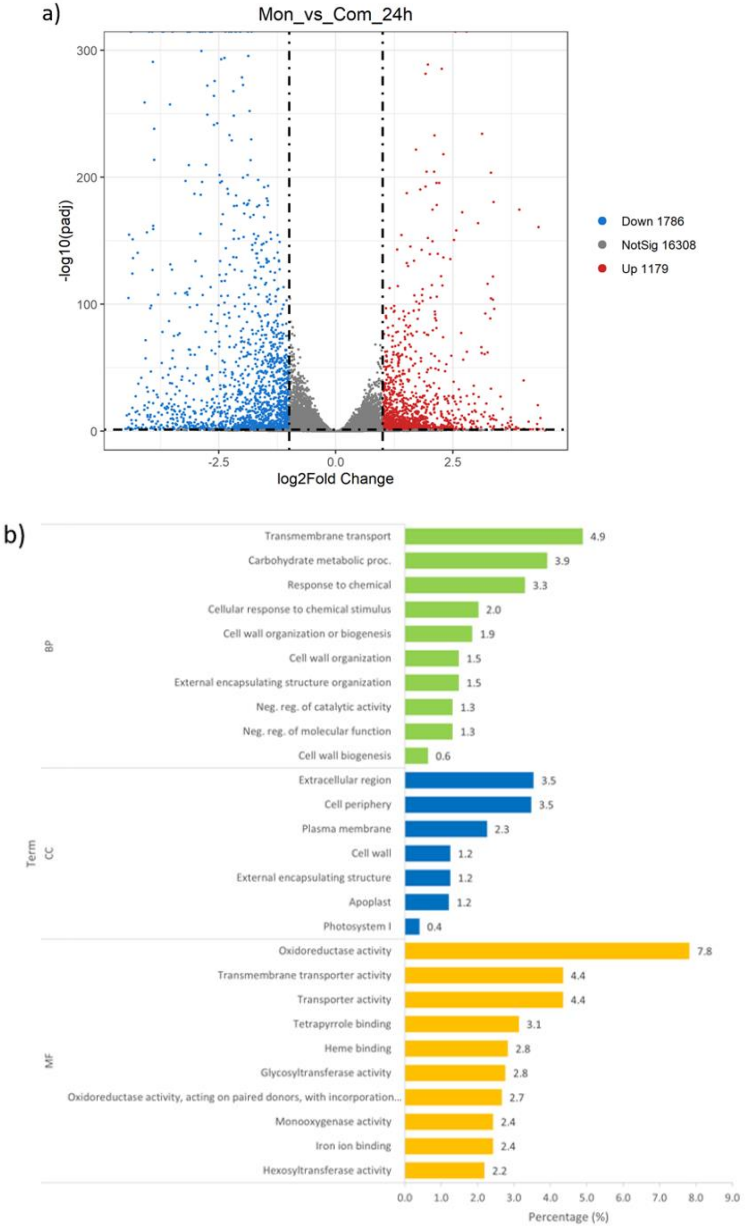


Figure 3.4 Integrative Genomics Viewer (IGV) tracks displaying sequencing read clusters of *S_{11}-RNase* and *S_7-RNase* genes from RNA-seq data generated from the styles of ComSI and MonSC clementine. The grey bars depict the number of the reads mapped to the reference. Alignment of the RNA mapping is shown below by pink and blue, representing the different read strands. No differences were shown in the number of reads mapped to *S_{11}-RNase* gene in the styles from ComSI and MonSC (a); there were significantly more reads mapped to *S_7-RNase* in the MonSC styles than in the ComSI styles (b).

Second, to further investigate the occurrence of genes responsible for the regulation of the *S_7-RNase*, an analysis of the DEGs detected at a threshold of Fold Change ≥ 2 and adjusted *P*-value ≤ 0.05 was carried out. Compared with ComSI, a total of 2.965 DEGs were detected in MonSC, of which 1.179 were up-regulated and 1.786 were down-regulated (Figure 3.5a).


a)
Mon_vs_Com_24h
-log10(padj)
log2Fold Change
Down 1786
NotSig 16308
Up 1179
b)
Transmembrane transport 4.9
Carbohydrate metabolic proc. 3.9
Response to chemical 3.3
Cellular response to chemical stimulus 2.0
Cell wall organization or biogenesis 1.9
Cell wall organization 1.5
External encapsulating structure organization 1.5
Neg. reg. of catalytic activity 1.3
Neg. reg. of molecular function 1.3
Cell wall biogenesis 0.6
Extracellular region 3.5
Cell periphery 3.5
Plasma membrane 2.3
Cell wall 1.2
External encapsulating structure 1.2
Apoplast 1.2
Photosystem I 0.4
Oxidoreductase activity 7.8
Transmembrane transporter activity 4.4
Transporter activity 4.4
Tetrapyrrole binding 3.1
Heme binding 2.8
Glycosyltransferase activity 2.8
Oxidoreductase activity, acting on paired donors, with incorporation... 2.7
Monooxygenase activity 2.4
Iron ion binding 2.4
Hexosyltransferase activity 2.2
BP
CC
MF
Term
Percentage (%)

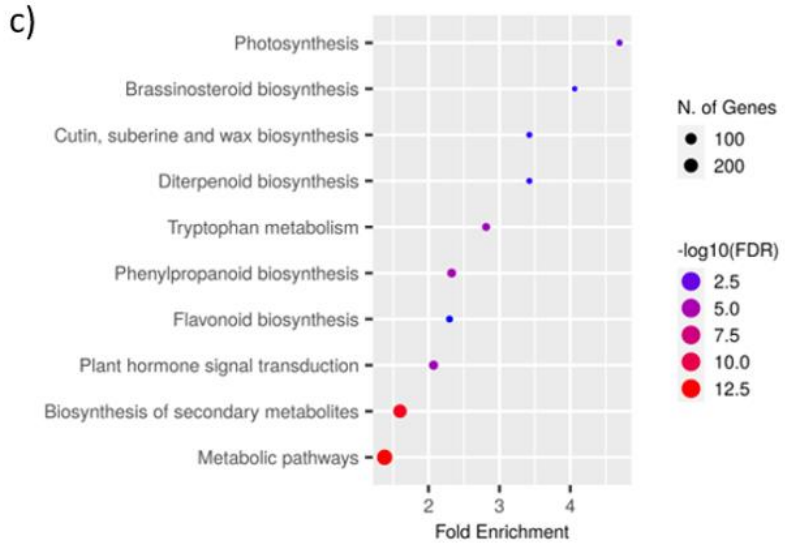


Figure 3.5 Differentially expressed genes (DEGs) in styles tissues from MonSC and ComSI at 24h after anthesis. (a): Volcano plot of DEGs determined by RNA-seq using the criteria *P*-value ≤ 0.05 and |log2 (Fold Change)| ≥ 1. Red dots indicate up-regulated genes and blue dots indicate down-regulated genes. (b): enriched GO terms in the "molecular function" (MF), "cellular component (CC) and "biological processes" (BP) categories of DEGs; (c) Top 10 significantly enriched KEGG pathways in the 2965 DEGs.

To understand the main biological functions associated with the DEGs, a Gene Ontology (GO) enrichment analysis was performed against the GO database using FDR ≤ 0.05 as the threshold (Figure 3.5b). The GO annotation assigned the DEGs to 271 GO terms, the 52.4% belongs to biological processes, 14 to cellular components (CC: 5.2%), and 115 to molecular functions (MF: 42.4%) (Figure 3.5b). The main biological functions in the BP category were 'Transmembrane transport' (GO:0055085, 4.9%), 'Carbohydrate metabolic process' (GO:0005975, 3.9%), and 'Response to chemical' (GO:0042221, 3.3%). In the CC category, the top three terms were 'Extracellular

region' (GO:0005576, 3.5%), 'Cell periphery' (GO:0071944, 3.5%), and 'Plasma membrane' (GO:0005886, 2.2%). In the MF category, the top three terms were 'Oxidoreductase activity' (GO:0016491, 7.8%), 'Transmembrane transporter activity' (GO:0022857, 4.3%), and 'Transporter activity' (GO:0005215, 4.3%, Figure 3.5b). To gain insight into the metabolic pathways associated with DEGs, a KEGG pathway enrichment analysis was carried out. In total, 770 DEGs were categorized into 27 KEGG pathways, 18 of which were significantly enriched (FDR $\leq$ 0.05). The main enriched pathways were the metabolic pathways (cic01100, 35.4%), the biosynthesis of secondary metabolites (cic01110, 23.4%), and the plant hormone signal transduction (cic04075, 5.7%, Figure 3.5c).

3.4.4 SNPs identification

The Illumina reads resulting from the RNA-seq of MonSC were aligned against the reference genome of ComSI (*C. clementina*-Phytozome v1.0) to detect SNPs or INDELS in genomic or regulatory regions that are expressed in MonSC. This analysis allowed the identification of 7.781 expressed genes characterized by one or more polymorphisms compared to the reference ComSI genome. Among those, 2.110 were characterized by the presence of SNPs predicted to have a high effect on the gene transduction (e.g. insertion or deletion of a stop codon), while 3.297 and 2.374 genes were mutations with medium or low impact. Figure 3.6 showed the coordinate of the detected SNPs in the ComSI genome; interestingly the chromosome characterized by the highest fraction of mutations (34.6% of the total) was scaffold 7. In particular, the SNPs are mainly located on the genomic region harbouring the *S_7-SRNase* suggesting the presence of a structural mutation.

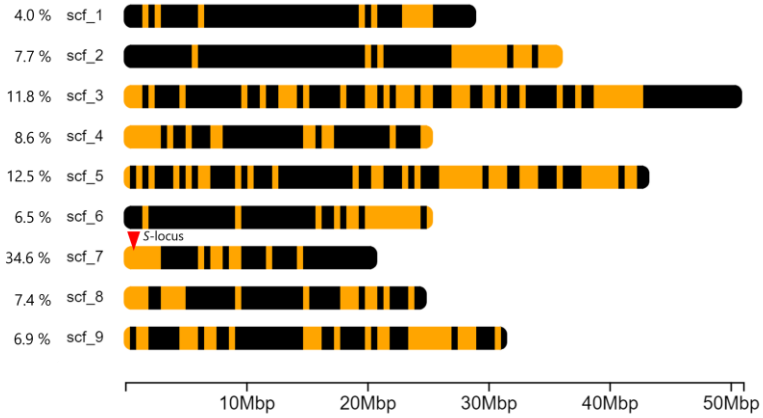


Figure 3.6 SNPs distributions (yellow bars) on MonSC transcriptome along the seven scaffolds; most of the SNPs are located in the region containing *S*-locus (red arrowhead).

3.5 Discussion

The presence of a GSI system in citrus has been recently confirmed by the discovery of several *S-RNases* in pummelo that were able to inhibit pollen tube growth (Liang et al. 2020). The identification of other *S-RNases* in Japanese cultivars (Honsho et al. 2021) together with studies combining transcriptomic, phylogenetic and genetic approaches studying the *S-RNases* segregation (Honsho et al. 2021; Ollitrault et al. 2021; Ren et al. 2020) offers new insights on citrus GSI mechanism.

In this study we first characterize the *S*-genotype of ComSI and MonSC that shared a S_7S_{11} genotype; being the clementine reference genome haploid, the entire *S*-locus for clementine was already mapped, but only S_{11}-*RNases* together with S_{11}-*SLF* genes were localized at the beginning of the pseudo-chromosome 7 of the clementine reference genome (Liang et al. 2020; Ramanauskas and

Igić 2017). First specific primers designed on the deposited *S-RNases* allowed the amplification of the two *S-RNases*. The S_7S_{11} genotype is attributed for the first time to MonSC and ComSI referring to two deposited sequences, S_7 (MN652903.1) and S_{11} (MN652907.1), submitted to GenBank in 2019 by Liang et al., 2020. Previous studies attributed to 'Clementine' and ComSC a S_3S_{11} genotype (Kim et al. 2020; Ollitrault et al. 2021) but, once each *S-RNase* is linked univocally to a deposited sequences, all new studies will follow these allele numeration and previous attribution should be interpreted according to the new classification.

All known *S-RNases* belong to the Class III of T2 RNase protein family (Igic and Kohn 2001) and they share several characteristics such as the locus architecture (Figure 3.2a), the expression patterns and similar isoelectric points (Ramanauskas and Igić 2017); all *S-RNases* contains two conserved amino acid sequences, CAS I ('F--HGLWPV') and CASII ('FW---W--HGS'), located respectively on or near C2 and C3 domains; they include two histidine residues, His46 and His109, that are essential for the ribonuclease activity (Honsho et al. 2021; Kawata et al. 1990; Parry et al. 1989). The *S_7-RNase* and *S_{11}-RNase* identified in this study shared these features; the only difference is found in the deduced amino acid sequence of MonSC *S_7-RNase* that reports a valine instead of an alanine at position 131. The difference is due to the presence of 'T' instead of an 'C' in the MonSC sequence, but the substitution involved two amino acids, both having an hydrophobic side chain and occurring in a hypervariable region, so we can hypothesize that this variation is not affecting *S_7-RNase* activity.

Previous works already demonstrated that the mutation between ComSI and MonSC affected the pistil function: histological analysis demonstrated that in MonSC both self- and cross-pollinated, pollen tubes reach the ovary, while in self- and cross-pollinated

ComSI pollen tubes arrest their growth in the upper or middle style (recognizing the pollen of the SC mutant as self-pollen, Distefano et al. 2009). Previous transcriptomic analysis identified several genes that are differentially expressed in ComSI and MonSC both.In the present work RNA-seq and qRT-PCR analysis revealed the lack of expression of S_7-*RNase* in MonSC that could be involved in the lack of self-incompatibility reaction (Figure 3.3 and 3.4).

SI was already studied comparing SI genotypes with their natural SC mutants. Hu and colleagues (2021) characterized the *S*-genotype of two pummelos ('Shatian' and 'Guiyou No.1'), and also in this case the loss of SI was due to a pistil-side mutation. Both pummelos had a S_1S_2 genotype for the *S*-locus, but the S_2-*RNase* was not expressed; the reason for the lack in S_2-*RNase* expression was not clarified even though nor structural variants, nor different level of methylation of cytosine in the two *S-RNases* were detected (Hu et al. 2021). Similar results were obtained by Honsho et al. (2021), in which work they focused on RNA-seq analysis of the cultivar Hyuganatsu (SI) and its natural SC mutant. The transcriptomic analysis revealed that one of the *S-RNase* (S_{15}-*RNase*) was down-regulated in the SC genotype. In the two works the SC trait seems to be associated with the downregulation of one of the two *S-RNases*, even though the mechanism responsible for the gene silencing was not clearly understood. Also, the S_m-*RNase* isolated from *C. sinensis* presenting a frameshift mutation in the coding sequence showed a low level of expression in the SC cultivar with respect to the SI counterpart (Liang et al. 2020). In these three works, the low expression of one of the *S-RNases* was detectable at balloon stage (Honsho et al. 2021), 1 day before anthesis (Hu et al. 2021) and from 5 days before the anthesis (Liang et al. 2020), only in female organs (stigma, style, and ovary tissues); in Hu et al. (2021) e Liang et al. (2020) data are followed also by the result of western blot analysis that are consistent with the transcriptomic profiles. In our work MonSC and ComSI pistils were

collected 24 h after anthesis, so our study confirms that the absence of one of the *S-RNase* allele in the SC cultivar is still present after the opening of the flower; this timing was chosen because the *S-RNases* were expressed before the anthesis (Liang et al. 2020, Hu et al. 2021, Honsho et al. 2021) and in self-pollinated pistils of ComSI, pollen tube elongation occurred from 48 h after pollination (Distefano et al. 2009) so the SI reaction should act in that moment.

Despite the lack of expression of S_7*-RNase* in MonSC that could be involved in the loss of SI, the real reason for the transition from SI to SC is still unclear; also, the lack of specific pollen inhibitory activity of S_m*-RNase* at the moment cannot be correlated with its attenuated expression in the self-compatible genotype (Liang et al. 2020).

Transcriptome analysis performed in pummelos by (Hu et al. 2021) let the identification of 10 different transcription factors that were differently expressed among the SC and the SI analysed cultivars: among them, *Cg2g033130 (*that corresponds to *LOC18040925*) was over-expressed in the SI genotype but did not pass the yeast one-hybrid assay proposed by the authors. Comparing our results with those reported in previous works, two transcripts were not significatively differentially expressed in our study and the remaining seven, including their candidate gene (CgHB40 or *Cg1g003830* that corresponds to *LOC18036748*), did not agree with the expression pattern indicated by the authors, in our analysis was more expressed by the SC genotype when for them was more abundant in the SI genotype and vice versa.

The different expression of *S*-allele in SC genotype with respect to the SI genotype has been detected also in other species. In almond (*Prunus amygdalus* Batsch) the presence of epigenetic changes in several cytosine residues were detected in the 5’ upstream

region of SC samples (Fernández i Martí, et al., 2014), while no difference was found in the coding or regulatory sequences of both SC and SI alleles nor in the whole chromosome region bordering the S-locus except for the differentially expressed *S-RNase* (Fernández i Martí et al. 2010); whole genome bisulfite sequencing of SC and SI pummelo cultivar resulted in no significant variation of the methylated cytosine (Hu et al. 2021).

Transcriptome comparison between pistils from ComSI and MonSC found that a total of 2.965 DEGs were present in MonSC (Figure 3.5); among these, 770 DEGs were categorized into 27 KEGG pathways and include metabolic biosynthesis of secondary metabolites and plant hormone signal transduction, suggesting that oxidoreductase activity and metabolic pathways may participate in the regulation of S_7*-RNase* expression; in our study, 'Oxidoreductase activity', 'Transmembrane transporter activity' and 'Transporter activity' were the top three terms present in the category of molecular function.

These data are in agreement with those found by Zhang et al. (2015) that performed an RNA-seq analysis on 'Xiangshui' SI seedless lemon and find that catalytic, transporter activity and binding were the main molecular function present, occurring in the cell, in the membrane and in organelle.

Subtractive hybridization libraries with cDNA microarray were employed to study the molecular mechanism involved in 'Wuzishatangju' (*C. reticulata* Blanco) SI mandarin (Miao et al. 2013, 2015). Results highlighted the involvement of genes that act by regulating signaling pathways, but also other processes like pollen development, receptor kinases, the ubiquitin pathway, calcium ion binding, gibberellin stimulus, and transcription (Miao et al. 2013, 2015).

The involvement of a signaling cascade with reactive oxygen

species (ROS) in the SI system has already be demonstrated in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) one of the *Brassicaceae* that, differently from *Citrus*, showed a SSI; in this species, self-pollen is rejected at the stigma level by the presence of high level of ROS present at the contact site of self-pollen grain that could immediately cause its arrest in SI genotype. Specific receptors, NADPH oxidases, respiratory burst oxidase homologous and GTPase regulate the level and the transport of ROS inside and between the cells (Zhang et al. 2021).

Our transcriptomic result suggest that oxidoreductase activity could participate also in the regulation of SI in citrus; recently a polyamine oxidase 2 (CrPAO2), responsible for spermine and spermidine oxidation leading to produce H2O2, was up-regulated in pollen of 'Wuzishatangju' SI mandarin respect to the SC mutant (Ren et al. 2020).

Montalt et al., (2021) suggested that the MonSC SC reaction may result from a mutation (either structural or caused by SNPs) or epigenetic variation of the genes that were located on scaffold 7 of the genome. In our study the majority of the mutations identified by transcriptome comparison affect scaffold 7 containing the *S*-locus, suggesting their involvement in the regulation of S_7*-RNase* expression.

3.6 Conclusion

SI is a key feature for many plant species to prevent inbreeding. In the present work the genetic mechanism of SI is investigated in ComSI clementine (SI) and in its SC mutant (MonSC). The design of specific primers allowed the characterization of the complete *S*-genotype of the samples, both showing a S_7S_{11} *S*-genotype. S_7*-RNase* in particular is here presented for the first time since the haploid reference genome of *C. clementina* reported only the S_{11}-

allele. Furthermore, the MonSC genome was assembled *de novo* with an overall quality that overcome those of the related species. To dissect the genetic regulation of SI, whole RNA was extracted from pistil 24 h after anthesis and the expression of the *S-RNase* was assessed through qRT-PCR. In parallel a RNA-seq analysis was carried out to provide a complete overview of the DEGs among the two samples. Both transcriptomic approaches were in agreement highlighting the lack of expression of S_7*-RNase* in MonSC. This result confirmed similar studies based on the comparison of SI and SC cultivars in pummelo, showing the lack of expression of one of the two *S-RNase* in the SC mutants. The RNA-seq analysis followed by the study of the gene ontology identified the highest fraction of DEGs among the oxidoreductase and transmembrane transport activity groups. Further studies will be required to validate the effective involvement of these genes in the mechanism of SI.

To better clarify the regulation of the gene expression, the RNA-seq data were also employed to detect SNPs in coding sequence between the two genotypes. The analysis allowed the identification of 7.781 genes characterized by the presence one or more polymorphisms. The majority of the mutations identified were located in the upper part of scaffold 7, the pseudo-chromosome containing the *S*-locus. This genomic region could be subjected to a structural mutation or can be an hotspot for recombination; further studies are required to better clarify the reason of this genetic divergence and to assess a relationship between these differences and the SI trait.

Studies on the genetic regulation of SI can provide novel molecular tools to researchers to better clarify the physiological regulation of SI and to breeders for the set-up of novel breeding programs aimed at the development of improved seedless varieties.

4. Genome editing applied to induce seedlessness in citrus

4.1 Abstract

Seedlessness is a desired characteristic in citrus fruits and it is particularly requested by consumers. For this reason in the past years many efforts have been done to obtain seedless fruit using conventional breeding techniques. The use of genome editing and, more generally, of new genomic techniques allows both the protection of the high quality of the selected cultivar to be improved as well the maintenance of the traditional genetic background often displayed by local varieties. Here CRISPR/Cas9 genome editing approach was applied to reduce seed presence and to produce edited citrus plants through the editing of *IKU1* gene; it codes for a protein that is involved in the development of the seed zygotic tissue and thus in the regulation of the seed size. 16 transgenic plants were obtained through *Agrobacterium*-mediated transformation, 1 'Sanguigno Vaccaro' sweet orange, 5 'Duncan' grapefruits and 10 'Carrizo' citrange. The construct pIKU-editing_GB was assembled using GoldenBraid technology and a tandem pair of single guide RNAs (sgRNA1 and sgRNA2) that were able to induce different types of mutation in *IKU1* gene, mostly insertion and deletion. Of the transformed plantlets 4 samples presented a big deletion between the two sgRNAs, while 2 other samples presented an inversion. The deduced amino acid sequence of the edited *IKU1* gene showed the introduction of stop codon responsible for the premature termination of the protein transduction. In one of the regenerated plantlets the VQ motif present in IKU1 protein was disrupted. The edited plants are now in the juvenile phase and further analysis of their flowers and fruits features would confirm the role of *IKU1* gene in *HAIKU* (*IKU*) pathway and its importance for the obtainment of seedless new cultivars.

Keywords: seed, HAIKU, CRISPR/Cas9, mutation, GoldenBraid

4.2 Introduction

The application of new biotechnological tools like the New Plants Breeding Techniques (NPBTs), (Eriksson et al. 2018b; Limera et al. 2017) can help to obtain novel varieties with the incorporation of the selected traits, while retaining the genetic background of the cultivar of origin; in fact NPBTs can help to overcome the limitations of conventional breeding that are expensive, long and, especially in citrus, are hampered by several reproductive biological features, including self-incompatibility, a high level of heterozygosity, a long juvenile period, large size and the lack of knowledge on how the most important horticultural traits are inherited (Poles et al. 2020).

Among NPBTs genome editing is one of the most effective tools for the accurate modification of specific sequences (Salonia et al. 2020). The technique is essentially based on the use of programmable nucleases that produce site-specific DNA double strand breaks (DSB) that can be repaired by the plants' own repair system leading to target mutation (Kim and Kim 2014; X. Liu et al. 2017). The first application of genome editing in citrus species (Table 4.1) was reported in 2014, when Jia and Wang transformed 'Valencia' sweet orange leaves and modified phytoene desaturase gene obtaining a mutated albino phenotype with a mutation rate of 3.2-3.9%. The same authors applied the same strategy to 'Duncan' grapefruit (Jia and Wang 2014b). Other examples of genome editing application in citrus considered the editing of genes involved in citrus canker susceptibility; Wang and colleagues (Wang et al., 2019) modified the transcription factor *CsWRKY22* and in the transgenic plants the development of canker symptoms was delayed. Other studies applied genome editing to the disease susceptibility gene *CsLOB1* gene (Table 4.1) The transformation experiments were done mostly using *Agrobacterium*-mediated transformation of epicotyl tissues (Dutt et al. 2022; Jia et al. 2016, 2022; Jia, Zhang, et al. 2017; Jia, Zou, et al. 2019; Jia, Orbovi, et al. 2019; Jia and Wang 2020a; Peng et al. 2017; Wang

et al. 2019; Zhu et al. 2019), Xcc-facilitated agroinfiltration (Jia et al. 2016, 2022; Jia, Zhang, et al. 2017; Jia, Orbovi, et al. 2019; Jia and Wang 2014b, 2014a, 2020a) and more recently the technique was applied also to citrus embryogenic cell cultures (Dutt et al. 2020) and protoplast (Mahmoud et al. 2022).

Table 4.1 Genome editing application in citrus. XFA = *Xanthomonas citri* ssp. citri (Xcc)-facilitated agroinfiltration, AMET = *Agrobacterium*-mediated epicotyl transformation, PPM =, protoplast PEG-mediated transformation, AMCC = *Agrobacterium*-mediated transformation of embryogenic cell cultures. EBEPthA4-CsLOB1 refers to the application of citrus to the EBE region of the LOB1 promoter of sweet orange; GFP = Green Fluorescent protein.

