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LA RECHERCHE AGRONOMIQUE POUR LE DÉVELOPPEMENT

Assessment of citrus reproductive biology for seedless mandarin production and its interaction with temperature

PhD THESIS PRESENTED BY

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Abstract

Since the origin of citrus, ancestral natural hybridizations, thousands of years of cultivation and admixture phenomenon have resulted in the complexity of the reproductive biology of today's cultivated citrus, which often involves female and male sterility, self-incompatibility, parthenocarpy and apomixis in different degrees. The global objective of this PhD was to increase our knowledge on different reproductive aspects that are crucial for citrus breeding and propagation and their interaction with environmental conditions.

Temperature in the progamic phase is critical for the success of plant sexual reproduction. Pollen grain germination, stigmatic receptivity and pollen tube growth are the main components of this phase. The analysis of temperature effect on the progamic phase of citrus was our first objective. We developed an innovative method based on the microscopic observation of transversal slices from pollinated pistils collected daily, allowing a more comprehensive analysis of the pollen tube growth kinetics and dynamics along the pistil. Using three compatible crosses within the *Citrus* genus, we evaluated, with this method, the effect of four temperature regimes in every process during the progamic phase. Warm temperatures reduced the time needed by pollen tubes to reach the ovules and also accelerate pistil degeneration while cold temperatures produced the opposite effects. However, pollen tubes were able to reach the ovules in all crosses studied. Interestingly, we observed for the first time in citrus both pollen germination and pollen tube growth at 10ºC.

Mandarins account for 24% of total citrus production and seedlessness is a crucial quality criterion for the mandarin fresh fruit market. Parthenocarpic ability (PA) is the key for seedless fruit production when it is coupled with self-incompatibility (SI) or sterility. The second objective of this PhD dissertation was to evaluate PA and SI for mandarin varieties with relevant characteristics as parents for seedless mandarin breeding. For this purpose, we developed an efficient protocol based on emasculation, hand self-pollination and hand cross-pollination. Pollen performance was analysed by histological observations, together with fruit set and seed production. Six different behaviors were observed among the nine varieties analysed. 'Clemenules' clementine and 'Moncada' mandarins were strictly self-incompatible with facultative and vegetative parthenocarpy; 'Imperial' mandarin and 'Ellendale' tangor displayed no strict self-incompatibility associated with facultative and vegetative parthenocarpy; 'Fortune' mandarin was self-incompatible with facultative and stimulative parthenocarpy; 'Campeona' and 'Salteñita' mandarins were self-compatible with vegetative parthenocarpy; 'Serafines' satsuma was associated with male sterility together with facultative and vegetative parthenocarpy; 'Monreal' clementine was self-compatible and non-parthenocarpic.

Beyond the critical importance of SI for seedless fruit production, SI is an obstacle for breeding programs based on hybridization as it reduces crossing possibilities. The third objective of this PhD dissertation was to compare the efficiency of the SI reaction breakdown by three factors previously identified in other plant species: temperature stress, bud pollination and polyploidization. The SI phenotype was characterized for two self-incompatible varieties 'Fortune' and 'Clemenules' by a histological study of pollen tube growth and ovule fertilization. A molecular analysis with SSRs and SNPs markers allowed us to demonstrate that all the obtained plants were zygotic from selfing. The

three methods were successful in recovering selfed plants, and bud pollination was the most efficient approach. Chromosome doubling was also efficient, but involved developing tetraploid plants. Cold temperature stress allowed us to obtain a few diploid selfed plants. However, this method proved much more complex to apply than bud pollination in specific breeding programs.

Marker-assisted selection (MAS) allows the selection of target genotypes at the seedling stage, making it a powerful tool, especially in tree species with long juvenile period, such as citrus. The 4th objective of this PhD dissertation focuses on the development of SNPs markers associated with polyembryony and male sterility. Polyembryony (a form of apomixis) is a desired trait for rootstock production, while monoembryony is researched for parents of sexual breeding projects. Male sterility is useful coupled with parthenocarpy for seedless fruit production and satsuma-derived nucleo-cytoplasmic male sterility (CMS) is the most prevalent in mandarins. In Chapter 4, a segregating offspring derived from 'Kiyomi' (a monoembryonic and CMS variety) × 'Murcott' (a polyembryonic and male fertile variety) was genotyped by genotyping-by-sequencing (GBS). It was also phenotyped for polyembryony and male sterility by the evaluation of the number of pollen grains per anther (NPGA) and apparent pollen fertility (APF). Through genetic association studies, we identified a genomic region in linkage group 1 associated with polyembryony and a genomic region on linkage group 8 associated with NPGA. SNP markers closely linked with implied genes were developed for the two genomic regions.

Overall, this PhD dissertation provides new insights into citrus reproductive biology and the influence of temperature on it. The knowledge generated will allow to implement breeding programs more efficiently, particularly those aimed at obtaining seedless varieties. This knowledge can eventually be used to respond to some of the most pressing challenges posed by the current global warming scenario.

Resumen

Desde el origen de los cítricos, hibridaciones naturales ancestrales, espontáneas o de cultivo a lo largo de miles de años, han ocasionado fenómenos de mezcla que han determinado la complejidad de la biología reproductiva de los cítricos cultivados hoy en día. El resultado final implica esterilidad femenina y masculina, autoincompatibilidad, partenocarpia y apomixis en diferentes grados. El objetivo global de esta tesis doctoral es aumentar el conocimiento sobre diferentes aspectos reproductivos que son cruciales para la mejora y propagación de los cítricos y su interacción con las condiciones ambientales.

La temperatura en la fase progámica es fundamental para el éxito de la reproducción sexual de las plantas. La germinación del grano de polen, la receptividad estigmática y el crecimiento del tubo polínico son los principales componentes de esta fase.

El primer objetivo de esta tesis doctoral ha sido el análisis del efecto de la temperatura en la fase progámica de los cítricos. Desarrollamos un método innovador basado en la observación microscópica de cortes transversales de pistilos polinizados recolectados diariamente, lo que permite un análisis más completo de la cinética y dinámica de crecimiento del tubo polínico a lo largo del pistilo. Usando tres cruces compatibles dentro del género Citrus, evaluamos, con este método, el efecto de cuatro regímenes de temperatura en cada proceso durante la fase progámica. Hemos comprobado que las temperaturas altas redujeron el tiempo que necesitaban los tubos polínicos para llegar a los óvulos y también aceleraron la degeneración del pistilo, mientras que las bajas temperaturas produjeron los efectos opuestos. Sin embargo, los tubos polínicos pudieron llegar a los óvulos en todos los cruces estudiados. Es interesante señalar que observamos por primera vez en cítricos tanto la germinación del polen como el crecimiento del tubo polínico a 10ºC.

Las mandarinas representan el 24% de la producción total de cítricos y la ausencia de semillas se valora como criterio de calidad en el mercado de la fruta fresca. La aptitud partenocárpica (AP) es la clave para la producción de frutos sin semillas cuando se combina con la autoincompatibilidad (AI) o la esterilidad. El segundo objetivo de esta tesis doctoral fue evaluar AP y AI para variedades de mandarina con características relevantes como progenitores para la mejora genética de mandarinas sin semillas. Para ello, desarrollamos un protocolo eficiente basado en la emasculación, la autopolinización manual y la polinización cruzada manual. El rendimiento del polen se analizó mediante observaciones histológicas, junto con el cuajado de frutos y la producción de semillas. Se observaron seis comportamientos diferentes entre las nueve variedades analizadas. Las mandarinas 'Clemenules' y 'Moncada' son estrictamente autoincompatibles con partenocarpia facultativa y vegetativa; El mandarino 'Imperial' y el tangor 'Ellendale' mostraron una autoincompatibilidad parcial y se clasificaron igualmente como variedades con partenocarpia facultativa y vegetativa; El mandarino 'Fortune' es autoincompatible y se le asignó partenocarpia facultativa y estimulativa; Las mandarinas 'Campeona' y 'Salteñita' son autocompatibles con partenocarpia vegetativa; satsuma 'Serafines' posee esterilidad masculina y se clasificó como partenocarpica facultativa y vegetative. Finálmente, la clementina 'Monreal' es autocompatible y no partenocárpica.

Subrayar que más allá de la importancia crítica de la AI para la producción de frutos sin semillas, la AI es un obstáculo para los programas de mejora genética basados en hibridación, ya que reduce las posibilidades de cruzamiento. Esta circunstancia motivó el planteamiento del tercer objetivo de esta tesis doctoral. Dicho objetivo fue comparar la eficiencia de la ruptura de la reacción de AI por tres factores previamente identificados en otras especies: estrés por temperatura, polinización de yemas florales y poliploidización. El fenotipo AI se caracterizó para dos variedades autoincompatibles 'Fortune' y 'Clemenules' mediante el estudio histológico del crecimiento de los tubos polínicos y mediante la obtención de frutos con o sin semillas. El análisis con marcadores moleculares SSRs y SNPs nos permitió demostrar que todas las plantas obtenidas eran de origen cigótico y producidas por autofecundación. Los tres métodos tuvieron éxito en la obtención de plantas autofecundadas, y la polinización de yemas florals resultó ser el método más eficiente. La duplicación de cromosomas también fue eficiente, pero implicó el desarrollo de plantas tetraploides. El estrés por bajas temperaturas nos permitió obtener unas pocas plantas autofecundadas diploides. Además, este método resulta mucho más complejo de aplicar que la polinización de yemas florales en programas de mejora genética específicos.

La selección asistida por marcadores (MAS) permite la selección de genotipos en la etapa de plántula, lo que la convierte en una herramienta poderosa, especialmente en especies de árboles con un largo período juvenil, como es el caso de los cítricos. El cuarto objetivo de esta tesis doctoral se centró por tanto en el desarrollo de marcadores SNPs asociados a poliembrionía y esterilidad masculina. La poliembrionía (una forma de apomixis) es un carácter deseado para la producción de portainjertos, mientras que la monoembrionía es interesante para parentales femeninos en proyectos de reproducción sexual. La esterilidad masculina es útil junto con la partenocarpia para la producción de frutos sin semillas, siendo la esterilidad masculina nucleocitoplasmática (CMS) derivada de satsuma la más frecuente en las mandarinas. En el Capítulo 4, una descendencia segregante derivada de 'Kiyomi' (una variedad monoembriónica y CMS) × 'Murcott' (una variedad poliembriónica y con fertilidad masculina) fue genotipada por GBS. También se fenotipó la poliembrionía y la esterilidad masculina. Esta última mediante la evaluación del número de granos de polen por antera (NPGA) y la fertilidad aparente del polen (APF). Mediante estudios exhaustivos de asociación genética, identificamos una región genómica en el grupo de ligamiento 1 asociado con poliembrionía y una región genómica en el grupo de ligamiento 8 asociado con NPG. Se desarrollaron marcadores SNP cercanos a genes que tienen funciones relacionadas con la esterilidad masculina y con la poliembrionia para las dos regiones genómicas.

Esta tesis doctoral proporciona nuevos conocimientos sobre la biología reproductiva de los cítricos y la influencia de la temperatura en ella. El conocimiento generado permitirá implementar de manera más eficiente los programas de mejora genética, particularmente aquellos destinados a la obtención de variedades sin semillas. Además, este conocimiento podrá utilizarse para ayudar en la respuesta a algunos de los desafíos más apremiantes que plantea el escenario actual de calentamiento global.

Resum

Des de que es van originar els cítrics, hibridacions naturals ancestrals, espontànies o de conreu, al llarg de milers d'anys, han ocasionat fenòmens de mescla que han determinat la complexitat de la biologia reproductiva dels cítrics actualment conreats. El resultat final implica sovint esterilitat femenina i masculina, autoincompatibilitat, partenocarpia i apomixi en diferents graus. L'objectiu global d'aquesta tesi va ser augmentar el coneixement de diferents aspectes reproductius dels cítrics —especialment en la seua interacció amb les condicions ambientals— crucials per a la seua millora genética, reproducció i propagació.

La temperatura en la fase progàmica és fonamental per a l'èxit de la reproducció sexual de les plantes. La germinació del gra de pol·len, la receptivitat estigmàtica i el creixement del tub pol·línic són els components principals d'aquesta fase.

El nostre primer objectiu ha estat l'anàlisi de l'efecte de la temperatura en la fase progàmica dels cítrics. Hem desenvolupat un mètode innovador basat en l'observació microscòpica de rodanxes transversals de pistils pol·linitzats recollits diàriament, que permet una anàlisi més completa de la cinètica i la dinàmica de creixement del tub pol·línic al llarg del pistil. Mitjançant tres creus compatibles dins del gènere Citrus, vam avaluar, amb aquest mètode, l'efecte de quatre règims de temperatura en cada procés durant la fase progàmica. Hem comprovat que les temperatures càlides redueixen el temps necessari per arribar als òvuls pels tubs de pol·len i que també acceleren la degeneració del pistil; mentre les temperatures fredes van produeixen els efectes contraris. No obstant això, els tubs pol·línics van poder arribar als òvuls en totes les creus estudiades. Curiosament, vam observar per primera vegada als cítrics tant la germinació del pol·len com el creixement del tub pol·línic a 10ºC.

Les mandarins representen el 24% de la producció total de cítrics i l'absència de llavors és un criteri de qualitat crucial en el mercat de la fruita fresca. La capacitat partenocàrpica (PA) és la clau per a la producció de fruites sense llavors quan s'acobla a l'autoincompatibilitat (SI) o l'esterilitat. El segon objectiu d'aquesta tesi doctoral va ser avaluar PA i SI per a varietats de mandarines amb característiques rellevants com a progenitors per a la millora genètica de mandarines sense llavors. Amb aquesta finalitat, hem desenvolupat un protocol eficient basat en l'emasculació, l'autopol·linització manual i la pol·linització creuada manual. El rendiment del pol·len es va analitzar mitjançant observacions histològiques, juntament amb la producció de fruites i llavors. Es van observar sis comportaments diferents entre les nou varietats analitzades. La clementina 'Clemenules' i la mandarina 'Moncada' són estrictament autoincompatibles amb partenocarpia facultativa i vegetativa; El mandarí 'Imperial' i el tangor 'Ellendale' van mostrar una autoincompatibilitat parcial i es van classificar igualment com a varietats amb partenocarpia facultativa i vegetativa; El mandarí 'Fortune' és autoincompatible i se li va assignar partenocàrpia facultativa i estimulativa; Les mandarines 'Campeona' i 'Salteñita' són autocompatibles amb partenocarpia vegetativa; la satsuma 'Serafines' posseeix esterilitat masculina i es va classificar com a partenocarpica facultativa i vegetative. Finalment, la clementina 'Monreal' és autocompatible i no partenocàrpica.

Subratllar que, enllà de la importància crítica del SI en la producció de fruites sense llavors, el SI és alhora un obstacle per als programes de millora basats en la hibridació, ja que redueix les possibilitats d'encreuament. Aquesta circumstància va motivar el

plantejament del tercer objectiu d'aquesta tesi doctoral. Aquest objectiu va ser comparar l'eficiència de la ruptura de la reacció SI per tres factors identificats prèviament en altres espècies: estrès per temperatura, pol·linització de rovells florals i poliploidització. El fenotip SI es va caracteritzar per a dues varietats autoincompatibles 'Fortune' i 'Clemenules' mitjançant un estudi histològic del creixement del tub pol·línic i la l'obtenció de fruits amb o sense llavors. L'anàlisi amb marcadors moleculars SSR i SNP ens va permetre demostrar que totes les plantes obtingudes eren d'origen zigòtic i produïdes per autofecundació. Els tres mètodes van tenir èxit en l'obtenció de plantes autofecundades, i la pol·linització de rovells florals va resultar ser el mètode més eficient. La duplicació de cromosomes també va ser eficient, però va implicar el desenvolupament de plantes tetraploides. L'estrès per baixes temperatures ens va permetre obtenir unes poques plantes autofecundades diploides. A més, aquest mètode resulta molt més complex d'aplicar que la pol·linització de rovells florals en programes de millora genètica específics.

La selecció assistida per marcadors (MAS) en l'etapa de plàntules permet la selecció de genotips diana, la qual cosa la converteix en una eina potent, especialment en espècies d'arbres amb període juvenil llarg, com els cítrics. El quart objectiu d'aquesta tesi doctoral se centra en el desenvolupament de marcadors SNPs associats a la poliembriona i l'esterilitat masculina. La poliembrionía (una forma d'apomixi) és un tret desitjat per a la producció de portaempelt, mentre que la monoembrionia es interessant per als progenitors femenins projectes de reproducció sexual. L'esterilitat masculina és útil juntament amb la partenocàrpia per a la producció de fruites sense llavors i l'esterilitat masculina nucleocitoplasmàtica (CMS) derivada de satsuma és la més freqüent en les mandarines. Al capítol 4, una descendència segregant derivada de 'Kiyomi' (una varietat monoembrionària i CMS) × 'Murcott' (una varietat poliembrionària i fèrtil masculina) va ser genotipada per GBS. També es va fenotipar per a la poliembrionia i l'esterilitat masculina mitjançant l'avaluació del nombre de grans de pol·len per antera (NPGA) i la fertilitat aparent de pol·len (APF). Estudis exhaustius d'associació genètica, vam identificar una regió genòmica al grup de lligament 1 associada a la poliembrionia i una regió genòmica al grup de lligament 8 associada amb NPG. Es van desenvolupar marcadors SNP estretament relacionats amb gens implicats per a les dues regions genòmiques.

Aquesta tesi doctoral proporciona nous coneixements sobre la biologia reproductiva dels cítrics i la influència de la temperatura sobre ella. El coneixement generat permetrà implementar de manera més eficient els programes de millora genètica, especialment aquells destinats a l'obtenció de varietats sense llavors. A més, aquest coneixement es podrà utilitzar en la complexa resposta a alguns dels reptes més urgents que planteja l'escenari actual d'escalfament global.

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Introduction index

Introduction

1. Citrus origin, evolution, and taxonomy

Citrus L. genus belongs to the *Rutaceae* family. This family encompasses six subfamilies, being *Aurantioideae* divided into two tribes, *Clauseneae* and *Citreae*. The last one is also divided into 3 subtribes, with the *Citrinae* subtribe comprising according to Swingle and Reece (1967) taxonomy the *Eremocitrus*, *Microcitrus*, *Clymenia*, *Fortunella*, *Poncirus* and *Citrus* genera. The most important citrus cultivars and rootstocks belong to the *Fortunella*, *Microcitrus*, *Poncirus* and *Citrus* genera. Ancestral natural hybridizations and thousands of years of cultivation, which included artificially crossed or selected varieties by humans, sexual compatibility between species and apomixis of many cultivars have given rise to a complex mixture of botanical characters that are very difficult to classify. Therefore, citrus phylogeny, genealogy, and taxonomy have been particularly controversial.

In the second half of the twentieth century (Swingle and Reece, 1967) and Tanaka (1977) proposed two important classifications. Swingle system recognizes 16 *Citrus* species while Tanaka system recognizes 162 species. The major discrepancies between these two classification systems are in mandarins: Swingle and Reece include three, whereas Tanaka includes 36. Both Swingle's and Tanaka's classifications are still widely used by the citrus scientific community, yet the definition and classification of the *Citrus* genus remain an issue open to discussion by taxonomists.

Indeed, two elements disagree with the circumscription of the genus *Citrus* as proposed by Swingle and Reece (1967). The first is the demonstrated sexual compatibility of the different species of the other "true citrus" genera with the species of *Citrus* as defined by Swingle and Reece (1967). Many fertile hybrids have been produced between *P. trifoliata* and several *Citrus* species and *Poncirus* is a very important genetic resource for rootstock breeding by "intergeneric" hybridization. Several hybrids have also been created between *Citrus* and *Microcitrus* species, *Eremocitrus glauca* or *Clymenia polyandra*. The second discordant element is the nonmonophyly of the chloroplast genomes of the Swingle *Citrus* species, revealed first by Bayer et al. (2009) and more recently from whole genome sequencing (WGS) resequencing data by Carbonell-Caballero et al. (2015). Moreover the "true citrus" group plus *Oxanthera* form a strongly supported clade, highly differentiated from the other Citreae genera (Bayer et al., 2009). These elements, and the very high synteny and collinearity observed between genetic maps of *Poncirus* and *Citrus* species (Bernet et al., 2010; C. Chen et al., 2008) and cytogenetic maps (da Costa Silva et al., 2015) strongly support the proposal of Mabberley (2004, 1998) and Zhang and Mabberley (2008) to integrate *Poncirus*, *Fortunella*, *Microcitrus*, *Eremocitrus* and *Clymenia* into the genus *Citrus*. According to the results of Bayer et al. (2009), *Oxanthera* may also be integrated into the *Citrus* genus (Ollitrault et al., 2020a). However, some other aspects regarding the specific subdivisions delimitations within the *Citrus* genus and the origin of admixture types proposed by Mabberley (2004, 1997) are not in agreement with recent molecular studies and Mabberley classification system is still incomplete (Ollitrault et al., 2020a).

The advent of new phylogenetic and phylogenomic data has revealed the origins and admixtures of modern cultivars and wild types. Considering the implication of reticulate evolution, partial apomixis and asexual diversification, Ollitrault et al. (2020a) propose a trinomial concept for citrus classification. For admixture taxa, the species rank is defined by their phylogenomic constitution. The variety rank is defined by the old independent

reticulation events from which groups of cultivars were differentiated by asexual mechanisms. These authors also point out that the available data are limited and further WGS studies are needed to establish a definitive classification of the *Citrus* genus. In this regard, knowledge of the reproductive biology of citrus is crucial.

Therefore, the Swingle and Reece (1967) classification of the true citrus group still remains popular in the citrus scientific community and we keep this classification system for the present manuscript.

Thanks to biochemical, morphological, and genomic analyses, nowadays there is a consensus that most of the cultivated citrus species arose from natural hybridizations events between only four ancestral species: *Citrus medica* L. (citron), *C. reticulata* Blanco (mandarin), *C. maxima* (L.) Osb. (pummelo) and *C. micrantha* Wester (a wild citrus Papeda) (Barkley et al., 2006; Barrett and Rhodes, 1976; Federici et al., 1998; Garcia-Lor et al., 2012; Nicolosi et al., 2000; P. Ollitrault et al., 2012b; Scora, 1975; Wu et al., 2018). These four ancestral species, sexually compatible among them, were differentiated by foundation effects in four different geographic zones and an allopatric evolution. Wu et al. (2018) proposed that citrus diversified through a rapid Southeast Asian radiation during the late Miocene (6-8 million years ago), when a marked weakening of the monsoons occurred. Citron evolved in north-eastern India and nearby regions of Myanmar and China. Pummelos originated in the Malay Archipelago and Indonesia. Mandarins were diversified over a region including Vietnam, southern China, and Japan and, *C. micrantha* is endemic of the Philippines islands. The secondary species, such as sweet orange (*C. sinensis* (L.) Osb.), sour orange (*C. aurantium* L.), grapefruit (*C. paradisi* Macf.), lemon (*C. limon* (L.) Burm.) and lime (*C. aurantifolia* (Christm.) Swing.) are the result of hybridization between these four ancestral species followed by a few interspecific recombinations and subsequent natural mutations. All these events have originated the wide genetic and phenotypic diversity among these species (Ahmed et al., 2019; Curk et al., 2016; Garcia-Lor et al., 2013b, 2012; Nicolosi et al., 2000; F. Ollitrault et al., 2012; P. Ollitrault et al., 2012a, 2012b; Oueslati et al., 2017; Wu et al., 2018). Oueslati et al. (2017) developed a workflow for phylogenomic inference from Genotyping by Sequencing (GBS) data and revealed the interspecific *C. maxima / C. reticulata* admixture along the genomes of modern mandarins of commercial importance, such as tangors (sweet orange × mandarin hybrids) and tangelos (mandarin × grapefruit hybrids), indicating that they were originated from hybridizations between ancestral or secondary species. Subsequently, apomixis and grafting fixed these interspecific heterozygous structures.

2. Current state of citrus cultivation

2.1. Economic importance and main commercial groups

Nowadays, citrus (together with bananas and plantains) is one of the most important fruit tree crops in the world. According to the latest FAO data (FAOSTAT, 2020), the total citrus world production in 2020 was about 159 million tons, cultivated in a total area of 10 million hectares spread over more than 140 countries.

From an economic viewpoint, citrus production is distributed in the following five groups established by FAO: (I) Oranges, within which the major cultivars are Common or Blonde

oranges, Navel oranges, and Pigmented or Blood oranges.; (II) tangerines, mandarins, clementines and satsumas; they are a group of smaller fruits, mostly easy-peelers in which morphological variations are larger than in any other citrus species due to cross-breeding with compatible citrus species; (III) lemons and limes, which are characterized by their high acidity; (IV) grapefruit group, including pummelo; and (V) other citrus fruits including bergamot (*C. bergamia*), citron, chinotto (*C. myrtifolia*) or kumquat (*Fortunella spp*).

Oranges represent the highest production with 48% of the total citrus production. 45% of the orange production is produced by Brazil, India, and China with 16.7, 9.9 and 7.7 million tons respectively. Tangerines, mandarins, clementines and satsumas represent 24% of the total citrus production. China is the largest mandarin producer with 23.3 million tons, followed by Spain and Turkey with 2.2 and 1.6 million tons respectively (FAOSTAT, 2020).

Spain produced 7 million tons of citrus in 2020, mainly oranges (50%) and mandarins (31%). Spain exports 51% of its total citrus production, which positions this country in the first rank for fresh fruit market exporters. The largest citrus producer region in Spain is the Valencian Comunity with 3.5 million tons, mainly mandarins (45%) and oranges (45%). Within the mandarin group production, 66% corresponds to clementines, 25% hybrids and 9% satsumas, which places it among the largest easy-peelers producers worldwide. Within the orange group production, 84% is based on Navel oranges followed by Common (15%) and Pigmented (1%) (MAPA, 2020).