Citrus species	Transformation method	Target gene	Reference
'Valencia' sweet orange	XFA	Citrus phytoene desaturase (CsPDS)	(Jia and Wang 2014a)
'Duncan grapefruit, 'Valencia sweet orange, 'Key' lime, 'Carrizo' citrange, Sour orance, 'Meiwa' kumquat	XFA	CsPDS	(Jia and Wang 2014b)
'Wanjincheng' orange	AMET	EBEPthA4-CsLOB1	(Peng et al. 2017)
'Duncan' grapefruit	XFA, AMET	EBEPthA4-CsLOB1 Type I	(Jia et al. 2016)
'Duncan' grapefruit	XFA, AMET	GFP, CsLOB1 Type I and Type II	(Jia et al. 2017)
'Mini citrus Hongkong' kumquat	AMET	GFP, CsLOB1	(Jia, Zou, et al. 2019)
'Wanjincheng' orange	AMET	CsWRY22	(Wang et al. 2019)
'Carrizo' citrange	AMET	GFP, CsPDS	(Zhang et al. 2017)
Pummelo	XFA, AMET	GFP, EBEPthA4-LOBP	(Jia and Wang 2020b)
'N7-3' seedless sweet orange	PPM	Nonexpressor of Pathogenesis-Related 3 (CsNPR3)	(Mahmoud et al. 2022)
'Duncan' grapefruit	XFA, AMET	CsPDS, GFP, EBEPthA4-CsLOBP	(Jia et al. 2019)
'EV2' sweet orange	AMCC	GFP, CsPDS	(Dutt et al. 2020)
Pummelo	XFA, AMET	GFP, EBEPthA4-LOBP	(Jia et al. 2022)

'Hamlin' sweet orange	AMET	Citrus sinensis TILLER ANGLE CONTROL 1 (CsTAC1)	(Dutt et al. 2022)
Pummelo	XFA, AMET	GFP, EBEPthA4-LOBP	(Jia and Wang 2020a)

Despite all these genome editing applications have focused on resistance to biotic and abiotic stresses, qualitative traits of the fruit and its nutraceutical properties are more and more considered. In this chapter we report an attempt to obtain new seedless genotypes.

Citrus seedless cultivar can contain no seed or less than 5, aborted or a significantly reduced number of seeds (Vardi et al. 2008; Varoquaux et al. 2000). Seedless fruits can be obtained from parthenocarpic cultivars that are self-incompatible and can be grown in blocks isolated from cross-pollinators or from cultivars that display male or female sterility (e.g. 'Satsuma' mandarin, 'Washington Navel' orange) or that are triploids ('Tahiti' lime and 'Orblanco'; Vardi et al., 2008). Seed production is controlled by many genes and the loss of seed in the fruits can be the result of many processes, for examples, male or female sterility (Vardi et al. 2008), self-incompatibility (Caruso et al. 2012), stenospermocarpy (Mesejo et al. 2014b) accompanied with parthenocarpy.

In this work we focused on *HAIKU* (*IKU*) pathway, that, together with phytohormones, regulates seed size affecting the development of the zygotic tissues (Li, Xu, and Li 2019).

The *Arabidopsis iku* (*IKU1* and *IKU2*) and *miniseed3* (*MINI3*) mutations specifically determine a reduction of seed size: in fact, they interest many features of endosperm development, causing a premature arrest of its growth which triggers precocious cellularization, restricts cell proliferation in the embryo and limits cell elongation of the maternally derived seed integument. In *iku* mutants, endosperm size is decreased at the globular stage, thus seeds from *iku* plants have an overall reduced mass (Garcia et al. 2003; Garcia,

Gerald, and Berger 2005; Luo et al. 2005). It has been demonstrated that *IKU1*, *IKU2* and *MINI3* genes act in the same pathway (Li et al. 2019): in particular *IKU2* and *MINI3* are both regulated by *SHORT HYPOCOTYL UNDER BLUE 1* (*SHB1*), which can binds to their promoters (Zhou et al. 2009). *IKU1* encodes a VQ motif protein, which, respect to these other genes, it is the only one expressed in early endosperm before cellularization, and it is essential for the action of *MINI3* gene (Luo et al. 2005; Wang et al. 2010).

In this study, we applied genome editing to citrus, in particular to 'Sanguigno Vaccaro' sweet orange, and to the model plants 'Carrizo' citrange and Duncan grapefruit: two sgRNAs were designed in order to disrupt *IKU1* gene and to verify that the mutation induced could result in anomalies in the endosperm growth and in the production of seedless fruits.

4.3 Materials and methods

4.3.1 Identification of IKU gene

The homologous sequence of Arabidopsis *IKU1* sequence (At2g35230) in Valencia genome orange 2.0 (www.citrusgenomedb.org) is the predicted mRNA of "LOC102627419". The sequence of *citrus* was used to design 2 single guides RNA (sgRNAs), using the web RNA design tool CRISPR-P 2.0 (http://cbi.hzau.edu.cn/cgi-bin/CRISPR; Lei et al., 2014; H. Liu et al., 2017).

The criteria used for the selection of the guides were: the possibility to have a double sgRNAs in tandem with a distance between both sgRNAs possibly of 300 bp; the on-target score higher than 0.50, the GC content higher than 50%; and the presence of a small number of off-targets. CRISPR-P 2.0 (http://cbi.hzau.edu.cn/cgi-bin/CRISPR Lei et al., 2014; H. Liu et al., 2017) and CRISPOR (http://crispor.tefor.net/Concordet & Haeussler, 2018) analysis

software were used to detect potential off-targets sequences.

4.3.2 Plasmid construction

The vector pIKU-editing_GB (Figure 4.1) was generated using GoldenBraid cloning system v3.0 (Vazquez-Vilar et al., 2016, https://gbcloning.upv.es/) and routinary enzyme digestion and ligation procedures. The 2 guides, 5'-GGTGGACGTTGGTTTCCCCT-3' (sgRNA1, on negative strand) 5'-GGTTTCGGAGGATTTTGTGG-3' (sgRNA2, on negative strand) were PCR-amplified using the respective primer couple (Table 4.2), each one inserting in a pDGB3-α vector. Assembly reactions were performed using *BsaI* (for pDGB3-α1 and pDGB3- α2) and *BsmBI* (for pDGB3-Ω1 and pDGB3-Ω2) as restriction enzymes. The 25 cycle digestion/ligation reactions consists in 37 °C for 20 min, 25 cycles at 37 °C for 3 min, 16 °C for 4 min and a final cycle of 37 °C for 5 min plus a step at 80 °C for 5 min to inactivates enzymes. The resulting mix was used to transform *Escherichia coli* JM109 chemical competent cells (Promega, USA); colony PCR analysis of verification were performed using VWR Taq DNA Polymerase (Life Science) following the manufacturer's instructions.

Each step of the GoldenBraid cloning was validated by checking the set of fragments generated using different restriction enzymes, in particular *Eco*RI to verify the correct assembly of pDGB3-α1 plasmid containing the sgRNA1, the U6 promoter and the RNA scaffold, *Hind*III to verify the correct assembly of either pDGB3-α1 plasmid containing the sgRNA2, the U6 promoter and the RNA scaffold or pDGB3-Ω2 containing the *hCas9* gene and the sgRNA1, and *Bam*HI to verify the correct assembly of pDGB3-Ω1R plasmid containing the *nptII* gene and the sgRNA2) (Promega). The general digestion protocol used for each restriction enzyme consists in 1 hour at 37°C of a mix with 2X enzyme buffer, 5U of restriction

enzyme, 1µg of DNA and 2µg of acetylated BSA in a final volume of 20µL; the product obtained was analysed on agarose 1.5% gel electrophoresis.

The final pIKU-editing_GB (Figure 4.1) assembly was confirmed by Sanger sequencing.

Figure 4.1 Schematic representation of the pIKU-editing_GB vector assembled for genome editing. RB and LB, right and left T-DNA borders, respectively; *Nos-P* and *Nos-T*, nopaline synthase promoter and terminator sequences, respectively; *nptII*, the selectable marker gene neomycin phosphotransferase II conferring resistance to the antibiotic kanamycin; a tandem pair of single guide RNAs (sgRNA1 and sgRNA2) driven by the *U626* promoter from *Arabidopsis* (U6-26-p); 35S-p, *CaMV 35S* promoter and human codon optimized Cas9 (Cas9). Blue arrows at the bottom of T-DNA indicate PCR primers used to amplify *nptII* and *Cas9* genes and the respective size of PCR amplicons.

After validation by sequencing, the pIKU-editing_GB was finally transferred to *A. tumefaciens* strain EHA105 by thermal shock: 1 ug of plasmid was added to competent cells and incubated for 2 min at –20 °C and then at 37 °C for 5 min. 1 mL of LB medium were added to the cells and, after a brief step of 2-4 h at 28°C, cells were centrifugated and pellet was resuspended in 10 µL of LB and cultures on selective medium consisting in LB added with 50 mg/L of kanamycin.

4.3.3 Plant materials

In vitro nucellar seedlings of 'Carrizo' citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.), 'Duncan' grapefruit (*C. paradisi* Macf.), and 'Sanguigno Vaccaro' sweet orange (*C. sinensis* L.) were used for genetic transformation. The seeds were extracted from fruit,

the outer seed coat removed and surface-sterilized as previously described (Dutt and Grosser 2009).

4.3.4 *Agrobacterium-mediated transformation*

Agrobacterium-mediated transformation of epicotyl explants was performed as previously described (Orbović and Grosser 2015) with the modified regeneration medium RM of Murashige and Skoog (MS) medium plus 1 mg/L of 6-Benzylaminopurine, 100 mg/L kanamycin, 500 mg/L of cefotaxime, 8 g/L of agar, pH 5.75. The leaves of kanamycin resistant shoots were screened by PCR to verify the integration of T-DNA cassette; transformed shoots of 'Duncan' grapefruit and 'Sanguigno Vaccaro' sweet orange were shoot-tip grafted on 'Carrizo' citrange seedlings grown *in vitro* as previously reported (Orbović and Grosser 2015) while 'Carrizo' transformed shoots were cultured in MS medium with 0.5 mg/L of 1-Naphthaleneacetic acid to induce rooting. Transformation efficiency (TE) was evaluated as the number of transformed shoots regenerated from inoculated explants.

4.3.5 *Genomic DNA extraction and PCR amplification*

The confirmation of the transgenic nature of regenerated shoots was done by PCR amplification. DNA was extracted from regenerated shoots following a modified CTAB protocol (Doyle and Doyle 1987); extracted DNA was quality checked through Nanodrop 1000 spectrophotometer (Thermo Scientific) and diluted to 20 ng/μL.

The presence of T-DNA cassette was confirmed by the amplification of both *nptII* and *Cas9* genes (Table 4.2). PCR analysis was performed using VWR Taq DNA Polymerase (Life Science) following the manufacturer's instructions. Reactions for *nptII and Cas3* amplification were carried out under following conditions: 95

°C for 2 min, 35 cycles of 95 °C for 25 s, primers annealing temperature for 30 s and 72 °C for 1.1 min, followed by 72 °C for 5 min.

Table 4.2 List of primer used for PCR amplifications. T.A. = annealing temperature; lowercase letters represent the Illumina adapter sequences necessary to generate the Illumina library

Primer Name	Sequence (5'-3')	Purpose	T.A.
sgRNA1-F	ATTGGTGGACGTTGGTTTCCCCT	pIKU-editing_GB assembly (sgRNA2)	62°C
sgRNA1-R	AAACAGGGGAAACCAACGTCCAC		
sgRNA2-F	ATTGGTTTCGGAGGATTTTGTGG	pIKU-editing_GB assembly (sgRNA1)	62°C
sgRNA2-R	AAACCCACAAAATCCTCCGAAAC		
NptF	CCTCAGCAATATCACGGGTAGC	Amplification of *nptII* gene	58°C
NptR	GGATCTCCTGTCATCTCACCTT		
Cas3F	GCCAGCCACTATGAAAAGCT	Amplification of *Cas9* gene	62°C
Cas3R	AATGTTTTCTGCCTGCTCCC		
Iku-regF	AGAGTCCATTGCACCAACCT	Amplification of sgRNAs region	57°C
Iku-regR	CTGTTGGGCATGTACAGGTG		
Adp_iku-regF	tcgtcggcagcgtcagatgtgtataagagacagAGA GTCCATTGCACCAACCT	Illumina sequencing	57°C
Adp_iku-regR	gtctcgtgggctcggagatgtgtataagagacagCT GTTGGGCATGTACAGGTG		
GG20F	ACCTTCACAAGAACCTTTGCC	Amplification of sgRNAs region	57°C
GG10R	TATTCCACGCCAAGTCCCAA		

PCR products were detected by electrophoresis on 1.5% agarose gels.

4.3.6 High-throughput sequencing (HTS) analysis

The genomic DNA of the transformed plants were used as template for the PCR amplification using Adp-iku-reg primers with overhang Illumina adapters (Table 4.2) and PCRBIO HS Taq Mix Red (PCR Biosystems Ltd., UK); PCR products were checked by electrophoresis on 1.5% agarose gels and and pooled in equimolar

way. Subsequently the final amplicon library were sequenced on an Illumina MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis Software 1.16.18).

Mutations were detected analysing raw paired-end reads with CRISPResso (http://crispresso.rocks/; Pinello et al., 2017) using default parameters. In few cases, the DNA of edited plants, already analysed with CRISPResso, was amplified using Iku-reg, GG10 and GG20 primers to check the mutation event present. Illumina reads were aligned against Valencia genome orange 2.0 (www.citrusgenomedb.org) and visualized into Integrative Genomics Viewer (IGV) tool. PCRs were performed using VWR Taq DNA Polymerase (Life Science) with the conditions described above.

4.3.7 Plant propagation and assay of mutant plant's phenotype

The edited plantlets were re-grafted on potted *P. trifoliata* seedlings in greenhouse to accelerate fruit production. At flowering stage all plants were manually cross-pollinated and the fruits derived were checked for seeds and ovules content in terms of number, shape and dimension.

4.4 Results and discussion

4.4.1 pIKU-editing_GB assembly

The pIKU-editing_GB assembly was checked and confirmed through Sanger sequencing. The 2 sgRNAs chosen possibly create a deletion of 327 bp in the first exon of *IKU1* gene; sgRNA1 had an on-target score of 0.55 and a percentage of GC content of 60%, while the values for sgRNA2 were respectively 0.62 and 50%. Both sgRNAs had off-target sites and, unfortunately, most of them present two or more mismatches on the 'PAM seed region', defined as the 10–12 base

pairs adjacent to the PAM; and most of these mismatches were present also in the "true seed region", defined as one to five base pairs of guide region proximal to the PAM (X. H. Zhang et al. 2015).

4.4.2 Generation of transgenic citrus plants

Regenerants buds resistant to kanamycin selection were screened by PCR to verify the integration of T-DNA, confirmed by the presence of both *nptII* and *Cas9* genes (Figure 4.2).

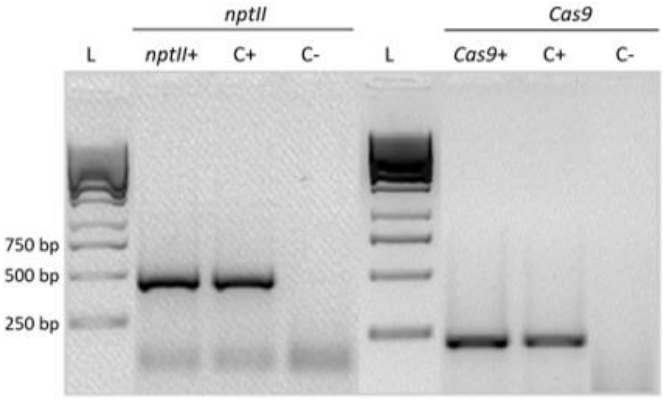


Figure 4.2 PCR screening of transgenic plants. Visualization on agarose gel of amplified PCR products of *nptII* (392 bp) and *Cas9* (213 bp) from leaves of a representative transgenic plant positive to both genes. pIKU-editing_GB plasmid extracted from *A. tumefaciens* EHA105 used for plant transformation was used as positive control (C+), while non transformed wild-type plant was used as negative control (C-). 1 Kb Ladder (L) (Thermo Scientific).

For experiment with 'Carrizo' citrange a frequency of transformed shoots of 37.1% and a TE of 42.5% were obtained; 7 transgenic shoots of 'Duncan' grapefruit were recovered from 22 regenerants analysed, resulting in a frequency of transformed shoots of 31.9% and a total TE of 3.5%. Percentages are comparable to the 47% obtained by Dutt & Grosser (2009), and the 41.3% reported in Cervera et al., (1998), while

for 'Duncan' grapefruit the value is a bit lower respect to the 40% TE (Dutt & Grosser, 2009) reported before for the transformation of this genotype. In 'Sanguigno Vaccaro' sweet orange experiment, the frequency of transformed shoots was 25.0% and TE of 1.3%, comparable to 23.8% and 25% reported for the transformation of other sweet oranges like 'Valencia' (Boscariol et al. 2003) and 'Hamlin' (Dutt & Grosser, 2009), respectively (Table 4.3). In our study the regeneration frequency (the number of shoots regenerated from inoculated explants) was around 8-10%, but problems with micrografting have led to the loss of the material before it was PCR-analysed.

Table 4.3 Transformation efficiency for pIKU-editing_GB transformation experiments.

Genotype	Shoot analysed by PCR	Positive shoots	Frequency of transformed shoots (%)[a]	Transformation efficiency (%)[b]
'Carrizo' citrange	229	85	37.1%	42.5%
'Duncan' grapefruit	22	7	31.9%	3.5%
'Sanguigno Vaccaro' sweet orange	8	2	25.0%	1.3%

[a] positive shoots of total shoots analysed
[b] positive shoots of 150 total explants inoculated

The PCR positive plantlets were transplanted to soil and transferred to greenhouse for the acclimatation.

4.4.3 *Characterization of IKU1 mutants*

IKU1 target region was screened in 16 transgenic citrus plants, 5 'Duncan' grapefruits, 10 'Carrizo' citrange and 1 'Sanguigno Vaccaro' sweet orange, respectively.

HTS analysis shows that both 'Duncan' grapefruit and 'Carrizo' citrange had a single nucleotide polymorphism at the 6th position of the sgRNA2 (Figure 4.3), while for sgRNA1 no differences in the sequence of the bases were found. This makes it possible to identify 2 forms (or aplotype) of *IKU1* gene and of sgRNA2, I type contained a 'T' while II type contained a 'C', like the original sgRNA2. A similar result was found in the 'Duncan' used for the editing of *CsLOB1* (Jia et al. 2016) and it is not surprisingly since grapefruit resulted from the hybridization between pummelo and sweet orange (Wu et al., 2014; Xu et al., 2013). Also 'Carrizo' citrange shows the 2 forms of the sgRNA2 (Figure 4.3) and this is compatible with its origin, in fact 'Carrizo' citrange has been obtained from a cross between *C. sinensis* and *P. trifoliata*.

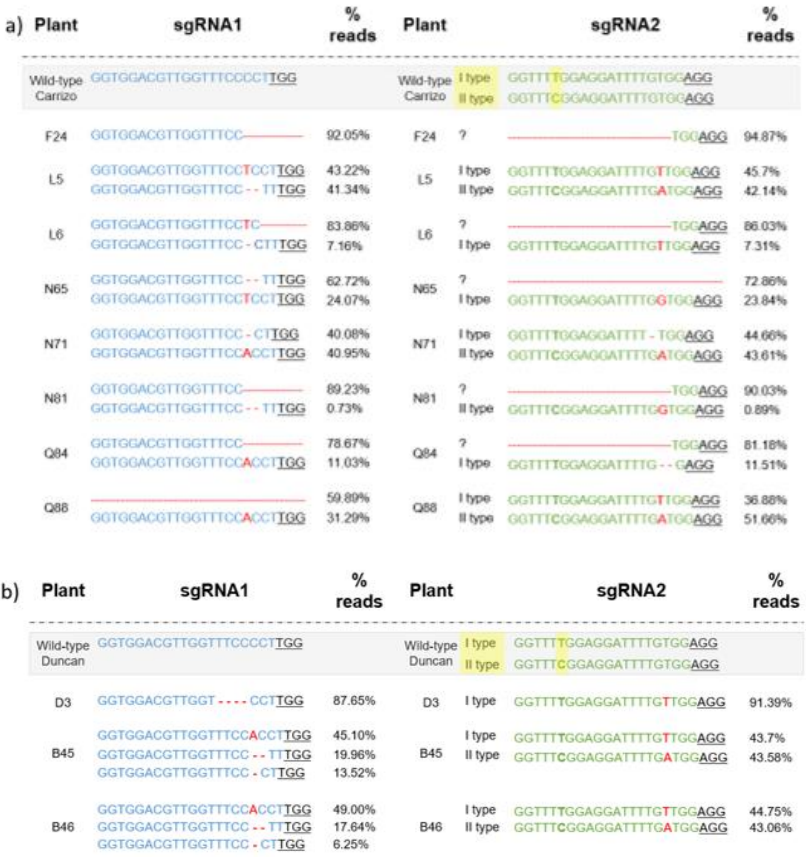
Plant	sgRNA1	% reads		sgRNA2	% reads
Wild-type Duncan	GGTGGACGTTGGTTTCCCCTTGG	83.81%	I type II type	GGTTTTGGAGGATTTTGTGGAGG GGTTTCGGAGGATTTTGTGGAGG	44.18% 43.58%
Wild-type Carrizo	GGTGGACGTTGGTTTCCCCTTGG	83.78%	I type II type	GGTTTTGGAGGATTTTGTGGAGG GGTTTCGGAGGATTTTGTGGAGG	44.19% 44.48%
Wild-type Vaccaro	GGTGGACGTTGGTTTCCCCTTGG	82.87%		GGTTTCGGAGGATTTTGTGGAGG	91.97%

Figure 4.3 sgRNAs sequences in the three genotypes transformed. For the sgRNA2, two forms of *IKU* gene, I type and II type, were found in 'Duncan' grapefruit and in 'Carrizo' citrange (highlighted in yellow), while for the sgRNA1, only one form was present; 'Sanguigno Vaccaro' sweet orange showed only I type for both sgRNAs.

On average 350.338 raw sequences were obtained among the three genotypes and a mean of 21.889 reads were aligned for each of the analysed plants. The *IKU1* region resulted edited in all samples; in F24, L6, N81 and Q84 the deletion of 327 bp, from sgRNA1 to sgRNA2, was present (Figure 4.4a, c). In 'Carrizo' citrange the most frequent mutations for both the sgRNA1 and sgRNA2 were small

insertion (+1) followed by large (-327 bp) and short (-1 and -2 bp) deletions (Figure 4.4c); in 'Duncan' grapefruit the most frequent mutation was a short insertion for sgRNA1 (Figure 4.4d). Considering both species, deletions prevailed in sgRNA1 while insertions prevailed in sgRNA2.

Editing occurred in Q87, D2 and DH2 showed a low percentage of mutated reads (Table 4.4); V107 and M44 had a more complicated editing pattern (Figure 4.5 and 4.6) so both these 5 samples were excluded form Figure 4.4.



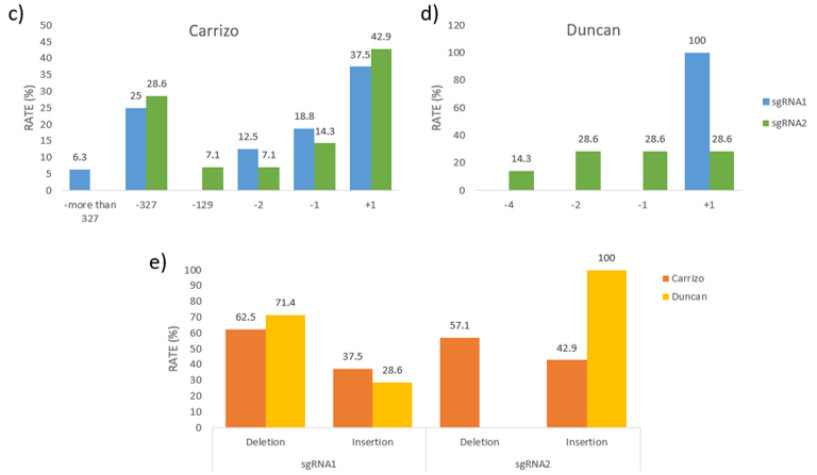


Figure 4.4 Targeted genome engineering in citrus plants. Representation of mutation events generated by the CRISPR/Cas9 system in *IKU1* gene for 'Carrizo' citrange (a), 'Duncan' grapefruit (b). The sgRNA1 target sequence is coloured in green, the sgRNA2 is coloured in blue. The modifications occurred are highlighted in red and deletions are represented by dashes. Rate of mutation sizes in 'Carrizo' citrange (c), 'Duncan' grapefruit (d) and rate of mutation types (e) that comprises all edited samples with the exclusion of D2, DH2, Q87 (that had low mutation reads), M44 and V107 (that had more complex editing pattern). Percentages in c, d and e were calculated by dividing the number of total events (of each mutation type and size, respectively) by the sum of total mutation events.

Among the samples showing several types of mutation, only 2 display a single event: the deletion of 327 bp for F24, and a deletion of 4 bp in sgRNA1 and a 'T' insertion in sgRNA2 for D3.

I type and II type showed a preferred type of mutation: the insertion in I type is mostly a 'T' with the exception of N65, while for II type is always an 'A' except for sample N81 that showed an insertion of 'G'. The only samples in which the editing affect only one form are F24 (unknown type) and D3 (I type) (Figure 4.4 a and b).
Samples that showed the 327 bp deletion (F24, L6, Q84 and N81), together with V107 sweet orange M44 and 'Carrizo' citrange showed

a complex CRISPResso profiles, so they were also screened using electrophoresis on 1.5% agarose gels and reads were visualized on IGV (Figure 4.5).