2.2. Biotic factors affecting citrus cultivation: diseases and pests

During the long history of citrus cultivation and spread throughout the world's tropic and subtropic regions, citrus have been affected by a significant number of pathogens and pests. Some of them pose a threat to citrus cultivation either in specific or wide cultivation areas.

Multiple pathogens including bacteria, fungi, viruses and viroids are responsible of citrus diseases. Currently, the most significant disease of citrus worldwide is Huanglongbing (HLB), also called greening. HLB is caused by the alpha-proteobacterium *Candidatus* Liberibacter, which is spread by the psyllid vectors *Diaphorina citri* Kuwayama and *Trioza erytreae* (Del Guercio). At least three species of the bacterium *Ca*. L. asiaticus, *Ca*. L. africanus, and *Ca*. L. americanus, are known to be associated with HLB being *Ca*. L. asiaticus the most destructive and widely distributed worldwide. Other important bacterial diseases are the citrus canker caused by *Xanthomonas citri* subsp. citri and the citrus variegated chlorosis caused by the proteobacter *Xylella fatidiosa* (Gabriel et al., 2020).

The most threatening fungi for citrus are *Alternaria* species –causing the brown spot, leaf spot and black rot diseases–, *Phytophtora* species –causing the root rot, foot rot, brown rot of fruits, canopy blight and damping-off diseases–, *Phyllosticta citricarpa* –causing the citrus black spot disease–, and *Plenodomus tracheiphilus* –causing the mal secco disease– (Batuman et al., 2020).

Regarding the citrus viruses and viroids, Zhou et al. (2020) reviewed a list of up to 30 virus and viroid diseases. Among them, the citrus tristeza virus (CTV), a *Closterovirus* naturally transmitted by several species of aphids, is particularly relevant. CTV was one of the most devastating citrus diseases with worldwide distribution. Since CTV is a

variety/rootstock association related disease, the use of resistant or tolerant rootstocks has protected the citrus industry in many countries (Moreno et al., 2008).

2.3. Abiotic factors affecting citrus cultivation in a global warming scenario

Prominent abiotic factors that affect or even restrict citrus cultivation are temperature, water availability, soil salinity, air humidity and CO_2, calcareous soils and boron disorders. Among them, temperature plays a significant role in the current global warming scenario. In general, cultivated citrus species adapt well to various climates, although they are limited by low-temperature (Vincent et al., 2020). This limitation causes that citrus production areas are located in the tropical and subtropical regions (approximately 40 degrees South and North latitude) including Southern Europe, subtropical North and South America, Southern Asia, South Africa, and Australia (Vincent et al., 2020).

The Intergovernmental Panel on Climate Change (IPCC) latest special report “Global warming of 1.5 ºC” of the Sixth Assessment Report (AR6) (IPCC, 2018) states that human activities are estimated to have caused approximately 1.0 ºC of global warming above pre-industrial levels. This report also estimates that global warming is likely to reach 1.5 ºC between 2030 and 2052 if it continues to increase at the current rate. Consequences due to global climate change are already effective in shaping the geographical distribution of the species (Saxe et al., 2001) and affect phenological traits of plants, in particular, those related to flowering (Cleland et al., 2007; De Ollas et al., 2019; Ibanez et al., 2010; Körner and Basler, 2010; Parmesan and Yohe, 2003; Root et al., 2003; Williams and Abberton, 2004).

Overall climate trends in citrus-producing regions vary according to geographical location. Mediterranean regions will likely undergo more frequent and lengthy droughts. Most coastal and arid to semiarid regions will face challenges related to salinity. Most regions will have less frequent freeze events, and nearly all citrus-producing regions will see increased average temperatures (Vincent et al., 2020). In addition to the urgent need to revert these trends by the achievement of sustainable development, future breeding programs may be forced to adapt their objectives to new environmental conditions revealing the great importance to advance the knowledge of how temperature influences citriculture and, in particular, the reproductive biology of citrus. Current knowledge of influence of temperature on the reproductive biology in citrus is discussed below (see 3.6).

3. Citrus reproductive biology

3.1. The flowers

Flowers are the reproductive structures of angiosperms (flowering plants) (Sauquet et al., 2017). In flowers takes place the haploid gametophyte generation –male pollen grains and female embryo sac–, the progamic phase, the fertilization and the zygotic phase (Ma and Sundaresan, 2010; Ohnishi and Kawashima, 2020). Flowering in citrus is related to the transition from juvenility to the mature phase. Flower development is a

multi-step process whose starting point is a vegetative shoot apical meristem. The first step, called floral induction, is the transformation of the shoot apical meristem into a floral meristem (a cluster of undifferentiated cells). The following steps result in the differentiation of floral structures (Liu et al., 2009; Zik and Irish, 2003).

Flower induction in citrus is regulated by either low temperatures or water deficit, depending on the growing zone. In subtropical zones, flower induction is regulated by low temperatures, while in tropical climates it is regulated by water deficit.

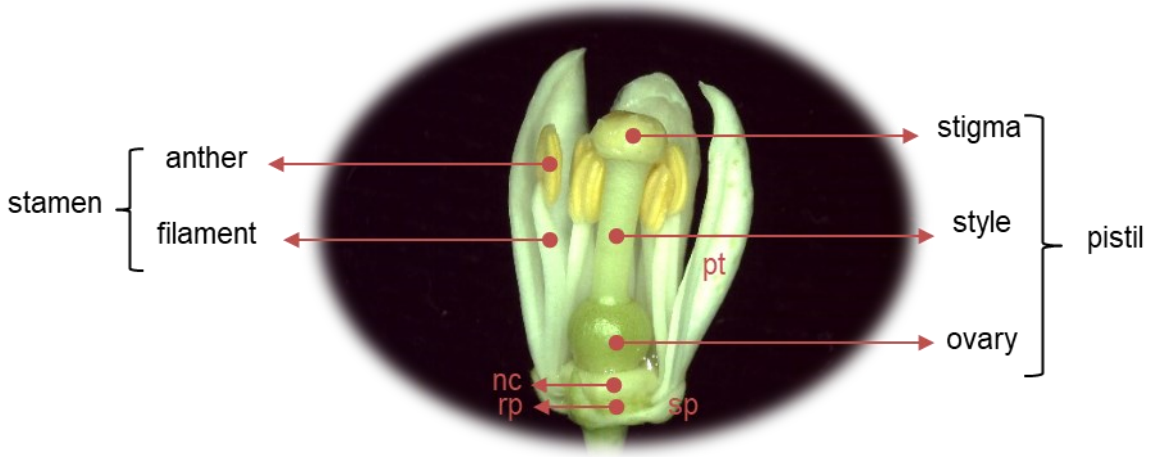
Flowering occurs in different types of inflorescences such as single terminal flower, axillary flower and shoots with and without leaves. Although the floral load and shooting pattern are characteristic of the genotype, citrus flowering is a complex process affected by several factors such as temperature, tree and shoot age, bud position, crop load, carbohydrate content and nutritional status, mainly nitrogen and hormonal factors such gibberellin (Agustí and Primo-Millo, 2020; Duarte and Guardiola, 1996; Iglesias et al., 2007; Krajewski and Rabe, 1995).

Flowering in citrus is usually very abundant. Fruit set values of 0.2 % of nearly 200.000 flowers produced by Navel sweet orange and 1% of nearly 75.000 flowers produced by Valencia sweet orange were reported by Erickson and Brannaman (1960). Floral load, inflorescence type and flower position are relevant factors in fruit set. Leafy shoots are most successful in fruit set and several flowers on the same axis trigger competition that affects fruit set (Agustí et al., 2003; Garcia-Papi and Garcia-Martinez, 1984a). Moreover, the presence of fertilized ovules has been shown to be crucial in determining fruit set in most plants (Gillaspy et al., 1993) and also in citrus (Bermejo et al., 2018).

Most flowers in citrus are hermaphrodite in which the four whorls or verticiles –calyx, corolla, androecium, and gynoecium– attached to the receptacle are distinguished. The calyx is formed by five sepals articulated at the basis and corolla is formed by five petals that surround the androecium and the gynoecium. Generally, petals are white colored, although in some species such lemons and citrons are purple tinged. The androecium is composed of stamens in a number from 20 to 40 depending on the species. Each stamen consists of an anther borne at the tip of a filament (Figure 1). Each anther is formed by two theca and each theca by two locules (or pollen sacs). Inside the locules is the mother cell that produces the pollen grains (microsporocyte) enclosed by the tapetum. In most citrus, pollen grains are binucleate (Cameron and Frost, 1968; Garavello et al., 2019), have a subreticular structure and are predominantly globose or ellipsoid in shape with four-five colporates. Pollen grain size varies among species and ranges from 25 to 35 μm, although exceptionally large pollen grains have been observed (Frost and Soost, 1968; Kozaki and Hirai, 1982).

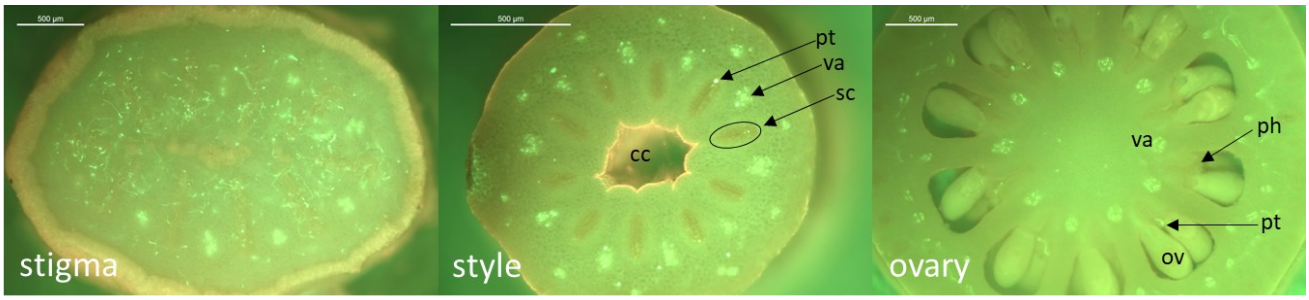
The gynoecium consists of a single pistil composed by a basal ovary, a columnar style, and distal stigma (Figure 1). The function of stigma is the reception of pollen; thus, the outermost surface of the stigma is coated with a secretion called stigma exudate as a source of water for pollen hydration and other chemical compounds that promote pollen grain germination and pollen tube growth. The style connects the stigma to the ovary and inside the style there is the same number of stylar canals as locules in the ovary (Figure 2). Distefano et al. (2011) reported that stylar canals become smaller as they descend towards the ovary due to a decrease in the number of cells bordering the stylar canals. These authors also pointed out that papillar hairs in the area where pollen tubes reach the ovaries appear to play a role similar to an obturator connecting the base of the style with the ovule.

Figure 1. Flower of 'Fortune' mandarin.



pt: petal; sp: sepal; nc: nectary; rp: receptable.

Figure 2. Transversal sections of the stigma, style and ovary.



pt: pollen tube; va: vascular axis; sc: stylar canal; cc: central canal; ph: papillar hairs; ov: ovule.

The ovary consists of a variable number of carpel leaves or locules. The single ovary growth results in the citrus fruit formation, named hesperidium, a modified berry, in which vesicles (juice sacs) and ovules (which in turn originate seeds) are formed inside locules. The ovules are anatropous, with axillary placentation. The female gametophyte, or embryo sac, follows the polygonum type arrangement (Frost and Soost, 1968) which is the most common pattern of embryo sac development in angiosperms. The embryo sac, consisting of eight nuclei in seven cells, develops within the ovule surrounded by the nucellus. The nucellus is surrounded by the inner and outer teguments. At the micropylar end three cells are identified, two peripheral cells become synergids and the other one differentiates into the egg cell. A large homodiploid central cell containing two haploid polar nuclei is in the center. At the chalazal end, opposite to the micropyle, the cluster of three cells differentiates into the antipodal cells.

Hermaphrodite flowers containing both female and male sex organs are the predominant form in angiosperms. Chasmogamy is the production of flowers that opens at maturity, exposing stamens and style to allow cross pollination. Chasmogamy is predominant in citrus. However, cleistogamy consisting of the self-pollination and fertilization on unopened flowers is characteristic in some citrus species such citron. In this regard, Curk et al. (2016) indicate that cleistogamy in citron is responsible for self-fertilization and thus the low heterozygosity and genetic diversity of this species observed in several molecular studies.

3.2. Progamic phase and double fertilization

Among the events that take place during the sexual reproduction process in plants, the progamic phase is crucial in achieving successful mating. The progamic phase runs from pollination to fertilization, so the interaction between the pistil and pollen performance, including stigmatic receptivity, pollen grain germination, pollen tube growth, and ovule degeneration takes place during the progamic phase. It has been widely reported that temperature plays a critical role in the progamic phase in plants (Hedhly et al., 2009).

For the reproductive process to be successful, pollen grains must germinate, and pollen tubes must grow to transport the male gametophytes to the ovules and fertilize them. After anthesis, flower development to senescence includes basipetal maturation beginning at the stigma and continuing to the ovary. Loss of stigmatic receptivity, abscission of the style and degeneration of the ovule are processes related to the pistillate senescence. This senescence does not depend on pollen performance, so the period in which pollination can be successful in mating is limited.

To express this situation in apple, Williams (1965) introduced the concept of effective pollination period (EPP). EPP is determined as longevity of the ovule minus the time elapsed between pollination and fertilization, and determines the number of days in which pollination can produce seed-bearing fruits. EPP has been analyzed in many fruit crops, and temperature appears as a crucial influencing factor (Sanzol and Herrero, 2001). In citrus, Mesejo et al. (2007) reported the influence of genotype on EPP under field conditions but information about how temperature affects EPP is scarce.

The progamic phase ends with the double fertilization (Figure 3). Double fertilization is a complex process completed over several steps. First, pollen tube is attracted into the embryo sac. Pollen tube is binucleate carrying a vegetative and a generative nucleus. The vegetative nucleus's mission is the pollen tube growth while the generative nucleus is divided resulting into the two male gametes. Once pollen tube is receipted in the embryo sac, pollen tube growth stops and the two male gametes are released into the degenerate synergid. In this step synergids play an important role. Then, the migration of the male gametes into the female gametes is produced giving rise to their recognition and fusion. One of the two male gametes fertilizes the egg-cell which results in the diploid embryo while the other male gamete fertilizes the two haploid polar nuclei to produce the triploid endosperm.

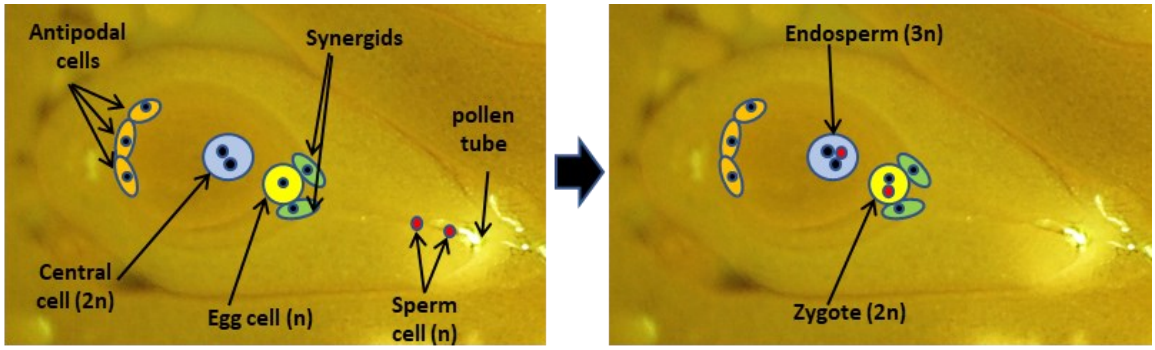


Figure 3. Double fertilization schematic representation in a ovule of 'Fortune' mandarin in which a pollen tube is accessing through the micropyle.

Finally, the reinitiation of the cell cycles leads to the onset of the zygotic life with the development of the endosperm and the embryo. The nucellus provides nutrients to the young embryo and the growing endosperm (Berger et al., 2008). Seed formation is the result of a successful mating for which both male and female fertility and sexual compatibility are required. The complexity of the biology of reproduction in citrus is characterized by the occurrence of apomixis, female sterility, male sterility and self-incompatibility. The following sub-section 3.3 deals with the seed formation and the apomixis. Sterility and self-incompatibility that prevent seed formation are discussed later in sub-sections 3.4 and 3.5, respectively.

3.3. Apomixis

Sexual reproduction in flowering plants is by seeds. The new plant formed by sexual reproduction begins as an embryo within the fertilized ovule which will give rise to the seed. Although seeds are usually of sexual origin, asexual seed formation, called apomixis, is also present in angiosperms. While sexual reproduction generates variation, apomixis produces progeny genetically identical to the female parent (Hand and Koltunow, 2014).

Apomixis has been observed in more than 400 plant species (S. Zhang et al., 2018). However apomixis is not very common in agriculturally important crops, with the exception of mango, apple and citrus (Dwivedi et al., 2010; Koltunow, 1993). Apomixis mechanisms are classified as gametophytic or sporophytic. In gametophytic apomixis, the embryo develops mitotically through the embryo sac (gametophyte). The mitotic development of the embryo sac, which avoids meiosis, is known as apomeiosis. Depending on the origin of the diploid precursor cell, apomeiotic development of the embryo sac is subdivided into two types: diplospory and apospory. In diplospory, the precursor is the megaspore mother cell (or a cell in its position). In apospory, the precursor is a diploid somatic cell positioned adjacent to the megaspore mother cell. In sporophytic apomixis, the embryo sac develops directly from diploid somatic cells (sporophyte) within the ovule (Hand and Koltunow, 2014).

In citrus, apomixis is sporophytic and is also called nucellar embryony or adventitious embryony. During sporophytic apomixis, the embryo sac develops following the sexual pathway typical of angiosperm. Besides, diploid nucellar cells surrounding the embryo sac differentiate and have an embryogenic cell fate. These initial embryonic cells begin mitosis forming multiple globular-shaped embryos that can develop to maturity only if the sexually derived embryo sac is fertilized, since both sexual and asexual embryos share the same nutritive endosperm. Thus, sporophytic apomixis can result in a seed containing a sexual embryo and multiple nucellar embryos, commonly referred to as polyembryony. The sexually derived embryo may or may not mature or germinate (Hand and Koltunow, 2014).

Most varieties of sweet oranges, lemons and grapefruits are apomictic. In mandarins apomictic and non-apomictic varieties are found. Practically all rootstocks used in citriculture are apomictic. Apomixis is very advantageous for the rootstock production since plants obtained from polyembryonic seeds are identical to the female parent. However, apomixis restricts the use of female parents in breeding programs. The impact of apomixis on breeding will be discussed later in this introduction (see 6.1).

On the basis of genomic analysis of primitive, wild and cultivated citrus, Wang et al. (2017) suggested that the emergence of apomixis took place during the citrus domestication process. These authors located a candidate region for apomixis to an 80-kb interval in chromosome 4 containing 11 genes. Among these 11 genes, a candidate gene *CitRWP* was identified for the single dominant allele responsible for polyembryony. *CitRWP* is more expressed in ovules of polyembryonic genotypes. A miniature inverted-repeat TE (MITE) insertion in the promoter region of *CitRWP* gene, which cosegregates with the polyembryonic phenotype, provides more evidence in support of the single dominant mutation model for the emergence of apomixis in mandarin (Shimada et al., 2018; Wang et al., 2017). Recently, Wang et al. (2022) focus on the genetics and evolution of apomixis in *Citrinae*. These authors suggest that parallel evolution of *Fortunella* and *Citrus* has driven the evolution of apomixis in these genera and reported that the MITE insertions were not associated with apomixis in *Poncirus* and its related individuals.

3.4. Mechanisms of female and male sterility in citrus

Sterility refers to the failure or inability of an individual to produce functional gametes, spores or sexual organs under given environmental conditions. It can affect both the male and female sexes.

Female sterility is the term used to refer seedlessness originating from the female counterpart. At diploid level, female sterility may be due to at least three mechanisms: (1) Degeneration of the embryo sac, as observed by Osawa (1912) in satsuma mandarin and navel sweet orange. (2) Failure of pistils to develop to the functional stage, as observed in lemon by Wilms et al. (1983). (3) Arrest of seed development at an earlier embryo stage, resulting in the formation of very small seeds with an immature coat, as observed by Yamasaki et al. (2007) in `Mukaku Kishu´, a bud variant of the seedy `Kinokuni´ mandarin (*C. kinokuni* hort. ex Tanaka). This mechanism is considered as the strictest female sterility in citrus (Yamamoto, 2014). Different studies have been focused on genetic control of the `Mukaku Kishu´ seedlessness and suggested that it is under the control of a single dominant gene (Nesumi, 2001; Yamasaki et al., 2007).

Despite the female sterility associated with satsuma, it should be noted that several varieties have been obtained in breeding programs around the world using satsuma as a seed parent, indicating that this trait could be influenced by environmental conditions. In Japan, where satsuma is one of the major citrus cultivars grown, many hybrids derived from satsuma have been obtained (Omura and Shimada, 2016). Among them ‘Kiyomi’ tangor (*C. unshiu* x *C. sinensis*) (Nishiura et al., 1983) is of particular relevance, not only because its great importance as a cultivated variety, but also because a large number of cultivars have been obtained from crosses using it as a seed parent (Omura and Shimada, 2016). Other examples of varieties obtained from satsuma as a seed parent in other countries are ‘Kara’ (*C. unshiu* x *C. nobilis*) and ‘Umatilla’ (*C. unshiu* x *C. sinensis*) in the USA (Hodgson, 1967), ‘Primosole’ (*C. unshiu* x *C. reticulata*), ‘Simeto’ (*C. unshiu* x *C. reticulata*), ‘Desiderio’ (*C. unshiu* x *C. clementina*) and ‘Belleza’ (*C. unshiu* x *C. reticulata*) (Tribulato and La Rosa, 1996, 1993) in Italy and ‘Queen’ (*C. unshiu* x unknown) (de Teresa, 2011) in Spain.

For male sterility, several levels and mechanisms have been reported in citrus at the diploid level. Chromosome aberration is an important phenomenon causing pollen sterility. Asynapsis, reciprocal translocation and failure of spindle formation have been

described in different citrus species. For example, failure of homologous chromosomes to pair or synapse during the first meiotic division has been identified in ‘Mukaku Yuzu’ and is genetically controlled, while in ‘Eureka’ lemon and `Mexican´ lime is induced by low temperature (Ollitrault et al., 2007b). However, nucleo-cytoplasmic male sterility (CMS) is the most prevalent system in citrus, and it has been proposed that satsuma and progenies derived from satsuma as female parent display CMS caused by the cooperative action of both cytoplasmic and nuclear genes. DNA marker analysis for nuclear and cytoplasmic genomes and genome-wide SNP marker analysis showed that CMS in satsuma was derived from its seed parent ‘Kishu’ mandarin (Goto et al., 2018; Shimizu et al., 2017, 2016). Goto et al. (2018, 2016) assessed male sterility in a satsuma-derived populations by measuring two parameters: (1) the number of pollen grains per anther and (2) apparent pollen fertility. They identified two QTLs linked to male sterility; MS-P1, which is a major QTL for reduced number of pollen grains per anther and MS-F1, associated to lower apparent pollen fertility.

Although satsuma is described as male sterile, it is possible to achieve crosses by hand-made pollinations with fertile satsuma pollen. In this regard, Yang and Nakagawa (1970, 1969) reported temperature adjustment during pollen development to increase pollen fertility. Others authors such as Vithanage (1991) reported two seeds per fruit when `Ellendale´ tangor was pollinated with satsuma under field conditions. All this suggest that male sterility in satsuma is partial and is influenced by both environmental conditions and genotype.

Comparison of transcript profiling between fertile genotypes and their male sterile types has provided a better understanding of gene regulation of male sterility in citrus. Male sterile types have been obtained either by mutation (see 6.2) or by cybridization (see 6.3). Regarding cybridization, Guo et al. (2004) recovered a sterile diploid cybrid (G1+HBP) with the nuclear and chloroplast genomes from leaf parent `Hirado Butan´ pummelo (HBP) and mitochondrial genome from the callus parent `Guoquing No.1´ satsuma (G1). Taking this recovered cybrid, Zheng et al. (2012) compared nuclear gene expression profiles of floral buds between male sterile cybrid pummelo (G1+HBP) and HBP fertile pummelo. These authors reported down-regulation expression of *PISTILLATA* and *APETALA3* genes in the cybrid pummelo and pointed to the dysfunctional mitochondria as the cause of male sterility. Later, Zheng et al. (2014) compared the proteome profile between the same sterile cybrid and fertile pummelos. These authors concluded that the failure of anther and pollen development in the sterile cybrid is associated with changes in protein expression under the control of both nuclear and mitochondrial genomes. Regarding the use of male sterile mutants, C. Zhang et al. (2018) analyzed the transcriptome and proteome profiling in different anther developmental stages between the male sterile 'Wuzi Ougan' (*C. suavissima* Hort. ex Tanaka 'seedless’ mutant type) and 'Ougan' (*C. suavissima*, wild type). These authors reported 487 differentially expressed proteins between them, most of them related with male sterility genes, including those for phenylpropanoid biosynthesis, flavonoid biosynthesis, and phenylalanine metabolism.