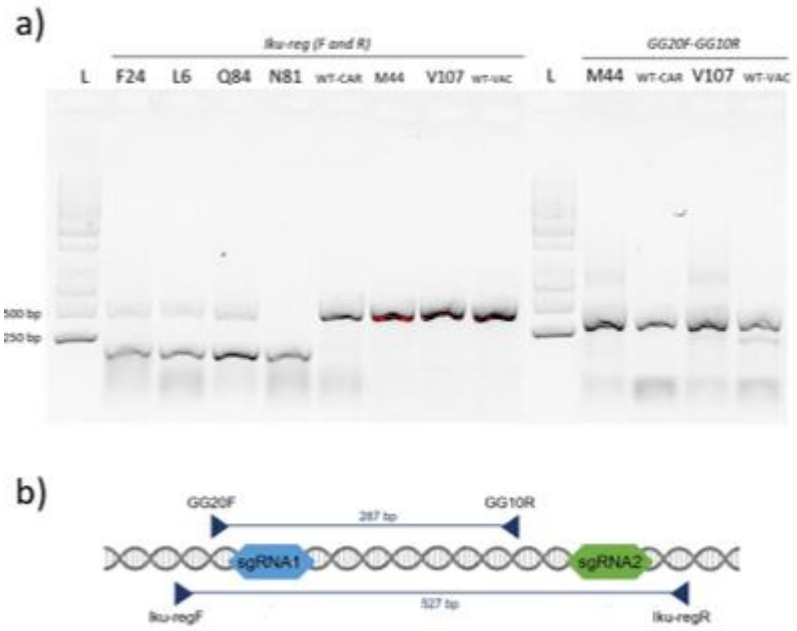
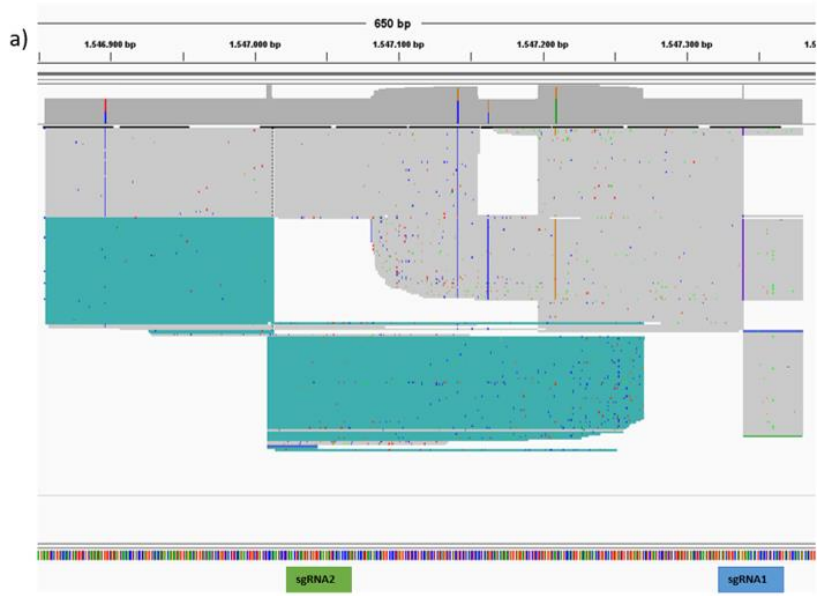


Figure 4.5 PCR screening of edited plants. Visualization on agarose gel of amplified PCR products (a) using Iku_regF, Iku_regR primers (527 bp), GG20F and GG10R (287 bp); primer positions were represented in the scheme (b). The samples amplified using Iku_reg primers included F24, L6, Q84 and N81 and showed a band at 109 bp, lower than positive control (WT-CAR), due to the 327 bp deletion occurred between sgRNA1 and sgRNA2; M44 and V107 showed the same profile of its control (WT-CAR and WT-VAC, respectively) also using GG20F and GG20R primers. 1 Kb Ladder (L) (Thermo Scientific).

F24, L6, Q84, and N81 showed a marked band at 109 bp, lower than positive control (WT-CAR), due to the 327 bp deletion occurred from the cutting site of sgRNA1 and sgRNA2 (Figure 4.5); M44 and

V107 showed the same profile of its control (WT-CAR and WT-VAC) so they were amplified also using GG20F and GG10R primers to check if an inversion was present; in that case no amplification would had occurred, but no difference were found. The reason of that could be traced back looking directly at the Illumina reads aligned on Valencia genome using IGV: automatically, the viewer depict in blue reads that showed a change of orientation read-strand, so between the sgRNAs an inversion had occurred, but only in a subset of reads (Figure 4.6).



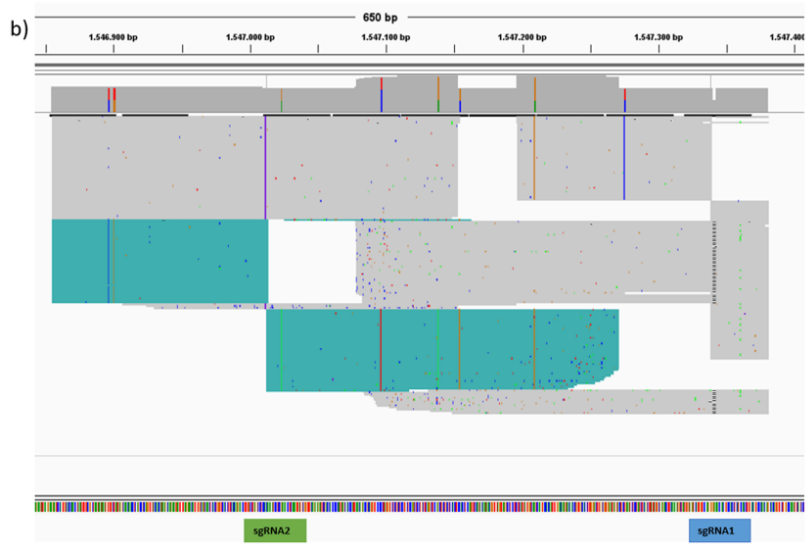


Figure 4.6 Illumina reads aligned on Valencia genome visualized on IGV tool. V107 (a) and M44 (b) reads were aligned and the reads depicted in blue colour indicate that an inversion has occurred, so they are reversed in the edited plants compared to the reference genome.

For both V107 and M44 the inversions did not occur in all reads, in fact the profiles of agarose gel resulted identical to those of the control and the amplification using GG20F-GG10R did not give the expected band of 287 bp (Figure 4.5). The percentage of mutated reads is high for both samples (85.35% for sgRNA1 and 87.38% for sgRNA2 for V107; 84.98% for sgRNA1 and 87.83% for sgRNA2 for M44) but it includes all reads that are different from reference. On the whole reads can be grouped into two group of different inversions (Figure 4.6, the two blue group) and in one small deletion (66 bp for M44 and 68 bp for V107) in both samples.

Using the reads alignment on Valencia genome it was possible to determine the amount of the deletion in sgRNA2 of N65 and the deletion in sgRNA1 of Q88 that were of 233 bp (from 52 bp before

sgRNA2 cut site) and 101bp (66 bp before the cut site), respectively. For edited plantlets, different percentage of mutated reads were obtained. Among the 'Duncan' samples, only D2 and DH2 showed low percentage of mutations of the reads, 31.29% and 46.69% respectively for the sgRNA1 and 26.99% and 19.03% for the sgRNA2, while the other 3 samples showed higher values. In particular, considering sgRNA2, the mutation affected only one form of sgRNA1 in D3 (I type), while for B45 and B46 the guide targeted both I and II type for a total of 88.15% and 87.81%, respectively (Table 4.4). Considering 'Carrizo' citrange samples, Q87 is the only one with a low percentage of mutated reads, 23.43% for sgRNA1 and 13.55% for sgRNA2; sgRNA mutations targeted both types of *IKU1* gene for L5 and Q88 sample, while in the other sample there were larger deletions, so it was not possible to identify if the mutation affected both forms. These results are comparable with other experiments of genome editing in citrus, especially those in which 'Duncan' was transformed; in particular, Jia et al. (2016) obtained 4 lines of edited plants that showed mutation only in *CsLOB1* I type and that were susceptible to *Xcc* infection, suggesting that only the biallelic mutation of the gene could generate a resistant plant. Subsequently, Xu et al. (2017) used a sgRNA to target a conserved region in both alleles; 6 lines were obtained with mutation rates between 23.80%-89.36%, but only the two with the higher mutation rate (89.36% and 88.79%) did not develop canker symptoms.

Table 4.4 Mutation rate of reads induced by CRISPR/Cas9.

Genotype	Plant ID	% of mutated reads	
		sgRNA1	sgRNA2
'Carrizo' citrange	F24	92.05%	94.87%
'Carrizo' citrange	L5	84.56%	87.84%
'Carrizo' citrange	L6	91.02%	93.34%

'Carrizo' citrange	M44	84.98%	87.83%
'Carrizo' citrange	N65	86.79%	96.7%
'Carrizo' citrange	N71	81.03%	88.27%
'Carrizo' citrange	N81	89.96%	90.89%
'Carrizo' citrange	Q84	89.7%	92.69%
'Carrizo' citrange	Q87	23.43%	13.55%
'Carrizo' citrange	Q88	91.18%	88.54%
'Duncan' grapefruit	D2	46.69%	26.99%
'Duncan' grapefruit	D3	87.65%	91.39%
'Duncan' grapefruit	DH2	31.29%	19.03%
'Duncan' grapefruit	B45	82.34%	88.15%
'Duncan' grapefruit	B46	79.82%	87.81%
'Sanguigno Vaccaro' sweet orange	V107	85.35%	87.38%

Comparable percentage of editing were obtained also in the present work for most of the samples of 'Duncan' grapefruit, 'Carrizo' citrange and 'Sanguigno Vaccaro' sweet orange; overall the percentage are higher for sgRNA2, greater than 92% for F24, L6, N65 and Q84, respect to sgRNA1 where the only sample with percentage greater than 92% is F24. The lowest percentage were obtained for sgRNA2, Q87, and DH2 showed percentage lower than 20% (13.55% and 19.03%, respectively, Table 4.4). In literature higher percentages of mutation rate are reported for the genome editing of sweet orange: Wang (et al., 2019) modified *CsWRKY22* gene obtaining three mutant lines with 85.7%, 79.2% and 68.2% mutation rates that displayed decreased susceptibility to citrus canker, but these values were calculated based on Sanger sequencing, and thus are not comparable with the ones obtained with HTS. Here also we used HTS on Illumina

MiSeq platform to estimate editing performance and respect to Sanger sequencing this method has shown to be more effective, rapid and cost-efficient, allowing the simultaneous visualization of a great number of clones for a single sample processed (Pompili et al. 2020). The use of double sgRNAs in tandem was employed since it could improve the success rate of targeted mutagenesis and generate large genomic deletion; in our study the hypothesis of creating a large deletion involving both sgRNAs was confirmed in 4 samples (F24, L6, Q84 and N81) and involved 327 bp, while 2 samples (V107 and M44) showed an inversion. By the simultaneous induction of DSBs within a chromosome, the area between the two DSBs is repaired by the NHEJ system: the segment can be inversely integrated into the genome, creating an inversion, or it can be lost, creating a deletion, that, if the segment is longer than 100 bp, will ensure gene knockout. (Mao et al. 2013; Shan et al. 2013; Upadhyay et al. 2013; Zhou et al. 2014).

Moreover, in some cases the use of two sgRNAs can ensure the success of the genome editing, especially if, differently from our results, one of the two sgRNA has a lower editing efficiency. In order to avoid this it is reported in citrus the possibility to evaluate the functionality of different sgRNAs that target the same gene, using the agroinfiltration approach facilitated through the use of *Xcc* infection (Jia et al. 2016; Jia, Zhang, et al. 2017; Jia et al. 2019; Jia and Wang 2014a).

Another issue in the application of genome editing is the occurrence of off-target, that are non-specific and mostly can induce unintended modifications in other than target loci. Based on sweet orange genome, the potential off-targets generated by pIKU-editing_GB were analysed using CRISPR-P and CRISPOR web-software. Two off-targets with very low Cutting Frequency Determination (CFD, 0.03 and 0.00) were present in sgRNA2, while 22 off-targets were present for sgRNA1. With the exception of two events of two and three mismatches, they

all comprise differences of four bases; among these, 4 had a CFD score lower than 0.05, and so are unlikely to be cleaved, while scoring the other 18 off-target events, only 5 had a CFD score higher than 0.20 and need to be checked to rule out the possibility that mutation in potential off-target sites could have occurred. These 5 present mismatches located both in the 'seed region' (8–12 PAM-proximal bases) and in the 'true seed region' (1–5 PAM-proximal bases) seem to be crucial to determine targeting specificity of the arginine-rich bridge helix within the recognition lobe of the *Cas9* protein (Nishimasu et al. 2014; Shan et al. 2013). Other mismatches that are located in the PAM distal sequence are more likely to be tolerated and seem not correlated with the proper functioning of the *sgRNA/Cas9* complex (Shan et al. 2013; Zhang et al. 2015).

4.4.4 Potential effect of genome editing on IKU protein

In our experiment the mutations that were induced by the sgRNA2 editing (the first guide that it is encountered considering the correct direction of *IKU1* protein translation) are sufficient to cause a large amino acids (aa) loss in the IKU1 protein: in the cases of insertion ('A', 'T' and 'G') or for the deletion of one or two bases ('G' or 'TG'), the deduced aa sequences showed frame-shift mutations with the introduction of stop codon responsible for the premature termination of the protein transduction. In addition, in L6, F24, N81 and Q84 the editing caused a loss of 109 aa, while the other part of the protein transduction is identical to the reference ones (Figure 4.7).

Type of mutation	Protein	Plant
IKU1 protein	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPQNPPKPQSMRLQKIRPPPLAPINRPHVPPMV SAPAPAPAQAPAAAPLPPPVPYNNGLVRPPPHYGQSMPQFGQVQPVAPWDLA WNSTAESPISAYMRDLQNAIVDHGPRGNQRPPHPPVHAQQAHVLGQIQPQPPS SGLLPNPPMPGFPTSRVNGPTPLMPNLPSPQMNGPGLLPSPTSQFLWPSPTNYM NLLSPQSPYPLLSPGVQFPPPLTPNFQFSPVAQSGILGPGPQPPPSPGLAFPLSPSGF FPIFSPRWRDQ*	Reference
A insertion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPSKSSETSKYAVAEDSTTPISTH*	B45, B46, L5, N71, Q88
T insertion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPTKSSETSKYAVAEDSTTPISTH*	D3, B45, L5, L6, Q88
G insertion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPPKSSETSKYAVAEDSTTPISTH*	N65, N81
G deletion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPKILRNLKVCGCRRFDHPH*	N71
TG deletion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPPKSSETSKYAVAEDSTTPISTH*	Q84
101 bp deletion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTEQTACSPYGLCSCSSSCSGSSCCSTSTSSSIQ*	N65
327 bp deletion	MDGSKNRHNNDHLGVNKMGKNIRKSPLHQPNFAANNNVANRQQPQPQVYNIS KNDFRNIVQQLTGSPSQEPLPRPP - - - - - - - 109 bp - - - - - - - GNQRPPHPPVH AQQAHVLGQIQPQPPSSGLLPNPPMPGFPTSRVNGPTPMPNLPSPQMNGPGLL PSPTSQFLWPSPTNYMNLLSPQSPYPLLSPGVQFPPPLTPNFQFSPVAQSGILGPG PQPPPSPGLAFPLSPSGFFPIFSPRWRDQ*	L6, F24, N81, Q84

Figure 4.7 The deduced amino acid sequences of the edited *IKU1* proteins. The translated IKU1 protein is compared with the reference protein and the possible scenarios induced by the CRISPR/Cas9 editing; the conserved VQ motif is highlighted in yellow, differences between the sequences are coloured in red, stop codons are represented by asterisks.

The sequence of the *IKU1* protein contains a short VQ motif that is conserved in other plant species such as *Vitis vinifera*, *Oryza sativa*, *Popolus trichoparpha*, *Gossypium hirsutum* and *Zea mays* (Wang et al., 2010); to characterize the function of the *IKU1* gene, Wang (et al., 2010) introduced constructs bearing mutations in each of the conserved regions of the *Arabidopsis iku1* mutant line; in particular aa replaced in the VQ motif region (58-61 aa) were not able to rescue the *iku1* mutant seed phenotype, suggesting an important role of this conserved region in seed development. Considering our editing

results, the deletion of 101 bp present in N65 ('Carrizo' citrange) caused the loss of 5 aa that are part of the VQ motif (Figure 4.7). Further mutations introduced by Wang et al. (2010) in other conserved region of the protein (between 12-15 aa for the N-terminus, 154-158 aa for VQ downstream region, 328-340 aa for the C-terminus of the protein) were able to restore the wild—type size of the seed. In our experiment the cut site of sgRNA2 is located at the 76 aa, 7 aa after the end of the VQ motif and 15 aa from the VQ aa. This region has not been investigated yet so it is possible that the modification induced by the CRISPR/Cas9 system, together with the premature codon stop inserted in the deduced sequences of the protein mutated, can affect the functionality of the protein and the size of the seeds produced.

4.5 Conclusion

CRISPR/Cas9 genome editing was applied to produce edited citrus plants through the transformation mediated by *Agrobacterium*. Three citrus species were edited using a tandem pair of sgRNAs (sgRNA1 and sgRNA2) that was able to induce different types of mutation in *IKU1* gene, mostly insertions and deletions; in four samples a deletion of 327 bp, from sgRNA1 to sgRNA2 was obtained and in two an inversion was present. The deduced aa sequence of the edited *IKU1* gene, coding for a protein that is involved in the development of the seed zygotic tissue and thus in the regulation of the seed size, showed the introduction of stop codon responsible for the premature termination of the protein transduction: Moreover, in one case (N65) the VQ motif was interrupted. Phenotype observations on the fruits obtained from these edited plants and especially of their ovules and seed content will confirm the role of *IKU1* gene in the corresponding pathway and its potential role for the obtainment of new seedless cultivars.

5. General Discussion

The development of seedless varieties is one of the main goals in citrus breeding programs worldwide. In fruits that are consumed fresh, the presence of seeds - often hard, in high number, rather big in size and characterized by an unpleasant taste - negatively influences the overall appreciation of the fruit. For this reason, consumers are willing to pay more for seedless fruits, especially for mandarins and mandarins like. Seedlessness can be the result of different mechanisms, including triploidy and male or female sterility, also these mechanisms can be coupled with parthenocarpy (if the fruit develops without ovules fertilisation), or stenospermocarpy (if the fruit contains partially formed seeds that have aborted after fertilization). Another mechanism associated with seedlessness can be the presence of self-incompatibility (SI) reaction that prevents seed formation, especially if the variety is cultivated in isolated blocks without cross pollination.

Along the years many efforts have been invested for the obtainment of seedless citrus varieties. Beside traditional techniques, such as hybridization, mutagenesis and selection, the application of New Genomic Techniques (NGTs) can now offer the possibility to obtain new varieties with the desired features. NGT approaches can in fact allow the introduction of precise modifications without altering the original genetic background of the considered variety. Among the NGTs, genome editing has been already successfully applied for the editing of genes involved in the susceptibility to citrus canker. Furthermore, genome editing represents an efficient tool for functional gene validation. The last decades experienced a tremendous increase in the technologies for genome sequencing, enabling the simultaneous interrogation of thousands of markers at a fraction of the costs of the first sequencing analysis two decades ago. Such fast development of the genotyping platforms paves the way to the analysis of the entire

genome and transcriptome of individuals of interest.

The presented thesis focuses on an integrated approach based on both the use of NGTs and whole-genome sequencing techniques to decipher the genetic mechanism of SI in clementine, one of the most common and valuable varietal groups in the Mediterranean area. The genetic analysis of SI 'Comune' clementine and of its natural SC mutant 'Monreal' allowed the characterization of the complete S-genotype of the two genotypes, both showing a S_7S_{11} S-genotype. Transcriptome comparison of the pistil of the two cultivars 24 h after anthesis revealed the lack of expression of *S_7-RNase* in 'Monreal', in agreement with already observed for other citrus species. RNA-seq analysis, followed by the study of the gene ontology, identified the highest fraction of DEGs among the oxidoreductase and transmembrane transport activity groups. In addition, 7,781 genes were characterized by the presence of one or more polymorphisms in their coding sequences among the two mutants. All together, the majority of the mutations identified were located in the upper part of scaffold 7, the one containing the S-locus and representing the main candidate region for finding possible genes responsible for SI regulation. Further studies will be required to identify a limited number of candidate genes to be validated, also through the use of NGTs, for their effective involvement in SI mechanism.

Another approach consisted in the use of CRISPR/Cas9 system that was successfully used to knock out, in some Citrus species, *IKU1*, a gene involved in the regulation of seed size. Since *IKU1* mutation in *Arabidopsis* specifically affects endosperm size leading to an overall reduced seed mass, genome editing was applied to the citrus homologous gene using a dual-single guide approach. Three seedy genotypes were transformed, including two model species and one sweet orange variety; sixteen plants were analysed confirming that *IKU1* gene and the translated protein were interrupted.

In particular, among the edited plantlets, 4 samples displayed a large deletion of 327 bp between the two cutting sites of sgRNA1 and sgRNA2, while 2 samples showed an inversion of the sequences between the two cutting- sites. Phenotypic evaluations, that are still undergoing due to plant juvenility phase, will help to understand the role of *IKU1* gene in *HAIKU* pathway and its potential for the obtainment of new seedless cultivars.

The work herein presented provide novel insights on the genetic mechanisms leading to seed formation and development in citrus. Despite many constraints reported for woody plants species, the availability of regeneration and transformation protocols optimized for the main commercialized cultivars, together with the possibility of accelerating the flowering and the fruiting of plants - for example using mature tissues or applying early flowering genes - are more and more facilitating the application of NGTs in citrus.

Of course the planning of a genome editing experiment, requires a deep knowledge of several aspects , including the choice of the RNA guide, and that of the most suitable selectable markers to be used.

Altogether, the results provided in this thesis demonstrate that the NGTs, especially the genome editing, can be easily used in citrus for the functional characterization of the genes involved in the obtainment of seedless fruits. Once the regulatory mechanisms of these pathway will be elucidated, these new informations, together with the key genes involved, would represent a novel step for cultivar genetic improvement. So, the application of NGTs will allow to overcome the limitation of conventional breeding techniques, leading to the modification into elite genotypes of specific traits, by inducing precise sequence mutation. In this way, a novel variety characterized by a superior agronomical trait like, for example, the absence of seeds, could be easily produced.

On the whole, the achievements of the present work, and especially the definition of the S allele genotype of two clementine varieties, the identification of the self-incompatibility mechanism in the same species, and the obtainment of some edited plantlets for a gene involved in seed development, offer an important contribution towards the possibility of obtaining high quality citrus varieties in which seedlessness can be coupled with other traits of agronomic interest especially fruit quality and stress tolerance.

References

Abouzari, Abouzar, and Nafiseh Mahdi Nezhad. 2016. "The Investigation of Citrus Fruit Quality. Popular Characteristic and Breeding." *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis* 64(3):725–40. doi: 10.11118/actaun201664030725.

von Aderkas, Patrick, and Jan M. Bonga. 2000. "Influencing Micropropagation and Somatic Embryogenesis in Mature Trees by Manipulation of Phase Change, Stress and Culture Environment." *Tree Physiology* 20(14):921–28. doi: 10.1093/treephys/20.14.921.

Agüero, Jesus, María del Carmen Vives, Karelia Velázquez, José Antonio Pina, Luis Navarro, Pedro Moreno, and Jose Guerri. 2014. "Effectiveness of Gene Silencing Induced by Viral Vectors Based on Citrus Leaf Blotch Virus Is Different in Nicotiana Benthamiana and Citrus Plants." *Virology* 460–461(1):154–64. doi: 10.1016/j.virol.2014.04.017.

Aleza, P., J. Juárez, M. Hernández, P. Ollitrault, and L. Navarro. 2012. "Implementation of Extensive Citrus Triploid Breeding Programs Based on 4x × 2x Sexual Hybridisations." *Tree Genetics and Genomes* 8(6):1293–1306. doi: 10.1007/s11295-012-0515-6.

Aleza, Pablo, José Cuenca, José Juárez, José A. Pina, and Luis Navarro. 2010. "'Garbí' Mandarin: A New Late-Maturing Triploid Hybrid." *HortScience* 45(1):139–41. doi: 10.21273/hortsci.45.1.139.

Ali, Shawkat, Abdul Mannan, Mohamed el Oirdi, Abdul Waheed, and Bushra Mirza. 2012. "Agrobacterium-Mediated Transformation of Rough Lemon (Citrus Jambhiri Lush) with Yeast HAL2 Gene." *BMC Research Notes* 5:1–8. doi: 10.1186/1756-0500-5-

285.

Almeida, Weliton A. B., Francisco A. A. Mourão Filho, Lilian E. Pino, Raquel L. Boscariol, Adriana P. M. Rodriguez, and Beatriz M. J. Mendes. 2003. "Genetic Transformation and Plant Recovery from Mature Tissues of Citrus Sinensis L. Osbeck." *Plant Science* 164(2):203–11. doi: 10.1016/S0168-9452(02)00401-6.

Alquezar, Berta, Maria J. Rodrigo, and Lorenzo Zacarías. 2008. "Regulation of Carotenoid Biosynthesis during Fruit Maturation in the Red-Fleshed Orange Mutant Cara Cara." *Phytochemistry* 69(10):1997–2007. doi: 10.1016/j.phytochem.2008.04.020.

de Azevedo, Fernando Alves, Francisco De Assis Alves Mourão Filho, Evandro Henrique Schinor, Luis Gustavo de Paoli, Beatriz Madalena Januzzi Mendes, Ricardo Harakava, Dean W. Gabriel, and Richard F. Lee. 2006. "GUS Gene Expression Driven by a Citrus Promoter in Transgenic Tobacco and 'Valencia' Sweet Orange." *Pesquisa Agropecuaria Brasileira* 41(11):1623–28. doi: 10.1590/S0100-204X2006001100008.

Ballester, A., M. Cervera, and L. Peña. 2007. "Efficient Production of Transgenic Citrus Plants Using Isopentenyltransferase Positive Selection and Removal of the Marker Gene by Site-Specific Recombination." *Plant Cell Reportsl* 738:191–200. doi: 10.17660/ActaHortic.2007.738.17.

Ballester, A., M. Cervera, and L. Peña. 2010. "Selectable Marker-Free Transgenic Orange Plants Recovered under Non-Selective Conditions and through PCR Analysis of All Regenerants." *Plant Cell, Tissue and Organ Culture* 102(3):329–36. doi: 10.1007/s11240-010-9737-1.

Ballester, Alida, Magdalena Cervera, and Leandro Peña. 2008. "Evaluation of Selection Strategies Alternative to NptII in Genetic Transformation of Citrus." *Plant Cell Reports* 27(6):1005–15. doi: 10.1007/s00299-008-0523-z.