In addition to the female and male sterility at diploid level described above, both male and female sterility are the main characteristics of citrus triploid hybrids. Cytogenetic studies have shown that during meiosis of citrus triploid hybrids, trivalent, bivalent and univalent associations are formed (Cameron and Frost, 1968) producing sterile gametes. Moreover, Fatta Del Bosco et al. (1992) have shown that the abortion of megasporogenesis from the first divisions of the embryo sac in the fertilized egg cell is

frequent. For this reason, citrus triploid hybrids are generally sterile, although they can occasionally produce fruits with very few seeds. Triploid plants are generally considered as an evolutionary dead-end, since they generally give rise to aneuploid gametes with very low fertility but are interesting to select triploid seedless varieties (Ollitrault et al., 2008; Otto and Whitton, 2000). The breeding strategies to develop triploid seedless varieties are described below (see 6.4).

3.5. Self-incompatibility

Self-incompatibility (SI), also called self-sterility, consist in the rejection of pollen from genetically related individuals. The end result of the SI reaction is that endogamy is prevented, which is crucial for the adaptation and evolution of species (Abdallah et al., 2020; Goldberg et al., 2010; Lin et al., 2015; Surridge, 2015). A distinction is made between gametophytic SI (GSI) and sporophytic SI (SSI). In GSI the pollen incompatibility is determined by the haploid pollen genotype while in SSI it is determined by the diploid male parent genotype.

Based on the self-compatibility(SC):self-incompatibility(SI) ratio obtained in citrus offspring from crosses between different genotypes, several authors proposed that SI is controlled by a single co-dominant *S* locus with multiple *S* alleles and suggested gametophytic SI (Kim et al., 2011; Soost, 1969, 1965; Vardi et al., 2000). Subsequent studies (Honsho et al., 2021; Liang et al., 2020) have provided evidence of GSI in citrus and demonstrated that GSI is based on S-RNase, which acts as a pistil *S* determinant by inhibiting pollen in an *S*-specific manner. Liang et al. (2020) provided evidence that SI is ancestral in citrus and identified a predominant single nucleotide mutation, S_m-RNases, in self-compatible citrus that provides a loss of SI. These authors located the SI locus at the beginning of the pseudo-chromosome 7 of the clementine reference genome. Based on the segregation distortion obtained from reciprocal crosses between two self-incompatible genotypes, Ollitrault et al. (2021) confirmed the gametophytic system and the location of the SI locus reported by Liang et al. (2020). Honsho et al. (2021) reported that T2 RNase gene in self-incompatible `Hyuganatsu´ (*C. tamurana* Hort. ex Tanaka) is significantly down-regulated in the styles of a self-compatible mutant of `Hyuganatsu´.

SI is prevalent in citrus, with pummelos and clementines being two major groups characterized by their SI. Among clementines, SC of `Monreal´ clementine is an exception and has been used to study the SI reaction in clementines. Distefano et al. (2009a) reported differences in pollen tubes growth and gene expression between self-pollinated flowers of `Comune´ and `Monreal´ clementines and Caruso et al. (2012) identified novel genes associated with self-pollen rejection by comparing the transcriptomes of stylar canal cells between these clementines. Another important group of self-incompatible genotypes are some mandarins, and several natural or artificial hybrids such as `Fortune´ (*C. clementina* x *C. tangerina*) and `Imperial´ (*C. reticulata* Blanco) mandarins and `Ellendale´ (*C. reticulata* x *C. sinensis*) tangor among others.

SI has been determined based on the observation of pollen tube growth and/or fruit production with or without seeds from self-pollinated flowers. It should be noticed that much research has been conducted to determine SI in many accessions and discrepant results have been reported in some of them. `Ellendale´ was described by Vardi et al. (2000) as a self-incompatible variety while Vithanage (1991) and Bono et al. (2000) reported fruits with seeds from self-pollinated flowers. For `Imperial´ mandarin, Wallace

and Lee (1999) reported SI as almost all pollen tubes stopped growing at the stigma and upper style of self-pollinated flowers, while Vithanage (1986) and Sykes, (2008a) reported SC as pollen tubes were observed growing into the self-pollinated styles and seeded fruits were obtained from self-pollinated flowers. Sykes, (2008a) suggested that the influence of environmental factors on SI reaction could be an explanation for these discrepancies. Subsequent studies have demonstrated that low temperature of 15ºC induced the breakdown of the SI reaction in clementine (Aloisi et al., 2020; Distefano et al., 2018). Beyond the influence of temperature, breakdown of SI was reported to be induced by bud pollination (Distefano et al., 2009b; Wakana et al., 2004) and by polyploidization (Yamashita et al., 1990) although these mechanisms have not been demonstrated formally in citrus until now.

Despite the extensive literature devoted to the SI in citrus, there are still many challenges. On the one hand, increasing the knowledge about SI in different mandarin genotypes to be used as parents in sexual hybridizations is crucial to improve breeding programs. On the other hand, increasing the knowledge about the breakdown of SI is crucial to expand crossing alternatives in breeding programs.

3.6. Impact of temperature on the reproductive biology

The sexual reproductive process can be divided into three consecutive developmental stages: gamete development, progamic phase and embryo development, which gives rise to the beginning of the next plant generation. The impact of temperature on each of these stages has been extensively researched in many plant genera showing the interdependence between them (Hedhly, 2011; Hedhly et al., 2009).

Effects of temperature stress on gamete development include flower production and quality, as well as number of pollen grains, morphology and chemical composition (Aloni et al., 2001; Distefano et al., 2018; Koti et al., 2005; Prasad et al., 2002; Sato et al., 2002; Warner and Erwin, 2005). Temperature stress on the progamic phase affects male (pollen viability, pollen germination and pollen tube growth rate), female (stigma receptivity and ovule viability) reproductive systems and their interaction (Cerović et al., 2000; Gao et al., 2014; Hedhly et al., 2005a, 2005b, 2004, 2003; Kakani et al., 2005; Pham et al., 2015; Radičević et al., 2016; Snider et al., 2011b, 2011a). Temperature stress during embryo development influence seed formation and subsequently seed germination rates, root elongation, plant biomass and cold acclimation, among others (Blödner et al., 2007; Johnsen et al., 2005; Lacey and Herr, 2000). In citrus, Bennici et al. (2019) have shown that temperature stresses during the flowering affect male gametophyte development, resulting in a drastic reduction in pollen performance.

Among the three developmental stages of the sexual reproductive process, the male gametophyte and the pistil interact during the progamic phase. It is therefore a crucial phase in which impact of temperature stress may affect successful mating. In citrus, Distefano et al. (2018, 2012) showed that temperature variation during this phase has a strong effect on pollen germination *in vitro* and on pollen–pistil interaction in detached flowers of three ancestral *Citrus* species. Aloisi et al. (2020) proposed that in *C. clementina* the involvement of transglutaminase during the SI response was similar to the one described for members of the Rosaceae family (Del Duca et al., 2010) and in other *Citrus* species (Gentile et al., 2012). Apart from these publications, research evaluating the influence of temperature on the progamic phase in citrus is still very scarce.

3.7. Histological techniques to observe pollen tube growth inside pistils

Attempts to observe pollen tubes inside pistils have been made since the beginning of the 20th century. Histological studies are usually performed following four steps: (1) fixation, (2) dissection, (3) staining and (4) observation.

The first step is the fixation of the sampled pistil in a killing solution which is usually based on alcohol together with other products such formalin or acetic acid. The pistils placed in this solution can be examined soon after collection or can be stored for a long time until examination. To observe the pollen tubes inside the pistil, this must be dissected or squashed to expose the internal structures to be observed. A staining solution is then added, and the pollen tubes can be observed under the microscope.

As for staining techniques, it is worth noting the extensive list of stains used to observe pollen tubes in styles, such as lactophenol-cotton blue (Datta and Naug, 1967; Rawlins, 1933; Watkins, 1925), safranina-O (Conn, 1925), acetocarmine (Chandler, 1931; Esser, 1955), lacmoid-martius-yellow (Nebel, 1931), fluorescein diacetate test (Heslop-Harrison and Heslop-Harrison, 1970) and aniline blue (Linskens FH, Esser, 1957). Mixed or modified techniques such safranina-aniline blue (Dionne and Spicer, 1958; Nair and Narasimhan, 1963) and aniline blue-calcofluor white (Jefferies and Belcher, 1974) have also been used. Some of these techniques offered limited achievements or were only suitable for a limited number of taxa. As an example, McGuire and Rick (1954) point out that lactophenol-cotton blue method stains both pollen tubes and stylar tissue cells deep blue. However, this method was slightly more accurate in species of *Lycopersicon* because pollen tubes can be differentiated from stylar tissue cells by their smaller diameter and the absence of cell walls.

Among the staining techniques mentioned above, aniline blue is the most commonly used to observe pollen tubes because callose plugs deposited during pollen tube growth stained with aniline blue are readily observable under fluorescence (Adhikari et al., 2020). Callose (a linear β-1,3-glucan with some 1,6 branches) is synthesized by plants at many locations throughout development and in response to biotic and abiotic stresses (Verma and Hong, 2001). Among the many locations where callose is synthetized by plants are the growing pollen tubes and the senescent stigma and ovules (Dumas and Knox, 1983).

In citrus, squashed pistils stained with aniline blue have been widely used to observe pollen tubes (Distefano et al., 2009a, 2009b; Eti and Stosser, 1992; Liang et al., 2020, 2017; Mesejo et al., 2013, 2007, 2006; Ngo et al., 2001; Sykes, 2008b, 2008a; Vithanage, 1991; Wallace and Lee, 1999; Yamamoto et al., 2006). Squashing is quick and easy to perform, but the shape and internal morphology of the pistil is altered. Therefore, the use of a rotary microtome to section the pistils while maintaining tissue morphology is necessary when the researcher requires more complete and detailed observations. In this regard, Distefano et al. (2011) used 10 µm thick cross sections of both methacrylate and paraffin-embedded pistils to describe in detail the anatomical features and pollen-pistil interactions in `Fortune´ and `Nova´ mandarins. However, this technique is difficult and time consuming.

4. Parthenocarpy

Parthenocarpy (from Greek *parthenos* virgin + *karpos* fruit) is defined as the development of fruit without fertilization and thus refer to the production of seedless fruits. In addition to parthenocarpy, seedless fruit production may be due to stenospermocarpy, in which fertilization occurs but fruits are seedless because the ovule or the embryo aborts without producing mature seeds (Picarella and Mazzucato, 2019; Varoquaux et al., 2000). In citrus, stenospermocarpy is uncommon. It has been occasionally observed in `Valencia´ sweet orange (Koltunow et al., 1995) and `Mukaku Kishu´ mandarin fruit (Yamasaki et al., 2009, 2007), and induced in `Nadorcott´ tangor (*C. reticulata* x *C. sinensis*) by interfering with cell division of newly fertilized ovule with maleic hydrazide (Mesejo et al., 2014).

Commonly, the term parthenocarpy is used in its broad sense to indicate both forms of apireny: parthenocarpy *sensu stricto* and stenospermocarpy (Picarella and Mazzucato, 2019). It refers to both parthenocarpic fruit (i.e. seedless fruits) and parthenocarpic varieties (i.e. varieties producing seedless fruits). Vardi et al. (2008) proposed four types of parthenocarpy in citrus. On the one hand, based on the mechanism that prevents fertilization, a distinction is made between obligate parthenocarpy for those varieties with female sterility, which always produce seedless fruits; and facultative parthenocarpy for self-incompatible or male sterile varieties, in which seed production depends on cross-pollination with compatible pollen sources. On the other hand, a distinction is made between autonomous parthenocarpy (also called vegetative parthenocarpy) to refer to the development of seedless fruits without the need for any external stimulus; and stimulative parthenocarpy, which requires the stimulus of pollination for seedless fruits to set.

The transition from ovaries to fruits is a complex process that includes the seed formation as a consequence of fertilization and embryonic development. Beyond their main reproductive function, seeds also act as a source of the phytohormones necessary for fruit growth (Ozga and Reinecke, 2003; Vardi et al., 2008). The ability to accumulate sufficient levels of phytohormones in developing ovaries without the need for seeds is known as parthenocarpic ability (PA) (Talon et al., 1990). Differences in PA quantification between satsuma and clementine have been reported. Satsuma shows high PA associated with high levels of endogenous gibberellin, while clementine shows low PA associated with low levels of endogenous gibberellin (Mesejo et al., 2016; Talon et al., 1992). In clementine the function of the seed as an endogenous source of phytohormones needed for fruit set can be supplied by exogenous application of gibberellin, which also prevents fertilization under cross pollination conditions (Mesejo et al., 2008).

Attempts to understand the genetic control of parthenocarpy have been reported based on the ratio of parthenocarpic hybrids obtained from different crosses. Based on data obtained from several crosses with satsuma, Vardi et al. (2000) proposed that parthenocarpy is determined by the action of three dominant complementary genes and that satsuma is homozygous for two of these genes and heterozygous for the other one. In a subsequent piece of research, Vardi et al. (2008) proposed that parthenocarpy is determined by at least two dominant complementary genes, with satsuma being heterozygous for these genes. On the basis of the offspring obtained from crossing

`Imperial´ mandarin and `Ellendale´ tangor, the hypothesis of three dominant genes was also suggested by Sykes (2008a).

The genetic control of parthenocarpy in citrus remains unclear. Moreover, the available information on parthenocarpy for many varieties is scarce. In some cases, it is even contradictory, as in the case of `Ellendale´, which has been classified as non-parthenocarpic (Vithanage, 1991), stimulating parthenocarpic (Vardi et al., 2000) and vegetative parthenocarpic (Sykes, 2008b).

5. Polyploidy

Polyploidy is the heritable condition of having more than two complete sets of chromosomes (Barker et al., 2016; Ramsey and Schemske, 2002). Polyploids are common among plants and should be considered as one of the most predominant modes of sympatric speciation, and therefore as an important component of plant evolution (Otto and Whitton, 2000). Autopolyploids are the result of the change in the ploidy level within a species, while allopolyploids result from two different genomes association through hybridization from unreduced gametes and subsequent chromosomal duplication. Each mechanism of polyploidization implies different genetic behaviour. As example for tetraploidization, autotetraploids contain four homologous chromosomes with equal matting opportunity during meiosis, producing multivalent associations and thus polysomic segregation. Allotetraploids contain two different sets of homologous chromosomes producing bivalents and thus disomic segregation.

For a long time, somatic chromosome doubling was considered by most authors as the main mechanism leading to polyploidy (Stebbins, 1971). However, Harlan and DeWet (1975) argued that spontaneous chromosome doubling is relatively rare, while sexual polyploidization by unreduced gametes seems to be much more frequent. These conclusions are now assumed to be the case in numerous plant species by several researchers (Bretagnolle and Thompson, 1995; Carputo et al., 2003; Dewitte et al., 2009; Honsho et al., 2016; Ramsey, 2007; Ramsey and Schemske, 1998; Stift et al., 2008; Wu et al., 2001).

Although diploidy is the general rule in citrus with a basic chromosome number of nine (x = 9) (Krug, 1943), few polyploid have been found in citrus (Iwasaki, 1943; Lapin, 1937; Longley, 1925). Examples of naturally occurring polyploids in *Aurantioideae* germplasm are triploid `Tahiti´ lime (*C. latifolia* Tan), tetraploid strains of *P. trifoliata*, allotetraploid *Clausena excavata*, tetraploid *Clausena harmandiana* and hexaploid *Glycosmis pentaphylla* (Ollitrault et al., 2007a).

Among polyploids, triploids and tetraploids are especially useful for citriculture. Triploid hybrids are used for seedlessness because they are generally considered sterile (see 3.4). Two of the first triploid varieties obtained were `Oroblanco´ and `Melogold´ grapefruits (*C. grandis* × *C. paradisi*) (Cameron and Burnett, 1978; Soost, 1987). Later, many triploid mandarin hybrids have been obtained from citrus breeding programs worldwide (see 6.4).

For tetraploid citrus origin, spontaneous duplication of chromosomes in nucellar cells seems to occur frequently in apomictic citrus genotypes and its frequency depends on genotypes and environment (Aleza et al., 2011; Cameron and Frost, 1968). In non-

apomictic genotypes, doubled-diploid plants are not found in the citrus germplasm, but can be artificially produced by treating micrografted shoot tips or embryogenic callus with colchicine and oryzalin to achieve chromosome doubling (Aleza et al., 2009b; Barrett, 1974; Gmitter et al., 1991). In addition to their use as parents in interploid hybridizations, tetraploids are also of great interest for use as rootstocks. In this regard, a wide range of phenotypic differences when compared to diploids have been reported (Ollitrault et al., 2020b). These include reduced tree size, larger stomata size with lower density, thicker and greener leaves, higher leaf water content, and thicker and smaller roots. Most of these differences result in better adaptation to unfavorable environmental conditions such as salt stress (Ruiz et al., 2016a, 2016c), drought (Allario et al., 2013; Oliveira et al., 2017), scion cold tolerance (Oustric et al., 2017) or boron excess (Ruiz et al., 2016b). Tetraploid plants can also be obtained by somatic hybridization which will be discussed later in this introduction (see 6.3).

6. Citrus breeding

Citriculture is based on grafted plants, with the scion (fruiting cultivar) budded on a rootstock. Scion and rootstock breeding programs vary according to the species and the characteristics of the growing areas. The challenge for breeders is the generation of new genotypes that meet market needs and show tolerance or resistance to biotic and abiotic stresses. In the following subsections, the breeding objectives are presented, as well as conventional methods and biotechnological tools. Finally, the breeding for seedless varieties, one of the main objectives of mandarin breeding and closely related to reproductive biology, is discussed.

6.1. Breeding objectives

Regarding the scion, two clearly differentiated markets are targeted: fresh fruit and processed juice. Both share the common objective of expanding the harvest period and specific objectives are usually addressed for each market. The juice market requires very productive varieties with high juice percentage and sugar content. Pigment composition is also important for high quality fresh juices. The fresh-fruit market requires varieties with high organoleptic (aroma, flavor, acidity, sugar), pomological (easy of peeling, seedlessness, external appearance) and nutritional (vitamin C, carotenoid and polyphenol contents) qualities and good postharvest performance. Seedlessness is an important criterion for the fresh fruit market (Ollitrault and Navarro, 2012). Resistance to some diseases is also integrated in scion breeding strategies such as alternaria for mandarins, anthracnose for limes, mal secco for lemons and Huanglongbing.

In the case of rootstock, the breeding objectives are adaptation to climatic and soil conditions (abiotic stress) and resistance or tolerance to soil pathogens and other microorganisms (biotic stress). In addition, the rootstock genotype can modify the behavior of the rootstock/scion interaction with respect to many characteristics such as tree vigor, productivity and fruit quality (sugar and acid content, fruit size and juice content). Rootstocks are propagated asexually by apomictic seeds, so it is important that new rootstocks have a high degree of apomixis, to avoid the germination of sexual embryos, which are difficult and costly to remove in nurseries (Ollitrault and Navarro, 2012). Cutting, layering and micropropagation are minor alternative techniques for

vegetative propagation of non-apomictic rootstocks although the last technique is currently having more importance due to the fact that some of the new rootstocks are difficult to produce from seeds.

6.2. Conventional breeding

Natural mutations occur frequently in cultivated citrus and modern citrus production has benefited from them more than any other tree crop. In fact, spontaneous mutation selection is considered as the oldest citrus breeding strategy and most cultivated varieties worldwide emerged from this process (Ollitrault and Navarro, 2012).

Induced mutagenesis is used to propagate and accelerate the natural mutation process. Although mutagenic treatment was first reported in the 1930s (Haskins and Moore, 1935; Moore and Haskins, 1932), it was not employed as a breeding tool until the late 1950s (Hensz, 1960), resulting in the first commercial released variety in 1970, the 'Star Ruby' grapefruit (Hensz, 1977). Gamma rays are the standard mutagen used, but other mutagenic agents, such as X-rays, fast and thermal neutrons, high-energy beams, UV light and chemical mutagens have also been employed (Caruso et al., 2020). In this process, budwoods are exposed to radiation that damages their DNA chromosomes and causes a wide range of random mutations. Once a new source of variation is identified, the desired trait can be fixed and propagated by clonal selection after dechimerization. The new variety is then grafted, and the stability of the novel trait is evaluated for 2-3 years (Raveh et al., 2020).

Along with mutation breeding and clonal selection, conventional breeding can be performed by sexual hybridization. Genetically, the most important difference between the two strategies is that new cultivars obtained from mutation have a genetic background similar to that of the parent, while hybridization produces populations with high genetic variability. In the establishment of a breeding program based on hybridization, some biological features that hinder the generation of improved cultivars should be taken into account, namely: juvenility, apomixis, sterility, self-incompatibility and heterozygosity.

The long juvenile period of citrus seedlings requires about 15 years for the selection and first evaluation phases of promising new cultivars. It is considered that practically 25-30 years are necessary from hybrid seed to commercial variety release (Caruso et al., 2020). Apomixis limits the use of many cultivars as seed parents, as zygotic embryo development is hindered by the presence of competitive nucellar embryos. Embryo rescue, culture and subsequent identification of the zygotic embryo with molecular markers allow hybrids to be obtained from polyembryonic seeds, but the large amount of time required to obtain a limited number of hybrids has restricted the use of this strategy. Sterility (male and/or female) and self-incompatibility may also limit the use of many elite cultivars either as male as or female parents. The high heterozygosity of most citrus cultivars results in a strong segregation leading to remarkable variation and new allelic combinations. This particularly precludes sexual breeding for species like *C. sinensis* arising from interspecific hybridization for which sexual segregation break the genomic structure sustaining the sweet orange ideotype. More generally the high level of segregation complicates citrus sexual breeding when considering the large number of traits that must be taken into account when selecting hybrids. Citrus breeding programs usually have selection criteria for more than 20 traits, most of which are under polygenic control and have not been fixed in breeding populations. Therefore, the probability of

obtaining a new hybrid that satisfies the necessary standards for all traits is extremely low. Consequently, large numbers of individual hybrids need to be evaluated to increase the probability of identifying new, truly improved candidate cultivars (Caruso et al., 2020). Once a new variety is created, grafting offers the opportunity to rapidly produce an unlimited number of genetically identical plants of the same variety without juvenile phase.

6.3. Biotechnological tools

In addition to the conventional breeding strategies mentioned above, the use of biotechnological tools has contributed significantly to citrus breeding programs. A very important area of plant biotechnology is tissue culture which allows the establishment and maintenance of plant tissues (callus, cells, protoplasts, etc.) and plant organs (embryos, shoots, roots, flower parts). Micropropagation, organogenesis and rooting are very useful regeneration methods. Beyond the fact that tissue culture plays a key role in the application of most biotechnological approaches within a breeding program, tissue culture is also a valuable source of genetic variation, as regenerated plants may show somaclonal variation (Germanà et al., 2020).

Gametic embryogenesis generates haploids with gametophytic chromosome number (n instead of 2n) and doubled haploids (haploids with chromosome doubling). This biotechnological technology improves the efficiency and speed in perennial crops by obtaining homozygosity at all loci in a single step (Ollitrault et al., 2020b). The haploid clementine obtained by Aleza et al. (2009a) was used for the citrus reference whole genome sequencing project performed by the citrus genome international consortium (Wu et al., 2014).

Somatic hybridization allows combining nuclear and cytoplasmic genomes in new patterns regardless sexual incompatibility between species. In citrus, protoplast fusion is generally performed using protoplasts isolated from embryogenic callus or cell suspension cultures of one parent that are fused with no-embryonic protoplasts derived from leaves of the second parent (Grosser et al., 1996). Thus, when two diploid genotypes are fused, tetraploid plants are obtained that fully contain the nuclear genomes of the two parents (Grosser et al., 2010; Grosser and Gmitter, 2011). This makes the addition of all dominant traits possible, regardless of the level of heterozygosity of the breeding material (Ollitrault and Navarro, 2012).

Methods used for protoplast fusion include PEG-induced fusion (Grosser and Gmitter Jr., 1990), electrofusion (Guo et al., 1998; Ollitrault et al., 1996), and electrochemical fusion (Olivares-Fuster et al., 2005). Since the first somatic hybrid obtained by protoplast fusion of *C. sinensis* + *P. trifoliata* (Ohgawara et al., 1985), somatic hybridization has become an integral part of many breeding programs worldwide. Somatic cybridization is a second outcome from the somatic hybridization procedure (Guo et al., 2013). The difference is that somatic hybrids contain the tetraploid hybrid nucleus, whereas cybrid plants contain the diploid nucleus from only one of the parents combined with cytoplasmic organelles of the second one.

Genetic transformation allows the incorporation of foreign DNA into known genotypes without altering their elite genetic background. Protoplast transformation, particle bombardment (biolistics) and *Agrobacterium*-mediated transformation have been used to obtain transgenic citrus. Among them, *Agrobacterium*-mediated transformation is the

most widely used due to the higher transformation efficiencies obtained in most cultivars (Peña et al., 1995b, 1995a; Yu et al., 2002). Because mature citrus tissues are recalcitrant to *Agrobacterium* infection, juvenile tissues are usually employed, resulting in the obtention of juvenile plants with a long time required to analyze mature traits. In this line, the direct transformation of mature tissues to obtain adult transgenic plants that flower and fruit in a short period of time (Cervera et al., 2008, 2005, 1998), is of great importance to speed up this process for citrus improvement. Emerging transformation strategies such as transient and tissue-localized transformation, and another new technologies, such as cisgenesis, intragenesis, and genome editing using clustered regularly interspaced short palindromic repeats (CRISPR) technology, are beginning to be used in breeding programs (reviewed in Conti et al. (2021)).

6.4. Breeding for seedlessness

Seedlessness is one of the most important characteristics for the fresh fruit market because consumers prefer seedless fruits. This fact, together with the great economic importance of citrus, has promoted the launching and implementation of numerous breeding programs worldwide aimed at obtaining new seedless varieties. Conventional breeding, including mutation and hybridization (see 6.2), have been key strategies for obtaining new seedless varieties.