Barbosa-Mendes, Janaynna Magalhães, Francisco de Assis Alves

Mourão Filho, Armando Bergamin Filho, Ricardo Harakava, Steven v. Beer, and Beatriz Madalena Januzzi Mendes. 2009. "Genetic Transformation of Citrus Sinensis Cv. Hamlin with HrpN Gene from Erwinia Amylovora and Evaluation of the Transgenic Lines for Resistance to Citrus Canker." *Scientia Horticulturae* 122(1):109–15. doi: 10.1016/j.scienta.2009.04.001.

Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, DA Romero, and Philippe Horvath. 2007. "CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes." *Science* 315(March):1709–12. doi: 10.1126/science.1138140.

Ben-Cheikh, Wadii, Joan Perez-Botella, Francisco R. Tadeo, Manuel Talon, and Eduardo Primo-Millo. 1997. "Pollination Increases Gibberellin Levels in Developing Ovaries of Seeded Varieties of Citrus." *Plant Physiology* 114(2):557–64. doi: 10.1104/pp.114.2.557.

Bennici, Stefania, Gaetano Distefano, Giuseppina las Casas, Mario Di Guardo, Giuseppe Lana, Ettore Pacini, Stefano la Malfa, and Alessandra Gentile. 2005. "Temperature Stress Interferes with Male Reproductive System Development in Clementine (Citrus Clementina Hort. Ex Tan.)." *Acta Radiologica* 0–30.

Bhattacharyya-Pakrasi, Maitrayee, Jianying Peng, J. Scott Elmer, Gary Laco, Ping Shen, Maria B. Kaniewska, Halina Kononowicz, Fujiang Wen, Thomas K. Hodges, and Roger N. Beachy. 1993. "Specificity of a Promoter from the Rice Tungro Bacilliform Virus for Expression in Phloem Tissues." *Plant Journal* 4(1):71–79. doi: 10.1046/j.1365-313X.1993.04010071.x.

Birch, R. G. 1997. "PLANT TRANSFORMATION: Problems and Strategies for Practical Application." *Annual Review of Plant Physiology and Plant Molecular Biology* 48(1):297–326. doi: 10.1146/annurev.arplant.48.1.297.

Biswas, Manosh Kumar, Mita Bagchi, Xiuxin Deng, and Lijun Chai. 2020. "Genetic Resources of Citrus and Related Genera." Pp. 23–31 in *The citrus genome*.

Boman, Hans G. 2003. "Antibacterial Peptides: Basic Facts and Emerging Concepts." *Journal of Internal Medicine* 254(3):197–215. doi: 10.1046/j.1365-2796.2003.01228.x.

Bortesi, Luisa, and Rainer Fischer. 2015. "The CRISPR/Cas9 System for Plant Genome Editing and Beyond." *Biotechnology Advances* 33(1):41–52. doi: 10.1016/j.biotechadv.2014.12.006.

Boscariol, R. L., Weliton A. B. Almeida, M. T. V. C. Derbyshire, F. A. A. Mourão Filho, and B. M. J. Mendes. 2003. "The Use of the PMI/Mannose Selection System to Recover Transgenic Sweet Orange Plants (Citrus Sinensis L. Osbeck)." *Plant Cell Reports* 22(2):122–28. doi: 10.1007/s00299-003-0654-1.

Boscariol, Raquel L., Mariza Monteiro, Elizabete K. Takahashi, Sabrina M. Chabregas, Maria Lucia C. Vieira, Luiz G. E. Vieira, Luiz F. P. Pereira, Francisco De A. A. Mourão Filho, Suane C. Cardoso, Rock S. C. Christiano, Armando Bergamin Filho, Janaynna M. Barbosa, Fernando A. Azevedo, and Beatriz M. J. Mendes. 2006. "Attacin A Gene from Tricloplusia Ni Reduces Susceptibility to Xanthomonas Axonopodis Pv. Citri in Transgenic Citrus Sinesis 'Hamlin.'" *Journal of the American Society for Horticultural Science* 131(4):530–36. doi: 10.21273/jashs.131.4.530.

Boscariol-Camargo, Raquel L., Marco A. Takita, and Marcos A. Machado. 2016. "Bacterial Resistance in AtNPR1 Transgenic Sweet Orange Is Mediated by Priming and Involves EDS1 and PR2." *Tropical Plant Pathology* 41(6):341–49. doi: 10.1007/s40858-016-0108-2.

Boszorádová, Eva, Jana Libantová, Ildikó Matušíková, and Jana Moravčíková. 2014. "Application of Arabidopsis Tissue-Specific CRUC Promoter in the Cre/LoxP Self-Excision

Strategy for Generation of Marker-Free Oilseed Rape: Potential Advantages and Drawbacks." *Acta Physiologiae Plantarum* 36(6):1399–1409. doi: 10.1007/s11738-014-1518-8.

Bové, J. M. 2006. "Huanglongbing: A Destructive, Newly-Emerging, Century-Old Disease of Citrus." *Journal of Plant Pathology* 88(1):7–37. doi: 10.4454/jpp.v88i1.828.

Cardoso, Suane Coutinho, Janaynna Magalhães Barbosa-Mendes, Raquel Luciana Boscariol-Camargo, Rock Seille Carlos Christiano, Armando Bergamin Filho, Maria Lúcia Carneiro Vieira, Beatriz Madalena Januzzi Mendes, and Francisco de Assis Alves Mourão Filho. 2010. "Transgenic Sweet Orange (Citrus Sinensis L. Osbeck) Expressing the Attacin a Gene for Resistance to Xanthomonas Citri Subsp. Citri." *Plant Molecular Biology Reporter* 28(2):185–92. doi: 10.1007/s11105-009-0141-0.

Caruso, Marco, Paz Merelo, Gaetano Distefano, Stefano la Malfa, Angela R. lo Piero, Francisco R. Tadeo, Manuel Talon, and Alessandra Gentile. 2012. "Comparative Transcriptome Analysis of Stylar Canal Cells Identifies Novel Candidate Genes Implicated in the Self-Incompatibility Response of Citrus Clementina." *BMC Plant Biology* 12. doi: 10.1186/1471-2229-12-20.

Caruso, Marco, Malcolm W. Smith, Yann Froelicher, Giuseppe Russo, and Frederick G. Gmitter. 2020. "Traditional Breeding." Pp. 129–48 in *The Genus Citrus*.

Caserta, R., N. S. Teixeira-Silva, L. M. Granato, S. O. Dorta, C. M. Rodrigues, L. K. Mitre, J. T. H. Yochikawa, E. R. Fischer, C. A. Nascimento, R. R. Souza-Neto, M. A. Takita, R. L. Boscariol-Camargo, M. A. Machado, and A. A. de Souza. 2020. "Citrus Biotechnology: What Has Been Done to Improve Disease Resistance in Such an Important Crop?" *Biotechnology Research and Innovation* (January). doi: 10.1016/j.biori.2019.12.004.

Cernadas, Raúl Andrés, Luciana Rodrigues Camillo, and Celso Eduardo Benedetti. 2008. "Transcriptional Analysis of the Sweet Orange Interaction with the Citrus Canker Pathogens Xanthomonas Axonopodis Pv. Citri and Xanthomonas Axonopodis Pv. Aurantifolii." *Molecular Plant Pathology* 9(5):609–31. doi: 10.1111/j.1364-3703.2008.00486.x.

Cervera, M., C. Ortega, A. Navarro, L. Navarro, and L. Peña. 2000. "Generation of Transgenic Citrus Plants with the Tolerance-to-Salinity Gene HAL2 from Yeast." *Journal of Horticultural Science and Biotechnology* 75(1):26–30. doi: 10.1080/14620316.2000.11511195.

Cervera, M., J. A. Pina, J. Juárez, L. Navarro, and L. Peña. 1998. "Agrobacterium-Mediated Transformation of Citrange: Factors Affecting Transformation and Regeneration." *Plant Cell Reports* 18(3–4):271–78. doi: 10.1007/s002990050570.

Cervera, Magdalena, José Juárez, Antonio Navarro, José A. Pina, Nuria Durán-Vila, Luis Navarro, and Leandro Peña. 1998. "Genetic Transformation and Regeneration of Mature Tissues of Woody Fruit Plants Bypassing the Juvenile Stage." *Transgenic Research* 7(1):51–59. doi: 10.1023/A:1008855922283.

Cervera, Magdalena, José Juárez, Luis Navarro, and Leandro Peña. 2005. "Genetic Transformation of Mature Citrus Plants." Pp. 177–88 in *Transgenic Plants. Methods and Protocols*. Vol. 286. Humana Press, Totowa. New Jersey.

Cervera, Magdalena, Antonio Navarro, Luis Navarro, and Leandro Peña. 2008. "Production of Transgenic Adult Plants from Clementine Mandarin by Enhancing Cell Competence for Transformation and Regeneration." *Tree Physiology* 28(1):55–66. doi: 10.1093/treephys/28.1.55.

Cervera, Magdalena, Luis Navarro, and Leandro Peña. 2009. "Gene Stacking in 1-Year-Cycling APETALA1 Citrus Plants for a Rapid Evaluation of Transgenic Traits in Reproductive Tissues."

Journal of Biotechnology 140(3–4):278–82. doi: 10.1016/j.jbiotec.2009.01.024.

Chao, Chih Cheng T. 2005. "Pollination Study of Mandarins and the Effect on Seediness and Fruit Size: Implications for Seedless Mandarin Production." *HortScience* 40(2):362–65. doi: 10.21273/hortsci.40.2.362.

Chao, Chih Cheng T., Jinggui Fang, and Pachanoor S. Devanand. 2005. "Long Distance Pollen Flow in Mandarin Orchards Determined by AFLP Markers - Implications for Seedless Mandarin Production." *Journal of the American Society for Horticultural Science* 130(3):374–80. doi: 10.21273/jashs.130.3.374.

Chavez, Dario J., and José X. Chaparro. 2011. "Identification of Markers Linked to Seedlessness in Citrus Kinokuni Hort. Ex Tanaka and Its Progeny Using Bulked Segregant Analysis." *HortScience* 46(5):693–97. doi: 10.21273/hortsci.46.5.693.

Chen, Xiuhua, Jinyoung Y. Barnaby, Aswathy Sreedharan, Xiaoen Huang, Vladimir Orbović, Jude W. Grosser, Nian Wang, Xinnian Dong, and Wen Yuan Song. 2013. "Over-Expression of the Citrus Gene CtNH1 Confers Resistance to Bacterial Canker Disease." *Physiological and Molecular Plant Pathology* 84(1):115–22. doi: 10.1016/j.pmpp.2013.07.002.

Cheng, Chunzhen, Yongyan Zhang, Jiawei Yang, and Yun Zhong. 2017. "Expression of Hairpin RNA (HpRNA) Targeting the Three CTV-Silencing Suppressor Genes Confers Sweet Orange with Stem-Pitting CTV Tolerance." *Journal of Horticultural Science and Biotechnology* 92(5):465–74. doi: 10.1080/14620316.2017.1304166.

Cheng, G. M., S. J. Huang, S. P. Chen, and J. Y. C. 1997. "Selection of Seedless 'Xuegan.'" *Fujian Fruits* 2(46).

Chhetri, Ashok, B. N. Hazarika, Thejangulie Angami, H. Lembisana Devi, . Lalhmingsanga, Athikho Kayia Alice, and Lakidon

Khonglah. 2019. “Overview and Applications of RNA Silencing in Fruit Crops.” *Current Journal of Applied Science and Technology* 33(5):1–7. doi: 10.9734/cjast/2019/v33i530094.

Chong-Pérez, Borys, Rafael G. Kosky, Maritza Reyes, Luis Rojas, Bárbara Ocaña, Marisol Tejeda, Blanca Pérez, and Geert Angenon. 2012. “Heat Shock Induced Excision of Selectable Marker Genes in Transgenic Banana by the Cre-Lox Site-Specific Recombination System.” *Journal of Biotechnology* 159(4):265–73. doi: 10.1016/j.jbiotec.2011.07.031.

Coletta-filho, Helvecio D., Matthew P. Daugherty, Cléderson Ferreira, and João R. S. Lopes. 2013. “Temporal Progression of ‘Candidatus Liberibacter Asiaticus’ Infection in Citrus and Acquisition Efficiency by Diaphorina Citri Helvecio.” (25):416–21.

Concordet, Jean Paul, and Maximilian Haeussler. 2018. “CRISPOR: Intuitive Guide Selection for CRISPR/Cas9 Genome Editing Experiments and Screens.” *Nucleic Acids Research* 46(W1):W242–45. doi: 10.1093/nar/gky354.

Cuellar, Wilmer, Amélie Gaudin, Dennis Solórzano, Armando Casas, Luis Ñopo, Prakash Chudalayandi, Giuliana Medrano, Jan Kreuze, and Marc Ghislain. 2006. “Self-Excision of the Antibiotic Resistance Gene NptII Using a Heat Inducible Cre-LoxP System from Transgenic Potato.” *Plant Molecular Biology* 62(1–2):71–82. doi: 10.1007/s11103-006-9004-3.

Cuenca, José, Andrés Garcia-Lor, Luis Navarro, and Pablo Aleza. 2018. “Citrus Genetics and Breeding.” Pp. 403–36 in *Advances in Plant Breeding Strategies: Fruits*. Vol. 3.

Dale, E. C., and D. W. Ow. 1991. “Gene Transfer with Subsequent Removal of the Selection Gene from the Host Genome.” *Proceedings of the National Academy of Sciences of the United States of America* 88(23):10558–62. doi: 10.1073/pnas.88.23.10558.

Dalla Costa, Lorenza, Stefano Piazza, Manuela Campa, Henryk Flachowsky, Magda Viola Hanke, and Mickael Malnoy. 2016. "Efficient Heat-Shock Removal of the Selectable Marker Gene in Genetically Modified Grapevine." *Plant Cell, Tissue and Organ Culture* 124(3):471–81. doi: 10.1007/s11240-015-0907-z.

Di Guardo, M., M. Moretto, M. Moser, C. Catalano, M. Troggio, Z. Deng, A. Cestaro, M. Caruso, G. Distefano, S. la Malfa, L. Bianco, and A. Gentile. 2021. "The Haplotype-Resolved Reference Genome of Lemon (Citrus Limon L. Burm f.)." *Tree Genetics and Genomes* 17(6). doi: 10.1007/s11295-021-01528-5

Distefano, Gaetano, Marco Caruso, Stefano la Malfa, Alessandra Gentile, and Eugenio Tribulato. 2009. "Histological and Molecular Analysis of Pollen-Pistil Interaction in Clementine." *Plant Cell Reports* 28(9):1439–51. doi: 10.1007/s00299-009-0744-9.

Distefano, Gaetano, Marco Caruso, Stefano La Malfa, Alessandra Gentile, and Eugenio Tribulato. 2009. "Histological and Molecular Analysis of Pollen–Pistil Interaction in Clementine." *Plant Cell Reports* 28:1439–51. doi: 10.1007/s00299-009-0744-9.

Distefano, Gaetano, Marco Cirilli, Giuseppina las Casas, Stefano la Malfa, Alberto Continella, Eddo Rugini, Brian Thomas, Guiyou Long, Alessandra Gentile, and Rosario Muleo. 2013. "Ectopic Expression of Arabidopsis Phytochrome B in Troyer Citrange Affects Photosynthesis and Plant Morphology." *Scientia Horticulturae* 159:1–7. doi: 10.1016/j.scienta.2013.04.022.

Distefano, Gaetano, Afif Hedhly, Giuseppina las Casas, Stefano la Malfa, Maria Herrero, and Alessandra Gentile. 2012. "Male-Female Interaction and Temperature Variation Affect Pollen Performance in Citrus." *Scientia Horticulturae* 140:1–7. doi:

10.1016/j.scienta.2012.03.011.

Distefano, Gaetano, Stefano la Malfa, Alessandro Vitale, Matteo Lorito, Ziniu Deng, and Alessandra Gentile. 2008. "Defence-Related Gene Expression in Transgenic Lemon Plants Producing an Antimicrobial Trichoderma Harzianum Endochitinase during Fungal Infection." *Transgenic Research* 17(5):873–79. doi: 10.1007/s11248-008-9172-9.

Dobin, Alexander, Carrie A. Davis, Felix Schlesinger, Jorg Drenkow, Chris Zaleski, Sonali Jha, Philippe Batut, Mark Chaisson, and Thomas R. Gingeras. 2013. "STAR: Ultrafast Universal RNA-Seq Aligner." *Bioinformatics* 29(1):15–21. doi: 10.1093/bioinformatics/bts635.

Domínguez, A., C. Fagoaga, L. Navarro, P. Moreno, and L. Peña. 2002. "Regeneration of Transgenic Citrus Plants under Non Selective Conditions Results in High-Frequency Recovery of Plants with Silenced Transgenes." *Molecular Genetics and Genomics* 267(4):544–56. doi: 10.1007/s00438-002-0688-z.

Domínguez, Antonio, Magdalena Cervera, Rosa M. Pérez, Juan Romero, Carmen Fagoaga, Jaime Cubero, María M. López, José A. Juárez, Luis Navarro, and Leandro Peña. 2004. "Characterisation of Regenerants Obtained under Selective Conditions after Agrobacterium-Mediated Transformation of Citrus Explants Reveals Production of Silenced and Chimeric Plants at Unexpected High Frequencies." *Molecular Breeding* 14(2):171–83. doi: 10.1023/B:MOLB.0000038005.73265.61.

Domínguez, Antonio, Alfonso Hermoso de Mendoza, José Guerri, Mariano Cambra, Luis Navarro, Pedro Moreno, and Leandro Peña. 2002. "Pathogen-Derived Resistance to Citrus Tristeza Virus (CTV) in Transgenic Mexican Lime (Citrus Arrurantifolia (Christ.) Swing.) Plants Expressing Its P25 Coat Protein Gene." *Molecular Breeding* 10(1–2):1–10. doi: 10.1023/A:1020347415333.

Doyle, J. J., and J. L. Doyle. 1987. "A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue." *PHYTOCHEMICAL BULLETIN* v.19(1):11–15.

Duan, Yan Xin, Jing Fan, and Wen Wu Guo. 2010. "Regeneration and Characterization of Transgenic Kumquat Plants Containing the Arabidopsis APETALA1 Gene." *Plant Cell, Tissue and Organ Culture* 100(3):273–81. doi: 10.1007/s11240-009-9646-3.

Duran-Vila, N., Y. Gogorcena, V. Ortega, J. Ortiz, and L. Navarro. 1992. "Morphogenesis and Tissue Culture of Sweet Orange (Citrus Sinensis (L.) Osb.): Effect of Temperature and Photosynthetic Radiation." *Plant Cell, Tissue and Organ Culture* 29(1):11–18. doi: 10.1007/BF00036140.

Duran-Vila N., Ortega V. &. Navarro L. 1989. "Morphogenesis and Tissue Cultures of Three Citrus Species." 133:123–33.

Dutt, M., G. Ananthakrishnan, M. K. Jaromin, R. H. Brlansky, and J. W. Grosser. 2012. "Evaluation of Four Phloem-Specific Promoters in Vegetative Tissues of Transgenic Citrus Plants." *Tree Physiology* 32(1):83–93. doi: 10.1093/treephys/tpr130.

Dutt, M., and J. W. Grosser. 2009. "Evaluation of Parameters Affecting Agrobacterium-Mediated Transformation of Citrus." *Plant Cell, Tissue and Organ Culture* 98(3):331–40. doi: 10.1007/s11240-009-9567-1.

Dutt, M., and J. W. Grosser. 2010. "An Embryogenic Suspension Cell Culture System for Agrobacterium-Mediated Transformation of Citrus." *Plant Cell Reports* 29(11):1251–60. doi: 10.1007/s00299-010-0910-0.

Dutt, M., M. Vasconcellos, and J. W. Grosser. 2011. "Effects of Antioxidants on Agrobacterium-Mediated Transformation and Accelerated Production of Transgenic Plants of Mexican Lime (Citrus Aurantifolia Swingle)." *Plant Cell, Tissue and Organ Culture* 107(1):79–89. doi: 10.1007/s11240-011-9959-x.

Dutt, M., M. Vasconcellos, K. J. Song, F. G. Gmitter, and J. W.

Grosser. 2010. "*In Vitro* Production of Autotetraploid Ponkan Mandarin (Citrus Reticulata Blanco) Using Cell Suspension Cultures." *Euphytica* 173(2):235–42. doi: 10.1007/s10681-009-0098-y.

Dutt, Manjul, Gary Barthe, Michael Irey, and Jude Grosser. 2015. "Transgenic Citrus Expressing an Arabidopsis NPR1 Gene Exhibit Enhanced Resistance against Huanglongbing (HLB; Citrus Greening)." *PLoS ONE* 10(9):1–17. doi: 10.1371/journal.pone.0137134.

Dutt, Manjul, Sadanand A. Dhekney, Leonardo Soriano, Raju Kandel, and Jude W. Grosser. 2014. "Temporal and Spatial Control of Gene Expression in Horticultural Crops." *Horticulture Research* 1(June):1–17. doi: 10.1038/hortres.2014.47.

Dutt, Manjul, Lígia Erpen, and Jude W. Grosser. 2018. "Genetic Transformation of the 'W Murcott' Tangor: Comparison between Different Techniques." *Scientia Horticulturae* 242(July):90–94. doi: 10.1016/j.scienta.2018.07.026.

Dutt, Manjul, Lamiaa M. Mahmoud, Yasser Nehela, Jude W. Grosser, and Nabil Killiny. 2022. "The Citrus Sinensis TILLER ANGLE CONTROL 1 (CsTAC1) Gene Regulates Tree Architecture in Sweet Oranges by Modulating the Endogenous Hormone Content." *Plant Science* 323(July):111401. doi: 10.1016/j.plantsci.2022.111401.

Dutt, Manjul, Zhonglin Mou, Xudong Zhang, Sameena E. Tanwir, and Jude W. Grosser. 2020. "Efficient CRISPR/Cas9 Genome Editing with Citrus Embryogenic Cell Cultures." *BMC Biotechnology* 20(1):1–7. doi: 10.1186/s12896-020-00652-9.

Dutt, Manjul, Daniel Stanton, and Jude W. Grosser. 2016. "Ornacitrus: Development of Genetically Modified Anthocyanin-Expressing Citrus with Both Ornamental and Fresh Fruit Potential." *Journal of the American Society for Horticultural Science* 141(1):54–61. doi:

10.21273/jashs.141.1.54.

Ebinuma, Hiroyasu, Koichi Sugita, Etsuko Matsunaga, and Mikiko Yamakado. 1997. “Selection of Marker-Free Transgenic Plants Using the Isopentenyl Transferase Gene.” *Proceedings of the National Academy of Sciences of the United States of America* 94(6):2117–21. doi: 10.1073/pnas.94.6.2117.

EFSA. 2012. *Scientific Opinion Addressing the Safety Assessment of Plants Developed Using Zinc Finger Nuclease 3 and Other Site-Directed Nucleases with Similar Function*. Vol. 10. doi: 10.2903/j.efsa.2012.2943.

EFSA. 2020. *Outcome of the Public Consultation on the Draft Scientific Opinion on the Applicability of the EFSA Opinion on Site-directed Nucleases Type 3 for the Safety Assessment of Plants Developed Using Site-directed Nucleases Type 1 and 2 and Oligonucleotide-dire*. Vol. 17. doi: 10.2903/sp.efsa.2020.en-1972.

EFSA. 2022a. *Criteria for Risk Assessment of Plants Produced by Targeted Mutagenesis , Cisgenesis and Intragenesis*. Vol. 20. doi: 10.2903/j.efsa.2022.7618.

EFSA. 2022b. *Evaluation of Existing Guidelines for Their Adequacy for the Food and Feed Risk Assessment of Genetically Modified Plants Obtained through Synthetic Biology*. Vol. 20. doi: 10.2903/j.efsa.2022.7410.

EFSA. 2022c. *Updated Scientific Opinion on Plants Developed through Cisgenesis and Intragenesis*. Vol. 20. doi: 10.2903/j.efsa.2022.7621.

Endo, Akira, Mikami Masafumi, Hidetaka Kaya, and Seiichi Toki. 2016. “Efficient Targeted Mutagenesis of Rice and Tobacco Genomes Using Cpf1 from Francisella Novicida.” *Scientific Reports* 6(September):1–9. doi: 10.1038/srep38169.

Endo, S., T. Kasahara, K. Sugita, and H. Ebinuma. 2002. “A New GST-MAT Vector Containing Both Ipt and IaaM/H Genes Can

Produce Marker-Free Transgenic Tobacco Plants with High Frequency." *Plant Cell Reports* 20(10):923–28. doi: 10.1007/s00299-001-0418-8.

Endo, Tomoko, Takehiko Shimada, Hiroshi Fujii, Yasushi Kobayashi, Takashi Araki, and Mitsuo Omura. 2005. "Ectopic Expression of an FT Homolog from Citrus Confers an Early Flowering Phenotype on Trifoliate Orange (Poncirus Trifoliata L. Raf.)." *Transgenic Research* 14(5):703–12. doi: 10.1007/s11248-005-6632-3.

Endo, Tomoko, Takehiko Shimada, Hiroshi Fujii, Takaya Moriguchi, and Mitsuo Omura. 2007. "Promoter Analysis of a Type 3 Metallothionein-like Gene Abundant in Satsuma Mandarin (Citrus Unshiu Marc.) Fruit." *Scientia Horticulturae* 112(2):207–14. doi: 10.1016/j.scienta.2006.12.042.

Enrique, Ramón, Florencia Siciliano, María Alejandra Favaro, Nadia Gerhardt, Roxana Roeschlin, Luciano Rigano, Lorena Sendin, Atilio Castagnaro, Adrian Vojnov, and María Rosa Marano. 2011. "Novel Demonstration of RNAi in Citrus Reveals Importance of Citrus Callose Synthase in Defence against Xanthomonas Citri Subsp. Citri." *Plant Biotechnology Journal* 9(3):394–407. doi: 10.1111/j.1467-7652.2010.00555.x.

Eriksson, Dennis, René Custers, Karin Edvardsson Björnberg, Sven Ove Hansson, Kai Purnhagen, Matin Qaim, Jörg Romeis, Joachim Schiemann, Stephan Schleissing, Jale Tosun, and Richard G. F. Visser. 2020. "Options to Reform the European Union Legislation on GMOs: Scope and Definitions." *Trends in Biotechnology* 38(3):231–34. doi: 10.1016/j.tibtech.2019.12.002.