Seedless fruit production is made possible by the presence of PA in citrus (see 4). In addition to PA, a key requirement for seedless fruit production is that some mechanism either prevents fertilization or causes abortion of newly fertilized ovules. The mechanisms that prevent fertilization –female sterility, male sterility and SI– are inherent to citrus (see 3.4 and 3.5), but female and male sterility can also be achieved by induced mutation (Bermejo et al., 2011; Goldenberg et al., 2014) and by triploidy (Navarro et al., 2015; Ollitrault et al., 2007a, 2007b).

In the case of induced mutation, chromosomal aberrations such as inversions or translocations caused by gamma irradiation often result in decreased fertility due to the high frequency of pollen and/or ovule abortion (Vardi et al., 2008). Exemples of seedless varieties obtained by mutation breeding include `Moncalina´ (Pardo et al., 2012), `Orri´ (Vardi et al., 2003) and `Tango´ (M L Roose and Williams, 2007).

Triploidy is also known to induce male and female sterility (Ollitrault et al., 2020b). Triploid plants can be recovered by sexual hybridization between two diploid parents from the union of an non-reduced megagametophyte with a haploid pollen grain (Aleza et al., 2010b; Cuenca et al., 2011), or by hybridization between diploid and tetraploid parents (Aleza et al., 2012b, 2012c). Many triploid mandarin hybrids have been obtained from citrus breeding programs worldwide, including `Shasta Gold´, `Tahoe Gold´ and `Yosemite Gold´ from the USA program (Williams and Roose, 2004); `Tacle´, `Clara´, `Mandared´, `Mandalate´, `Sweet Sicily´ and `Early Sicily´ from the Italian program (Recupero et al., 2005; Russo et al., 2015); and `Garbí´, `Safor´ and `Alborea´ from the Spanish program (Aleza, 2015; Aleza et al., 2010a; Cuenca et al., 2010).

Male sterility has also been achieved producing cybrids by protoplast fusion. As noted above (see 6.3), in citrus, cybrids can derive as subproducts from somatic hybridization and are generally composed of the nuclear genome of the leaf parent, the mitochondrial genome of the callus or cell suspension parent, and a randomly inherited chloroplast genome (Aleza et al., 2016; Cabasson et al., 2001; Moreira et al., 2000). This material

open new avenues for seedless breeding (Ollitrault and Navarro, 2012) and particularly raise the possibility of transferring the male-sterile cytoplasm of satsuma mandarin to seeded cultivars with attractive fruit quality (see 3.4). Several researchers successfully introduced male-sterile satsuma mandarin mtDNA into different seeded cultivars by symmetric somatic hybridization (Cai et al., 2007; Guo et al., 2004; Tokunaga et al., 1999; Xiao et al., 2014; Zheng et al., 2012). Applications of somatic hybridization to citrus scion improvement also include the production of quality tetraploid breeding parents that can be used in interploid crosses to generate seedless triploids (Grosser et al., 2010, 2000) and the direct production of triploids by haploid + diploid fusion (Kobayashi et al., 1997; Ollitrault et al., 2000).

7. Molecular tools for citrus breeding and genetics

7.1. Molecular markers

Molecular markers are very useful in genetics because their capability to distinguish between genotypes. First markers used to distinguish among different plant varieties were secondary metabolites such as anthocyanins or phenolics. However, enzyme markers (allozymes and isozymes) are considered the first true molecular markers to be established (Grover and Sharma, 2016). The arrival of DNA manipulation techniques promoted a shift from enzyme-based to DNA-based markers. DNA marker technology started with the development of Restriction Fragment Length Polymorphism (RFLPs) and since them, wide variety of DNA-based markers has been developed, either PCR-based or non-PCR-based. Among PCR-based markers, Random Amplified Polymorphic DNA (RAPDs), Sequence Characterized Amplified Regions (SCARs), Intersimple sequence repeat (ISSRs), Cleaved Amplified Polymorphic Sequences (CAPs), Simple Sequence Repeats (SSRs or microsatellites) and Single Nucleotide Polymorphism (SNPs) have been some of the most used for plant genetic analysis (Agarwal et al., 2008; Grover and Sharma, 2016; Schlötterer, 2004).

Exemples of the use of molecular markers in citrus are phylogenetic studies, germplasm characterization, distinction between zygotic and nucelar plantules, marker assisted selection and genetic mapping. For that moleculars markers used include among others: Isozymes (Roose, 1988; Torres et al., 1982, 1978), RAPDs (Randomly Amplified PolymorphicDNAs; (Luro et al., 1994; Xiao et al., 1995), ISSRs (Inter-Simple SequenceRepeats; (Fang et al., 1998, 1997; Fang and Roose, 1997), AFLPs (Amplified Fragment LengthPolymorphisms; (de Oliveira et al., 2007; Fang et al., 2009; Liang et al., 2006), RFLPs (Restriction Fragment Length Polymorphisms; (Durham et al., 1992; Federici et al., 2000, 1998; Luro et al., 1996) and SSRs (Simple SequenceRepeats or microsatellites; (Barkley et al., 2006; Chen et al., 2006; Froelicher et al., 2008; Kijas et al., 1995; Liang et al., 2015; Liu et al., 2013; Luro et al., 2008; Ollitrault et al., 2010; Shimizu et al., 2016).

In recent years, molecular tagging techniques have undergone a major evolution with high-throughput sequencing and the decreasing costs of detection methodologies. The expansion and availability of sequencing data has enabled the development of SNP (Single Nucleotide Polymorphisms) markers, which have now emerged as an

indispensable tool in genetic applications and breeding programs. A SNP marker is defined as an allelic variation of a single base between two haplotypes of the same individual or between homologous chromosomes of a population of individuals. These markers are present in high abundance and their distribution is relatively uniform in a genome. SNPs genotyping microarrays have been used for genetic diversity studies (Fujii et al., 2013; P. Ollitrault et al., 2012b) and saturated genetic mapping (P. Ollitrault et al., 2012a; Yu et al., 2016).

Cuenca et al. (2011) demonstrates that the KASPar SNP genotyping technique, combined with the cluster analysis method, enables the efficient assignment of heterozygous allelic configuration within polyploid populations, which has been successfully applied to study the mechanisms underlaying unreduced gametes formation and segregation pattern of doubled-diploid citrus genotypes (Aleza et al., 2016; Cuenca et al., 2015; Garavello et al., 2020; Rouiss et al., 2018).

In the framework of this thesis, we will use SSRs and SNPs markers for the genetic analysis of recovered plants. SSRs markers are short tandem repeated motifs that may vary in the number of repeats at a given locus. They are codominants, high polymorphic, abundant, widely dispersed across the genome, easily scored and highly reproducible (Garcia-Lor et al., 2013b; Lu et al., 2015). In addition SSRs coupled with MAC-PR (Microsatellite DNA Allele Counting-peak Ratios) (Esselink et al., 2004) allows estimating the allele dosage in polyploids genotypes. This methodology has been validate for citrus by Cuenca et al. (2011). SNPs are single base-pair differences in the DNA sequence. They are bialellic, codominant, highly abundant and widely dispersed across the genome and highly reproducible (Brookes, 1999; Garcia-Lor et al., 2013a).

7.2. Whole genome sequencing and resequencing data

High-throughput sequencing techniques have made it possible to obtain reference sequences assembled into pseudochromosomes for five citrus species. These WGS (Whole Genome Sequencing) sequences constitute a valuable genomic resource for citrus genetics and improvement. The genome of sweet orange (*C. sinensis*) was the first genotype to be fully sequenced in citrus (Xu et al., 2013). Shortly thereafter, the International Citrus Genome Consortium (ICGC) involving several international laboratories published the reference sequence of the complete clementine genome (Wu et al., 2014). This was followed by the reference sequence of pummelo (*C. maxima*) genome (Wang et al., 2017), *P. trifoliata* (Peng et al., 2020), lemon (Guardo et al., 2021) and *Fortunella hindsii* (Wang et al., 2022). The availability of these reference sequences is a valuable resource for studying citrus genetics and for improving the exploitation of genetic diversity in breeding programs. Reference genomes are also very useful in resequencing projects that have developed in recent years.

De novo assemblies in scaffolds have also been published for a citrus relative, Atalantia buxifolia (Wang et al., 2017) and four species of the *Citrus* genus: mandarin (Wang et al., 2018), citron (Wang et al., 2017), *C. ichangensis* (Wang et al., 2017) and satsuma mandarin (Shimizu et al., 2017).

In parallel many genomes have undergone complete genome re-sequencing. These include a large number of species in the genus *Citrus* (mandarins, grapefruits, citrons, clementines, sweet oranges, sour oranges, lemons, limes, pummelos) as well as a few accessions from related genera (*Poncirus, Fortunella, Eremocitrus* and *Microcitrus*).

These re-sequencing data have shed light on the phylogenetic origin of many modern varieties and allowed deciphering their interspecific mosaic structures throughout the genome (Wu et al., 2021, 2018, 2014). The sequence data set was made available in the National Center of Biotechnology Information (NCBI) database. The re-sequencing of the whole genome remains very costly for studies on large populations. This is why other less expensive methods, such as genotyping by sequencing which reduces the complexity of the genome studied, are commonly used.

7.3. Genome-wide genotyping by Sequencing

Advances in next-generation sequencing (NGS; also known as high-throughput sequencing), the time-efficient and the sharp decrease in the cost, have led to the development of large-scale sequencing arrays based on reduced genome representations by using restriction enzymes. NGS provide thousands of markers densely covering the genome without the need for designing DNA markers beforehand. Examples include restriction site-associated DNA sequencing (RADseq) (Davey et al., 2011; Davey and Blaxter, 2010), diversity array technology sequencing (DArTseq) (Sansaloni et al., 2011) and genotyping by sequencing (GBS) (Elshire et al., 2011).

In citrus GBS, RAD-sequencing and DARTSeq have been successfully developed and used to study germplasm diversity and decipher its phylogenomic structures (Ahmed et al., 2019; Oueslati et al., 2017; Penjor et al., 2014), genome mapping (Curtolo et al., 2017b; Guo et al., 2015; Huang et al., 2018; Ollitrault et al., 2021) as well as QTL (Curtolo et al., 2017a) and GWAS (Imai et al., 2018) analyses. In this tesis we will use GBS for genotyping a F1 population obtained from Kiyomi x Murcott cross.

7.4. Linkage map and marker-trait association studies

A very important outbreak for efficient use of molecular markers in genetics and breeding is the availability of genetically mapped codominant markers anchored with the physical sequence. Generally, markers segregate in a mendelian fashion although distorted segregation ratios may be encountered. Citrus, with a basic chromosome number of 9, has a relatively small genome size. Haploid genomes of *C. sinensis* and *C. clementina* are, respectively, 380 Mb and 370 Mb of size (Arumuganathan and Earle, 1991).

Early citrus linkage maps were constructed using isoenzyme, RFLPs, RADPs and AFLPs markers and most of them suffered the dominant nature of some markers (RAPDs and AFLPs) and also from the limited analyzed hybrids and markers provided. So far, there are a reasonable number of linkage maps for citrus which are continually updated with the accumulation of knowledge and advancement of technologies. (Shimizu et al., 2020). The two-way pseudo-testcross mapping strategy was implemented for genetic mapping of progenies resulting from crosses between two heterozygous parents (Ritter et al., 1990). This strategy has been used in several high density mapping studies in citrus (Guo et al., 2015; Huang et al., 2018; Ollitrault et al., 2021; P. Ollitrault et al., 2012a). P. Ollitrault et al. (2012a) established the clementine reference genetic linkage map with 961 markers (677 SNPs, 258 SSRs y 26 Indels) spanning 1084.1 cM. These reference map has been used to enable the chromosome assembly of the reference whole genome citrus sequence (Wu et al., 2014) . Other saturated genetic maps followed: sweet orange (Xu et al., 2013), mandarin (Curtolo et al., 2017a, 2017b; Ollitrault et al., 2021; Shimada et al., 2014), grapefruit (Guo et al., 2015) and *Poncirus* (Curtolo et al., 2017b; Huang et

al., 2018). Foremost use for linkage maps is to identify genomic regions associated with specific phenotypic traits. Detection of major genes and QTLs controlling traits is based on the linkage disequilibrium between closely linked loci. Significant genetic association may be interpreted as either direct association, in which the genotyped molecular marker is the true causal variant conferring phenotypic variation; or indirect association, in which a molecular marker in linkage disequilibrium with the true causal variant is genotyped. Distinguishing between direct and indirect association is challenging and may require resequencing of the candidate region, dense genotyping of all available markers, or functional studies to confirm the role of a putative mutation in the phenotypic trait (Lewis and Knight, 2012). The most important application for marker-trait association studies is the marker-assisted selection (MAS). MAS is a key in breeding programs, particularly in tree species with long juvenile period such citrus, because selection of target genotypes can be carried out at the seedling stage.

In citrus, QTL analyses have located chromosomal regions controlling salinity tolerance (Raga et al., 2016; Tozlu et al., 2000, 1999), Tristeza resistance (Asins et al., 2012, 2004), *Alternaria* Brown Spot resistance (Cuenca et al., 2016, 2013b; Dalkilic et al., 2005), *phytophtora* resistance (Lima et al., 2018; Siviero et al., 2006), nematode resistance (Ling et al., 2000), and most recently, HLB tolerance in *P. trifoliata* (Huang et al., 2018). QTLs associated with fruit quality (Asins et al., 2015; Curtolo et al., 2017a; Yu et al., 2016; Y. Yu et al., 2017) and seed number and yield (García et al., 2000) could be identified. A QTL controlling leaf abscission, a characteristic trait of the genus *Poncirus*, was also identified (Xu et al., 2021). Today, the citrus genome database (https://www.citrusgenomedb.org/) lists 673 QTL identified in citrus. An alternative approach to QTL analysis is the Genome Wide Association Study (GWAS). It involves the analysis of germplasm collections and very high-density genotyping, or even complete genome resequencing data. GWAS studies are still very limited in citrus. They concern fruit quality on panels, mixing traditional germplasm and individuals from recombinant families (Imai et al., 2018; Minamikawa et al., 2017).

Mechanisms involved in the biology of reproduction have also been tagged with molecular markers including apomixis (García et al., 1999; Kepiro and Roose, 2010; Nakano et al., 2013, 2012; Wang et al., 2017) and male sterility (Goto et al., 2018). The genetic determinism of female sterility, self-incompatibility and parthenocarpy remains unanswered and studies to improve knowledge on the phenotypic are required to be applied in association studies.

8. Objectives of the PhD dissertation and research questions

Reproductive biology behavior is a key component for efficient breeding programs. It is particularly important for seedless mandarin breeding for which male and female sterility, self-incompatibility (SI) and parthenocarpic ability (PA) are directly linked with the production of seedless fruits, while apomixis is a constraint for the production of hybrids. These characters, as well as the success of the progamic phase, can be affected by environmental conditions. The overall objective of this PhD dissertation is to increase our knowledge on different reproductive aspects that are crucial for seedless mandarin breeding and propagation, and for understanding their interaction with environmental

conditions that could become critical in the context of global climate change. This PhD dissertation is structured around the following four specific objectives.

Objective 1. To generate comprehensive and nature-representative knowledge about the influence of temperature on citrus progamic phase applicable to future breeding programs in a climate change context.

Knowledge of how temperature influences the progamic phase is very important to adapt breeding programs in the current climate change context, and also to establish improved pollination protocols based on controlled environmental conditions. Previous information about the influence of temperature on citrus progamic phase is scarce, in particular, results in connection with the female counterpart. As for the male counterpart, previous research has shown the strong effect of temperature on pollen grain germination *in vitro* and pollen tube growth observed in squashed pistils from detached flowers. However, the observation of pollen tube growth on cross slices from pistils collected daily is necessary to assess pollen tube growth kinetics and dynamics along the pistil. The implementation of such procedure together with *in planta* pollen grain germination, stigmatic receptivity and ovule degeneration evaluations will allow us to carry out a more comprehensive and nature-representative analysis of the influence of temperature on citrus progamic phase.

Objective 2. To evaluate PA and SI in nine mandarin varieties with relevant characteristics as parents for seedless mandarin breeding.

PA coupled with SI is one of the main mechanisms for seedlessness in mandarins. A thorough review of the research devoted to some genotypes of this mechanism evidences that the information available now is still insufficient and, in some cases, contradictory. Therefore, it is necessary to increase such knowledge to improve the selection of parents for seedless breeding. For this purpose, we will develop a protocol based on emasculation, hand self-pollination and hand cross-pollination. Through this protocol we will evaluate the PA, the SI and the requirement of pollination stimuli for seedless fruit production in nine mandarin varieties selected due to their importance as parents for mandarin breeding programs: 'Clemenules' and 'Monreal' clementines (*C. clementina* Hort. ex Tan.), 'Campeona' (*C. nobilis* Lour.), 'Imperial' (*C. reticulata* Blanco), 'Salteñita' (*C. deliciosa* Ten.), 'Fortune' (*C. clementina* × *C. tangerina*), and 'Moncada' (*C. clementina* × (*C. unshiu* × *C. nobilis*)) mandarins, 'Ellendale' tangor (*C. reticulata* × *C. sinensis*), and 'Serafines' satsuma (*C. unshiu* (Mak.) Marc.).

Objective 3. To compare the effectiveness of the SI reaction breakdown caused by temperature stress, bud pollination and polyploidization.

Breakdown of the SI reaction allows to obtain new populations from selfing of self-incompatible parents thus expanding cross possibilities in breeding programs. Few studies have focused only partially on the breakdown of the SI reaction in citrus and no complete work has evaluated in depth the breakdown of SI through temperature stress, bud pollination and tetraploidy. Therefore, the question of which of these three strategies is the most effective in obtaining a high number of hybrids from selfing remains to be answered. To address this issue, we will evaluate the breakdown of the SI reaction

caused by temperature stress, bud pollination and tetraploidy in two varieties: ‘Clemenules’ clementine and ‘Fortune’ mandarin. In addition, we will recover seeds from the three different strategies and will assess their genetic origin.

Objective 4. To develop SNP markers associated with polyembryony and male sterility.

In recent years, molecular tagging techniques have evolved and SNP markers have emerged as an indispensable tool in genetic applications and breeding programs. Genomic regions associated with polyembryony and male sterility have been reported, but to our knowledge there are no SNP markers developed for these traits. The development of such markers associated with polyembryony and male sterility will be very useful for marker-assisted selection (MAS). Using a segregating progeny derived from ‘Kiyomi’ tangor (a monoembryonic and CMS variety) × ‘Murcott’ tangor (a polyembryonic and male fertile variety), we will carry out a genetic association study, performing genotyping by sequencing (GBS) and phenotyping for both polyembryony and male sterility. SNP markers closely linked to these traits will be developed using KASPar technology and their technical validity and efficiency to select the targeted phenotype will be tested.

Chapters

Chapter 1. Influence of temperature on the progamic phase in citrus

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Abstract

Temperature in the progamic phase is critical for the success of plant sexual reproduction, and new knowledge is needed to optimise breeding programmes to obtain new varieties that adapt to a climate change scenario. Using three male donors and one female recipient in the genus *Citrus*, we evaluated the effect of four temperature regimes on each process in the progamic phase. An innovative method based on microscopic observations of cross sections from pollinated pistils collected daily allowed a comprehensive analysis of pollen tube growth (dynamics and kinetics) along the pistil. Pollen grain germination and stigmatic receptivity were evaluated directly on the stigma, which offers more accurate information than previously reported *in vitro* experiments. Our results showed that warm temperatures reduce the time needed by pollen tubes to reach ovules and accelerate pistil degeneration, while cold temperatures produce the opposite effects. Interestingly, we observed both pollen grain germination and pollen tube growth at 10ºC, which have not been observed in previous studies in citrus. At this temperature, the differences observed in both pollen grain germination and pollen tube growth for different genotypes reflect the adaptation of their sporophytic generation to low temperatures which would enable gametophytic screening to be used as a tool to select better adapted genotypes to different temperature conditions. The differences observed in the growth rates between pollen tubes in each genotype-temperature combination provide an opportunity to explore additional gametophytic selection in this reproductive phase. The capacity to respond to temperature changes in the progamic phase to ensure mating can be useful for breeding programs that focus on obtaining better adapted populations to different temperature conditions.

Keywords: Pollen tube growth dynamics and kinetics; stigmatic receptivity; ovule degeneration; breeding programmes; climate change; plant reproduction

1. Introduction

Temperature is one of the main environmental conditions that influence the success of plant sexual reproduction (Iizumi et al., 2017; Zhao et al., 2017). Several studies report on the impact of temperature on gametophytic generation and the progamic phase. The effect of high temperatures can be observed on both female and male gametes. However, most previous studies have focused on the temperature effect on the morphology, chemical composition, and functionality of pollen grains (Aloni et al., 2001; Distefano et al., 2018; Koti et al., 2005; Lora et al., 2009; Prasad et al., 2002; Sato et al., 2002). Other studies report on sporophytic generation from the postzygotic stage to the reproductive phase (Hedhly, 2011; Hedhly et al., 2009; Sage et al., 2015; Zinn et al., 2010).

The progamic phase, which elapses from pollination to fertilization, is one of the most critical phases among the events that take place during the sexual reproduction process in plants. It is a period in which specific interactions between the male gametophyte and the pistil occur. This phase is crucial to achieve successful mating and is extremely vulnerable to prevailing environmental conditions. Temperature strongly affects each process in the progamic phase; i.e. stigmatic receptivity, pollen grain germination, pollen tube growth and ovule degeneration (Hedhly, 2011). These processes influence the

effective pollination period (EPP), defined for the first time by Williams (1965) in apple as ovule longevity minus the time between pollination and fertilization. The EPP determines the number of days on which pollination is able to produce non-parthenocarpic fruits. This period has been analysed in many fruit crops, and temperature appears as a crucial influential factor (Sanzol and Herrero, 2001). In citrus, the influence of genotype on EPP under field conditions has been reported by Mesejo et al. (2007), but there is very little information available about how temperature affects this period.

Between pollination and fertilization, pollen grains germinate on the receptive stigma surface and grow through the pistil to reach a viable ovule. In addition, male-female compatibility is needed for fertilization to occur. In the case of many citrus genotypes, a gametophytic self-incompatibility system is common, which arrests pollen tube development in the style (Soost, 1965). Therefore, a compatible cross is required to complete ovule fertilization and subsequent seed formation.

By taking advantage of staining techniques to observe pollen tubes inside pistils, early reports evaluated pollen tube growth at increasing temperatures in *Datura stramonium* (Buchholz and Blakeslee, 2006) and *Oenothera organensis* (Lewis, 1942). Since then, the effect of temperature in the progamic phase has been extensively studied in many herbaceous species, (Coast et al., 2016; Elgersma et al., 1989; Kakani et al., 2005; Koti et al., 2005; Liu et al., 2006; Matsumoto et al., 2012; Mckee and Richards, 1998; Shivanna et al., 1991; Snider et al., 2011a, 2011b) as well as woody species and tree crops (Acar and Kakani, 2010; Gao et al., 2014; Hedhly et al., 2005b, 2005a, 2004; Huang et al., 2010; Jefferies et al., 1982; Jefferies and Brain, 1984; Kakani et al., 2002; Koubouris et al., 2009; Luza et al., 1987; Nygaard, 1969; Pham et al., 2015; Radičević et al., 2016; Sedgley, 1977).

Regarding the female counterpart, the evolution of flowers after anthesis until senescence includes basipetal maturation that starts at the stigma and continues downwardly to the ovary. These changes are developmentally regulated and do not, therefore, depend on the action of pollen tubes (Distefano et al., 2011). The pistil senescence process includes loss of stigmatic receptivity, style abscission and ovule degeneration. The influence of temperature on stigmatic receptivity has been reported in woody species, such as sweet cherry (Hedhly et al., 2003), peach (Hedhly et al., 2005a), and cherimoya (Lora et al., 2011), while the influence of temperature on ovule degeneration has been described in plum, and sweet and sour cherry cultivars (Beppu et al., 2001; Cerović et al., 2000; Postweiler et al., 1985).

Citrus (*Citrus* spp.) is the leading fruit crop worldwide, whose production amounts to more than 146 million tons (FAOSTAT, 2020) in more than 100 countries with tropical and subtropical climates (between 40º N and 40º S, approx.), and even in colder areas like Japan and the Jeju Island in South Korea. Studies on global climate change predict an increase in average temperatures between 0.3 and 4.8ºC in 80 years (IPCC, 2014), and in the temperature range amplitude, which could limit plant cultivation in some areas. The consequences of the global climate change are already affecting phenological plant traits, especially those related to flowering (Hedhly et al., 2009; Springate and Kover, 2014), and also shifts the expected geographical distribution in natural ecosystems (Corlett and Westcott, 2013; Singer et al., 2016). In this context, the environmental conditions of the main citrus production areas will change and citrus-growing areas may be extended. Thus breeding programs based on sexual hybridisations could take into account new environmental conditions. Very few studies have evaluated the influence of

temperature on the progamic phase in citrus, especially those related with the female parent. Distefano et al., (2012) showed that temperature variation in this phase has a strong effect on pollen germination *in vitro* and on the pollen-pistil interaction in detached flowers of three ancestral citrus species. However, new methods based on *in planta* evaluations need to be implemented to characterise the influence of temperature on the progamic phase in citrus, specifically in a more comprehensive and nature-representative way.

Knowledge of how temperature influences the progamic phase in citrus is most important to adapt breeding programmes to a climate change context, and to also establish improved pollination protocols based on controlled environmental conditions. Citrus breeding programmes based on sexual hybridisations have been developed worldwide at both the diploid and triploid levels. At the Instituto Valenciano de Investigaciones Agrarias (IVIA), we have been performing a large-scale triploid breeding programme based on sexual hybridisation since 1996, with more than 16,000 hybrids obtained from more than 300 parental combinations (Navarro et al., 2015). The experience acquired over more than 20 years of hybridisations reveals the importance of temperature in the progamic phase and of the male-female interaction on hybrid production. Indeed major variations between different years and locations have been observed in terms of the number of hybrids recovered from the same hybridisation, which evidences the influence of environmental conditions on pollen and pistil performance (Aleza et al., 2010b, 2012c, 2012b).

This paper evaluates the influence of temperature on both male and female parts in the progamic phase of citrus. Experiments were performed *in planta* under three constant temperature regimes, 10ºC, 20ºC and 30ºC, representing cool to hot spring temperatures and the field conditions in the Mediterranean region of Moncada, Valencia, Spain. Alternatively to classic whole pistils staining and squashing protocols, we made histological observations on several cross sections along pistils. This new methodology allowed us to perform a more comprehensive analysis of the dynamics and kinetics of pollen tube growth. The objective of this study was to generate knowledge about the influence of temperature on the progamic phase applicable to future breeding programmes in a climate change context.

2. Material and Methods

2.1. Plant Material

The influence of temperature on the progamic phase was evaluated using ‘Clemenules’ clementine (*C. clementina*), ‘Pineapple’ sweet orange (*C. sinensis* (L.) Osb.) and ‘Ichang’ papeda (*C. ichangensis* Swing) as the male parents, all crossed with ‘Fortune’ mandarin (*C. clementina* x *C. tangerina*) as the female parent. ‘Clemenules’ clementine is the most representative mandarin cultivar grown in Spain. ‘Pineapple’ sweet orange is a widely used cultivar for juice production, and is reported to be more sensitive to frost than most other varieties. ‘Ichang’ papeda is a remarkable plant, reported to be the most cold-resistant of all the evergreen species in the citrus group (Hodgson, 1967). The female parent ‘Fortune’ mandarin is a high quality variety that is widely used as a female parent in breeding programmes due to not only its late maturity and fruit quality, but also

because it produces a high frequency of unreduced gametes for triploid hybrid production (Aleza et al., 2010b).

2.2. Experimental Procedures for Pollinations

As our research focused on the progamic phase, experiments were carried out from pollination. The development of sexual organs of both the male and female parents took place herein under the same field conditions (FC), and no differences during gametogenesis due to temperature were assumed between genotypes.

Eight adult 'Fortune' mandarin trees grown in containers under FC were used for the experiments. During the flowering period, six of them were moved by placing two of them in culture chambers in each studied temperature regime: 10(±2)ºC; 20(±2)ºC, 30(±2)ºC. The remaining two were left under FC. The average temperature under FC within the experimental time frame was 18.5ºC, with a typically gradual increase of up to 30ºC in the daytime and one less than 10ºC at night. To perform hand-pollinations, anthers were removed from the flowers of the donors randomly harvested at the balloon stage and were dried in Petri dishes over silica gel in a desiccator at room temperature. Dehiscence occurred after one to two days, and the dehiscent anthers were used to pollinate the emasculated flowers at anthesis of the 'Fortune' mandarin plants placed under the different temperature regimes. The pollinated flowers were labelled and bagged to avoid any undesired cross-pollination. The pistils from the pollinated flowers were fixed in FAA solution (formalin, glacial acetic acid, 70% ethanol, 1:1:18, v/v) (Johansen, 1940) and stored at 4ºC until the histological observations. The time that elapsed from pollination to pistil fixation in FAA differed for each experiment, as described below.

2.3. Histological and Microscopic Observations

In order to evaluate pollen grain germination, pollen tube growth, stigmatic receptivity and ovule degeneration, histological preparations were performed. The pistils fixed in FAA were submerged three times in water for one hour. Pistil length was recorded and they were sliced into 14 cross-sections (0-13) using a sharp blade. Stigmas were sliced into two sections (0-2), styles into eight sections (2-10) and ovaries into four sections (10-13) (Figure 1). Slices were then stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens FH, Esser, 1957) and preparations were observed under a Leica MZ16FA stereomicroscope equipped with GFP1 epifluorescence.

As slicing is an innovative method for pollen tube growth characterisations, we compared slicing and the classical squashing approach in our initial research process stages to ensure that observations were consistent. Having verified this methodological consistency, pollen tube growth observations based on slicing were made.

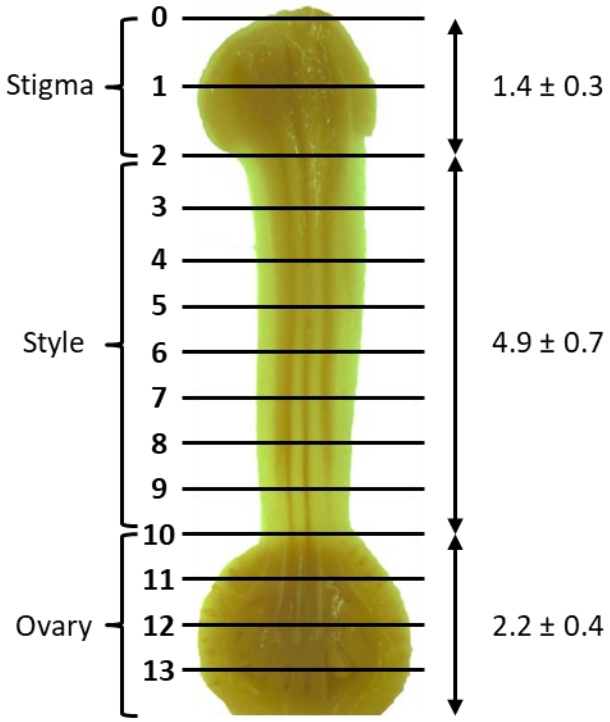

Figure 1. Pistil longitudinal section. The cross-sections used in this study are indicated with lines from 0 to 13. Length in mm (mean ± SD) of the stigma, style and ovary.

2.4. Evaluation of Pollen Grain Germination (PGG) in planta

To evaluate the effect of temperature and genotype on PGG *in planta*, five flowers of the 'Fortune' mandarin trees placed at 10ºC, 20ºC and 30ºC (FC were not used for the PGG evaluation) were hand-pollinated with pollen from the three male parents. The pollinated flowers were sampled 12-24 hours after pollination. PGG was quantified directly on the stigma surface where pollen germination took place. Then the stigma surface was squashed to count the germination of pollen grains (Figure 2). Pollen grains were scored as germinated when pollen tube length exceeded the diameter of its pollen grain. Five stigmas for each genotype-temperature combination were used and at least 500 pollen grains per stigma were counted with the ImageJ2 software (Schindelin et al., 2015).

2.5. Evaluation of Pollen Tube Growth (PTG) in planta

Five pollinated pistils of each cross and temperature regime (10ºC, 20ºC, 30ºC and FC) were sampled sequentially on ten consecutive days, starting on the day after pollination. The histological observations of the fixed pistils were made to track PTG *in planta* by scoring the number of pollen tubes observed in each pistil section. PTG dynamics was determined by the five maximum values for the number of pollen tubes observed in each pistil section during the ten-day sampling period. PTG kinetics was determined by the pistil section reached by pollen tubes daily. Ovary sections were excluded from the PTG analysis.

2.6. Stigmatic Receptivity

Fifty flowers of the 'Fortune' mandarin cultivated at 10ºC, 20ºC, 30ºC and FC were emasculated and labelled at the balloon stage. Five of the labelled flowers for each temperature regime were pollinated sequentially on ten consecutive days, starting on the day of anthesis. 'Ichang' papeda was used as the pollen donor for this experiment. On day one after pollination, pistils were fixed in FAA solution and stored at 4ºC until the histological observations. For each sample, the percentage of the germinated pollen grains and the growth capacity of pollen tubes were evaluated. The percentage of the germinated pollen grains was scored by squashing the stigma surface as described before for PGG (Figure 2). The growth capacity of pollen tubes was evaluated by counting the number of pollen tubes growing in the middle section of the stigma (Section 1; Figure 1). A comparison between the samples pollinated on different numbers of days after anthesis in each temperature regime was made to evaluate changes in stigmatic receptivity.

2.7. Ovule Degeneration and Style Abscission

Ovule degeneration is associated with the presence of callose in their chalazal region, whose fluorescence can be observed by aniline blue staining (Mesejo et al., 2006; L. Zhang et al., 2018). To assess the influence of temperature on ovule life span, 20 ovules of the 'Fortune' mandarin for each temperature-day combination were isolated from the previously stained ovaries. The isolated ovules were squashed to clearly observe fluorescence without other surrounding tissues interacting.

In order to analyse the temperature effect on style abscission, 10 flowers were tagged on the day of anthesis in the 'Fortune' mandarin trees under the four temperature regimes of the study, and the changes in these flowers were monitored daily for 10 days. The day when the abscission line appeared and its distance from the style-ovary junction were recorded.

2.8. Statistical Analyses

Data were confirmed to fit the normal distribution and outlier values based on box plots were removed prior to further analyses. The experimental design was double factorial. Analyses of variance and LSD multiple range tests were performed using version 16.1.03 of the Statgraphics Centurion XVI statistical software package.

3. Results

3.1. Pollen Grain Germination (PGG) in planta

For all nine genotype-temperature combinations, pollen grain germinated and pollen tubes grew between the finger-like papillae of the stigma surface accessing inside stigma (Figure 2). Both genotype and temperature, as well as the genotype-temperature interactions, had a significant effect on PGG *in planta*, as the ANOVA revealed (Supplementary Material).

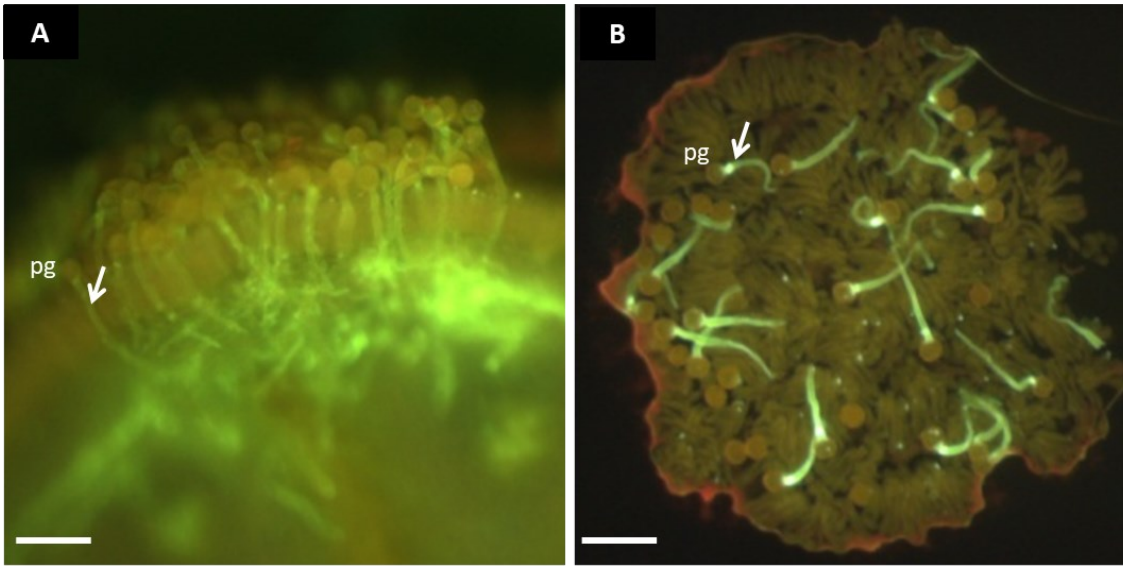

Figure 2. *In planta* pollen grain germination of 'Clemenules' clementine on the stigma surface of 'Fortune' mandarin 24 h after pollination at 10 °C.(A) Accumulation of the germinated pollen grains and pollen tubes growing between the finger-like papillae of the stigma surface (Section 0 in Figure 1). (B) Squash of the stigma surface on which pollen grains and pollen tubes can be individually observed. Pollen tubes are marked by an arrow; pg: pollen grain. Stigma surface stained with aniline-blue. Scale bar: 100 µm.

Results were expressed as a percentage of the germinated pollen grains (Table 1). A different behaviour was revealed among genotypes and was temperature-dependent. In 'Pineapple' sweet orange, the lowest percentage was observed at 10ºC, while no significant differences were detected between 20ºC and 30ºC. In 'Clemenules' clementine, differences between 20ºC and 30ºC were observed for PGG, while no significant differences were noted between these two temperatures and 10ºC. For 'Ichang' papeda, the lowest percentage was 30ºC, and no significant differences were found between 10ºC and 20ºC (Table 1).

The comparison made between genotypes at all three temperatures showed significant differences at both 10ºC and 20ºC. At these temperatures, 'Pineapple' sweet orange always displayed the lowest percentage, 'Clemenules' clementine obtained intermediate values and the highest percentage went to 'Ichang' papeda. Conversely, no differences were observed between genotypes at 30ºC (Table 1).

Table 1. Influence of temperature and genotype on the percentage of pollen grain germination in planta.

Genotypes	10ºC	20ºC	30ºC
'Pineapple'	42±3.6 (a_p ; a_{10})	51±6.9 (b_p ; a_{20})	54±7.7 (b_p ; a_{30})
'Clemenules'	64±3.4 (ab_c ; b_{10})	70±6.3 (b_c ; b_{20})	60±2.3 (a_c ; a_{30})
'Ichang'	81±2.3 (b_i ; c_{10})	84±7.3 (b_i ; c_{20})	61±2.8 (a_i ; a_{30})

The percentage of germinated pollen grains is given as the mean±SD (n=5). In brackets, significant differences between temperatures for the same genotype and between genotypes for the same temperature are indicated by different letters in the first and second positions, respectively (p=0.05, Fisher LSD). For each genotype-temperature combination, the initial letters of the genotype and temperature value are added as a subscript to make viewing easy.

3.2. Pollen Tube Growth (PTG) Dynamics in planta

The syncarpous gynoecium of the 'Fortune' mandarin is composed of 9-15 carpels fused together. Each carpel has an independent stylar canal starting from a common stigma surface that leads to a locule in the ovary. Each locule contains 4±1 ovules, resulting in an average of 45 ovules per ovary (data not shown). The whole pistil length measured an average of 8.5 mm with 1.4±0.3 mm for stigmas, 4.9±0.7 mm for styles and 2.2±0.4 mm for ovaries (Figure 1).

In our experiments, high PGG rates were produced. Although the initiation of stylar canals takes place on the upper stigma, we observed many pollen tubes to grow outside stylar canals (Figure 3A). From the stigma-style junction, the number of pollen tubes growing outside the stylar canals decreased (Figure 3B); and along the lower half of the style (Figure 3C), only pollen tubes growing inside stylar canals were observed in all the genotype-temperature combinations, which were subsequently able to reach the locules (Figure 3D).

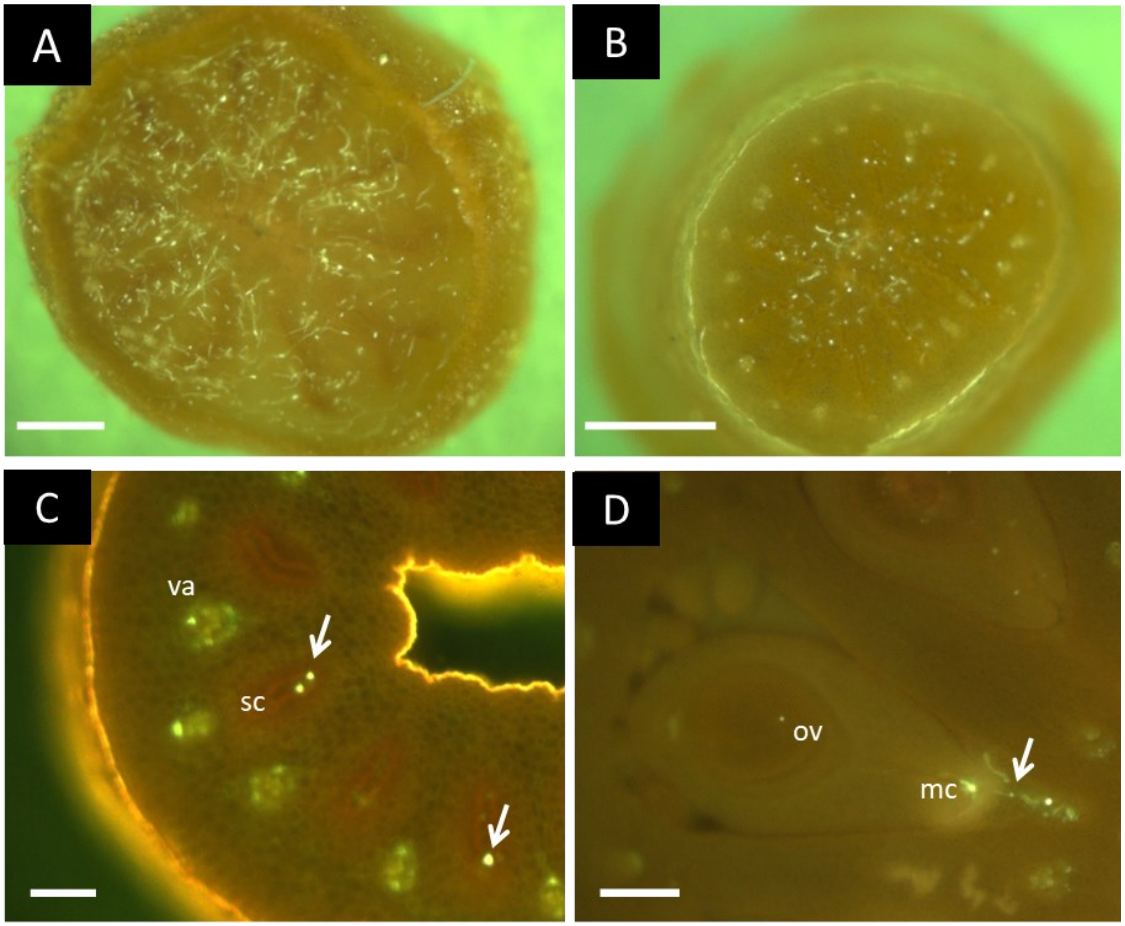

Figure 3. Cross-sections showing pollen tube growth in the 'Fortune' mandarin pistil. (A) Massive pollen tube growth throughout the middle section of the stigma. (B) Pollen tubes growth in the upper style. (C) pollen tubes growing inside the stylar canals in the middle section of the style. (D) Pollen tube accessing the ovule through the micropyle. Figures A, B, C and D correspond to Sections 1–3–6 and 12 in Figure 1, respectively. Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule; mc: micropyle. Sections of the pistil stained with aniline-blue. Scale bar: A,B = 500 µm; C,D = 100 µm.

Regarding the quantification of PTG, the three pollen genotypes showed massive PTG in the stigma at the four studied temperatures, which decreased along the style and depended differently on both genotype and temperature (Figure 4). As a result, the ANOVA revealed significant differences among temperature, genotype and their interaction in the maximum number of pollen tubes observed at the style-ovary junction (Supplementary Material).

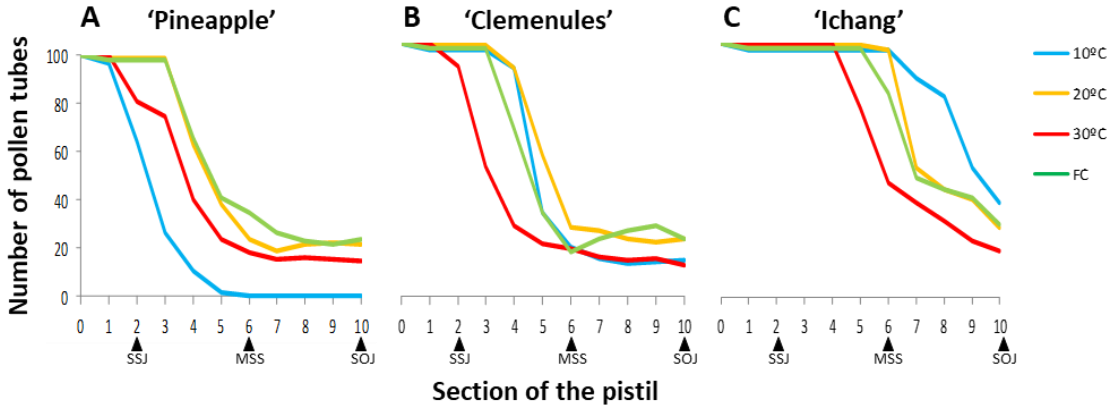

Figure 4. Pollen tubes growth dynamics for all three crosses evaluated in the four temperature regimes.Dynamics is expressed as the average of the five maximum values of the number of pollen tubes observed in each cross-section for 10 days after pollination for (A) 'Pineapple' sweet orange; (B) 'Clemenules' clementine; and (C) 'Ichang' papeda. More than 100 pollen tubes observed in one slice were recorded as 100. The X-axis shows the sections of the pistil (displayed in Figure 1). As a reference, the stigma-style junction (SSJ), middle section of the style (MSS) and style-ovary junction (SOJ) are pointed out below the corresponding pistil section.

In 'Pineapple' sweet orange (Figure 4A), the number of pollen tubes lowered from the stigma-style junction at 10ºC and 30ºC, and many pollen tubes (more than 100) were observed between sections 0 and 3 at 20ºC and under FC. At the style-ovary junction (Table 2), no pollen tubes were observed at 10ºC, whereas fewer pollen tubes were noted at 30ºC compared to 20ºC and the FC. In 'Clemenules' clementine (Figure 4B), the reduction in the number of pollen tubes started at the stigma-style junction at 30ºC, while many pollen tubes were observed between sections 0 and 3 at 10ºC, 20ºC and for the FC. At the style-ovary junction (Table 2), the maximum number of pollen tubes was significantly lower at 10ºC and 30ºC than at 20ºC and under FC, similarly to 'Pineapple' genotype. For 'Ichang' papeda, a large amount of pollen tubes was observed between sections 0 and 4-5 at 30ºC and under FC, respectively, and down to the middle of the style at 10ºC and 20ºC (Figure 4C). At the style-ovary junction (Table 2), major differences were observed between the lowest value at 30ºC and the highest one at 10ºC, whereas intermediate values were recorded at 20ºC and under FC.

The days elapsing from pollination to the maximum number of pollen tubes observed at the style-ovary junction differed depending on both genotype and temperature. This timeframe was three days in 'Ichang' papeda at 20ºC and 30ºC and in 'Clemenules' clementine at 30ºC, four days in 'Pineapple' sweet orange at 20ºC and 30ºC and in 'Clemenules' clementine at 20ºC, and five, six and seven days in 'Ichang' papeda, 'Clemenules' clementine and 'Pineapple' sweet orange, respectively, under FC, and was 10 days in 'Clemenules' clementine and 'Ichang' papeda at 10ºC.

Table 2. Maximum number of pollen tubes of the genotypes studied at different temperatures at the style-ovary junction of 'Fortune' mandarin pistils for 10 days after pollination.

Genotypes	10ºC	20ºC	30ºC	Field Conditions
'Pineapple'	0±0.0 (a_p ; a_{10})	22±2.7 (c_p ; a_{20})	15±1.7 (b_p ; ab_{30})	24± 1.1 (c_p ; a_{FC})
'Clemenules'	14±2.0 (a_c ; b_{10})	23±4.3 (b_c ; a_{20})	12±1.7 (a_c ; a_{30})	23±1.8 (b_c ; a_{FC})
'Ichang'	37±6.0 (c_i ; c_{10})	27±8.4 (ab_i ; a_{20})	18±5.2 (a_i ; b_{30})	28±6.8 (bc_i ; a_{FC})

The maximum number of pollen tubes is given as mean±SD (n=5). In brackets, significant differences between temperatures for the same genotype and between genotypes for the same temperature are indicated by different letters in the first and second positions, respectively (p=0.05 Fisher LSD). For each genotype-temperature combination, the initial letters of the genotype and temperature value are added as a subscript to make viewing easy.

3.3. Pollen Tube Growth (PTG) Kinetics in planta

The fixation of samples every day from pollination allowed us to analyse the daily progression of pollen tubes from the stigma surface through the pistil. Significant genotype and temperature effects on the section reached by pollen tubes were found on the first three days after pollination, while the effect on day four was predominantly temperature-dependent. From day five, pollen tubes reached the ovaries at 20ºC, 30ºC and under FC regardless of genotype, whereas differences were genotype- and day-dependent at 10ºC (Figure 5 and Supplementary Material).

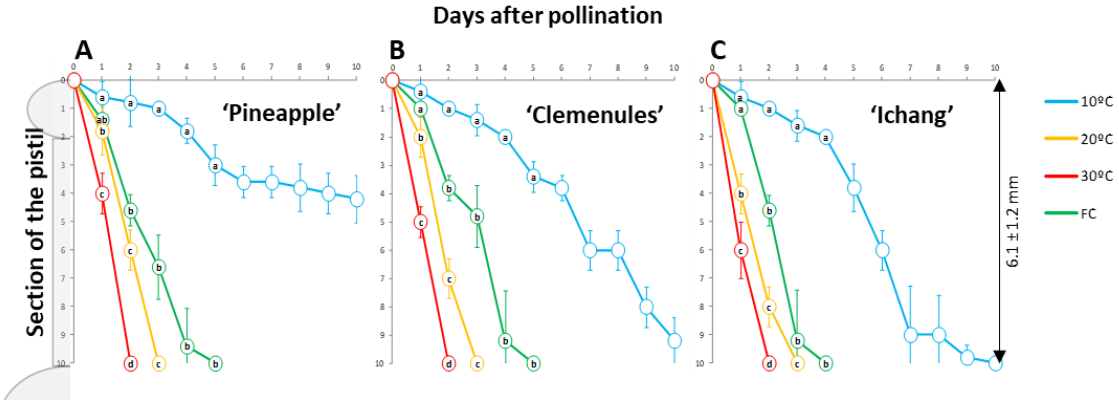

Figure 5. Pollen tubes growth kinetics for all three crosses evaluated in the four temperature regimes. The results are expressed as the pistil section reached daily by pollen tubes (mean ± SD) for (A) 'Pineapple' sweet orange; (B) 'Clemenules' clementine; and (C) 'Ichang' papeda. Different letters in circles indicate significant differences between temperatures for the same genotype and day (0.05% Fisher LSD). Pistil is represented on the Y-axis to easily view the results, and the longitude (mean ± SD) from the stigma surface to the bottom style is shown to the right of the figure.