Eriksson, Dennis, Wendy Harwood, Per Hofvander, Huw Jones, Peter Rogowsky, Eva Stöger, and Richard G. F. Visser. 2018a. "A Welcome Proposal to Amend the GMO Legislation of the EU." *Trends in Biotechnology* 36(11):1100–1103. doi:

10.1016/j.tibtech.2018.05.001.

Eriksson, Dennis, Wendy Harwood, Per Hofvander, Huw Jones, Peter Rogowsky, Eva Stöger, and Richard G. F. Visser. 2018b. "A Welcome Proposal to Amend the GMO Legislation of the EU." *Trends in Biotechnology* 36(11):1100–1103. doi: 10.1016/j.tibtech.2018.05.001.

Eriksson, Dennis, Drew Kershen, Alexandre Nepomuceno, Barry J. Pogson, Humberto Prieto, Kai Purnhagen, Stuart Smyth, Justus Wesseler, and Agustina Whelan. 2019. "A Comparison of the EU Regulatory Approach to Directed Mutagenesis with That of Other Jurisdictions, Consequences for International Trade and Potential Steps Forward." *New Phytologist* 222(4):1673–84. doi: 10.1111/nph.15627.

Erpen Dalla Corte, L., B. M. .. J. Mendes, F. A. A. Mourão Filho, J. W. Grosser, and M. Dutt. 2020. "Functional Characterization of Full-Length and 5′ Deletion Fragments of Citrus Sinensis-Derived Constitutive Promoters in Nicotiana Benthamiana." *In Vitro Cellular and Developmental Biology - Plant*. doi: 10.1007/s11627-019-10044-0.

Erpen, L., E. C. R. Tavano, R. Harakava, M. Dutt, J. W. Grosser, S. M. S. Piedade, B. M. J. Mendes, and F. A. A. Mourão Filho. 2018. "Isolation, Characterization, and Evaluation of Three Citrus Sinensis-Derived Constitutive Gene Promoters." *Plant Cell Reports* 37(8):1113–25. doi: 10.1007/s00299-018-2298-1.

Esmaeilnia, Ehsan, and Ali Dehestani. 2015. "*In Vitro* Plant Regeneration from Mature Tissues of Thomson Navel Sweet Orange (Citrus Sinensis L. Osbeck.)." *Biharean Biologist* 9(1):9–14.

European Commission. 2021. *Study on the Status of New Genomic Techniques under Union Law and in Light of the Court of Justice Ruling in Case C-528/16*. Vol. SWD(2021).

van Ex, Frédéric, Dimitri Verweire, Martine Claeys, Ann Depicker,

and Geert Angenon. 2009. “Evaluation of Seven Promoters to Achieve Germline Directed Cre-Lox Recombination in Arabidopsis Thaliana.” *Plant Cell Reports* 28(10):1509–20. doi: 10.1007/s00299-009-0750-y.

Fagoaga, Carmen, Carmelo López, Alfonso Hermoso de Mendoza, Pedro Moreno, Luis Navarro, Ricardo Flores, and Leandro Peña. 2006. “Post-Transcriptional Gene Silencing of the P23 Silencing Suppressor of Citrus Tristeza Virus Confers Resistance to the Virus in Transgenic Mexican Lime.” *Plant Molecular Biology* 60(2):153–65. doi: 10.1007/s11103-005-3129-7.

Fagoaga, Carmen, Francisco R. Tadeo, Domingo J. Iglesias, Laura Huerta, Ignacio Lliso, Ana M. Vidal, Manuel Talon, Luís Navarro, José L. García-Martínez, and Leandro Peña. 2007a. “Engineering of Gibberellin Levels in Citrus by Sense and Antisense Overexpression of a GA 20-Oxidase Gene Modifies Plant Architecture.” *Journal of Experimental Botany* 58(6):1407–20. doi: 10.1093/jxb/erm004.

Fagoaga, Carmen, Francisco R. Tadeo, Domingo J. Iglesias, Laura Huerta, Ignacio Lliso, Ana M. Vidal, Manuel Talon, Luís Navarro, José L. García-Martínez, and Leandro Peña. 2007b. “Engineering of Gibberellin Levels in Citrus by Sense and Antisense Overexpression of a GA 20-Oxidase Gene Modifies Plant Architecture.” *Journal of Experimental Botany* 58(6):1407–20. doi: 10.1093/jxb/erm004.

FAO, Food and Agriculture Organization of the United Nations. 2021. *Citrus Fruits Fresh and Processed - Statistical Bulletin 2020*. Rome. doi: 10.12987/9780300242546-011.

FAOSTAT. 2018. “FAOSTAT Database Results.Pdf.” *FAOSTAT Database Result 2018*.

Fernández i Martí, Angel, Thomas M. Gradziel, and Rafel Socias i Company. 2014. “Methylation of the Sf Locus in Almond Is Associated with S-RNase Loss of Function.” *Plant Molecular*

Biology 86(6):681–89. doi: 10.1007/s11103-014-0258-x.

Fernández i Martí, Àngel, Toshio Hanada, José M. Alonso, Hisayo Yamane, Ryutaro Tao, and Rafel Socias i Company. 2010. "The Almond Sf Haplotype Shows a Double Expression despite Its Comprehensive Genetic Identity." *Scientia Horticulturae* 125(4):685–91. doi: 10.1016/j.scienta.2010.05.024.

Fidel, Lena, Mira Carmeli-Weissberg, Yosef Yaniv, Felix Shaya, Nir Dai, Eran Raveh, Yoram Eyal, Ron Porat, and Nir Carmi. 2016. "Breeding and Analysis of Two New Grapefruit-Like Varieties with Low Furanocoumarin Content." *Food and Nutrition Sciences* 07(02):90–101. doi: 10.4236/fns.2016.72011.

Figueiredo, Jose F. L., Patrick Römer, Thomas Lahaye, James H. Graham, Frank F. White, and Jeffrey B. Jones. 2011. "Agrobacterium-Mediated Transient Expression in Citrus Leaves: A Rapid Tool for Gene Expression and Functional Gene Assay." *Plant Cell Reports* 30(7):1339–45. doi: 10.1007/s00299-011-1045-7.

Flor, H. H. 1971. "Current Status of the Gene-for-Gene Concept." *Annual Review of Phytopathology* 9:275–96.

Furman, Nicolás, Ken Kobayashi, Maria Cecilia Zanek, Javier Calcagno, Maria Laura García, and Alejandro Mentaberry. 2013. "Transgenic Sweet Orange Plants Expressing a Dermaseptin Coding Sequence Show Reduced Symptoms of Citrus Canker Disease." *Journal of Biotechnology* 167(4):412–19. doi: 10.1016/j.jbiotec.2013.07.019.

Gambetta, Giuliana, Alfredo Gravina, Carolina Fasiolo, Cecilia Fornero, Sebastián Galiger, Cristian Inzaurralde, and Florencia Rey. 2013. "Self-Incompatibility, Parthenocarpy and Reduction of Seed Presence in 'Afourer' Mandarin." *Scientia Horticulturae* 164:183–88. doi: 10.1016/j.scienta.2013.09.002.

Garcia, Damien, Jonathan N. Fitz Gerald, and Frédéric Berger. 2005. "Maternal Control of Integument Cell Elongation and Zygotic

Control of Endosperm Growth Are Coordinated to Determine Seed Size in Arabidopsis." *Plant Cell* 17(1):52–60. doi: 10.1105/tpc.104.027136.

Garcia, Damien, Virginie Saingery, Pierre Chambrier, Ulrike Mayer, Gerd Jürgens, and Frédéric Berger. 2003. "Arabidopsis Haiku Mutants Reveal New Controls of Seed Size by Endosperm." *Plant Physiology* 131(4):1661–70. doi: 10.1104/pp.102.018762.

Ge, Steven Xijin, Dongmin Jung, Dongmin Jung, and Runan Yao. 2020. "ShinyGO: A Graphical Gene-Set Enrichment Tool for Animals and Plants." *Bioinformatics* 36(8):2628–29. doi: 10.1093/bioinformatics/btz931.

Gentile, A., Z. Deng, S. la Malfa, G. Distefano, F. Domina, A. Vitale, G. Polizzi, M. Lorito, and E. Tribulato. 2007. "Enhanced Resistance to Phoma Tracheiphila and Botrytis Cinerea in Transgenic Lemon Plants Expressing a Trichoderma Harzianum Chitinase Gene." *Plant Breeding* 126(2):146–51. doi: 10.1111/j.1439-0523.2007.01297.x.

Gentile, A., Z. N. Deng, S. la Malfa, F. Domina, C. Germanà, and E. Tribulato. 2004. "Morphological and Physiological Effects of RolABC Genes into Citrus Genome." *Acta Horticulturae* 632:235–42. doi: 10.17660/ActaHortic.2004.632.29.

Gentile, Alessandra, Ziniu Deng, Stefano la Malfa, Fausta Domina, C. Germanà, and Eugenio Tribulato. 2004. "Morphological and Physiological Effects of RolABC Genes into Citrus Genome." *Acta Horticulturae* 632:235–42.

Ghorbel, R., A. Dominguez, L. Navarro, and L. Pena. 2000. "High Efficiency Genetic Transformation of Sour Orange (Citrus Aurantium) and Production of Transgenic Trees Containing the Coat Protein Gene of Citrus Tristeza Virus." *Tree Physiology* 20(17):1183–89. doi: 10.1093/treephys/20.17.1183.

Ghorbel, R., J. Juárez, L. Navarro, and L. Peña. 1999. "Green Fluorescent Protein as a Screenable Marker to Increase the

Efficiency of Generating Transgenic Woody Fruit Plants." *Theoretical and Applied Genetics* 99(1–2):350–58. doi: 10.1007/s001220051244.

Ghorbel, Riadh, Stefano la Malfa, Maria M. López, Annik Petit, Luis Navarro, and Leandro Peña. 2001. "Additional Copies of VirG from PTiBo542 Provide a Super-Transformation Ability to Agrobacterium Tumefaciens in Citrus." *Physiological and Molecular Plant Pathology* 58(3):103–10. doi: 10.1006/pmpp.2000.0318.

Gill, Komaljeet, Pankaj Kumar, Anshul Kumar, Bhuvnesh Kapoor, Rajnish Sharma, and Ajay Kumar Joshi. 2022. "Comprehensive Mechanistic Insights into the Citrus Genetics, Breeding Challenges, Biotechnological Implications, and Omics-Based Interventions." *Tree Genetics and Genomes* 18(2). doi: 10.1007/s11295-022-01544-z.

Gong, Xiao Qing, and Ji Hong Liu. 2013. "Genetic Transformation and Genes for Resistance to Abiotic and Biotic Stresses in Citrus and Its Related Genera." *Plant Cell, Tissue and Organ Culture* 113(2):137–47. doi: 10.1007/s11240-012-0267-x.

Gottwald, Tim R., Renato B. Bassanezi, and São Paulo. 2007. "Citrus Huanglongbing: The Pathogen and Its Impact Plant Health Progress Plant Health Progress." *Plant Health Progress* (September):36. doi: 10.1094/PHP-2007-0906-01-RV.Introduction.

Grosser, J. W., P. Ollitrault, and O. Olivares-Fuster. 2000. "Invited Review: Somatic Hybridization in Citrus: An Effective Tool to Facilitate Variety Improvement." *In Vitro Cellular and Developmental Biology - Plant* 36(6):434–49. doi: 10.1007/s11627-000-0080-9.

Grosser, Jude W., and Fred G. Gmitter. 2011. "Protoplast Fusion for Production of Tetraploids and Triploids: Applications for Scion and Rootstock Breeding in Citrus." *Plant Cell, Tissue and Organ*

Culture 104(3):343–57. doi: 10.1007/s11240-010-9823-4.

Guo, Wenwu, Yanxin Duan, Oscar Olivares-Fuster, Zhencai Wu, Covadonga R. Arias, Jacqueline K. Burns, and Jude W. Grosser. 2005. "Protoplast Transformation and Regeneration of Transgenic Valencia Sweet Orange Plants Containing a Juice Quality-Related Pectin Methylesterase Gene." *Plant Cell Reports* 24(8):482–86. doi: 10.1007/s00299-005-0952-x.

Hamzeh, Shiva, Mostafa Motallebi, Mohammad Reza Zamani, and Zahra Moghaddassi Jahromi. 2015. "Selectable Marker Gene Removal and Expression of Transgene by Inducible Promoter Containing FFDD Cis-Acting Elements in Transgenic Plants." *Iranian Journal of Biotechnology* 13(3):1–9. doi: 10.15171/ijb.1099.

Hao, Guixia, Marco Pitino, Yongping Duan, and Ed Stover. 2016. "Reduced Susceptibility to Xanthomonas Citri in Transgenic Citrus Expressing the FLS2 Receptor from Nicotiana Benthamiana." *Molecular Plant-Microbe Interactions* 29(2):132–42. doi: 10.1094/MPMI-09-15-0211-R.

Hao, Guixia, Ed Stover, and Goutam Gupta. 2016. "Overexpression of a Modified Plant Thionin Enhances Disease Resistance to Citrus Canker and Huanglongbing (HLB)." *Frontiers in Plant Science* 7(JULY2016):1–11. doi: 10.3389/fpls.2016.01078.

He, Yongrui, Shanchun Chen, Aihong Peng, Xiuping Zou, Lanzheng Xu, Tiangang Lei, Xiaofeng Liu, and Lixiao Yao. 2011a. "Production and Evaluation of Transgenic Sweet Orange (Citrus Sinensis Osbeck) Containing Bivalent Antibacterial Peptide Genes (Shiva A and Cecropin B) via a Novel Agrobacterium-Mediated Transformation of Mature Axillary Buds." *Scientia Horticulturae* 128(2):99–107. doi: 10.1016/j.scienta.2011.01.002.

He, Yongrui, Shanchun Chen, Aihong Peng, Xiuping Zou, Lanzheng Xu, Tiangang Lei, Xiaofeng Liu, and Lixiao Yao. 2011b.

"Production and Evaluation of Transgenic Sweet Orange (Citrus Sinensis Osbeck) Containing Bivalent Antibacterial Peptide Genes (Shiva A and Cecropin B) via a Novel Agrobacterium-Mediated Transformation of Mature Axillary Buds." *Scientia Horticulturae* 128(2):99–107. doi: 10.1016/j.scienta.2011.01.002.

Hijaz, Faraj, Yasser Nehela, Shelley E. Jones, Manjul Dutt, Jude W. Grosser, John A. Manthey, and Nabil Killiny. 2018. "Metabolically Engineered Anthocyanin-Producing Lime Provides Additional Nutritional Value and Antioxidant Potential to Juice." *Plant Biotechnology Reports* 12(5):329–46. doi: 10.1007/s11816-018-0497-4.

Hong, Y., Y. R. He, S. C. Chen, and et al. 2000. "New Gene Transformation Technique Forcommercial Citrus Cultivars. Chinese J. Trop. Crops 2, 37–41." *Chinese J. Trop. Crops* 15(3–2):37–41.

Honsho, Chitose, Koichiro Ushijima, Misa Anraku, Shuji Ishimura, Qibin Yu, Frederick G. Gmitter, and Takuya Tetsumura. 2021. "Association of T2/S-RNase With Self-Incompatibility of Japanese Citrus Accessions Examined by Transcriptomic, Phylogenetic, and Genetic Approaches." *Frontiers in Plant Science* 12(February):1–17. doi: 10.3389/fpls.2021.638321.

Hu, Jianbing, Qiang Xu, Chenchen Liu, Binghao Liu, Chongling Deng, Chuanwu Chen, Zhuangmin Wei, Muhammad Husnain Ahmad, Kang Peng, Hao Wen, Xiangling Chen, Peng Chen, Robert M. Larkin, Junli Ye, Xiuxin Deng, and Lijun Chai. 2021. "Downregulated Expression of S 2 -RNase Attenuates Self-Incompatibility in 'Guiyou No. 1' Pummelo." *Horticulture Research* 8(1). doi: 10.1038/s41438-021-00634-8.

Hu, Xixun, Chun Wang, Qing Liu, Yaping Fu, and Kejian Wang. 2017. "Targeted Mutagenesis in Rice Using CRISPR-Cpf1 System." *Journal of Genetics and Genomics* 44(1):71–73. doi:

10.1016/j.jgg.2016.12.001.

Hu, Yang, Junli Zhang, Hongge Jia, Davide Sosso, Ting Li, Wolf B. Frommer, Bing Yang, Frank F. White, Nian Wang, and Jeffrey B. Jones. 2014. "Lateral Organ Boundaries 1 Is a Disease Susceptibility Gene for Citrus Bacterial Canker Disease." *Proceedings of the National Academy of Sciences of the United States of America* 111(4). doi: 10.1073/pnas.1313271111.

Igic, Boris, and Joshua R. Kohn. 2001. "Evolutionary Relationships among Self-Incompatibility RNases." *Proceedings of the National Academy of Sciences of the United States of America* 98(23):13167–71. doi: 10.1073/pnas.231386798.

Iglesias, Domingo J., Manuel Cercós, José M. Colmenero-Flores, Miguel A. Naranjo, Gabino Ríos, Esther Carrera, Omar Ruiz-Rivero, Ignacio Lliso, Raphael Morillon, Francisco R. Tadeo, and Manuel Talon. 2007. "Physiology of Citrus Fruiting." *Brazilian Journal of Plant Physiology* 19(4):333–62. doi: 10.1590/S1677-04202007000400006.

Ignatiadis, Nikolaos, Bernd Klaus, Judith B. Zaugg, and Wolfgang Huber. 2016. "Data-Driven Hypothesis Weighting Increases Detection Power in Genome-Scale Multiple Testing." *Nature Methods* 13(7):577–80. doi: 10.1038/nmeth.3885.

Jia, Hongge, Vladimir Orbovi, and Nian Wang. 2019. "CRISPR-LbCas12a-Mediated Modification of Citrus." *Plant Biotechnology Journal* 17:1928–37. doi: 10.1111/pbi.13109.

Jia, Hongge, Vladimir Orbovic, Jeffrey B. Jones, and Nian Wang. 2016. "Modification of the PthA4 Effector Binding Elements in Type I CsLOB1 Promoter Using Cas9/SgRNA to Produce Transgenic Duncan Grapefruit Alleviating XccΔpthA4: DCsLOB1.3 Infection." *Plant Biotechnology Journal* 14(5):1291–1301. doi: 10.1111/pbi.12495.

Jia, Hongge, and Nian Wang. 2014a. "Targeted Genome Editing of Sweet Orange Using Cas9/SgRNA." *PLoS ONE* 9(4). doi:

10.1371/journal.pone.0093806.

Jia, Hongge, and Nian Wang. 2014b. "Xcc-Facilitated Agroinfiltration of Citrus Leaves: A Tool for Rapid Functional Analysis of Transgenes in Citrus Leaves." *Plant Cell Reports* 33(12):1993–2001. doi: 10.1007/s00299-014-1673-9.

Jia, Hongge, and Nian Wang. 2020a. "Generation of Homozygous Canker-resistant Citrus in the T0 Generation Using CRISPR-SpCas9p." *Plant Biotechnology Journal* 1–3. doi: 10.1111/pbi.13375.

Jia, Hongge, and Nian Wang. 2020b. "Generation of Homozygous Canker-Resistant Citrus in the T0 Generation Using CRISPR-SpCas9p." *Plant Biotechnology Journal* 18(10):1990–92. doi: 10.1111/pbi.13375.

Jia, Hongge, Yuanchun Wang, Hang Su, Xiaoen Huan, and Nian Wang. 2022. "LbCas12a-D156R Efficiently Edits LOB1 Effector Binding Elements to Generate Canker-Resistant Citrus Plants." *Cells* 11(3). doi: 10.3390/cells11030315.

Jia, Hongge, Jin Xu, Vladimir Orbović, Yunzeng Zhang, and Nian Wang. 2017. "Editing Citrus Genome via SaCas9/SgRNA System." *Frontiers in Plant Science* 8(December):1–9. doi: 10.3389/fpls.2017.02135.

Jia, Hongge, Yunzeng Zhang, Vladimir Orbović, Jin Xu, Frank F. White, Jeffrey B. Jones, and Nian Wang. 2017. "Genome Editing of the Disease Susceptibility Gene CsLOB1 in Citrus Confers Resistance to Citrus Canker." *Plant Biotechnology Journal* 15(7):817–23. doi: 10.1111/pbi.12677.

Jia, Hongge, Xiuping Zou, Vladimir Orbovic, and Nian Wang. 2019. "Genome Editing in Citrus Tree with CRISPR/Cas9." Pp. 235–41 in *Qi Y. (eds) Plant Genome Editing with CRISPR Systems. Methods in Molecular Biology*. Vol. 1917. Humana Press, New York, NY.

Jinek, Martin, Krzysztof Chylinski, Ines Fonfara, Michael Hauer,

Jennifer A. Doudna, and Emmanuelle Charpentier. 2012. “A Programmable Dual-RNA – Guided.” 337(August):816–22.

Joersbo, Morten, Lain Donaldson, Jette Kreiberg, Steen Guldager Petersen, Janne Brunstedt, and Finn T. Okkels. 1998. “Analysis of Mannose Selection Used for Transformation of Sugar Beet.” *Molecular Breeding* 4(2):111–17. doi: 10.1023/A:1009633809610.

Joldersma, Dirk, and Zhongchi Liu. 2018. “The Making of Virgin Fruit: The Molecular and Genetic Basis of Parthenocarpy.” *Journal of Experimental Botany* 69(5):955–62. doi: 10.1093/jxb/erx446.

Kawata, Yasushi, Fumio Sakiyama, Fumiaki Hayashi, and Yoshimasa Kyogoku. 1990. “Identification of Two Essential Histidine Residues of Ribonuclease T2 from Aspergillus Oryzae.” *European Journal of Biochemistry* 187(1):255–62. doi: 10.1111/j.1432-1033.1990.tb15303.x.

Khan, Ehsan Ullah, Xing Zheng Fu, and Ji Hong Liu. 2012. “Agrobacterium-Mediated Genetic Transformation and Regeneration of Transgenic Plants Using Leaf Segments as Explants in Valencia Sweet Orange.” *Plant Cell, Tissue and Organ Culture* 109(2):383–90. doi: 10.1007/s11240-011-0092-7.

Khan, Ehsan Ullah, Xing Zheng Fu, Jing Wang, Qi Jun Fan, Xiao San Huang, Ge Ning Zhang, Jie Shi, and Ji Hong Liu. 2009. “Regeneration and Characterization of Plants Derived from Leaf *in Vitro* Culture of Two Sweet Orange (Citrus Sinensis (L.) Osbeck) Cultivars.” *Scientia Horticulturae* 120(1):70–76. doi: 10.1016/j.scienta.2008.10.004.

Kim, Hyongbum, and Jin Soo Kim. 2014. “A Guide to Genome Engineering with Programmable Nucleases.” *Nature Reviews Genetics* 15(5):321–34. doi: 10.1038/nrg3686.

Kim, Jeong il, and Jae Yean Kim. 2019. “New Era of Precision Plant

Breeding Using Genome Editing." *Plant Biotechnology Reports* 13(5):419–21. doi: 10.1007/s11816-019-00581-w.

Kim, Jung Hee, Etty Handayani, Akira Wakana, Mayumi Sato, Mai Miyamoto, Riko Miyazaki, Xiaohui Zhou, Kaori Sakai, Yuki Mizunoe, Masayoshi Shigyo, and Jun ichiro Masuda. 2020. "Distribution and Evolution of Citrus Accessions with S3 and/or S11 Alleles for Self-Incompatibility with an Emphasis on Sweet Orange [Citrus Sinensis (L.) Osbeck; Sf S3 or Sf S3sm]." *Genetic Resources and Crop Evolution* 67(8):2101–17. doi: 10.1007/s10722-020-00964-x.

Kobayashi, A. K., J. C. Bespalhok, L. F. P. Pereira, and L. G. E. Vieira. 2003. "Plant Regeneration of Sweet Orange (Citrus Sinensis) from Thin Sections of Mature Stem Segments." *Plant Cell, Tissue and Organ Culture* 74(1):99–102. doi: 10.1023/A:1023375526781.

Kobayashi, Adilson K., Luiz Gonzaga E. Vieira, João Carlos Bespalhok Filho, Rui Pereira Leite, Luiz Filipe P. Pereira, Hugo Bruno C. Molinari, and Viviani v. Marques. 2017. "Enhanced Resistance to Citrus Canker in Transgenic Sweet Orange Expressing the Sarcotoxin IA Gene." *European Journal of Plant Pathology* 149(4):865–73. doi: 10.1007/s10658-017-1234-5.

Kobayashi, Shozo, and Hirofumi Uchimiya. 1989. "Expression and Integration of a Foreign Gene in Orange (Citrus Sinensis Osb.) Protoplasts by Direct DNA Transfer." *The Japanese Journal of Genetics* 64(2):91–97. doi: 10.1266/jjg.64.91.

Kolmogorov, Mikhail, Jeffrey Yuan, Yu Lin, and Pavel A. Pevzner. 2019. "Assembly of Long, Error-Prone Reads Using Repeat Graphs." *Nature Biotechnology* 37(May). doi: 10.1038/s41587-019-0072-8.

Koltunow, A. M., K. Soltys, N. Nito, and S. McClure. 1995. "Anther, Ovule, Seed, and Nucellar Embryo Development in Citrus Sinensis Cv. Valencia." *Canadian Journal of Botany*

73(10):1567–82. doi: 10.1139/b95-170.

Koltunow, AM, P. Brennan, and S. Protopsaltis. 2000. “Regeneration of West Indian Limes (Citrus Aurantifolia) Containing Genes for Decreased Seed Set.” in *Proc. Int. Symp. on Citrus Biotecnology*.

Kopertekh, L., K. Schulze, A. Frolov, D. Strack, I. Broer, and J. Schiemann. 2010. “Cre-Mediated Seed-Specific Transgene Excision in Tobacco.” *Plant Molecular Biology* 72(6):597–605. doi: 10.1007/s11103-009-9595-6.

Kuc, Joseph. 1982. “Immunity to Plant Disease.” *BioScience* 32(11):854–60.