The pollen tubes of the three male genotypes displayed a similar behaviour by reaching more basal pistil sections on a daily basis at higher temperatures, which resulted in a shorter time for pollen tubes to reach the ovary. At 30ºC, pollen tubes reached the bottom of the style two days after pollination, whereas at 20ºC they took one more day regardless of genotype (Figure 5). However, under FC 'Ichang' papeda needed four days to get to the bottom of the style, whereas 'Pineapple' and 'Clemenules' reached it on day five (Figure 5).

The biggest differences were observed at 10°C, as only the 'Ichang' papeda pollen tubes reached the bottom of the style in 10 days, whereas no 'Pineapple' and 'Clemenules'

pollen tubes arrived at the style-ovary junction. ‘Clemenules’ pollen tubes reached pistil section 9, whereas ‘Pineapple’ pollen tubes arrived only at section 4 (Figure 5).

3.4. Stigmatic Receptivity

During the 10 experimental days, the reduction observed in germinated pollen grains and the quantity of pollen tubes growing across the middle section of the stigma evidenced a reduction in stigmatic receptivity, which was noticeably influenced by temperature (Figure 6, Supplementary Material).

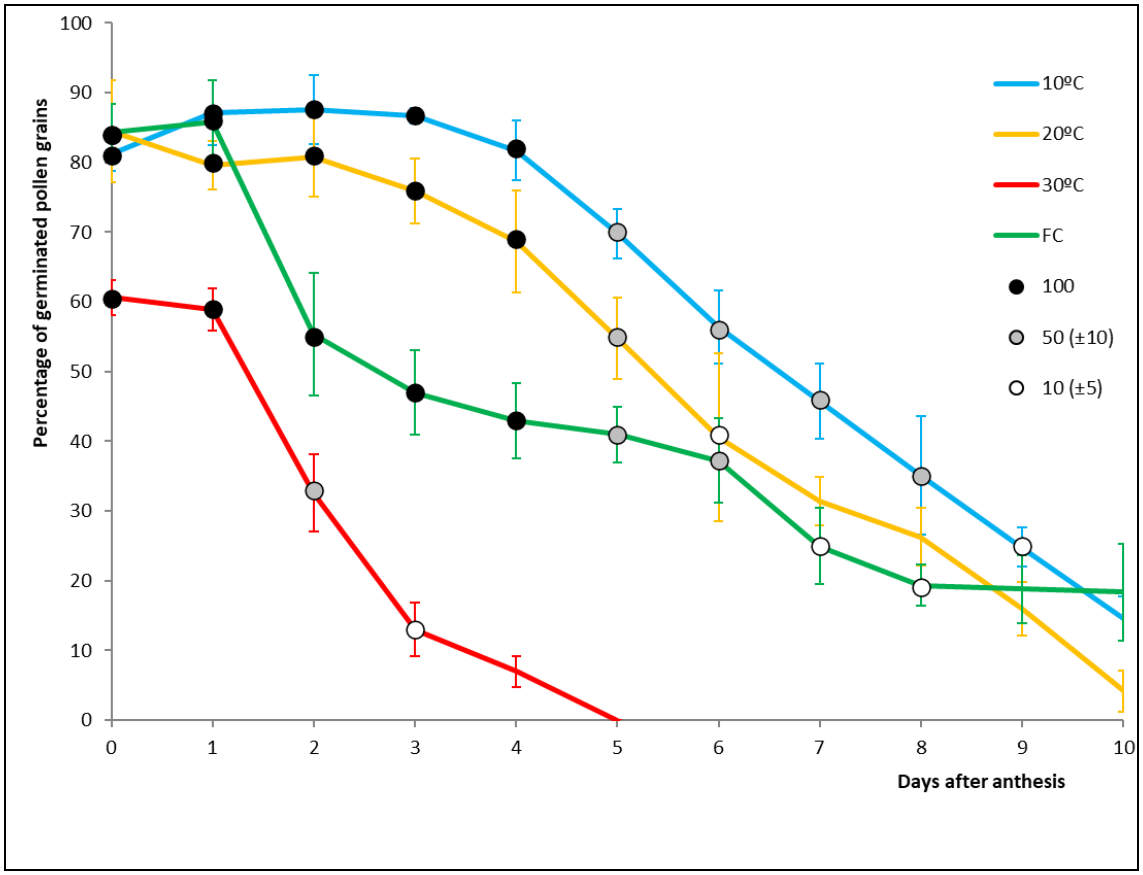

Figure 6. Receptivity of the ‘Fortune’ mandarin stigmas pollinated with ‘Ichang’ papeda, expressed as a percentage of pollen grain germination (mean ± SD on the Y-axis) and quantity of pollen tubes observed in the middle section of the stigma (circles) from the day of anthesis (0 on the X-axis) until 10 days after anthesis at 10 °C, 20 °C, 30 °C and under the field conditions (FC). The LSD multiple range test for the variable number of pollen tubes growing in the middle section of the stigma for each temperature identified four homogeneous groups, represented with different filled circles: black represents more than 100 pollen tubes growing in the middle section of the stigma, grey denotes 50 and white indicates 10 (mean ± SD). No circle means that zero pollen tubes were observed in the middle section of the stigma.

The flowers that pollinated at anthesis showed 61% of germinated pollen grains at 30ºC, and more than 80% did so at 10ºC, 20ºC and under FC, and many pollen tubes were observed to grow inside the stigma. At 30ºC, a rapid drop in both the germinated pollen grains and the quantity of pollen tubes growing along the stigma took place and resulted in a 13% of the germinated pollen grains to be coupled with a few pollen tubes growing in the stigmas that pollinated 3 days after anthesis. For the other three temperatures, although the reduction in the germinated pollen grains gradually occurred on the 10

experimental days, no pollen tubes growing along the stigma were observed in the flowers pollinated 7, 9 and 10 days after anthesis at 20ºC, under FC and 10ºC, respectively (Figure 6). Thus the germination that took place during these days seemed non-effective in fecundation terms.

The results of this experiment indicate that stigmatic receptivity was strongly influenced by high temperature, and notably lowered the percentage of the germinated pollen grains and the number of pollen tubes growing in the middle section of the stigma on the first three days after anthesis. In contrast, cold temperatures (10ºC) prolonged the stigmatic receptivity period, with more than 30% of the pollen tubes germinated and many pollen tubes (50±10) growing in the middle section of the stigma eight days after anthesis.

3.5. Ovule Degeneration and Stylar Abscission

The collected data about ovule degeneration and the appearance of the style abscission line (SAL) on 10 consecutive days after anthesis in the four studied temperature regimes showed that temperature significantly influenced both processes (Figs. 7-8, Supplementary Material). Ovule degeneration is associated with the presence of callose fluorescence (Figures 7A-B) and the first symptoms (with 10% degenerating ovules) were observed four days after anthesis at 30ºC, five days at 20ºC and seven days under FC. At 10ºC, no ovule degeneration took place during the 10-day observation period. Conversely at 30ºC, all the ovules were degenerated on day seven after anthesis, while the percentage of ovule degeneration was 77% and 20%, respectively, at 20ºC and under FC (Figure 8A).

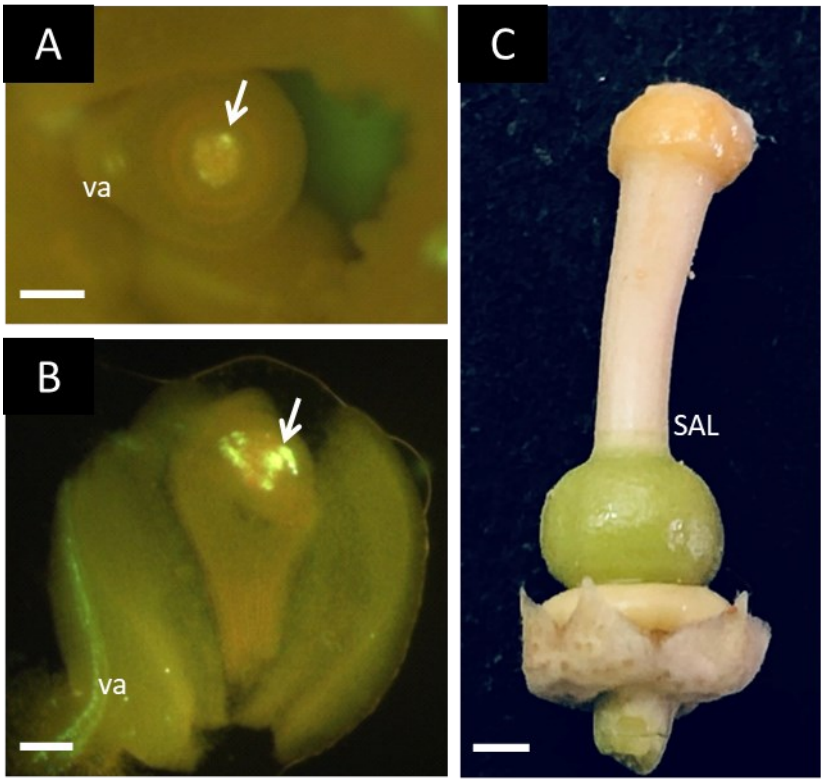

Figure 7. 'Fortune' mandarin ovule degeneration and style abscission five days after anthesis at 30 °C. (A) cross-section of one ovule with degeneration symptoms; (B) Ovule squashed with the presence of strong fluorescence, which indicates the degeneration process; (C) Pistil with the appearance of the style abscission line (SAL). Arrows indicate ovule degeneration fluorescence. va: vascular axis. (A,B) Ovules stained with aniline-blue. Scale bar: A,B = 100 µm; C = 1000 µm

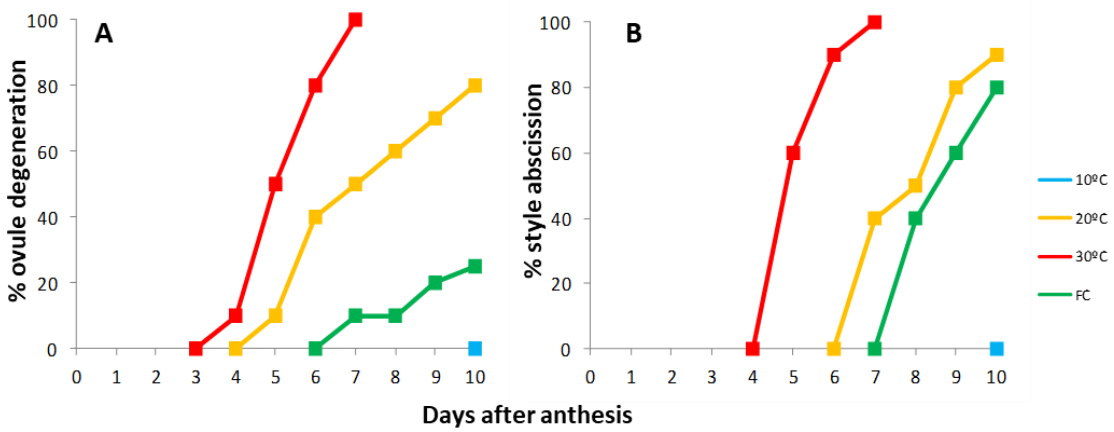

Figure 8. Pistil degeneration of 'Fortune' mandarin from 0 to 10 days after anthesis at 10 °C, 20 °C, 30 °C and under FC. Values are expressed as (A) a percentage of ovule degeneration and (B) a percentage of the pistils with a style abscission line.

Regarding stylar abscission (Figure 7C), no SAL was observed until four days after anthesis at 30ºC, six days at 20ºC and seven days under FC. At 10ºC, no SAL was observed during the 10-day observation period. At 30ºC and five days after anthesis, the SAL was observed in 60% of the styles and all the styles showed the SAL on day seven after anthesis. At 20ºC and under FC, about 40% of the styles showed the SAL on day seven and eight after anthesis, respectively, whereas the percentage of SAL was 90% and 80%, respectively, after 10 days (Figure 8B). No differences in the distance from the SAL to the ovary were observed between the styles subjected to different temperatures (data not shown).

These results revealed that the first ovule degeneration symptoms occurred earlier than the first appearance of the SAL. The biggest difference was recorded under FC, with notable differences between the ovule degeneration and SAL percentages. For example, 10 days after anthesis, the ovule degeneration and SAL percentages were more than 20% and 80%, respectively, but no differences were observed at low temperatures (Figure 8).

4. Discussion

Temperature stress is a key parameter in the progamic phase in plants. Our results offer accurate knowledge about the influence of temperature in the progamic phase by dissecting the effects of both temperature and genotype on the male donor and the effect of temperature on the female recipient.

4.1. Temperature and Genotype Influence on the Male Donor: Pollen Grain Germination (PGG) and Pollen Tube Growth (PTG)

For male parents, our results showed the influence of temperature and genotype, and their interaction, on both PGG and PTG, as well as differences in the optimal temperature for PGG and PTG kinetics, which fall in line with the independence of these processes as reported previously (Distefano et al., 2012; Kakani et al., 2002; Mckee and Richards,

1998). The methodology formerly reported to evaluate PGG is based on *in vitro* tests. Distefano et al. (2012) analysed PGG *in vitro* of different citrus species from 10ºC to 30ºC, and found that no PGG was produced at 10ºC. However, we observed PGG at 10ºC for the three studied genotypes. This discrepancy may be due to the fact that we analysed PGG directly on the stigma surface *in planta* instead of testing PGG *in vitro*. Differences in the pollen germination rates between *in vitro* and *in vivo* tests have been reported in tobacco (Shivanna et al., 1991) and sweet cherry (Hormaza and Herrero, 1999), and suggest that *in vitro* germination media do not provide the optimal conditions offered by the stigma. In contrast, the pollen grain germination analysis performed *in planta* is a more accurate method of testing actual pollen performance because germination occurs in the stigmatic secretion, which is composed of lipids, polysaccharides and proteins (Cresti et al., 1982; Rejón et al., 2014, 2013), and plays an important role in pollen adhesion and germination (Distefano et al., 2011).

Among the 12 analysed pollen-temperature combinations, the biggest differences in both PGG and PTG were observed between ‘Pineapple’ sweet orange and ‘Ichang’ papeda at 10ºC. The worst pollen performance observed in ‘Pineapple’ sweet orange, in contrast to the best performance noted in ‘Ichang’ papeda, may be associated with the previously reported high sensitivity to frost of ‘Pineapple’ sweet orange and the cold-resistance of ‘Ichang’ papeda (Hodgson, 1967). However, no significant differences were noted between genotypes at the warmest temperature in our study (30°C).

Bono et al. (2000) reported the number of seeds in the ‘Fortune x Clemenules’ cross under FC which was 26 seeds/fruit on average. This is consistent with our observation of the 23 pollen tubes of the ‘Clemenules’ clementine at the style-ovary junction of ‘Fortune’ mandarin under FC. This finding supports the possibility that the pollen tubes at the style base could be used to estimate seed quantity in the sexual hybridisations among citrus species, provided that no mechanism intervenes to jeopardise embryo development and seed formation.

The average temperature under FC (18.5ºC) was similar to 20ºC in the growth chamber. However, the PTG kinetics results obtained for these temperatures significantly differed. This could be due to a drop in temperature around 10ºC for several hours a day in the FC regime, which slowed down the PTG kinetics. Despite differences in the PTG kinetics, no differences were observed in the maximum number of pollen tubes that reached the style-ovary junction, which indicated that in fertilization terms, PTG behaviour may be inferred by evaluating constant temperature regimes with similar temperature averages to those of the studied FC.

In other species, the response of PGG and PTG to temperature stress has been used to screen genotypes that are tolerant to both high and cold temperatures, and to also transfer this tolerance to offspring (Domínguez et al., 2005; Kakani et al., 2005, 2002; Liu et al., 2006; Zamir et al., 1982). Previous results suggest that temperature stress in the reproductive phase produces a natural selection of the best-adapted pollen tubes (Hedhly et al., 2009), and a significant correlation between male gametophyte and sporophyte behaviour for temperature stress has been confirmed (Hebbar et al., 2018; Hedhly, 2011; Hormaza and Herrero, 1992). Our experimental design allowed us to observe the daily progress of PTG (kinetics and dynamics) and thus showed a timeframe for each genotype between the day when the first pollen tubes reached the ovary (which could correspond to the best-adapted gametes to the prevailing temperature) and the day on which the maximum pollen tubes reached the ovary. This observation opens up the possibility to limit fertilization to the gametes displaying the best behaviour upon

temperature stress by removing part of the pistil (stigma and/or style) soon after the first pollen tubes reach the ovary. Previous studies have reported seed formation from early removed stigmas in rice (S.-Q. Chen et al., 2008). In citrus, our preliminary observations indicate that pollen tubes are capable of fertilising ovules in early removed pistils (stigma and/or style), but the ability to obtain seeded fruits after this procedure remains unknown. This method could provide an opportunity to explore gametophytic selection pressure in the progamic phase, in addition to parental selection based on our above-mentioned results.

Knowledge about how temperature affects pollen performance can be useful for better planning the number of pollinations in breeding programmes based on sexual hybridisations, and to also improve hybrid production by choosing the most favourable time and location to perform pollination depending on temperature forecasts. Our breeding programme in Spain includes three locations available to perform pollinations: a region located in the province of Huelva on the Atlantic coast with warm temperatures; another area in Valencia characterised by more moderate temperatures; and a third one north of the province of Castellón characterised by colder temperatures during the flowering period of citrus fruits between April and May. Therefore, these results can be applied to perform further pollinations under the most favourable conditions.

4.2. Temperature Influence on the Female Recipient: Stigma Receptivity, Ovule Degeneration and Style Abscission

Stigmatic receptivity and ovule degeneration are key points in regulating the interaction between male and female reproductive phases, and have important consequences on the EPP. Both issues are influenced by environmental conditions and temperature has a clear effect on the modulation of these processes (Cerović et al., 2000; Lora et al., 2011). In citrus, information about stigma receptivity and ovule degeneration is scarce and non-existent regarding the influence of temperature on them. Regarding style abscission, previous visual observations have suggested that high temperatures accelerate this process (Estornell et al., 2016). Indeed, the above authors considered that the differences in the style abscission timing of *C. sinensis* and *C. bergamia* between two consecutive flowering seasons were due to the distinct average temperatures between both seasons.

In all temperature regimes dealt with in this study, pistil senescence started with loss of stigmatic receptivity, followed by ovule degeneration and finally by style abscission from the ovary. Our results showed that temperature had a clear effect on pistil degeneration in ‘Fortune’ mandarin. Warm temperature regimes shortened the stigmatic receptivity period and the ovule life span, and anticipated style abscission from the ovary, whereas the cold temperature regime had the opposite effect.

This work provides new information about how temperature affects the stigmatic receptivity in the progamic phase in citrus. The percentage of germinated pollen grains progressively lowered as flowers were pollinated on subsequent post-anthesis days. Likewise, we noticed that the number of pollen tubes growing in the stigma decreased. In all temperature regimes in this study, the growth ability of pollen tubes is lost before the ability of pollen grain to germinate. Similar results have been found in sweet cherry (Hedhly et al., 2003) and peach (Hedhly et al., 2005a).

Under FC, the drop in stigmatic receptivity in the flowers pollinated 7-8 days after anthesis and the total stigmatic degeneration in the flowers pollinated 9-10 days after anthesis, according to our results, agree with the seed set (7 seeds in the flowers pollinated 8 days after anthesis and no seeds in the flowers pollinated 10 days after anthesis) resulting from delaying pollinations using the 'Clemenules x Fortune' cross under FC (Mesejo et al., 2007). In addition, 20% of the ovules degenerated 10 days after anthesis under the FC similarly to those observed by the same authors in 'Clemenules' under FC.

As we have shown, the stigma of 'Fortune' mandarin is receptive at anthesis. This is of much practical value for citrus breeding programmes based on sexual hybridisation since effective pollination can be performed when flowers are at anthesis, which facilitates such process. This also occurs in other woody species like peach, sweet cherry and kiwi (Sanzol and Herrero, 2001), whereas post-anthesis maturation is required for optimal stigma receptivity in almond (YI et al., 2006). In addition, knowledge about the amount of time pollination can be delayed from anthesis (which depends on temperature, as we have shown in this piece of research) without significantly reducing the quantity of obtained seeds allows flexibility in decision-making during the flowering period in breeding programmes.

4.3. Pollen and Pistil Synchronic Response to Temperature Stress Enable Mating

Our method consisted of cultivating whole plants in culture chambers and observing the cross-sections of the pistils fixed daily for 10 days from pollination. This method enabled us to acquire more comprehensive knowledge about the pollen-pistil interaction in the progamic phase. In this study, pollen tubes were able to reach the ovules in all the evaluated combinations on the 10 experimental days, except for the 'Fortune' x 'Pineapple' cross at 10ºC combination. However, as neither ovule degeneration nor pistil abscission were observed at this temperature, it could be possible that pollen tubes of 'Pineapple' sweet orange reached ovaries of 'Fortune' mandarin after the 10 days of the experiment. Our results show that when performing pollinations at anthesis, the studied crosses were able to successfully perform the progamic phase with temperature changes by maintaining the male-female synchrony described as being necessary for successful mating (Herrero, 2003). This plasticity is reflected by the fact that citrus plants are cultivated in 147 countries around the world (FAOSTAT, 2020), and at between approximately latitudes of 40°N and 40°S that comprise tropical, subtropical and colder areas. Knowledge about the influence of temperature in the progamic phase on citrus plants is of much interest as climate change during the flowering season can alter the progamic phase and, consequently, the reproductive process.

Temperature affects both PGG and PTG dynamics and kinetics because high and low temperatures respectively accelerate and decelerate these processes. Regarding the female parent, high temperatures accelerate stigma and ovule development, which results in a shorter period of stigmatic receptivity and a shorter ovule life span, whereas low temperatures extend both processes and, thus, also the EPP. These results allow us to suggest that temperature stress from 10ºC to 30ºC has a complementary effect on both male and female parents by accelerating or decelerating the progamic phase. Nevertheless, as highlighted by Hedhly et al. (2009) and as observed herein, the response to stress caused by temperature is genotype-dependent. This means that

variations within the optimal range for each genotype may alter, or even interrupt the reproduction process and lead to a low or null seed content and/or fruit set. In citrus fruits, especially mandarins, parthenocarpy is a common phenomenon and seed production is not necessary to obtain good yields in most varieties. However, this decoupling would hamper large populations being obtained in breeding programmes based on sexual hybridisations, where it is necessary to recover large numbers of seeds for specific male and female combinations.

5. Conclusions

Despite the strong influence of temperature in the progamic phase, the evaluated crosses are capable of responding to environmental changes and ensuring good fertilization levels.

The results of this paper can be useful for improving pollination efficiency and adapting breeding programmes to the temperature forecasts during the pollination period. Our results also suggest that pollen performance-based screening may be a useful strategy to select better adapted citrus genotypes to different environmental conditions, and also to explore gametophytic selection within genotypes.

In future research, it would be relevant to investigate the influence of temperature and genotype during gametogenesis. If coupled with the results obtained for the progamic phase, such investigation could be useful for enhancing the efficiency of citrus breeding programmes based on sexual hybridisation; in particular, those whose aim is to obtain new varieties that can adapt to both colder areas and current areas in the process of becoming warmer as a result of global climate change.

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Supplementary information

Supplementary Table 1. Analysis of variance of PGG in vivo 24 h after pollination. Percentage of pollen grains germinated for the independent variables genotype and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Genotype	5351.24	2	2675.62	92.09	0.0000
Temperature[1]	775.511	2	387.756	13.35	0.0000
Gen:Temp	1476.22	4	369.056	12.7	0.0000
Residuals	1046	36	29.0556		

Statistical significance for p<0.05. Independent variables: Genotype ('Pineapple' sweet orange, 'Clemenules' clementine and 'Ichang' papeda), Temperature (10ºC, 20ºC, 30ºC). [1] FC were not used in this analysis.

Supplementary Table 2. Analysis of variance of PTG kinetics from days 1 to 4 after pollination. Pistil section reached by the pollen tubes for the independent variables genotype and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Day=1					
Genotype	10.4333	2	5.21667	12.78	0.0000
Temperature	177.917	3	59.3056	145.24	0.0000
Gen:Temp	15.0333	6	2.50556	6.14	0.0001
Residuals	19.6	48	0.408333		
Day=2					
Genotype	5.2	2	2.6	8.91	0.0005
Temperature	934.8	3	311.6	1068.34	0.0000
Gen:Temp	8	6	1.33333	4.57	0.0010
Residuals	14	48	0.291667		
Day=3					
Genotype	22.3	2	11.15	16.12	0.0000
Temperature	1114.13	3	371.378	536.93	0.0000
Gen:Temp	44.3667	6	7.39444	10.69	0.0000
Residuals	33.2	48	0.691667		
Day=4					
Genotype	4.43333	2	2.21667	2.74	0.0745
Temperature	1049.2	3	349.733	432.66	0.0000
Gen:Temp	8.5	6	1.41667	1.75	0.1292
Residuals	38.8	48	0.808333		

Statistical significance for p<0.05. Independent variables: Genotype ('Pineapple' sweet orange, 'Clemenules' clementine and 'Ichang' papeda), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Supplementary Table 3. Analysis of variance of PTG kinetics at 10ºC. Pistil section reached by pollen tubes for the independent variables genotype and day.