LeBlanc, Chantal, Fei Zhang, Josefina Mendez, Yamile Lozano, Krishna Chatpar, Vivian F. Irish, and Yannick Jacob. 2018. “Increased Efficiency of Targeted Mutagenesis by CRISPR/Cas9 in Plants Using Heat Stress.” *Plant Journal* 93(2):377–86. doi: 10.1111/tpj.13782.

Lei, Y., L. Lu, H. Y. Liu, S. Li, F. Xing, and C. Ling-Ling. 2014. “CRISPR-P : A Web Tool for Synthetic Single-Guide RNA Design of CRISPR-System in Plants.” *Molecular Plant* 7:1494–96. doi: 10.1093/mp/ssu044.

Lewin, I. J., and S. P. Monselise. 1976. “Further Studies on the Reduction of Seeds in Mandarins by NAA Sprays.” *Scientia Horticulturae* 4:229–34.

Li, D. D., W. Shi, and X. X. Deng. 2002. “Agrobacterium-Mediated Transformation of Embryogenic Calluses of Ponkan Mandarin and the Regeneration of Plants Containing the Chimeric Ribonuclease Gene.” *Plant Cell Reports* 21(2):153–56. doi: 10.1007/s00299-002-0492-6.

Li, D. D., W. Shi, and X. X. Deng. 2003a. “Factors Influencing Agrobacterium-Mediated Embryogenic Callus Transformation of Valencia Sweet Orange (Citrus Sinensis) Containing the PTA29-Barnase Gene.” *Tree Physiology* 23(17):1209–15. doi: 10.1093/treephys/23.17.1209.

Li, D. D., W. Shi, and X. X. Deng. 2003b. "Factors Influencing Agrobacterium-Mediated Embryogenic Callus Transformation of Valencia Sweet Orange (Citrus Sinensis) Containing the PTA29-Barnase Gene." *Tree Physiology* 23(17):1209–15. doi: 10.1093/treephys/23.17.1209.

LI, Fang, Su ming DAI, Zi niu DENG, Da zhi LI, Gui you LONG, Na LI, Yi LI, and Alexandra Gentile. 2017. "Evaluation of Parameters Affecting Agrobacterium-Mediated Transient Expression in Citrus." *Journal of Integrative Agriculture* 16(3):572–79. doi: 10.1016/S2095-3119(16)61460-0.

Li, Na, Ran Xu, and Yunhai Li. 2019. "Molecular Networks of Seed Size Control in Plants." *Annual Review of Plant Biology* 70(1):435–63. doi: 10.1146/annurev-arplant-050718-095851.

Liang, Mei, Zonghong Cao, Andan Zhu, Yuanlong Liu, Mengqin Tao, Huayan Yang, Qiang Xu, Shaohua Wang, Junjie Liu, Yongping Li, Chuanwu Chen, Zongzhou Xie, Chongling Deng, Junli Ye, Wenwu Guo, Qiang Xu, Rui Xia, Robert M. Larkin, Xiuxin Deng, Maurice Bosch, Vernonica E. Franklin-Tong, and Lijun Chai. 2020. "Evolution of Self-Compatibility by a Mutant Sm -RNase in Citrus." *Nature Plants* 6(2):131–42. doi: 10.1038/s41477-020-0597-3.

Liang, Zhen, Kunling Chen, Tingdong Li, Yi Zhang, Yanpeng Wang, Qian Zhao, Jinxing Liu, Huawei Zhang, Cuimin Liu, Yidong Ran, and Caixia Gao. 2017. "Efficient DNA-Free Genome Editing of Bread Wheat Using CRISPR/Cas9 Ribonucleoprotein Complexes." *Nature Communications* 8:6–10. doi: 10.1038/ncomms14261.

Liao, Yang, Gordon K. Smyth, and Wei Shi. 2014. "FeatureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features." *Bioinformatics* 30(7):923–30. doi: 10.1093/bioinformatics/btt656.

Limera, Cecilia, Silvia Sabbadini, Jeremy B. Sweet, and Bruno

Mezzetti. 2017. “New Biotechnological Tools for the Genetic Improvement of Major Woody Fruit Species.” *Frontiers in Plant Science* 8(August):1–16. doi: 10.3389/fpls.2017.01418.

Liu, H. .., Y. .. Ding, Y. Zhou, W. Jin, K. Xie, and L. L. Chen. 2017. “CRISPR-P 2.0 : An Improved CRISPR-Cas9 Tool for Genome Editing in Plants.” *Molecular Plant* 10:530–32. doi: 10.1016/j.molp.2017.01.003.

Liu, Xuejun, Chuanxiao Xie, Huaijun Si, and Jinxiao Yang. 2017. “CRISPR/Cas9-Mediated Genome Editing in Plants.” *Methods* 121–122:94–102. doi: 10.1016/j.ymeth.2017.03.009.

Livak, Kenneth J., and Thomas D. Schmittgen. 2001. “Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method.” *Methods* 25(4):402–8. doi: 10.1006/meth.2001.1262.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. “Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2.” *Genome Biology* 15(12):1–21. doi: 10.1186/s13059-014-0550-8.

Luo, Ming, Elizabeth S. Dennis, Frederic Berger, William James Peacock, and Abed Chaudhury. 2005. “MINISEED3 (MINI3), a WRKY Family Gene, and HAIKU2 (IKU2), a Leucine-Rich Repeat (LRR) KINASE Gene, Are Regulators of Seed Size in Arabidopsis.” *Proceedings of the National Academy of Sciences of the United States of America* 102(48):17531–36. doi: 10.1073/pnas.0508418102.

Lusser, Maria, Claudia Parisi, Damien Plan, and Emilio Rodríguez-Cerezo. 2012. “Deployment of New Biotechnologies in Plant Breeding.” *Nature Biotechnology* 30(3):231–39. doi: 10.1038/nbt.2142.

Lyoyd, G. B., and B. H. McCown. 1980. “Commercially Feasible Micropropagation of Mountain Laurel (Kalmia Latifolia) by Use of Shoot Tip Culture.” *Proc. Int. Plant Propagators Soc.*

30:421–37.

Lyznik, L. A., W. J. Gordon-Kamm, and Y. Tao. 2003. “Site-Specific Recombination for Genetic Engineering in Plants.” *Plant Cell Reports* 21(10):925–32. doi: 10.1007/s00299-003-0616-7.

Mahmoud, Lamiaa M., Prabhjot Kaur, Daniel Stanton, Jude W. Grosser, and Manjul Dutt. 2022. “A Cationic Lipid Mediated CRISPR/Cas9 Technique for the Production of Stable Genome Edited Citrus Plants.” *Plant Methods* 18(1):1–14. doi: 10.1186/s13007-022-00870-6.

Malnoy, M., J. P. Reynoird, E. E. Borejsza-Wysocka, and H. S. Aldwinckle. 2006. “Activation of the Pathogen-Inducible Gst1 Promoter of Potato after Elicitation by Venturia Inaequalis and Erwinia Amylovora in Transgenic Apple (Malus x Domestica).” *Transgenic Research* 15(1):83–93. doi: 10.1007/s11248-005-2943-7.

Malnoy, Mickael, Roberto Viola, Min Hee Jung, Ok Jae Koo, Seokjoong Kim, Jin Soo Kim, Riccardo Velasco, and Chidananda Nagamangala Kanchiswamy. 2016. “DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins.” *Frontiers in Plant Science* 7(DECEMBER2016):1–9. doi: 10.3389/fpls.2016.01904.

Manni, Mosè, Matthew R. Berkeley, Mathieu Seppey, Felipe A. Simão, and Evgeny M. Zdobnov. 2021. “BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic, Prokaryotic, and Viral Genomes.” *Molecular Biology and Evolution* 38(10):4647–54. doi: 10.1093/molbev/msab199.

Mao, Yanfei, Hui Zhang, Nanfei Xu, Botao Zhang, Feng Gou, and Jian Kang Zhu. 2013. “Application of the CRISPR-Cas System for Efficient Genome Engineering in Plants.” *Molecular Plant* 6(6):2008–11. doi: 10.1093/mp/sst121.

Marques, Natália T., Gustavo B. Nolasco, and JoséP P. Leitão. 2011.

"Factors Affecting *in Vitro* Adventitious Shoot Formation on Internode Explants of Citrus Aurantium L. Cv. Brazilian." *Scientia Horticulturae* 129(2):176–82. doi: 10.1016/j.scienta.2011.03.018.

Martínez de Alba, Angel Emilio, Emilie Elvira-Matelot, and Hervé Vaucheret. 2013. "Gene Silencing in Plants: A Diversity of Pathways." *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* 1829(12):1300–1308. doi: 10.1016/j.bbagrm.2013.10.005.

Marutani-Hert, Mizuri, Kim D. Bowman, Greg T. McCollum, T. Erik Mirkov, Terence J. Evens, and Randall P. Niedz. 2012. "A Dark Incubation Period Is Important for Agrobacterium-Mediated Transformation of Mature Internode Explants of Sweet Orange, Grapefruit, Citron, and a Citrange Rootstock." *PLoS ONE* 7(10):1–11. doi: 10.1371/journal.pone.0047426.

Mendes, B. M. J., S. C. Cardoso, R. L. Boscariol-Camargo, R. B. Cruz, F. A. A. Mourão Filho, and A. Bergamin Filho. 2010. "Reduction in Susceptibility to Xanthomonas Axonopodis Pv. Citri in Transgenic Citrus Sinensis Expressing the Rice Xa21 Gene." *Plant Pathology* 59(1):68–75. doi: 10.1111/j.1365-3059.2009.02148.x.

Mesejo, Carlos, Amparo Martínez-Fuentes, Carmina Reig, and Manuel Agustí. 2008. "Gibberellic Acid Impairs Fertilization in Clementine Mandarin under Cross-Pollination Conditions." *Plant Science* 175(3):267–71. doi: 10.1016/j.plantsci.2008.04.008.

Mesejo, Carlos, Amparo Martínez-Fuentes, Carmina Reig, Fernando Rivas, and Manuel Agustí. 2006. "The Inhibitory Effect of CuSO4 on Citrus Pollen Germination and Pollen Tube Growth and Its Application for the Production of Seedless Fruit." *Plant Science* 170(1):37–43. doi: 10.1016/j.plantsci.2005.07.023.

Mesejo, Carlos, Natalia Muñoz-Fambuena, Carmina Reig, Amparo

Martínez-Fuentes, and Manuel Agustí. 2014a. “Cell Division Interference in Newly Fertilized Ovules Induces Stenospermocarpy in Cross-Pollinated Citrus Fruit.” *Plant Science* 225:86–94. doi: 10.1016/j.plantsci.2014.05.019.

Mesejo, Carlos, Natalia Muñoz-Fambuena, Carmina Reig, Amparo Martínez-Fuentes, and Manuel Agustí. 2014b. “Cell Division Interference in Newly Fertilized Ovules Induces Stenospermocarpy in Cross-Pollinated Citrus Fruit.” *Plant Science* 225:86–94. doi: 10.1016/j.plantsci.2014.05.019.

Mesejo, Carlos, Roberto Yuste, Carmina Reig, Amparo Martínez-Fuentes, Domingo J. Iglesias, Natalia Muñoz-Fambuena, Almudena Bermejo, Ma Antonietta Germanà, Eduardo Primo-Millo, and Manuel Agustí. 2016. “Gibberellin Reactivates and Maintains Ovary-Wall Cell Division Causing Fruit Set in Parthenocarpic Citrus Species.” *Plant Science* 247:13–24. doi: 10.1016/j.plantsci.2016.02.018.

Meyer, P., and H. Saedler. 1996. “Homology-Dependent Gene Silencing in Plants.” *Annual Rev. Plant Physiol. Plant Mol. Biol.* 47:23–48.

Miao, Hongxia, Yonghua Qin, Jaime A. Teixeira da Silva, Zixing Ye, and Guibing Hu. 2013. “Identification of Differentially Expressed Genes in Pistils from Self-Incompatible Citrus Reticulata by Suppression Subtractive Hybridization.” *Molecular Biology Reports* 40(1):159–69. doi: 10.1007/s11033-012-2045-6.

Miao, Hongxia, Zixing Ye, Guibing Hu, and Yonghua Qin. 2015. “Comparative Transcript Profiling of Gene Expression between Self-Incompatible and Self-Compatible Mandarins by Suppression Subtractive Hybridization and CDNA Microarray.” *Molecular Breeding* 35(1). doi: 10.1007/s11032-015-0204-x.

Miles, John S., and John R. Guest. 1984. “Nucleotide Sequence and Transcriptional Start Point of the Phosphomannose Isomerase

Gene (ManA) of Escherichia Coli." *Gene* 32(1–2):41–48. doi: 10.1016/0378-1119(84)90030-1.

Miyamoto, Y., A. Masunaka, T. Tsuge, M. Yamamoto, K. Ohtani, T. Fukumoto, K. Gomi, T. L. Peever, and Kazuya Akimitsu. 2008. "Functional Analysis of a Multicopy Host-Selective ACT-Toxin Biosynthesis Gene in the Tangerine Pathotype of Alternaria Alternata Using RNA Silencing." *Molecular Plant-Microbe Interactions* 21(12):1591–99. doi: 10.1094/MPMI-21-12-1591.

Miyata, Luzia Yuriko, Ricardo Harakava, Liliane Cristina Libório Stipp, Beatriz Madalena Januzzi Mendes, Beatriz Appezzato-da-Glória, and Francisco de Assis Alves Mourão Filho. 2012. "GUS Expression in Sweet Oranges (Citrus Sinensis L. Osbeck) Driven by Three Different Phloem-Specific Promoters." *Plant Cell Reports* 31(11):2005–13. doi: 10.1007/s00299-012-1312-2.

Molinari, Hugo Bruno Correa, Celso Jamil Marur, João Carlos Bespalhok Filho, Adilson Kenji Kobayashi, Marcos Pileggi, Rui Pereira Leite Júnior, Luiz Filipe Protasio Pereira, and Luiz Gonzaga Esteves Vieira. 2004. "Osmotic Adjustment in Transgenic Citrus Rootstock Carrizo Citrange (Citrus Sinensis Osb. x Poncirus Trifoliata L. Raf.) Overproducing Proline." *Plant Science* 167(6):1375–81. doi: 10.1016/j.plantsci.2004.07.007.

Montalt, Rafael, María Carmen Vives, Luis Navarro, Patrick Ollitrault, and Pablo Aleza. 2021. "Parthenocarpy and Self-Incompatibility in Mandarins." *Agronomy* 11(10):1–18. doi: 10.3390/agronomy11102023.

Moravčíková, Jana, Eva Vaculková, Miroslav Bauer, and Jana Libantová. 2008. "Feasibility of the Seed Specific Cruciferin C Promoter in the Self Excision Cre/LoxP Strategy Focused on Generation of Marker-Free Transgenic Plants." *Theoretical and Applied Genetics* 117(8):1325–34. doi: 10.1007/s00122-008-0866-4.

Muccilli, Vera, Alessandro Vitale, Ling Sheng, Alessandra Gentile, Nunzio Cardullo, Corrado Tringali, Cinzia Oliveri, Rosa la Rosa, Mario Di Guardo, Stefano la Malfa, Ziniu Deng, and Gaetano Distefano. 2020. "Substantial Equivalence of a Transgenic Lemon Fruit Showing Postharvest Fungal Pathogens Resistance." *Journal of Agricultural and Food Chemistry* 68(12):3806–16. doi: 10.1021/acs.jafc.9b07925.

Murashige, T., and F. Skoog. 1962. "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Culture." *Physiologia Plantarum* 15:473–79.

Murashige, T., and D. P. H. Tucker. 1969. "Growth Factor Requirements of Citrus Tissue Culture." *Proc First Intl Citrus Symp* 3:1155–1161.

Narayan, Reena D., Leila M. Blackman, Weixing Shan, and Adrienne R. Hardham. 2010. "Phytophthora Nicotianae Transformants Lacking Dynein Light Chain 1 Produce Non-Flagellate Zoospores." *Fungal Genetics and Biology* 47(8):663–71. doi: 10.1016/j.fgb.2010.04.008.

Navarro-García, Nuria, Asunción Morte, and Olaya Pérez-Tornero. 2016. "*In Vitro* Adventitious Organogenesis and Histological Characterization from Mature Nodal Explants of Citrus Limon." *In Vitro Cellular and Developmental Biology - Plant* 52(2):161–73. doi: 10.1007/s11627-015-9743-4.

Newbigin, Ed, Marilyn A. Anderson, and Adrienne E. Clarke. 1993. "Gametophyte Self-Incompatibility Systems." *Plant Cell* 5(10):1315–24. doi: 10.1105/tpc.5.10.1315.

Nishikawa, Fumie, Tomoko Endo, Takehiko Shimada, Hiroshi Fujii, Tokurou Shimizu, Yasushi Kobayashi, Takashi Araki, and Mitsuo Omura. 2010. "Transcriptional Changes in CiFT-Introduced Transgenic Trifoliate Orange (Poncirus Trifoliata L. Raf.)." *Tree Physiology* 30(3):431–39. doi: 10.1093/treephys/tpp122.

Nishikawa, Fumie, Tomoko Endo, Takehiko Shimada, Hiroshi Fujii, Tokurou Shimizu, and Mitsuo Omura. 2008. "Isolation and Characterization of a Citrus FT/TFL1 Homologue (CuMFT1), Which Shows Quantitatively Preferential Expression in Citrus Seeds." *Journal of the Japanese Society for Horticultural Science* 77(1):38–46. doi: 10.2503/jjshs1.77.38.

Nishimasu, Hiroshi, F. Ann Ran, Patrick D. Hsu, Silvana Konermann, Soraya I. Shehata, Naoshi Dohmae, Ryuichiro Ishitani, Feng Zhang, and Osamu Nureki. 2014. "Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA." *Cell* 156(5):935–49. doi: 10.1016/j.cell.2014.02.001.

Odell, Joan T., Ferenc Nagy, and Nam Hai Chua. 1985. "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter." *Nature* 313(6005):810–12. doi: 10.1038/313810a0.

de Oliveira, M. L., C. C. de Lima Silva, V. Y. Abe, M. G. C. Costa, R. A. Cernaddas, and C. E. Benedetti. 2013. "Increased Resistance against Citrus Canker Mediated by a Citrus Mitogen-Activated Protein Kinase." *Molecular Plant-Microbe Interactions* 26:1190–99.

de Oliveira, ML P., MG Cardoso Costa, CV da Silva, and W. Campos Otoni. 2010. "Growth Regulators, Culture Media and Antibiotics in the *in Vitro* Shoot Regeneration from Mature Tissue of Citrus Cultivars." *Pesquisa Agropecuária Brasileira* 45(7):654–60.

Ollitrault, Patrick, Dalel Ahmed, Gilles Costantino, Jean Charles Evrard, Celine Cardi, Pierre Mournet, Aude Perdereau, and Yann Froelicher. 2021. "Segregation Distortion for Male Parents in High Density Genetic Maps from Reciprocal Crosses between Two Self-Incompatible Cultivars Confirms a Gametophytic System for Self-Incompatibility in Citrus." *Agriculture (Switzerland)* 11(5). doi: 10.3390/agriculture11050379.

Omar, A. A., W. Y. Song, and J. W. Grosser. 2007. "Introduction of

Xa21, a Xanthomonas-Resistance, Gene from Rice, into 'Hamlin' Sweet Orange [Citrus Sinensis (L.) Osbeck] Using Protoplast- GFP Co-Transformation or Single Plasmid Transformation." *Journal of Horticultural Science and Biotechnology* 82(6):914–23. doi: 10.1080/14620316.2007.11512326.

Omar, Ahmad A., Manjul Dutt, Frederick G. Gmitter, and Jude W. Grosser. 2016. "Chapter 13 Somatic Embryogenesis : Still a Relevant Technique in Citrus Improvement." in *In Vitro Embryogenesis in Higher Plants, Methods in Molecular Biology*. Vol. 1359.

Omar, Ahmad A., Mayara M. Murata, Hesham A. El-Shamy, James H. Graham, and Jude W. Grosser. 2018. "Enhanced Resistance to Citrus Canker in Transgenic Mandarin Expressing Xa21 from Rice." *Transgenic Research* 27(2):179–91. doi: 10.1007/s11248-018-0065-2.

Orbovic, V., A. Shankar, ME Peeples, C. Hubbard, and J. Zale. 2015. "Citrus Transformation Using Mature Tissue Explants." Pp. vii–viii in *Agrobacterium protocols: vol. 2, Methods in molecular biology (Clifton, N.J.)*. Vol. 1224.

Orbović, Vladimir, Milica Ćalović, Manjul Dutt, Jude W. Grosser, and Gary Barthe. 2015. "Production and Characterization of Transgenic Citrus Plants Carrying P35 Anti-Apoptotic Gene." *Scientia Horticulturae* 197:203–11. doi: 10.1016/j.scienta.2015.09.038.

Orbović, Vladimir, Jeb S. Fields, and James P. Syvertsen. 2017. "Transgenic Citrus Plants Expressing the P35 Anti-Apoptotic Gene Have Altered Response to Abiotic Stress." *Horticulture Environment and Biotechnology* 58(3):303–9. doi: 10.1007/s13580-017-0196-z.

Orbović, Vladimir, and Jude W. Grosser. 2015. "Citrus Transformation Using Juvenile Tissue Explants." in

Agrobacterium protocols: vol. 2, Methods in molecular biology (Clifton, N.J.). Vol. 1224, edited by K. Wang. Springer Singapore.

Otero, Alvaro, and Fernando Rivas. 2017. "Field Spatial Pattern of Seedy Fruit and Techniques to Improve Yield on 'Afourer' Mandarin." *Scientia Horticulturae* 225(December 2016):264–70. doi: 10.1016/j.scienta.2017.06.067.

Paiva, Paulo Eduardo Branco, Tânia Cota, Luís Neto, Celestino Soares, José Carlos Tomás, and Amílcar Duarte. 2020. "Water Vapor Pressure Deficit in Portugal and Implications for the Development of the Invasive African Citrus Psyllid Trioza Erytreae." *Insects* 11(4):1–11. doi: 10.3390/insects11040229.

Parry, Simon, Ed Newbigin, Graeme Currie, Antony Bacic, and David Oxley. 1989. "Identification of Active-Site Histidine Residues of a Self-Lncompatibility Ribonuclease from a Wild Tomato '." (1 997):1421–29.

Pena, L., M. Cervera, C. Fagoaga, R. Perez, Juan Romero, José Juarez, J. A. Pina, and L. Navarro. 2004. "Agrobacterium-Mediated Transformation of Citrus." Pp. 145–58 in *Transgenic Crops of the World*, edited by K. A. Publishers.

Pena, Leandro, Magdalena Cervera, Carmen Fagoaga, Juan Romero, Alida Ballester, Nuria Soler, Elsa Pons, Ana Rodr, and Josep Peris. 2008. "Citrus." P. 61 in *Transgenic Tropical and Subtropical Fruits and Nuts. Edited.*

Pena, Leandro, Magdalena Cervera, José Juarez, Antonio Navarro, Carmen Ortega, José Pina, Nuria Duran-Vila, and Luis Navarro. 1998. "Procedure for the Genetic Transformation of Adult Plants of Woody Species." *Office* 42(19):1–47.

Peña, Leandro, Magdalena Cervera, José Juárez, Carmen Ortega, JoséA A. Pina, Nuria Durán-Vila, and Luis Navarro. 1995. "High Efficiency Agrobacterium-Mediated Transformation and Regeneration of Citrus." *Plant Science* 104(2):183–91. doi:

10.1016/0168-9452(94)04021-8.

Peña, Leandro, Mar Martín-Trillo, José Juárez, José A. Pina, Luis Navarro, and José M. Martínez-Zapater. 2001. "Constitutive Expression of Arabidopsis LEAFY or APETALA1 Genes in Citrus Reduces Their Generation Time." *Nature Biotechnology* 19(3):263–67. doi: 10.1038/85719.

Peng, Aihong, Shanchun Chen, Tiangang Lei, Lanzhen Xu, Yongrui He, Liu Wu, Lixiao Yao, and Xiuping Zou. 2017. "Engineering Canker-Resistant Plants through CRISPR/Cas9-Targeted Editing of the Susceptibility Gene CsLOB1 Promoter in Citrus." *Plant Biotechnology Journal* 15(12):1509–19. doi: 10.1111/pbi.12733.

Peng, Aihong, Xiuping Zou, Lanzhen Xu, Yongrui He, Tiangang Lei, Lixiao Yao, Qiang Li, and Shanchun Chen. 2019. "Improved Protocol for the Transformation of Adult Citrus Sinensis Osbeck 'Tarocco' Blood Orange Tissues." *In Vitro Cellular & Developmental Biology - Plant*. doi: 10.1007/s11627-019-10011-9.

Peng, Jian Ling, Zhi Long Bao, Ping Li, Guang Yong Chen, Jin Sheng Wang, and Han Song Dong. 2004. "HarpinX00 and Its Functional Domains Activate Pathogen-Inducible Plant Promoters in Arabidopsis." *Acta Botanica Sinica* 46(9):1083–90.

Pinello, L., M. C. Canver, M. D. Hoban, S. H. Orkin, D. B. Kohn, D. E. Bauer, and G. C. Yuan. 2017. "Analyzing CRISPR Genome Editing Experiments with CRISPResso." *Nature Biotechnology* 34(7):695–97. doi: 10.1038/nbt.3583.Analyzing.

Poles, Lara, Concetta Licciardello, Gaetano Distefano, Elisabetta Nicolosi, Alessandra Gentile, and Stefano la Malfa. 2020. "Recent Advances of *in Vitro* Culture for the Application of New Breeding Techniques in Citrus." *Plants* 9(8):1–25. doi: 10.3390/plants9080938.

Pompili, Valerio, Lorenza Dalla Costa, Stefano Piazza, Massimo Pindo, and Mickael Malnoy. 2020. "Reduced Fire Blight Susceptibility in Apple Cultivars Using a High-Efficiency CRISPR/Cas9-FLP/FRT-Based Gene Editing System." *Plant Biotechnology Journal* 18(3):845–58. doi: 10.1111/pbi.13253.

Pons, Elsa, Berta Alquézar, Ana Rodríguez, Patricia Martorell, Salvador Genovés, Daniel Ramón, María Jesús Rodrigo, Lorenzo Zacarías, and Leandro Peña. 2014. "Metabolic Engineering of β-Carotene in Orange Fruit Increases Its in Vivo Antioxidant Properties." *Plant Biotechnology Journal* 12(1):17–27. doi: 10.1111/pbi.12112.