	Sum Sq	Df	Mean Sq	F value	P value
Genotype	12.9513	2	6.4756	199.94	0.0000
Day	10.0553	5	2.01106	62.09	0.0000
Gen:Day	2.9694	10	0.29694	9.17	0.0000
Residuals	2.33192	72	0.03238		

Statistical significance for p<0.05. Independent variables: Genotype ('Pineapple' sweet orange, 'Clemenules' clementine and 'Ichang' papeda), Day (from day 5 to 10 after pollination).

Supplementary Table 4. Analysis of variance of PTG dynamics for 10 days after pollination. Maximum number of pollen tubes observed in the bottom style for the independent variables genotype and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Genotype	1712.13	2	856.067	47.67	0.0000
Temperature	1072.98	3	357.661	19.92	0.0000
Gen:Temp	2015.07	6	335.844	18.7	0.0000
Residuals	862	48	17.9583		

Statistical significance for p<0.05. Independent variables: Genotype ('Pineapple' sweet orange, 'Clemenules' clementine and 'Ichang' papeda), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Supplementary Table 5. Analysis of variance of Stigmatic receptivity of 'Fortune' mandarin. Number of pollen tubes growing in the middle section of the stigma for the independent variables day and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Day	10.216	10	1.0216	359.95	0.0000
Temperature	5.31619	2	2.6581	936.55	0.0000
Day:Temp	2.32122	20	0.116061	40.89	0.0000
Residuals	0.37464	132	0.00283818		

Statistical significance for p<0.05. Independent variables: Day (from day 1 to 10 after pollination), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Supplementary Table 6. Analysis of variance of Stigmatic receptivity of 'Fortune' mandarin. Pollen Grain Germination for the independent variables day and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Day	118284	10	11828.4	484.77	0.0000
Temperature	62504.4	3	20834.8	853.89	0.0000
Day:Temp	17712	30	590.401	24.2	0.0000
Residuals	4294.4	176	24.4		

Statistical significance for p<0.05. Independent variables: Day (from day 1 to 10 after pollination), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Supplementary Table 7. Analysis of variance of ovule degeneration of 'Fortune' mandarin. Percentage of ovules degenerating for the independent variables day and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Day	19.027	10	1.9027	33.62	0.0000
Temperature	16.4128	3	5.47093	96.68	0.0000
Day:Temp	14.883	30	0.496098	8.77	0.0000
Residuals	22.409	396	0.0565884		

Statistical significance for p<0.05. Independent variables: Day (from day 1 to 10 after pollination), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Supplementary Table 8. Analysis of variance of style abscission of 'Fortune' mandarin. Percentage of styles with the SAL for the independent variables day and temperature.

	Sum Sq	Df	Mean Sq	F value	P value
Day	27.2902	10	2.72902	63.17	0.0000
Temperature	14.271	3	4.75699	110.1	0.0000
Day:Temp	18.0198	30	0.600659	13.9	0.0000
Residuals	17.109	396	0.0432045		

Statistical significance for p<0.05. Independent variables: Day (from day 1 to 10 after pollination), Temperature (10ºC, 20ºC, 30ºC and field conditions; FC).

Chapter 2. Parthenocarpy and Self-Incompatibility in Mandarins

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Abstract

Citrus reproductive biology is complex. One of its characteristic features is parthenocarpy that enables seedless fruit production. Citrus parthenocarpy and self-incompatibility knowledge is only partial and sometimes discrepant. Increasing such knowledge is relevant for better managing cultivated varieties and improving the selection of parents in breeding strategies to recover seedless varieties such as mandarins. This work develops an efficient protocol to characterize self-incompatibility and different parthenocarpy types based on emasculation, hand self-pollination, and hand cross-pollination. It analyzes fruit setting and seed production coupled with histological pollen performance observations. We analyzed the reproductive behavior of nine mandarin varieties with relevant characteristics as parents for seedless mandarin breeding. 'Clemenules' clementine and 'Moncada' mandarins were strictly self-incompatible with facultative and vegetative parthenocarpy; 'Imperial' mandarin and 'Ellendale' tangor displayed no strict self-incompatibility associated with facultative and vegetative parthenocarpy; 'Fortune' mandarin was self-incompatible with facultative and stimulative parthenocarpy; 'Campeona' and 'Salteñita' mandarins were self-compatible with vegetative parthenocarpy; 'Serafines' satsuma was associated with male sterility together with facultative and vegetative parthenocarpy; and 'Monreal' clementine was self-compatible and nonparthenocarpic. Our protocol can be applied for screening of mandarin germplasm and to characterize new parents. Reproductive behavior knowledge is important for optimizing seedless mandarin breeding programs based on diploidy, triploidy, or induced mutagenesis.

Keywords: citrus; seedless; parthenocarpic ability; pollination; fruit setting; breeding

1. Introduction

Seedlessness is one of the most important characteristics for citrus fresh-fruit markets because consumers do not accept seedy fruit. Breeding programs that aim to obtain seedless varieties focus mainly on mandarins, which are a large and diverse group with wide genetic variability (Garcia-Lor et al., 2015). Seedlessness in citrus differs among genotypes, and is sometimes altered by environmental conditions and cross-pollination success (Ollitrault et al., 2007b).

Two main seedlessness types have been described in plants: (1) parthenocarpy in sensu stricto, which occurs when the ovary develops directly without fertilization and produce seedless fruits and (2) stenospermocarpy, in which fruits are seedless because the ovule/embryo aborts without producing mature seed (Picarella and Mazzucato, 2019). In citrus, stenospermocarpy is uncommon (Mesejo et al., 2014) and ovule sterility or lack of its fecundation (self-incompatibility or no compatible viable pollen) coupled with parthenocarpy is necessary for the production of seedless fruits.

Ovule sterility, lack of fecundation, or no seed development at early stages often prevents seed formation and different mechanisms have been reported in citrus (Ollitrault et al., 2007b; Yamamoto, 2014). Ovule sterility can be originated in flowers whose pistils do not develop up to the functional stage (Wilms et al., 1983). For example, in nonfunctional pistils of lemon (*Citrus limon* (L.) Burm f.), possible blocking of further

stigma and style development is associated with the presence or absence of receptive embryo sacs in the ovule (Wilms et al., 1983). Osawa (1912) observed degeneration of the embryo sac in both navel sweet orange (*C. sinensis* (L.) Osbeck) and satsuma mandarin (*C. unshiu* (Mak.) Marc.) and Wong (Wong, 1939) reported chromosome aberrations into the embryo sac as the responsible mechanism of low seed number in hand-pollinated flowers of 'Valencia' sweet orange and 'Marsh' grapefruit (*C. paradisi* Macf.). In addition to ovule sterility, other mechanisms have been described in citrus originating seedless citrus fruits coupled with parthenocarpy. 'Mukaku Kishu' mandarin (*C. kinokuni* hort. ex Tanaka) produces seedless fruits because the seed development is arrested at the early stage of development (Yamasaki et al., 2009, 2007). However, for easy reading of the manuscript, hereinafter with the term female sterility we refer to the different mechanisms that prevent seeds formation in citrus fruits: (1) the failure of the pistil development up to the functional stage, (2) ovule sterility or (3) the early stage arrest of the seed development.

Male sterility prevents seed formation in the absence of cross-pollination and prevents pollen flow toward neighbor orchards. In citrus, various kinds of male sterility related to chromosome aberration occur at the diploid level and result in different degrees of pollen fertility (Ollitrault et al., 2007b). The most marked male sterility in citrus is due to the gene–cytoplasmic interaction, as in satsumas where male sterility is associated with failed pollen grain development and scant viability (Goto et al., 2016). Recently, two QTLs related to male sterility have been reported; MS-P1, which is a major QTL for reducing the number of pollen grains per anther and MS-F1, related to lower apparent pollen fertility (Goto et al., 2018).

Both female and male sterility can be achieved by gamma irradiation (Bermejo et al., 2011; Goldenberg et al., 2014) and triploidy (Navarro et al., 2015; Ollitrault et al., 2007a, 2007b). In fact, triploid hybrids have very little pollen and poor ovule fertility, and are generally considered sterile (Otto and Whitton, 2000), although they can occasionally produce very few seeds. Triploid citrus plants can be recovered from sexual hybridization between two diploid parents from the union of an unreduced megagametophyte with a haploid pollen (Aleza et al., 2010b; Cuenca et al., 2011), or by hybridization between diploid and tetraploid parents (Aleza et al., 2012c, 2012b).

Self-incompatibility, also called self-sterility, causes self-pollen rejection and prevents seed formation in the absence of cross-pollination. A recent report indicates that citrus gametophytic self-incompatibility is based on S-RNases, which act as S genes determinants in inhibiting pollen tube growth (Liang et al., 2020). The most important self-incompatible horticultural citrus groups are pummelos (*C. maxima* (Burm.) Merr.), clementines (*C. clementina* Hort. ex Tan.), and several natural or artificial mandarin hybrids (Liang et al., 2020; Yamamoto et al., 2006; S. Zhang et al., 2018).

The term 'Parthenokarpie' (which literally means 'virgin fruit') was introduced in 1902 to refer to seedless fruit production (Vardi et al., 2000). Seeds are the consequence of fertilization and embryo development, however most citrus genotypes presents sporophytic apomixis and produce polyembryonic seeds by adventitious embryony. Even in this case, nucellar embryos depends on sexual reproduction to produce endosperm for nucellar embryo growth and development and seed set (Esen and Soost, 1977; Koltunow, 1993). Apart from their reproductive function, seeds act as a source of phytohormones required for fruit development (Ozga and Reinecke, 2003; Vardi et al., 2008). The ability to accumulate sufficient levels of phytohormones in developing ovaries with no need for seeds is, therefore, a condition for natural seedless fruit production,

which is known as parthenocarpic ability (PA) (Talon et al., 1990). Different degrees of PA have been reported in citrus and PA is associated with high levels of endogenous gibberellins (GAs) measured in nonfertilized developing ovaries (Mesejo et al., 2016, 2013; Talon et al., 1992).

Four parthenocarpy types have been described in citrus (Vardi et al., 2008). A distinction is made between obligatory parthenocarpy for those varieties that always produce seedless fruit, and facultative parthenocarpy in which seedless fruit is produced when cross-pollination with compatible sources of pollen is prevented. Moreover, a distinction is made between vegetative parthenocarpy (also called autonomous or autonomic parthenocarpy) to refer to seedless fruit developing without requiring any external stimulus, and stimulative parthenocarpy that requires the pollination stimulus for seedless fruit set.

In citrus, the molecular mechanisms controlling parthenocarpy are poorly understood. On the basis of the different ratio of parthenocarpic hybrids obtained for several progenies of satsuma with different second parents, Vardi et al. (2000) suggested that parthenocarpy was due to the action of three dominant complementary genes and that two of these genes were in homozygosity for parthenocarpy and one in heterozygosity in satsuma. This hypothesis for three dominant genes for parthenocarpy in citrus is in agreement with the conclusion of Sykes (2008a) based on the parthenocarpic segregation ratio in a diploid segregating progeny recovered between 'Imperial' (*C. reticulata* Blanco) and 'Ellendale' (*C. reticulata* × *C. sinensis*). However, Vardi et al. (2008) proposed that parthenocarpy in satsuma was controlled by at least two dominant complementary genes, with satsuma being heterozygous for these genes. These contradictory hypotheses point out that parthenocarpy is a character that needs to be studied in more detail to understand how it is determined in citrus. High-density genotyping of segregating progenies for parthenocarpy, coming from different genetic pools, should allow us to decipher the genetic determinism of parthenocarpy and to identify candidate genes. In this regard, knowledge of PA is crucial in selecting parents that could be used to obtain segregating progenies for parthenocarpy.

Current parthenocarpy information for many genotypes is inaccurate and sometimes discrepant between authors. All this indicates the complexity of the factors affecting citrus reproductive biology. This work focuses on nine different varieties selected for their importance as parents for mandarin breeding programs (Navarro et al., 2015): 'Clemenules' and 'Monreal' clementines, 'Campeona' (*C. nobilis* Lour.), 'Imperial', 'Salteñita' (*C. deliciosa* Ten.), 'Fortune' (*C. clementina* × *C. tangerina*), and 'Moncada' [*C. clementina* × (*C. unshiu* × *C. nobilis*)] mandarins, 'Ellendale' tangor, and 'Serafines' satsuma.

Clementine, which was recovered from a 'Common' mandarin chance seedling, is the main mandarin varietal group cultivated in the Mediterranean Basin. As a result of spontaneous budsports mutations from the original 'Commune' clementine, many different clementine varieties have been selected, with 'Clemenules' as the main variety cultivated in Spain for its exceptional fruit quality. Discrepancies are found in the literature between the stimulative parthenocarpy proposed by Vardi et al. (2000) and the vegetative parthenocarpy put forward by Mesejo et al. (2013). Regarding 'Monreal', a self-compatible natural mutation of the 'Commune' clementine, previous research points out the need of seeds for fruit to set (Garcia-Papi and Garcia-Martinez, 1984b). However, the possibility of seedless fruit setting by a pollination stimulus without fecundation has not yet been explored in this variety.

‘Campeona’ and ‘Ellendale’ produce fruit with a medium to high caliber and a late and very late harvesting period, respectively, whereas ‘Imperial’ and ‘Salteñita’ mandarins are characterized by their particular fruit flavor and aroma. ‘Fortune’ and ‘Moncada’ produce fruits with excellent organoleptical qualities and a very late harvesting period. Altogether, the seeds of all these varieties, except for ‘Salteñita’, are monoembryonic, which facilitates the recovery of new hybrids by sexual hybridization when used as female parents. These traits make these varieties very interesting breeding parents.

Previous information reports that ‘Fortune’ and ‘Moncada’ mandarins are self-incompatible (Yamamoto et al., 2006), but no information about the requirement of a pollination stimulus for fruit set has yet been reported. With ‘Campeona’ and ‘Salteñita’ mandarins, the scientific literature only reports that they are seedy varieties (Hodgson, 1967), but knowledge about PA goes unnoticed. Regarding both the ‘Imperial’ mandarin and ‘Ellendale’ tangor, previous research offers discrepant results about their PA and self-incompatibility (Sykes, 2008b, 2008a; Sykes and Possingham, 1992; Vardi et al., 2000; Vithanage, 1991, 1986; Wallace and Lee, 1999)

‘Serafines’ is a spontaneous mutation of the ‘Owari’ satsuma characterized by its very late harvesting period, from January to the end of February, which is an outstanding satsuma variety feature. Satsumas are characterized by their male and female sterility and parthenocarpy (Ollitrault et al., 2007b), but no specific information about ‘Serafines’ is available.

Increasing knowledge about the parthenocarpy and self-incompatibility of different mandarin genotypes to be used as parents in sexual hybridizations is crucial to improve breeding program efficiency. In this research, we assessed the self-compatibility or self-incompatibility system, the PA and the requirement of pollination stimuli for the seedless fruit production of the aforementioned mandarin varieties to classify them according to the above-described parthenocarpy types and established a relatively simple protocol to evaluate these characters in new potential parents. For this purpose, we evaluated the data obtained for fruit setting, seed production and histological observations of pollen performance obtained from emasculated, hand self-pollinated and hand cross-pollinated flowers.

2. Material and Methods

2.1. Plant Material

Nine mandarin varieties were used to carry out the present study. These varieties were grown in plots of the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (IVIA), located in Moncada, Valencia (Spain) and had the following accession numbers: ‘Clemenules’ (IVIA-022), ‘Monreal’ (IVIA-459), ‘Campeona’ (IVIA-193), ‘Imperial’ (IVIA-576), ‘Salteñita’ (IVIA-361), ‘Fortune’ (IVIA-080), ‘Moncada’ (IVIA-421), ‘Ellendale’ (IVIA-194), and ‘Serafines’ (IVIA-256). Four trees of each variety were used, and all were healthy (Navarro et al., 2002) and cultivated following the same agronomic practices in a Mediterranean climate.

2.2. Experimental Procedure

Terminal flowers in pre-anthesis from shoots bearing one flower and leaves were used to perform the following treatments: emasculation (E), emasculation and hand self-pollination (SP), and emasculation and hand cross-pollination (CP). To avoid any undesired pollination, flowers were bagged after treatments. For the CP treatment, 'Fortune' was used as apollen source for its high pollination aptitude and 'Clemenules' was utilized to cross-pollinate 'Fortune'. To perform hand-pollination, pollen grains from flowers in pre-anthesis were harvested by covering all the flowering period. Anthers were removed from stamens and placed in Petri dishes on silica gel at room temperature until dehiscence. Then pollen was used for pollination. Ten days after treatment, five SP and five CP flowers per tree from each variety were fixed in FAA solution (formalin, glacial acetic acid, 70% ethanol, 1:1:18, *v*/*v*) (Johansen, 1940) and stored at 4 °C until histological observations were made. The other flowers were left for fruit set. Fruits were collected when ripe. The data for fruit setting and number of seeds per fruit were recorded.

In order to ensure consistent results, a large number of treated flowers were needed, which involved using four trees per variety to carry out our study. Once tree effect in the obtained results was discarded, treated flowers were analyzed together. The number of flowers employed in each treatment per variety and the number of fruits obtained are shown in Supplementary Table S1. In all, 6792 flowers (320 for histological observations, plus 6472 for fruit set) were used in this work. The results obtained from the above-described treatments enabled us to assess the self-compatibility or self-incompatibility and the PA in eight of the studied varieties. For 'Monreal', an additional CP treatment with the pollen of the 'Oroblanco' (*C. grandis* × *C. paradisi*) triploid hybrid was necessary to assess PA.

2.3. Histological Observations

The pistils fixed in FAA 10 days after SP and CP treatments were used for histological observations. Pollen performance in planta, including pollen germination and pollen tube growth, was analyzed under a Leica MZ16FA epifluorescence stereomicroscope equipped with GFP1 filter (excitation filter 395–455 nm and barrier filter 480 LP). For that purpose, pistils were submerged 3 times in water for 1 h. Afterward, pistils were sliced into 14 cross-sections using a sharp blade. Stigmas were sliced into two sections, styles into eight sections, and ovaries into four sections following the methodology described by Montalt et al. (Montalt et al., 2019). Sections were stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens FH, Esser, 1957). Pollen germination was observed on the stained stigma surfaces and pollen tubes identified by its callose plugs fluorescence (Adhikari et al., 2020).

2.4. Statistical Analysis

In order to evaluate self-incompatibility, the average number of seeds per fruit between treatments for each variety was compared. The data of the dependent variable number of seeds per fruit were confirmed to fit the normal distribution, and outlier values based on box plots were removed prior to further analyses. Analyses of variance and LSD multiple range tests were performed for comparisons using the Statgraphics Centurion

XVI statistical software package, version 16.1.03. To evaluate PA, a fruit setting comparison between treatments was analyzed in each genotype. To this end, the proportions of fruit setting obtained in each treatment were compared by pairs. Each proportion was a binomial variable with two possible outcomes: 'successful fruit setting' and 'failed fruit setting'. The 95% confidence interval obtained for the differences between treatments is shown in Supplementary Table S2.

2.5. Genetic Analysis with Simple Sequence Repeat (SSR) Markers

The plants recovered from the SP treatment performed in 'Imperial' and 'Ellendale' were genotyped using 13 heterozygous SSR markers located on the reference Clementine genetic map (P. Ollitrault et al., 2012a). Of them, height heterozygous markers for each variety were selected to characterize their progenies. Detailed information about all the used markers is given in Supplementary Table S3.

Genomic DNA was extracted from leaves of the samples described above using a Plant DNeasy kit from Qiagen Inc. (Valencia, CA, USA) following the manufacturer's protocol and measured using a spectrophotometer (NanoDrop 2000C, Thermo Fisher Waltham, MA, U.S.). The samples were diluted with sterile water (Sigma-Aldrich, Co., Gillingham UK) at a concentration of 10 ng/mL and stored at 20 °C until use. Polymerase chain reactions (PCRs) were performed using SSR markers with a Thermocycler rep gradient S (Eppendorf©. Hamburg, Germany) according to the following protocol: reaction volume, 15 µL containing 0.5 µL of 1 U/µL of Taq DNA polymerase (Fermentas© Waltham, MA, U.S.), 3 µL of citrus template DNA (10 ng/µL), 1.5 µL of 2 µM welled (Sigma© Burlington, MA, U.S.) dye-labeled forward primer, 1.5 µL of 2 µM non-dye-labeled reverse primer, 0.2 µM of each dNTP, 1.5 µL of PCR reaction buffer 10x, and 0.45 µL of 50 mM $MgCl_2$. The cycling program was set as follows: denaturation for 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C or 55 °C, 30 s at 72 °C; and a final elongation step of 8 min at 72 °C. Separation was carried out by capillary gel electrophoresis using a Genetic Analysis System 8000 (Beckman Coulter Inc. Brea, CA, U.S.). PCR products were initially denatured at 90 °C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were size-based on a DNA size standard (400 bp). The GenomeLabTM v.10.0 (Beckman Coulter Inc.) genetic analysis software was used for data collection.

3. Results and Discussion

We first analyzed whether self-incompatibility prevented self-fertilization in each variety. Next, we assessed if the studied varieties had PA and if a pollination stimulus was required for seedless fruit to set. Based on the obtained results, we discuss the self-compatibility or self-incompatibility reactions observed, the parthenocarpy type in each studied variety and their implications in breeding programs aimed to obtain seedless varieties by triploidization or induced mutagenesis.

3.1. Self-Incompatibility

In order to determine self-compatibility or self-incompatibility, we observed the presence or absence of pollen tubes reaching the ovaries in the histological sections of the flowers

fixed 10 days after SP (Figure 1), as well as the production of seeded or seedless fruit from SP (Table 1).

In ‘Monreal’, ‘Campeona’ and ‘Salteñita’, the histological sections of SP flowers showed pollen tubes growing through the pistil and reaching the ovaries in all the analyzed flowers of the three varieties (Figure 1 and Supplementary Figure S1) and all the recovered fruits contained seeds (Table 1). No differences were seen in ‘Monreal’ when comparing the average seed number between SP (22.5 seeds per fruit) and CP (21 seeds per fruit) treatments. With ‘Campeona’ (4.9 seeds per fruit from SP vs. 9.9 seeds per fruit from CP) and ‘Salteñita’ (11 seeds per fruit from SP vs. 16.5 seeds per fruit from CP), the fruits obtained from the SP flowers presented lower seed numbers than those from the CP flowers (Table 1). Taking together, the results obtained for seed production and pollen tubes growth in the pistils of the self-pollinated flowers, we concluded that ‘Monreal’, ‘Campeona’ and ‘Salteñita’ are self-compatible.

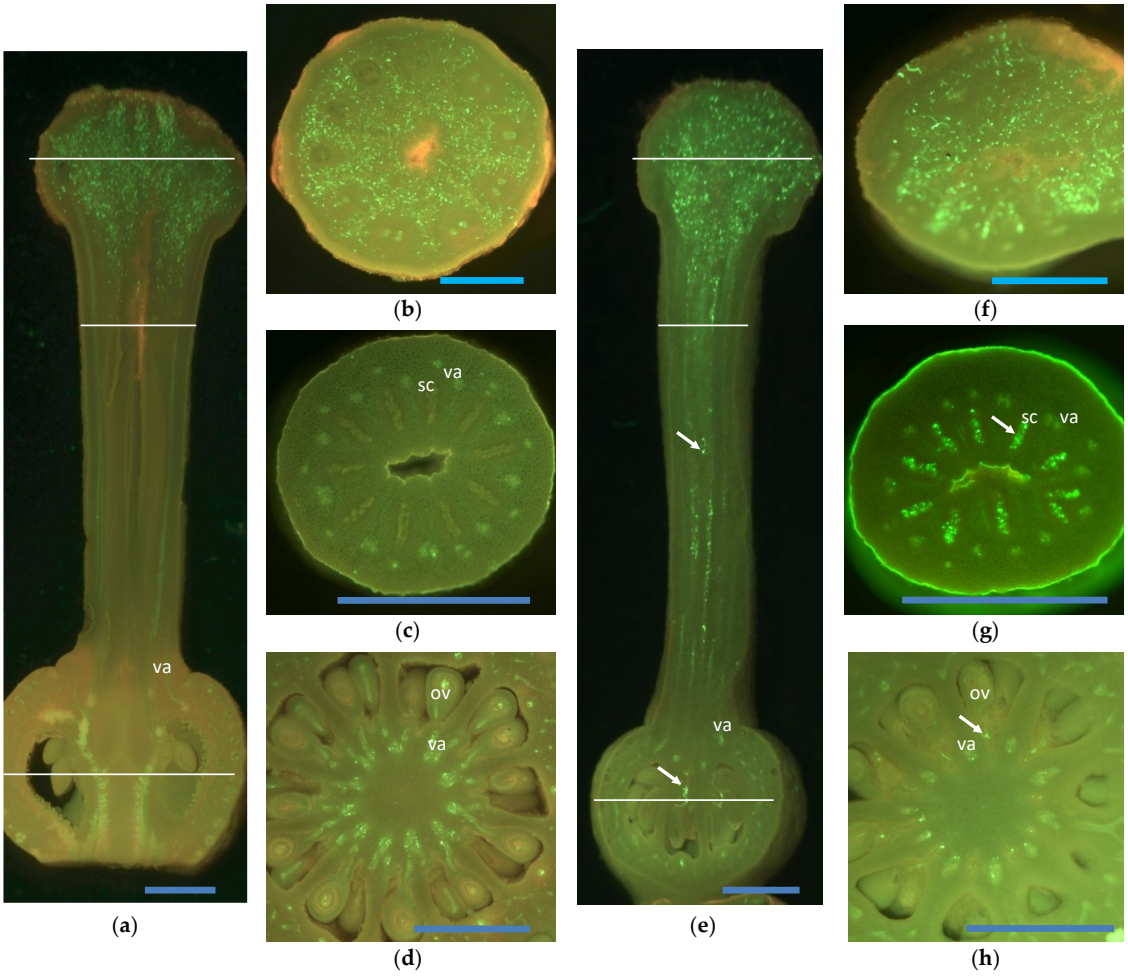

Figure 1. Histological sections of self-pollinated pistils. (a–d) Self-incompatible pistil 10 days after self-pollination. (a) Longitudinal section of the entire pistil in which high quantity pollen tubes were observed thorough the stigma whose growth further stopped in the upper style. (e–h) Self-compatible pistil 10 days after self-pollination. (e) Longitudinal section of the entire pistil in which high quantity pollen tubes were observed throughout the stigma, along the entire style and reached ovaries. The white lines on the longitudinal pistil sections indicate the transversal section that corresponds to the middle stigma (b,f), upper style (c,g), and ovary (d,h). Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule. Scale bars are depicted by blue lines: (a,e) 1 mm; (b–g) 0.5 mm. Pictures of ‘Fortune’ mandarin and ‘Monreal’ clementine are shown as examples of self-incompatible and self-compatible varieties, respectively.