Pontier, Dominique, Laurence Godiard, Yves Marco, and Dominique Roby. 1994. "Hsr203J, a Tobacco Gene Whose Activation Is Rapid, Highly Localized and Specific for Incompatible Plant/Pathogen Interactions." *The Plant Journal* 5(4):507–21. doi: 10.1046/j.1365-313X.1994.5040507.x.

Qiu, Wenming, Juliana Soares, Zhiqian Pang, Yixiao Huang, Zhonghai Sun, Nian Wang, Jude Grosser, and Manjul Dutt. 2020. "Potential Mechanisms of AtNPR1 Mediated Resistance against Huanglongbing (HLB) in Citrus." *International Journal of Molecular Sciences* 21(6):1–17. doi: 10.3390/ijms21062009.

Ramanauskas, Karolis, and Boris Igić. 2017. "The Evolutionary History of Plant T2/S-Type Ribonucleases." *PeerJ* 2017(9). doi: 10.7717/peerj.3790.

Raveh, Eran, Livnat Goldenberg, Ron Porat, Nir Carmi, Alessandra Gentile, and Stefano la Malfa. 2020. "Conventional Breeding of Cultivated Citrus Varieties." Pp. 33–48 in.

Ren, Yi, Jiayan Pan, Zhike Zhang, Jietang Zhao, Xinhua He, Yonghua Qin, and Guibing Hu. 2020. "Identification of an Up-Accumulated Polyamine Oxidase 2 in Pollen of Self-Incompatible 'Wuzishatangju' Mandarin Using Comparative Proteomic Analysis." *Scientia Horticulturae* 266(October

2019):109279. doi: 10.1016/j.scienta.2020.109279.

Reyes, Carina Andrea, Agustina de Francesco, Eduardo José Peña, Norma Costa, María Inés Plata, Lorena Sendin, Atilio Pedro Castagnaro, and María Laura García. 2011. "Resistance to Citrus Psorosis Virus in Transgenic Sweet Orange Plants Is Triggered by Coat Protein-RNA Silencing." *Journal of Biotechnology* 151(1):151–58. doi: 10.1016/j.jbiotec.2010.11.007.

Robertson, CJ, X. Zhang, S. Gowda, Vladimir Orbović, WO Dawson, and Mou Z. 2018. "Overexpression of the Arabidopsis NPR1 Protein in Citrus Confers Tolerance to Huanglongbing." *Journal of Citrus Pathology* 0–8.

Rodríguez, Ana, Magdalena Cervera, Josep Enric Peris, and Leandro Peña. 2008. "The Same Treatment for Transgenic Shoot Regeneration Elicits the Opposite Effect in Mature Explants from Two Closely Related Sweet Orange (Citrus Sinensis (L.) Osb.) Genotypes." *Plant Cell, Tissue and Organ Culture* 93(1):97–106. doi: 10.1007/s11240-008-9347-3.

Russo, G., G. Reforgiato Recupero, and S. Recupero. 2004. "New Triploid Hybrids of Citrus in Italy." P. 399:401 in *Proc Int Soc Citric*.

Salonia, Fabrizio, Angelo Ciacciulli, Lara Poles, Helena Domenica Pappalardo, Stefano la Malfa, and Concetta Licciardello. 2020. "New Plant Breeding Techniques in Citrus for the Improvement of Important Agronomic Traits. A Review." *Frontiers in Plant Science* 11(August):1–15. doi: 10.3389/fpls.2020.01234.

Sander, Jeffry D., and J. Keith Joung. 2014. "CRISPR-Cas Systems for Genome Editing, Regulation and Targeting." *Nature Biotechnology* 32(4):347–55. doi: 10.1038/nbt.2842.CRISPR-Cas.

Sauer, Norbert, and Jürgen Stolz. 1994. "SUC1 and SUC2: Two Sucrose Transporters from Arabidopsis Thaliana; Expression and Characterization in Baker's Yeast and Identification of the

Histidine-tagged Protein." *The Plant Journal* 6(1):67–77. doi: 10.1046/j.1365-313X.1994.6010067.x.

Savage, E. M., and F. E. Gardner. 1965. *The Troyer and Carrizo Citranges*. Vol. 50.

Schaad, Norman W., Elena Postnikova, George Lacy, Aaron Sechler, Irina Agarkova, Paul E. Stromberg, Verlyn K. Stromberg, and Anne K. Vidaver. 2006. "Emended Classification of Xanthomonad Pathogens on Citrus." *Systematic and Applied Microbiology* 29(8):690–95. doi: 10.1016/j.syapm.2006.08.001.

Schmulling, Thomas, Jeff Schell, and Angelo Spena. 1989. "Promoters of the RolA, B and C Genes of Agrobacterium Rhizogenes Are Differentially Regulated in Transgenic Plants." *The Plant Cell* 1:665–70.

Schouten, Henk J., Frans A. Krens, and Evert Jacobsen. 2006. "Cisgenic Plants Are Similar to Traditionally Bred Plants: International Regulations for Genetically Modified Organisms Should Be Altered to Exempt Cisgenesis." *EMBO Reports* 7(8):750–53. doi: 10.1038/sj.embor.7400769.

Sendín, L. N., M. P. Filippone, I. G. Orce, L. Rigano, R. Enrique, L. Peña, A. A. Vojnov, M. R. Marano, and A. P. Castagnaro. 2012. "Transient Expression of Pepper Bs2 Gene in Citrus Limon as an Approach to Evaluate Its Utility for Management of Citrus Canker Disease." *Plant Pathology* 61(4):648–57. doi: 10.1111/j.1365-3059.2011.02558.x.

Sendin, Lorena Noelia, and María Paula Filippone. 2019. "The Genetic Transformation of Sweet Orange (Citrus Sinensis L. Osbeck) for Enhanced Resistance to Citrus Canker." in *Transgenic Plants. Methods and Protocols*.

Sendin, Lorena Noelia, Ingrid Georgina Orce, Rocío Liliana Gómez, Ramón Enrique, Carlos Froilán Grellet Bournonville, Aldo Sergio Noguera, Adrián Alberto Vojnov, María Rosa Marano, Atilio Pedro Castagnaro, and María Paula Filippone. 2017.

"Inducible Expression of Bs2 R Gene from Capsicum Chacoense in Sweet Orange (Citrus Sinensis L. Osbeck) Confers Enhanced Resistance to Citrus Canker Disease." *Plant Molecular Biology* 93(6):607–21. doi: 10.1007/s11103-017-0586-8.

Shan, Qiwei, Yanpeng Wang, Jun Li, Yi Zhang, Kunling Chen, Zhen Liang, Kang Zhang, Jinxing Liu, Jianzhong Jeff Xi, Jin Long Qiu, and Caixia Gao. 2013. "Targeted Genome Modification of Crop Plants Using a CRISPR-Cas System." *Nature Biotechnology* 31(8):686–88. doi: 10.1038/nbt.2650.

Shi, Y., M. B. Wang, K. S. Powell, E. van Damme, V. A. Hilder, A. M. R. Gatehouse, D. Boulter, and J. A. Gatehouse. 1994. "Use of the Rice Sucrose Synthase-1 Promoter to Direct Phloem-Specific Expression of β-Glucuronidase and Snowdrop Lectin Genes in Transgenic Tobacco Plants." *Journal of Experimental Botany* 45(5):623–31. doi: 10.1093/jxb/45.5.623.

Simão, Felipe A., Robert M. Waterhouse, Panagiotis Ioannidis, Evgenia v Kriventseva, and Evgeny M. Zdobnov. 2015. "BUSCO: Assessing Genome Assembly and Annotation Completeness with Single-Copy Orthologs." *Bioinformatics* 31(19):3210–12. doi: 10.1093/bioinformatics/btv351.

Singerman, Ariel, and Michael E. Rogers. 2020. "The Economic Challenges of Dealing with Citrus Greening: The Case of Florida." *Journal of Integrated Pest Management* 11(1). doi: 10.1093/jipm/pmz037.

Singh, Sandeepa, and Manchikatla Venkat Rajam. 2010. "Highly Efficient and Rapid Plant Regeneration in Citrus Sinensis." *Journal of Plant Biochemistry and Biotechnology* 19(2):195–202. doi: 10.1007/bf03263340.

Soler, Nuria, Montserrat Plomer, Carmen Fagoaga, Pedro Moreno, Luis Navarro, Ricardo Flores, and Leandro Peña. 2012. "Transformation of Mexican Lime with an Intron-Hairpin Construct Expressing Untranslatable Versions of the Genes

Coding for the Three Silencing Suppressors of Citrus Tristeza Virus Confers Complete Resistance to the Virus." *Plant Biotechnology Journal* 10(5):597–608. doi: 10.1111/j.1467-7652.2012.00691.x.

Soriano, Leonardo, Eveline Carla da Rocha Tavano, Marcelo Favaretto Correa, Ricardo Harakava, Beatriz Madalena Januzzi Mendes, and Francisco de Assis Alves Mourão Filho. 2019. "*In Vitro* Organogenesis and Genetic Transformation of Mandarin Cultivars." *Revista Brasileira de Fruticultura* 41(2):1–11. doi: 10.1590/0100-29452019116.

Sorkina, Alina, Gabriel Bardosh, Yong Zhong Liu, Ifat Fridman, Ludmila Schlizerman, Naftali Zur, Etti Or, Eliezer E. Goldschmidt, Eduardo Blumwald, and Avi Sadka. 2011. "Isolation of a Citrus Promoter Specific for Reproductive Organs and Its Functional Analysis in Isolated Juice Sacs and Tomato." *Plant Cell Reports* 30(9):1627–40. doi: 10.1007/s00299-011-1073-3.

Spreen, Thomas H., Zhifeng Gao, Waldir Fernandes, and Marisa L. Zansler. 2020. "Global Economics and Marketing of Citrus Products." Pp. 471–93 in *The Genus Citrus*.

Sugita, K., E. Matsunaga, and H. Ebinuma. 1999. "Effective Selection System for Generating Marker-Free Transgenic Plants Independent of Sexual Crossing." *Plant Cell Reports* 18(11):941–47. doi: 10.1007/s002990050688.

Talon, Manuel, Lorenzo Zacarias, and Eduardo Primo-Millo. 1992. "Gibberellins and Parthenocarpic Ability in Developing Ovaries of Seedless Mandarins." *Plant Physiology* 99(4):1575–81. doi: 10.1104/pp.99.4.1575.

Tan, Bin, Ding Li Li, Shi Xiao Xu, Gai En Fan, Jing Fan, and Wen Wu Guo. 2009. "Highly Efficient Transformation of the GFP and MAC12.2 Genes into Precocious Trifoliate Orange (Poncirus Trifoliata [L.] Raf), a Potential Model Genotype for Functional

Genomics Studies in Citrus." *Tree Genetics and Genomes* 5(3):529–37. doi: 10.1007/s11295-009-0206-0.

Tang, Xu, Levi G. Lowder, Tao Zhang, Aimee A. Malzahn, Xuelian Zheng, Daniel F. Voytas, Zhaohui Zhong, Yiyi Chen, Qiurong Ren, Qian Li, Elida R. Kirkland, Yong Zhang, and Yiping Qi. 2017. "A CRISPR-Cpf1 System for Efficient Genome Editing and Transcriptional Repression in Plants." *Nature Plants* 3(September). doi: 10.1038/nplants.2017.18.

Tavano, Eveline Carla da Rocha, Ligia Erpen, Bianca Aluisi, Ricardo Harakava, João Roberto Spotti Lopes, Maria Lucia Carneiro Vieira, Sônia Maria De Stefano Piedade, Beatriz Madalena Januzzi Mendes, and Francisco de Assis Alves Mourão Filho. 2019. "Sweet Orange Genetic Transformation with the Attacin A Gene under the Control of Phloem-Specific Promoters and Inoculation with Candidatus Liberibacter Asiaticus." *Journal of Horticultural Science and Biotechnology* 94(2):210–19. doi: 10.1080/14620316.2018.1493361.

Tong, Zhu, Bin Tan, Jiancheng Zhang, Zhiyong Hu, Wenwu Guo, and Xiuxin Deng. 2009. "Using Precocious Trifoliate Orange (Poncirus Trifoliata [L.] Raf.) to Establish a Short Juvenile Transformation Platform for Citrus." *Scientia Horticulturae* 119(3):335–38. doi: 10.1016/j.scienta.2008.08.035.

Tribulato, E., and G. la Rosa. 1993. "Primosole e Simeto: Due Nuovi Ibridi Di Mandarino." *Italus Hortus* 12:125.

United States Department of Agriculture, National Agricultural Statistics Service (USDA-NASS). 2019. *Florida Citrus Statistics 2017-2018*.

Upadhyay, Santosh Kumar, Jitesh Kumar, Anshu Alok, and Rakesh Tuli. 2013. "RNA-Guided Genome Editing for Target Gene Mutations in Wheat." *G3: Genes, Genomes, Genetics* 3(12):2233–38. doi: 10.1534/g3.113.008847.

Vardi, Aliza, Ilan Levin, and Nir Carmi. 2008. "Induction of

Seedlessness in Citrus: From Classical Techniques to Emerging Biotechnological Approaches." *Journal of the American Society for Horticultural Science* 133(1):117–26. doi: 10.21273/jashs.133.1.117.

Varoquaux, Fabrice, Robert Blanvillain, Michel Delseny, and Patrick Gallois. 2000. "Less Is Better: New Approaches for Seedless Fruit Production." *Trends in Biotechnology* 18(11):233–42.

Vazquez-Vilar, Marta, Joan Miquel Bernabé-Orts, Asun Fernandez-del-Carmen, Pello Ziarsolo, Jose Blanca, Antonio Granell, and Diego Orzaez. 2016. "A Modular Toolbox for GRNA-Cas9 Genome Engineering in Plants Based on the GoldenBraid Standard." *Plant Methods* 12(1):1–12. doi: 10.1186/s13007-016-0101-2.

Velázquez, Karelia, Jesús Agüero, María C. Vives, Pablo Aleza, José A. Pina, Pedro Moreno, Luis Navarro, and José Guerri. 2016. "Precocious Flowering of Juvenile Citrus Induced by a Viral Vector Based on Citrus Leaf Blotch Virus: A New Tool for Genetics and Breeding." *Plant Biotechnology Journal* 14(10):1976–85. doi: 10.1111/pbi.12555.

Walker, Bruce J., Thomas Abeel, Terrance Shea, Margaret Priest, Amr Abouelliel, Sharadha Sakthikumar, Christina A. Cuomo, Qiandong Zeng, Jennifer Wortman, Sarah K. Young, and Ashlee M. Earl. 2014. "Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement." *PLoS ONE* 9(11). doi: 10.1371/journal.pone.0112963.

Wang, Aihua, Damien Garcia, Hongyu Zhang, Ke Feng, Abed Chaudhury, Fred Berger, William James Peacock, Elizabeth S. Dennis, and Ming Luo. 2010. "The VQ Motif Protein IKU1 Regulates Endosperm Growth and Seed Size in Arabidopsis." *Plant Journal* 63(4):670–79. doi: 10.1111/j.1365-313X.2010.04271.x.

Wang, Lijuan, Shanchun Chen, Aihong Peng, Zhu Xie, Yongrui He, and Xiuping Zou. 2019. "CRISPR/Cas9-Mediated Editing of CsWRKY22 Reduces Susceptibility to Xanthomonas Citri Subsp. Citri in Wanjincheng Orange (Citrus Sinensis (L.) Osbeck)." *Plant Biotechnology Reports* 13(5):501–10. doi: 10.1007/s11816-019-00556-x.

Wang, Lun, Fa He, Yue Huang, Jiaxian He, Shuizhi Yang, Jiwu Zeng, Chongling Deng, Xiaolin Jiang, Yiwen Fang, Shaohua Wen, Rangwei Xu, Huiwen Yu, Xiaoming Yang, Guangyan Zhong, Chuanwu Chen, Xiang Yan, Changfu Zhou, Hongyan Zhang, Zongzhou Xie, Robert M. Larkin, Xiuxin Deng, and Qiang Xu. 2018. "Genome of Wild Mandarin and Domestication History of Mandarin." *Molecular Plant* 11(8):1024–37. doi: 10.1016/j.molp.2018.06.001.

Wang, Xia, Yuantao Xu, Siqi Zhang, Li Cao, Yue Huang, Junfeng Cheng, Guizhi Wu, Shilin Tian, Chunli Chen, Yan Liu, Huiwen Yu, Xiaoming Yang, Hong Lan, Nan Wang, Lun Wang, Jidi Xu, Xiaolin Jiang, Zongzhou Xie, Meilian Tan, Robert M. Larkin, Ling Ling Chen, Bin Guang Ma, Yijun Ruan, Xiuxin Deng, and Qiang Xu. 2017. "Genomic Analyses of Primitive, Wild and Cultivated Citrus Provide Insights into Asexual Reproduction." *Nature Genetics* 49(5):765–72. doi: 10.1038/ng.3839.

Wu, G. Albert, Simon Prochnik, Jerry Jenkins, Jerome Salse, Uffe Hellsten, Florent Murat, Xavier Perrier, Manuel Ruiz, Simone Scalabrin, Javier Terol, Marco Aurélio Takita, Karine Labadie, Julie Poulain, Arnaud Couloux, Kamel Jabbari, Federica Cattonaro, Cristian del Fabbro, Sara Pinosio, Andrea Zuccolo, Jarrod Chapman, Jane Grimwood, Francisco R. Tadeo, Leandro H. Estornell, Juan v. Muñoz-Sanz, Victoria Ibanez, Amparo Herrero-Ortega, Pablo Aleza, Julián Pérez-Pérez, Daniel Ramón, Dominique Brunel, François Luro, Chunxian Chen, William G. Farmerie, Brian Desany, Chinnappa Kodira, Mohammed

Mohiuddin, Tim Harkins, Karin Fredrikson, Paul Burns, Alexandre Lomsadze, Mark Borodovsky, Giuseppe Reforgiato, Juliana Freitas-Astúa, Francis Quetier, Luis Navarro, Mikeal Roose, Patrick Wincker, Jeremy Schmutz, Michele Morgante, Marcos Antonio MacHado, Manuel Talon, Olivier Jaillon, Patrick Ollitrault, Frederick Gmitter, and Daniel Rokhsar. 2014. "Sequencing of Diverse Mandarin, Pummelo and Orange Genomes Reveals Complex History of Admixture during Citrus Domestication." *Nature Biotechnology* 32(7):656–62. doi: 10.1038/nbt.2906.

Wu, Guohong Albert, Javier Terol, Victoria Ibanez, Antonio López-García, Estela Pérez-Román, Carles Borredá, Concha Domingo, Francisco R. Tadeo, Jose Carbonell-Caballero, Roberto Alonso, Franck Curk, Dongliang Du, Patrick Ollitrault, Mikeal L. Roose, Joaquin Dopazo, Frederick G. Gmitter, Daniel S. Rokhsar, and Manuel Talon. 2018. "Genomics of the Origin and Evolution of Citrus." *Nature* 554(7692):311–16. doi: 10.1038/nature25447.

Wu, H., Y. Acanda, M. Canton, and J. Zale. 2019. "Efficient Biolistic Transformation of Immature Citrus Rootstock Using Phosphomanose- Isomerase Selection." *Plants* 8(390):2–11.

Wu, Hao, Yosvanis Acanda, Hongge Jia, Nian Wang, and Janice Zale. 2016. "Biolistic Transformation of Carrizo Citrange (Citrus Sinensis Osb. × Poncirus Trifoliata L. Raf.)." *Plant Cell Reports* 35(9):1955–62. doi: 10.1007/s00299-016-2010-2.

Xu, Qiang, Ling Ling Chen, Xiaoan Ruan, Dijun Chen, Andan Zhu, Chunli Chen, Denis Bertrand, Wen Biao Jiao, Bao Hai Hao, Matthew P. Lyon, Jiongjiong Chen, Song Gao, Feng Xing, Hong Lan, Ji Wei Chang, Xianhong Ge, Yang Lei, Qun Hu, Yin Miao, Lun Wang, Shixin Xiao, Manosh Kumar Biswas, Wenfang Zeng, Fei Guo, Hongbo Cao, Xiaoming Yang, Xi Wen Xu, Yun Jiang Cheng, Juan Xu, Ji Hong Liu, Oscar Junhong Luo, Zhonghui Tang, Wen Wu Guo, Hanhui Kuang, Hong Yu Zhang,

Mikeal L. Roose, Niranjan Nagarajan, Xiu Xin Deng, and Yijun Ruan. 2013. "The Draft Genome of Sweet Orange (Citrus Sinensis)." *Nature Genetics* 45(1):59–66. doi: 10.1038/ng.2472.

Xu, Rongfang, Ruiying Qin, Hao Li, Dongdong Li, Li Li, Pengcheng Wei, and Jianbo Yang. 2017. "Generation of Targeted Mutant Rice Using a CRISPR-Cpf1 System." *Plant Biotechnology Journal* 15(6):713–17. doi: 10.1016/j.tplants.2017.05.001.

Yamasaki, Atsu, Akira Kitajima, Norihiro Ohara, Mitsutoshi Tanaka, and Kojiro Hasegawa. 2007. "Histological Study of Expression of Seedlessness in Citrus Kinokuni 'Mukaku Kishu' and Its Progenies." *Journal of the American Society for Horticultural Science* 132(6):869–75. doi: 10.21273/jashs.132.6.869.

Yamasaki, Atsu, Akira Kitajima, Norihiro Ohara, Mitsutoshi Tanaka, and Kojiro Hasegawa. 2009. "Characteristics of Arrested Seeds in Mukaku Kishu-Type Seedless Citrus." *Journal of the Japanese Society for Horticultural Science* 78(1):61–67. doi: 10.2503/jjshs1.78.61.

Yan, Liuhua, Shaowei Wei, Yaorong Wu, Ruolan Hu, Hongju Li, Weicai Yang, and Qi Xie. 2015. "High-Efficiency Genome Editing in Arabidopsis Using YAO Promoter-Driven CRISPR/Cas9 System." *Molecular Plant* 8(12):1820–23. doi: 10.1016/j.molp.2015.10.004.

Yang, L., C. J. Xu, G. B. Hu, and K. S. Chen. 2007. "Establishment of an Agrobacterium-Mediated Transformation System for Fortunella Crassifolia." *Biologia Plantarum* 51(3):541–45. doi: 10.1007/s10535-007-0117-6.

Yang, Li, Chunhua Hu, Na Li, Jiayin Zhang, Jiawen Yan, and Ziniu Deng. 2011. "Transformation of Sweet Orange [Citrus Sinensis (L.) Osbeck] with PthA-Nls for Acquiring Resistance to Citrus Canker Disease." *Plant Molecular Biology* 75(1):11–23. doi: 10.1007/s11103-010-9699-z.

Yang, Li, Wei Hu, Yuming Xie, Yi Li, and Ziniu Deng. 2016. "Factors

Affecting Agrobacterium-Mediated Transformation Efficiency of Kumquat Seedling Internodal Stem Segments." *Scientia Horticulturae* 209:105–12. doi: 10.1016/j.scienta.2016.06.018.

Yin, Xiaojia, Akshaya K. Biswal, Jacqueline Dionora, Kristel M. Perdigon, Christian P. Balahadia, Shamik Mazumdar, Caspar Chater, Hsiang Chun Lin, Robert A. Coe, Tobias Kretzschmar, Julie E. Gray, Paul W. Quick, and Anindya Bandyopadhyay. 2017. "CRISPR-Cas9 and CRISPR-Cpf1 Mediated Targeting of a Stomatal Developmental Gene EPFL9 in Rice." *Plant Cell Reports* 36(5):745–57. doi: 10.1007/s00299-017-2118-z.

Zaidi, Syed Shan e. Ali, Magdy M. Mahfouz, and Shahid Mansoor. 2017. "CRISPR-Cpf1: A New Tool for Plant Genome Editing." *Trends in Plant Science* 22(7):550–53. doi: 10.1016/j.tplants.2017.05.001.

Zetsche, Bernd, Jonathan S. Gootenberg, Omar O. Abudayyeh, Ian M. Slaymaker, Kira S. Makarova, Patrick Essletzbichler, Sara E. Volz, Julia Joung, John van der Oost, Aviv Regev, Eugene v. Koonin, and Feng Zhang. 2015. "Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System." *Cell* 163(3):759–71. doi: 10.1016/j.cell.2015.09.038.

Zhang, Fei, Chantal LeBlanc, Vivian F. Irish, and Yannick Jacob. 2017. "Rapid and Efficient CRISPR/Cas9 Gene Editing in Citrus Using the YAO Promoter." *Plant Cell Reports* 36(12):1883–87. doi: 10.1007/s00299-017-2202-4.

Zhang, Lili, Jiabao Huang, Shiqi Su, Xiaochun Wei, Lin Yang, Huanhuan Zhao, Jianqiang Yu, Jie Wang, Jiyun Hui, Shiya Hao, Shanshan Song, Yanyan Cao, Maoshuai Wang, Xiaowei Zhang, Yanyan Zhao, Zhiyong Wang, Weiqing Zeng, Hen Ming Wu, Yuxiang Yuan, Xiansheng Zhang, Alice Y. Cheung, and Qiaohong Duan. 2021. "FERONIA Receptor Kinase-Regulated Reactive Oxygen Species Mediate Self-Incompatibility in Brassica Rapa." *Current Biology* 31(14):3004-3016.e4. doi:

10.1016/j.cub.2021.04.060.

Zhang, Shuwei, Feng Ding, Xinhua He, and Cong Luo. 2015. "Characterization of the ' Xiangshui ' Lemon Transcriptome by de Novo Assembly to Discover Genes Associated with Self-incompatibility." *Mol Genet Genomics* 290:365–75. doi: 10.1007/s00438-014-0920-7.

Zhang, Xiao Hui, Louis Y. Tee, Xiao Gang Wang, Qun Shan Huang, and Shi Hua Yang. 2015. "Off-Target Effects in CRISPR/Cas9-Mediated Genome Engineering." *Molecular Therapy - Nucleic Acids* 4(11):e264. doi: 10.1038/mtna.2015.37.

Zhang, Xudong, Marta I. Francis, William O. Dawson, James H. Graham, Vladimir Orbović, Eric W. Triplett, and Zhonglin Mou. 2010. "Over-Expression of the Arabidopsis NPR1 Gene in Citrus Increases Resistance to Citrus Canker." *European Journal of Plant Pathology* 128(1):91–100. doi: 10.1007/s10658-010-9633-x.

Zhang, Yingxiao, Aimee A. Malzahn, Simon Sretenovic, and Yiping Qi. 2019. "The Emerging and Uncultivated Potential of CRISPR Technology in Plant Science." *Nature Plants* 5(8):778–94. doi: 10.1038/s41477-019-0461-5.

Zhang, Yong yan, Dong min Zhang, Yun Zhong, Xiao jun Chang, Min lun Hu, and Chun zhen Cheng. 2017. "A Simple and Efficient in Planta Transformation Method for Pommelo (Citrus Maxima) Using Agrobacterium Tumefaciens." *Scientia Horticulturae* 214:174–79. doi: 10.1016/j.scienta.2016.11.033.

Zhou, Huanbin, Bo Liu, Donald P. Weeks, Martin H. Spalding, and Bing Yang. 2014. "Large Chromosomal Deletions and Heritable Small Genetic Changes Induced by CRISPR/Cas9 in Rice." *Nucleic Acids Research* 42(17):10903–14. doi: 10.1093/nar/gku806.

Zhou, Yun, Xiaojuan Zhang, Xiaojun Kang, Xiangyu Zhao, Xiansheng Zhang, and Min Ni. 2009. "Short Hypocotyl Under

Blue1 Associates with Miniseed3 and Haiku2 Promoters in Vivo to Regulate Arabidopsis Seed Development." *Plant Cell* 21(1):106–17. doi: 10.1105/tpc.108.064972.

Zhu, Chenqiao, Xiongjie Zheng, Yue Huang, Junli Ye, Peng Chen, Chenglei Zhang, Fei Zhao, Zongzhou Xie, Siqi Zhang, Nan Wang, Hang Li, Lun Wang, Xiaomei Tang, Lijun Chai, Qiang Xu, and Xiuxin Deng. 2019. "Genome Sequencing and CRISPR/Cas9 Gene Editing of an Early Flowering Mini-Citrus (Fortunella Hindsii)." *Plant Biotechnology Journal* 17(11):2199–2210. doi: 10.1111/pbi.13132.

Zhuang, L., L. Rongyao, Q. Xuxiong, C. Maoxin, L. Tao, and C. Tengtu. 1994. "Study on Laser-Mutagenesis of Shatian Pumello." *Laser Biol*.

Zi-xing, Y., Z. Tai, X. Jian-kai, L. Zhi-da, H. Gui-bing, Z. Zhaoqi, J. Zuo-liang, C. Yu-cheng, C. Guo-liang, and C. Li-xiong. 2006. "Wuzishatangju, a New Mandarin Cultivar." *J Fruit Sci* 23:149–50.

Zou, Xiuping, Xueyou Jiang, Lanzhen Xu, Tiangang Lei, Aihong Peng, Yongrui He, Lixiao Yao, and Shanchun Chen. 2017. "Transgenic Citrus Expressing Synthesized Cecropin B Genes in the Phloem Exhibits Decreased Susceptibility to Huanglongbing." *Plant Molecular Biology* 93(4–5):341–53. doi: 10.1007/s11103-016-0565-5.

Zou, Xiuping, Aihong Peng, Lanzhen Xu, Xiaofeng Liu, Tiangang Lei, Lixiao Yao, Yongrui He, and Shanchun Chen. 2013. "Efficient Auto-Excision of a Selectable Marker Gene from Transgenic Citrus by Combining the Cre/LoxP System and Ipt Selection." *Plant Cell Reports* 32(10):1601–13. doi: 10.1007/s00299-013-1470-x.

Zou, Xiuping, Erling Song, Aihong Peng, Yongrui He, Lanzhen Xu, Tiangang Lei, Lixiao Yao, and Shanchun Chen. 2014. "Activation of Three Pathogen-Inducible Promoters in

Transgenic Citrus (Citrus Sinensis Osbeck) after Xanthomonas Axonopodis Pv. Citri Infection and Wounding." *Plant Cell, Tissue and Organ Culture* 117(1):85–98. doi: 10.1007/s11240-013-0423-y.

USDA (2020). Citrus Production by the USA States. https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail/?chartId=98417 [accessed on 30 October 2022]

PREHLB (2022). www.prehlb.eu [accessed on 30 October 2022]

Annexes

Articles in ISI journals

- Salonia F., Ciacciulli A., Pappalardo H. D., Poles L., Pindo M., Larger S., Caruso P., Caruso M., Licciardello C. "A dual sgRNA-directed CRISPR/Cas9 construct used for increasing lycopene accumulation in pigmented citrus fruits" - Frontiers in Plant Science, 4382. (publication in progress)– http://doi: 10.3389/fpls.2022.975917

- Di Pierro E.A., Franceschi P., Endrizzi I., Farneti B., Poles L., Masuero D., Khomenko I., Trenti F., Marrano A., Vrhovsek U., Gasperi F., Biasioli F., Guella G., Bianco L., Troggio M. "Valorization of Traditional Italian Walnut (Juglans regia L.) Production: Genetic, Nutritional and Sensory Characterization of Locally Grown Varieties in the Trentino Region" - Plants (2022), 11, 1986 - https://doi.org/10.3390/plants11151986

- Salonia F., Ciacciulli A., Poles L., Pappalardo H.D., La Malfa S., Licciardello C. "New Plant Breeding Techniques in Citrus for the Improvement of Important Agronomic Traits. A Review". Front. Plant Sci. 11:1234. (2020). http://doi.org/10.3389/fpls.2020.01234

- Poles L., Licciardello C., Distefano G., Nicolosi E., Gentile A., La Malfa S. "Recent Advances of In Vitro Culture for the Application of New Breeding Techniques in Citrus" – Plants 9(8), 938 (2020). https://doi.org/10.3390/plants9080938

- Poles L., Gentile A., Giuffrida A., Valentini L., Endrizzi E., Apreae E., Gasperi F., Distefano G., Artioli G., La Malfa S., Costa F., Lovatti L., Di Guardo M. "Role of fruit flesh cell morphology and MdPG1 allelotype in influencing juiciness and texture properties in apple" - Postharvest Biology and Technology,

(2020). https://doi.org/10.1016/j.postharvbio.2020.111161

- Padmarasu S., Sargent D.J., Patocchi A., Troggio M., Baldi P., Linsmith G., Poles L., Jänsch M., Kellerhals M., Tartarini S., Velasco R. "Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for Rvi12 (Vb) based apple scab resistance" - Molecular Breeding, (2018) 38:73. https://doi.org/10.1007/s11032-018-0825-y
- Di Guardo M., Bink M.C., Guerra W., Letschka Y., Lozano L., Busatto N., Poles L., Tadiello A., Bianco L., Visser R., Van de Weg E., Costa F. "Deciphering the genetic control of fruit texture in apple by multiple family-based analysis and genome-wide association" - J Exp Bot (2017) erx017. doi: https://doi.org/10.1093/jxb/erx017
- Chagné D., Kirk C., How N., Whitworth C., Fontic C., Reig G., Sawyer G., Rouse S., Poles L., Gardiner S., Kumar S., Espley R., Volz R., Troggio M, Iglesias I. "A functional genetic marker for apple red skin coloration across different environments" - Tree Genetics & Genomes (2016) 12: 67. https://doi.org/10.1007/s11295-016-1025-8
- Buti M., Poles L., Caset D., Magnago P., Fernandez Fernandez F., Colgan RJ., Velasco R., Sargent DJ. "Identification and validation of a QTL influencing bitter pit symptoms in apple (*Malus pumila*)" - Mol Breeding (2015) 35: 29. https://doi.org/10.1007/s11032-015-0258-9

Articles in conference proceedings or national journals

- Poles L., Ciacciulli A., Pappalardo H. D., Salonia F., Distefano G., Gentile A., Caruso M., Larger S., Pindo M., La Malfa S., Licciardello C. "Genome editing of IKU1 to obtain citrus seedless

fruits" Acta Horticolture submitted

- Poles L., Modica G., Camilleri G., Vecchio L., Sipione A., Arcidiacono F., La Malfa S., Continella A., Gentile A. "Stem cuttings and micropropagation protocols for Bitters rootstock large scale production" Acta Horticolture submitted
- Poles L., Micheletti D., Banchi E., Bianco L., Costa F., Lovatti L., Velasco R. "Genetic diversity investigation of the apple germplasm available at the Fondazione Edmund Mach" - Acta Horticolture. (2018) 1203, 155-164, https://doi.org/10.17660/ActaHortic.2018.1203.23
- Banchi E., Poles L., Magnago P., Pindo M., Costa F., Velasco R., Sargent D.J. "A cost-effective strategy for marker assisted selection (MAS) in apple (M. pumila Mill.): The experience from the fondazione edmund Mach programme for resistance and quality traits" - Acta Horticolture (2015), 1100: 85-89 - doi: 10.17660/ActaHortic.2015.1100.10 handle: http://hdl.handle.net/10449/22997
- Peressotti E., Dolzani C., Poles L., Banchi E., Stefanini M., Salamini F., Velasco R., Vezzulli S., Riaz S., Walker M.A., Reisch B.I., Van de Weg W.E., Bink M.C.A.M. "A first pedigree-based analysis (PBA) approach for the dissection of disease resistance traits in grapevine hybrids." - Acta Horticolture (2015). 1082, 113-12. doi: 10.17660/ActaHortic.2015.1082.15
- Peressotti E., Dolzani C., Poles L., Malfatti S., Velasco R., Vezzulli S. "High-throughput phenotyping for downy mildew resistance applied to marker assisted pre-breeding in grapevine." - VII GDPM Congress, 30th June – 4th July 2014, Vitoria/Gasteiz (Spain)
- Poles L. "Recupero e valorizzazione della Susina di Dro DOP". -

Frutticoltura (2018) n.8, 78-80

- Poles L., Lovatti L. "Breeding e selezione assistita: esperienze del CIF di Trento". - Frutticoltura (2018) n.10, 26-29

Posters

Poles L., Bennici S., Di Guardo M., Ereddia V., Licciardello G., La Malfa S., Gentile A. and Distefano G. "Integrated approaches to investigate the genetic bases of Citrus clementina self-incompatibility", LXVI SIGA Annual Congress, 6th-9th September 2022, Piacenza (Italy)

Poles L., Modica G., Camilleri G., Vecchio L., Sipione A., Arcidiacono F., La Malfa S., Continella A., Gentile A. "Stem cuttings and micropropagation protocols for Bitters rootstock large scale production", XIV International Citrus Congress, 6th-11th November 2022, Mersin (Turkey)

Poles L., Ciacciulli A., Pappalardo H. D., Salonia F., Distefano G., Gentile A., Caruso M., Larger S., Pindo M., La Malfa S., Licciardello C. "Genome editing of IKU1 to obtain Citrus seedless fruits", XIV International Citrus Congress, 6th-11th November 2022, Mersin (Turkey)

Poles L., Ciacciulli A., Pappalardo D.H., Salonia F., Distefano G., Gentile A., La Malfa S., Licciardello C."Genome editing applied to citrus to induce Seedlessness", LXIV SIGA Annual Congress, 14th-16th September 2021, Online (Winner of Poster Competition at LXIV SIGA Annual Congress)

Ciacciulli A., Poles L., Pappalardo H. D., Salonia, Licciardello C. "The effect of the light on the control of anthocyanin pigmentation of fruits, flowers, and shoots of Citrus and relatives", LXIV SIGA Annual Congress, 14th- 16th September 2021, Online (Winner of

Poster Competition at LXIV SIGA Annual Congress)

Salonia F., Ciacciulli A., Pappalardo H. D., Poles L., La Malfa S., Licciardello C. "Dati preliminari sull'utilizzo di due approcci di editing genico al fine di coniugare la presenza di licopene e di antocianine in frutti di arancio dolce", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Pappalardo H. D., Ciacciulli A., Poles L., Salonia F., Licciardello C. "Ruolo dello zucchero e del gelificante nella capacità rigenerativa di Citrus", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Pappalardo H. D., Ciacciulli A., Poles L., Salonia F., Licciardello C. "Valutazione di diversi tipi di espianto in Citrus per la trasformazione mediata da Agrobacterium tumefaciens", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Salonia F., Ciacciulli A., Amenta M., Pappalardo H. D., Poles L., Caruso M., Russo G., La Malfa S., Licciardello C. "Analisi quali-quantitativa di frutti di arancio, pompelmo e pummelo caratterizzati dalla presenza di licopene", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Poles L., Gentile A., Giuffrida A., Valentini L., Endrizzi E., Aprea E., Gasperi F., Distefano G., Artioli G., La Malfa S., Costa F., Lovatti L., Di Guardo M. "Ruolo della morfologia cellulare e del gene MdPG1 nel determinare le caratteristiche di texture e di succosità in melo", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Poles L., Ciacciulli A., Salonia F., Pappalardo H. D., Distefano G., Gentile A., La Malfa S., Licciardello C. "Genome editing applicato agli agrumi per l'induzione di apirenia nei mandarini", XIII Giornate Scientifiche SOI, 22nd -23rd June 2021, Catania (Italy)

Poles L., Gentile A., Giuffrida A., Valentini L., Endrizzi E., Aprea E.,

Gasperi F., Distefano G., Artioli G., La Malfa S., Costa F., Lovatti L., Di Guardo M. "Role of fruit flesh cell morphology and MdPG1 allelotype in influencing juiciness and texture properties in apple" - 7th International Horticulture research Conference 1st- 30th July 2020, Online (Winner of the Third Prize in the Poster Competition, 279 participants)

Ciacciulli A., Pappalardo H.D., Salonia F., Poles L., Arlotta C., Caruso M., Licciardello C. "The base editing approach to enrich orange fruit in nutraceuticals" - 7th International Horticulture research Conference 1st- 30th July 2020, Online

Pappalardo H.D., Ciacciulli A., Salonia F., Poles L., Licciardello C. "Preliminary results on the regeneration and transformation of citrus varieties addressed to produce fruits with improved traits" - 7th International Horticulture research Conference 1st- 30th July 2020, Online

Salonia F., Ciacciulli A., Poles L., Pappalardo H.D., Arlotta C., La Malfa S., Licciardello C. "Target and base editing approaches to induce lycopene accumulation in anthocyanin-rich sweet oranges" - 7th International Horticulture research Conference 1st- 30th July 2020, Online

Poles L., Lovatti L. "Novel apple cultivars in Trentino, the case if the red-fleshed apple" - 10th International Workshop on Anthocyanins (IWA), 9th-11th September 2019, San Michele all'Adige TN (Italy)

Ciacciulli A., Salonia F., Poles L., Pappalardo H.D., Caruso M., Caruso P., Russo M.P., Catalano C., Russo G., Licciardello C. "A cisgenesis and target editing approach to improve health properties of citrus fruits combining anthocyanins and lycopene" - 10th International Workshop on Anthocyanins (IWA), 9th-11th September 2019, San Michele all'Adige TN (Italy)

Catalano C., Salonia F., Ciacciulli A., Russo M.P., Poles L., Caruso

P., Distefano G., Caruso M., Russo G., Licciardello C. "A gene-specific approach illustrates the anthocyanins tissue-specificity on Citrus species and related genera" - 10th International Workshop on Anthocyanins (IWA), 9th-11th September 2019, San Michele all'Adige TN (Italy)

Poles L., Stefani E., Larger S., Lovatti L. "Genetic identification of cv 'Susina di Dro' (Prunus domestica L.) ecotype using microsatellites" - 9th International Rosaceae Genomics Conference (RGC9), 26th-30th June 2018, Nanjing (China)

Poles L., Calzà M., Chagné D., Padmarasu S., Kòirk C., Troggio M., Magnago P., Velasco R. "Marker Assisted Selection (MAS) in apple: case studies for red skin coloration and Rvi12 (Vb) scab resistance" - 8th International Rosaceae Genomics Conference (RGC8), 21st-23rd June 2016, Angers (France)

Peressotti E., Poles L., Dolzani C., Arrigoni E., Van de Weg E., Bink M., Velasco R., Vezzulli S. "Downy mildew resistance QTL identification in multiple inter-specific populations of grapevine: a Pedigree-Based Analysis (PBA) approach". X international symposium on grapevine physiology and botechnology – 13rd-18th June 2016, Verona (Italy)

Peressotti E., Dolzani C., Banchi E., Poles L., Buonassisi D., Migliaro D., Arrigoni E., Vecchione A., Zulini L., Van De Weg W.E., Bink M.C.A.M., Stefanini M., Velasco R., Vezzulli S. "Innovative strategies towards marker-assisted (pre-)breeding for disease resistance in grapevine"- SIBV-SIGA Congress, 8th-11th September 2015, Milano (Italy)

Peressotti E., Dolzani C., Poles L., Malfatti S., Velasco R., Vezzulli S. "Highthroughput phenotyping for downy mildew resistance applied to marker assisted pre-breeding in grapevine" - VII GDPM Congress, 30th June – 4th July 2014, Vitoria/Gasteiz (Spain)

Buti M., Caset D., Poles L., Magnago P., Chagne D., Kumar S., Velasco R., Sargent DJ. "Mapping and genetic dissection of QTL influencing bitter pit symptoms in apple (Malus×domestica)" - 7th International Rosaceae Genomics Conference RGC7, 24th -26th June 2014, Seattle – Washington (USA)

Banchi E., Poles L., Magnago P., Pindo M., Costa F., Velasco R., Sargent DJ. "A cost-effective strategy for marker assisted selection (MAS) in apple (M. Pumila Mill.): the experience from the Fondazione Edmund Mach programme for resistance e quality traits" – III International Symposium on Molecolar Markers in Horticulture, 25th-27th September 2013, Riva del Garda TN (Italy)

Oral presentation

- Bennici S., Poles L., Di Guardo M., Percival-Alwyn L., Licciardello C., Distefano G., Salonia F., Caccamo M., Gentile A., La Malfa S. 'Next-generation sequencing technologies reveal novel candidate genes responsible for self-incompatibility in Citrus clementine' - XIV International Citrus Congress, 6th-11th November 2022, Mersin (Turkey) [Presenting author]
- Salonia F, Ciacciulli A, Pappalardo HD, Poles L, La Malfa S, Licciardello C. "Genome editing approaches to induce lycopene accumulation in anthocyanin-rich sweet orange varieties", LXIV SIGA Annual Congress, 14th- 16th September 2021, Online [Presenting author]
- Salonia F., Ciacciulli A., Pappalardo H. D., Poles L., Arlotta C., Caruso P., Russo M. P., Russo G., Caruso M., Licciardello C. "Genetic improvement of Citrus fruits rich in anthocyanins and lycopene through modern biotechnology approaches", Plant and Animal Genome XXVIII Conference, 11th-15th January 2020, San Diego (USA). [Presenting author]

- Poles L, Micheletti D, Banchi E, Bianco L, Costa F, Lovatti L, Velasco R. "Genetic diversity investigation of the apple germplasm available at the Fondazione Edmund Mach" - IV International Symposium on Molecular Markers in Horticulture, 7th -10th March 2017, Napier (New Zealand). [Presenting author]

Attendance to seminars and courses

- '*Managing biological data with R (40hrs)*' 22nd February - 4th March2022 – Department of Agricultural, Food and environmental (University of Catania)

- *'Tecnologie di Evoluzione Assistita: CRISPR/Cas9 in piante agrarie' theoretical and practical course organized by SIGA (Italian Society of Agricultural Genetics)*, 31st August-3rd September 2021,Verona (Italy)

- *'CAD, GIS and ICT for Participatory Mapping & Disseminating Science Course',* 8th-18th February 2021 - Department of Agricultural, Food and environmental (University of Catania)

- *'Industrial Biotechnology' an online course (12 hrs)* authorized by University of Manchester and offered through Coursera

-*'Introduction to Introduction to Genomic Technologies'* an online course (6 hrs) authorized by Johns Hopkins University and offered through Coursera

- *'Plant Epigenetics: Basics, Applications and* Methodologies' Online Training School organized by EPI-CATCH COST Action, 28th - 30th June 2021

-*'Contribution of RNAi to sustainable agriculture, food, safety and security'* group of webinars organized by iPlanta COST Action 1st, 7th and 14th December 2020

-*'Aspetti fisiologici e genetici della biologia riproduttiva degli*

agrumi' – Dott. Stefania Bennici , webinar 31st May 2021

- *'Sapere tradizionale e genomica: un connubio possibile? Suggestioni dall'Etiopia'* Prof. Mario Enrico Pè, webinar 28th May 2021

- *'Biometry and data analysis'* 18th-22nd November (40 hrs) – Department of Agricultural, Food and environmental (University of Catania)

- *'Academic Literacy'* an online course (22 hrs) authorized by Moscow Institute of Physics and Technology and offered through Coursera

- *'Programming for Everybody (Getting Started with Python)'* an online course (19 hrs) authorized by University of Michigan and offered through Coursera

- *'Data Science Math Skills' an online course (13 hrs)* authorized by Duke University and offered through Coursera

- *2nd Cost IPlanta Training School (COST ACTION CA15223) "RNAi application; from lab to field",* 27th -28th September 2018, Rothamsted Research, Harpenden (UK)

Attendance to conferences

- 4th Joint Meeting of Agriculture-oriented PhD Programs UniCT, UniFG and UniUD, 3rd -7th October 2022, Paluzza UD (Italy)
- LXVI SIGA Annual Congress, 6th-9th September 2022, Piacenza (Italy)
- LXIV SIGA Annual Congress, 14th- 16th September 2021 (Online)
- XIII Giornate Scientifiche SOI – "I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana", 22nd -23rd June 2021, Catania (Italy)
- Web Workshop 'Young Scientists for Plant Health' organized by SIGA Young group, 16th December 2020 (Online)

- 2nd Joint meeting of Agriculture-oriented PhD programs UniCT, UniFG and UniUD, 14th -16th September 2020, Catania (Italy)
- SIGA Young Web Meeting, 7th July 2020 (Online)
- 7th International Horticulture research Conference 1st- 30th July 2020 (Online)
- 10th International Workshop on Anthocyanins (IWA), 9th -11th September 2019, San Michele all'Adige TN (Italy)
- Final Conference ORPRAMed Project: Risk assessment of introduction of *Xanthomonas citri* subsp. Citri through commercial trade of ornamental rutaceous plants in the Mediterranean basin – Giarre, CT (Italy), 3rd June 2019
- WG2 Meeting on RNAi applications (COST ACTION CA15223), 26th September 2018, Rothamsted Research, Harpenden (UK)
- 9th International Rosaceae Genomics Conference (RGC9), 26th - 30th June 2018, Nanjing (China)
- IV International Symposium on Molecular Markers in Horticulture, 7th -10th March 2017, Napier (New Zealand)
- LX SIGA annual congress- Società Italiana di Genetica Agraria, 13rd-16th September 2016, Catania (Italy)
- 8th International Rosaceae Genomics Conference (RGC8), 21st-23rd June 2016, Angers (France)
- Congresso SIBV-SIGA - Società Italiana di Biologia Vegetale e Genetica Agraria, 8th -11th September 2015, Milano (Italy)
- III International Symposium on Molecular Markers in Horticulture, 25th -27th September 2013, Riva del Garda TN (Italy)

Previous working experiences

From July 2021 to January 2023 - Department of Agricultural, Food and Environmental (Di3A) University of Catania, Italy
Holder of Assegno di ricerca (Project VIVAiCITRUS - Introduzione nel sistema vivaistico di nuovi portinnesti di elevato valore

agronomico e di protocolli innovativi di propagazione per l'agrumicoltura siciliana. D.D.S. 2476/2020 del 28/04/2020, CUP G68H20000300009)

Brief project description: Large scale micropropagation of citrus CTV-resistant rootstocks, C22 (Bitters), C54 (Carpenter) and C57 (Furr); evaluation of their performance in combination with both sweet oranges and ornamental citrus, in presence of mycorrhizal symbiosis or in water stress

From February 2019 to July 2021 - Research Centre for Olive, Citrus and Tree (CREA-OFA), Acireale CT, Italy

Holder of Assegno di ricerca (Project CITRUS BIOTECH - citrus improvement by sustainable biotechnologies. L. 28/12/2015, n.208, art. 1, cc. 665-667)

Brief project description: Identification of candidate genes involved in sterility mechanism (e.g. self-incompatibility) in mandarins and mandarins-like to be used in CRISPR/Cas9 system for seedless fruit production.

- morphological and histological analysis of citrus mutant flower apparatus
- Development and assembly of CRISPR/Cas9 constructs
- Citrus stable transformation mediated by Agrobacterium tumefaciens
- Optimization of regeneration protocol for citrus commercial varieties

From April 2015 to December 2018 - Innovation Fruit Consortium (CIF), Trento, Italy

Technician, project 'AppleBerry' (Autonomous Province of Trento)

Brief project description:

- Marker Assisted Selection (MAS), screening and developing of novel markers for apple scab resistance and for other traits of agronomical interest to improve Edmund Mach Foundation (FEM) apple breeding program.
- Fingerprint analysis on different fruit species ranging from apple to plum and walnut; characterization of the whole FEM apple germplasm collection and curation of a web interface

database collecting all genetic profiles
- Field and trial evaluation in apple experimental orchards
- Coordinator of WPs 2, 5, 6 of the project entitled "Frutticoltura alternativa sostenibile" (FAS -CUP C45B18000120008) a rural development program financed by the European Agricultural Fund for Rural Development (EAFRD).

From April 2013 to April 2014 - Edmund Mach Foundation (FEM), San Michele all'Adige, TN (Italy)
Research fellowship

Brief project description:

- MAS, screening of apple seedlings using SSR and SCAR markers on several disease resistance and fruit quality traits.
- QTL analysis on grape using pedigree-based analysis (PBA) approach: identification of the favorable alleles involved in downy mildew resistance.

From November 2012 to April 2013 - Research Centre for Viticulture and Enology (CREA-VIT), Conegliano, TV (Italy)
Grant scholarship ("Tirocinio Formativo"):

Brief project description:

- Diagnostic and biological analysis (PCR, ELISA test) for the identification of pathogens (phytoplasmosis, bacteria and fungi) on grape.
- Analysis for the traceability of Agrobacterium vitis, the predominant species causing grape crown gall.
- Grapes fingerprint analysis with SSRs markers