Table 1. Self-compatibility (SC) or self-incompatibility (SI) classification based on pollen tube growth and number of seeds per fruit obtained from hand self-pollination (SP) and hand cross-pollination (CP) treatments in each variety.

		Pollen Tubes Growth		Number of Seeds per Fruit	
	SC/SI	**SP**	**CP**	**SP**	**CP**
'Clemenules'	SI	0	100	0	24.3 ± 2.6
'Monreal'	SC	100	100	22.5 ± 3.6 (a)	21.0 ± 4.4 (a)
'Campeona'	SC	100	100	4.9 ± 1.8 (a)	9.9 ± 2.9 (b)
'Imperial'	SI	15	100	0.8 ± 1.4 (a)	9.3 ± 2.5 (b)
'Salteñita'	SC	100	100	11.0 ± 4.3 (a)	16.5 ± 4.1 (b)
'Fortune'	SI	0	100	0	20.4 ± 5.6
'Moncada'	SI	0	100	0	11.6 ± 5.2
'Ellendale'	SI	10	100	0.7 ± 1.0 (a)	33.1 ± 5.8 (b)
'Serafines'	NA	0	100	0	5.3 ± 2.1

Pollen tubes growth is expressed as the percentage of pistils in which pollen tubes were observed reaching the ovaries. Number of seeds per fruit are given as the mean ± SD (n = 16 to 40 depending on the number of fruits obtained in the treatment). For each variety, significant differences (p = 0.05 Fisher LSD) between treatments are indicated by different letters in brackets.

Regarding 'Clemenules', 'Fortune' and 'Moncada', the histological observations of the self-pollinated flowers showed high pollen grain germination on stigma surfaces and pollen tube growth in the bottom half of the stigma. Nevertheless, pollen tubes stopped growing in the upper style part and no pollen tubes were identified in ovaries (Figure 1 and Supplementary Figure S1). All the recovered fruits from the SP treatment were seedless (Table 1). These results testify a self-incompatible system in 'Clemenules', 'Fortune' and 'Moncada'.

Clementines have been studied in detail for their self-incompatibility reaction using histological approaches (Distefano et al., 2009a, 2009b; Eti and Stosser, 1992; Yamamoto et al., 2006). 'Monreal' display different features than other clementines. Distefano et al. (Distefano et al., 2009a) already observed that it was self-compatible, with similar results to those obtained in the present work. 'Monreal' was obtained from a spontaneous mutation of 'Commune' clementine later known as 'Fina' clementine which is a self-incompatible variety (Distefano et al., 2009a). Liang et al. (2020) identified the S-RNase gene and eleven *S-locus F-box* (SLF) genes on scaffold 7 of *C. clementina* reference genome implicated on self-incompatibility system. These findings suggest that the self-compatibility reaction in 'Monreal' may result from a mutation or epigenetic variation on these genes. A frameshift mutation in one S-RNase has been described to result in a loss of self-incompatibility in the genus *Citrus* (Liang et al., 2020). With 'Fortune', our results coincide with the SI previously reported by Yamamoto et al. (2006) and Distefano et al. (2009b).

In 'Imperial' and 'Ellendale', the histological observations displayed similar results in both varieties (Supplementary Figure S2). Pollen tubes were observed throughout the stigma, and the growth of most of them stopped in the upper style, although some pollen tubes reached ovaries in small percentages (15% for 'Imperial', 10% for 'Ellendale') of the self-pollinated flowers (Table 1). Unlike the other varieties studied, 'Imperial' and 'Ellendale' produced both seedless and low-seeded fruits from SP. The percentage of low-seeded fruit was 23% for 'Imperial' and 54% for 'Ellendale' and the seed number per seeded fruit was 2.3 ± 1.6 and 5.7 ± 6.2 respectively. This resulted in the values of 0.8 and 0.7 seeds

per fruit shown on Table 1 for 'Imperial' and 'Ellendale' respectively. When taking into account both histological observations and poor seed production from the self-pollinated flowers, we demonstrated that some pollen tubes were able to reach ovaries, fertilize ovules and then produce seeds, which suggests no strict self-incompatibility in these two varieties under our field conditions. To rule out any uncontrolled pollination hypothesis, 12 seeds obtained from the SP treatment of each variety were cultivated under greenhouse conditions (Aleza et al., 2012c), and six and 11 plantlets were recovered from 'Imperial' mandarin and 'Ellendale' respectively. The progenies of each variety were analyzed with eight heterozygous markers for the female parent (Aleza et al., 2011; Cuenca et al., 2011; Froelicher et al., 2008; Garcia-Lor et al., 2012; Kamiri et al., 2011; Kijas et al., 1997) and all plantlets displayed only alleles from the female parent in either heterozygosity or homozygosity (Figure 2, Supplementary Tables S3 and S4). These results indicate that the obtained plantlets originated from selfing.

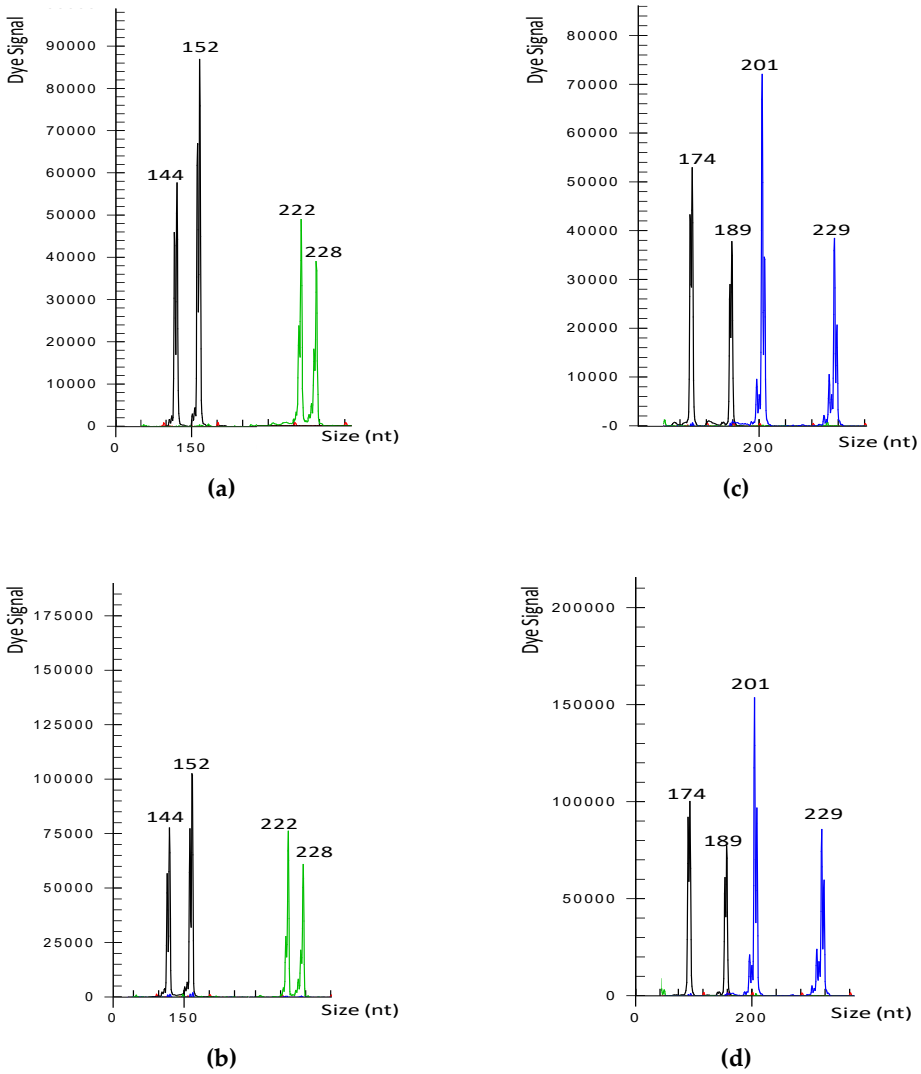

Figure 2. Electropherograms obtained using the mCrCIR05A05 (black) and mCrCIR03G05 (green) SSR markers for 'Imperial' mandarin (a), a diploid hybrid recovered from the self-pollination of 'Imperial' (b), the MEST15 (black) and Ci02D04 (blue) loci for 'Ellendale' tangor (c), and a diploid hybrid recovered from the self-pollination of 'Ellendale' tangor (d). Numbers indicate the size of alleles in nucleotides (nt) for each variety.

Our results are in line with the low-seeded fruits obtained from self-pollinated flowers in 'Ellendale' reported by Vithanage (1991) even though this author considered 'Ellendale' as a highly self-compatible variety. However, Vardi et al. (2000) considered 'Ellendale' as self-incompatible variety. Based on the ratio 1:1 (SC:SI) of the hybrids obtained from Satsuma x 'Ellendale' cross, this author suggested two different self-incompatible alleles (S_Y and S_Z) in 'Ellendale' which differs from the S_X allele proposed for Satsuma. Recently, based on segregation distortion for male parents in high density genetic maps from reciprocal crosses between 'Ellendale' and 'Fortune', Ollitrault et al. (2021) concluded that 'Ellendale' and 'Fortune' share a common self-incompatible allele and confirmed the location of the S locus at the beginning of the chromosome 7 of the Clementine reference genome. From the haplotype sequence analysis on the surrounding genomic region the same authors concluded that 'Ellendale' share the same two self-incompatible alleles as Clementine as already proposed by Kim et al. (2020) from crosses with homozygous lines for self-incompatible alleles.

In 'Imperial', based on the observation of pollen tube growth and the production of seeded or seedless fruit, discrepant results have been reported regarding SI. On the one hand, Wallace and Lee (1999) recorded some pollen tubes (0.2 ± 0.1) at the basis of the style and low-seeded fruits (less than one seed per fruit) form selfed flowers. However, these authors considered 'Imperial' to be self-incompatible since almost all pollen tubes were arrested in the stigma and upper style of self-pollinated flowers and very low number of seeds were obtained. On the other hand, Vithanage (1986) and (Sykes (2008a) concluded this variety was self-compatible since pollen tubes were observed growing into the self-pollinated styles and seeded fruits were obtained from self-pollinated flowers. In this regard, Sykes (2008b) suggested that the environment might affect self-pollination in 'Imperial' because these experiments were performed in different locations.

Taking advantage of staining techniques to observe pollen tubes growth inside pistils, different authors have demonstrated that in citrus, self-incompatibility reaction takes place from the stigma to the ovary (Ngo et al., 2001; Yamamoto et al., 2006) although in mandarin and mandarin hybrids several authors agreed that self-incompatibility reaction is originated in the first half of the style (Distefano et al., 2009b, 2009a; Eti and Stosser, 1992; Yamamoto et al., 2006). These studies have been carried out in different years and in different countries with different environmental conditions (Catania, Italy; Adana, Turkey; Kagoshima, Japan; Moncada, Spain) and all of them coincided with the SI classification of clementines and 'Fortune'. Taking into account that environment may affect self-pollination in 'Imperial', as has been suggested by Sykes (2008b), and the stability of the self-incompatibility reaction in clementines and 'Fortune' reported in the different locations mentioned above, it seems plausible to hypothesize that the impact of environmental change on the SI reaction is dependent on the considered genotype. Beyond the stability of the SI reaction displayed in clementines and 'Fortune' under field conditions at different locations, previous research performed in these two varieties under controlled environmental conditions indicate that constant high and low temperatures appear to have an effect on the self-incompatibility reaction by affecting the place where pollen tubes are arrested (Distefano et al., 2012). Recently, Aloisi et al. (2020) indicated that temperature contributed to a different activation of the self-incompatibility reaction in *C. clementina*, occurring at an optimal temperature of 25 °C and bypassed at 15 °C. The incompatible reaction resulted in enhancement of both transglutaminase enzyme activity and levels of conjugated polyamines when compared

to cross-pollination (Aloisi et al., 2020). However, more research is needed to understand how environmental conditions can influence the self-incompatibility reaction in citrus.

In ‘Serafines’, pale-colored anthers were obtained, which produced very scant pollen with very poor viability. Notwithstanding, we used these anthers to perform the SP treatment. We observed very few pollen tubes at the bottom of the stigma (Supplementary Figure S1), which is in line with the reduced pollen germination of satsuma reported by Vithanage (1991). However, in our observations, no pollen tubes were found in the upper style (Supplementary Figure S1). The seedless fruit set obtained from SP was, therefore, a consequence of poor pollen performance. Therefore, the self-incompatibility of ‘Serafines’ could not be assessed. Vardi et al. (2000) considered satsuma as self-compatible and heterozygous with a self-compatible allele and a self-incompatible one. However, the authors did not give information on the variety used for their study.

In those varieties that produced seedless fruits from SP treatment, the seedy fruit production obtained from CP treatment indicates the potential to produce both seedless and seeded fruits, indicating that parthenocarpy itself is independent from the fertilization process and seed formation. This uncoupling of the reproductive and fruiting processes has been reported previously in ‘Fortune’ by Distefano et al. (2011).

3.2. Assessing Parthenocarpic Ability (PA) and Testing the Pollination Stimulus Requirement for Fruit to Set

Under natural conditions, PA in self-incompatible genotypes is easily identified by avoiding cross-pollination. However, in self-compatible genotypes, PA can only be ascertained by emasculating and bagging (treatment E). As emasculating and bagging prevent pollination stimulus, the fruit set obtained from E (which is mandatory seedless) was assessed to check the pollination stimulus requirement for fruit to set. The fruit setting percentage from E obtained in ‘Clemenules’ (15%), ‘Campeona’ (9%), ‘Imperial’ (19%), ‘Salteñita’ (5%), ‘Moncada’ (34%), ‘Ellendale’ (5%) and ‘Serafines’ (64%) (Table 2) indicated that these varieties had PA and pollination stimulus was not required for fruit to set.

Table 2. Fruit setting percentage obtained in each treatment.

	E	SP	CP
‘Clemenules’	15 (a)	16 (a)	84 (b)
‘Monreal’	0	68 (a)	74 (a)
‘Campeona’	9 (a)	42 (b)	56 (c)
‘Imperial’	19 (a)	33 (b)	67 (c)
‘Salteñita’	5 (a)	58 (b)	70 (c)
‘Fortune’	0	9 (a)	63 (b)
‘Moncada’	34 (a)	33 (a)	72 (b)
‘Ellendale’	5 (a)	5 (a)	60 (b)
‘Serafines’	64 (a)	66 (a)	65 (a)

E: Emasculation; SP: hand self-pollination; CP: hand cross-pollination. For each variety, significant differences (95% confidence interval for fruit setting proportions) between treatments are indicated by different letters in brackets.

In the self-incompatible varieties ‘Clemenules’, ‘Ellendale’, and ‘Moncada’, the fruit setting comparison between treatments E and SP did not show any statistical differences (Table 2). This similar fruit setting obtained from treatments E and SP indicated not only that no pollination stimulus was required for fruit set, but also pollination stimulus did not increase fruit setting. In ‘Fortune’, no fruit was obtained from E, but the fruit setting from SP was 9% (Table 2) and all the recovered fruit was seedless (Table 1). This scenario indicated that ‘Fortune’ had PA and pollination stimulus was necessary for fruit to set.

‘Monreal’ was unable to produce fruit from emasculated flowers. Due to its self-compatibility, we used pollen from the ‘Oroblanco’ triploid hybrid to evaluate the ability of ‘Monreal’ to produce seedless fruit when seed formation was avoided and pollination stimulus was maintained. We pollinated 140 emasculated flowers and bagging. Ten of them were fixed in FAA 10 days after hand-cross pollination and were used to perform histological observations. High quantity pollen grains were observed on the stigmatic surface, which resulted in poor pollen germination and pollen tube growth. Only in two of the 10 observed pistils a few pollen tubes reached ovaries (Figure 3). The remaining 130 flowers were left for assessing fruit setting and seed content. Eight fruit were obtained and they all contained seeds with an average of three seeds per fruit. The fact that no seedless fruit was obtained by any treatment suggests lack of PA in ‘Monreal’.

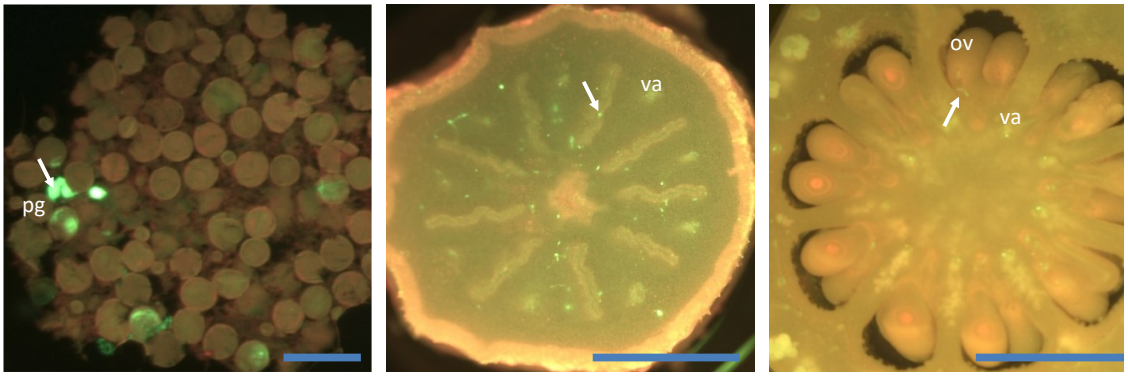

Figure 3. Histological observations in the ‘Monreal’ clementine x ‘Oroblanco’ triploid hybrid 10 days after pollination. (a) Large quantity of pollen grains observed on the stigma surface displaying poor pollen germination; (b) very low quantity pollen tubes growing throughout the bottom half of the stigma; (c) one pollen tube reached ovules. Pollen tubes marked by an arrow; pg: pollen grain; va: vascular axis; ov: ovule. Scale bars are denoted by blue lines: (a) 100 µm; (b) and (c) 1 mm.

Apart from identifying PA based on the ability to produce seedless fruit or not, as discussed above, a high or low degree of PA was assessed by comparing the fruit setting percentages between treatments E that produced only seedless fruit and CP that produced only seeded fruit.

In ‘Clemenules’, ‘Campeona’, ‘Imperial’, ‘Salteñita’, ‘Fortune’, ‘Moncada’, and ‘Ellendale’, the fruit setting percentages obtained from CP were higher than those obtained from E (and SP in ‘Fortune’) (Table 2), which indicates that the presence of fertilized ovules strongly influenced fruit set. In contrast, ‘Serafines’ showed no differences in the fruit setting percentages between E (64%) and CP (65%) (Table 2), which implies greater PA in this variety. When comparing satsuma and clementine, previous research has associated high levels of endogenous GAs in developing ovaries of the satsuma with

greater PA, whereas clementine produced lower GA levels and less PA (Mesejo et al., 2016; Talon et al., 1992). Our results displayed 'Serafines' as the variety with greater PA in which seed production did not appear crucial for fruit set. Therefore, the comparison of fruit setting between E and CP offers a methodology to identify citrus genotypes with different PA levels.

In citrus, competition between flowers results in a marked drop of flowers and fruitlets (Agustí et al., 1982). Together with several factors that affect fruit setting, such as floral load, inflorescence typeb and flower position (Garcia-Papi and Garcia-Martinez, 1984a), conducting more work using a large quantity of flowers on different trees is necessary to assess reliable PA data. The results presented herein are supported by the large quantity of treated flowers and, thus, provide consistent PA data.

3.3. Parthenocarpic Classification

We classified the parthenocarpy of each variety according to the four types described in citrus by Vardi et al. (2008). To classify each variety as either vegetative parthenocarpy, which allows seedless fruit to set with no external stimuli, or stimulative parthenocarpy, we tested whether pollination stimulus was necessary for seedless fruit to set. Regarding the distinction made between facultative and obligatory parthenocarpy, the conditions under which seedless fruit production occurs are crucial. Facultative parthenocarpy produces seedless fruit when cross-pollination with compatible pollen is avoided, and corresponds to self-incompatible or male sterility varieties. Obligatory parthenocarpy always produces seedless fruit regardless of pollination conditions and, therefore, corresponds to varieties with female sterility. Beyond the poor pollen performance displayed by 'Serafines', all the studied varieties are male and female fertile ones and bear seeded fruit when cross-pollinated. Therefore, self-incompatibility for these varieties is key to produce seedless fruit under natural conditions.

'Clemenules' and 'Moncada' have self-incompatibility and produced seedless fruit from SP treatment (Table 1), which reveals facultative parthenocarpy. In both varieties, fruit setting percentage obtained when pollination stimulus was removed (E treatment) was similar to those obtained from SP treatment (Table 2) and consequently, displayed vegetative parthenocarpy. Therefore, we classified 'Clemenules' and 'Moncada' as facultative and vegetative parthenocarpic. With 'Clemenules', this result agrees with the pollination-independent proposed by Mesejo et al. (2013) but challenges the classification of stimulative parthenocarpy, as proposed by Vardi et al., (2000).

'Fortune', as 'Clemenules' and 'Moncada', displayed a self-incompatibility reaction and produced seedless fruits from SP treatment (Table 1). In contrast, no fruit was obtained when the pollination stimulus was removed (E treatment in Table 2). This denotes that 'Fortune' requires a pollination stimulus to set fruit. Therefore, apart from the facultative parthenocarpy related to self-incompatibility, we classified 'Fortune' as having stimulative parthenocarpy. To the best of our knowledge, we are the first to report the requirement of a pollination stimulus for fruit to set in 'Fortune'.

'Imperial' and 'Ellendale' produced 19% and 5% of fruit setting respectively after removing the pollination stimulus (E treatment in Table 2), and all fruits were seedless. The percentage of fruit setting after SP treatment was 33% and 5% respectively (Table 2). In 'Imperial', 23% of fruits recovered from SP treatment contained seeds and the other 77% were seedless. Regarding 'Ellendale', 54% of fruits recovered from SP treatment

contained seeds and the rest were seedless. The average number of seeds per fruit was 0.8 and 0.7 for ‘Imperial’ and ‘Ellendale’ respectively (Table 1). Therefore, we classified ‘Imperial’ and ‘Ellendale’ as facultative and vegetative parthenocarpic, but point out that low-seeded fruit can be produced even if cross-pollination is avoided. With ‘Imperial’, previous studies (Sykes, 2008b; Sykes and Possingham, 1992; Vithanage, 1986; Wallace and Lee, 1999) coincided with our results and reported PA in this variety. Furthermore, Sykes (2008b) reported vegetative (autonomic) parthenocarpic fruit development. In contrast, ‘Ellendale’ has been studied by different authors who reached quite opposite conclusions. Vithanage (1991) classified it as nonparthenocarpic, Vardi et al. (2000) as stimulative parthenocarpic, and Sykes (2008b) reported vegetative (autonomic) parthenocarpic fruit development. Our results obtained under environmental conditions of Valencia and from more than 800 flowers enabled us to classify ‘Ellendale’ with facultative and vegetative parthenocarpy, which rules out the hypotheses proposed by the first two authors, but agrees with Sykes, (2008b).

For ‘Campeona’ and ‘Salteñita’ fruit setting percentages from SP treatment were respectively 42% and 58% (Table 2). Given their self-compatibility, all fruits recovered were seeded with an average of 4.9 seeds per fruit in ‘Campeona’ and 11 seeds per fruit in ‘Salteñita’ (Table 1). However, we found that they were able to produce seedless fruit when self-pollination was avoided by emasculating and bagging (E treatment), with a fruit setting percentage of 9% and 5% for ‘Campeona’ and ‘Salteñita’ respectively (Table 2), which means that they possess PA and do not need a pollination stimulus to set seedless fruit. As the term parthenocarpy is used to refer to seedless fruit production, self-compatible varieties can be classified as nonparthenocarpic. However, as ‘Campeona’ and ‘Salteñita’ possess PA, classifying these varieties as nonparthenocarpic can be confusing. The fact that in natural conditions, self-compatible genotypes produce seedy fruit even if cross pollination is avoided and seedless fruit can be recovered only from emasculation, which has to be performed by hand, explains why the scientific literature only reports them as seedy varieties (Hodgson, 1967), but information about PA goes unnoticed. We classify ‘Campeona’ and ‘Salteñita’ as self-compatible varieties with PA, which, therefore, provides relevant information on the parthenocarpy of these varieties.

For ‘Serafines’, we obtained practically the same fruit setting percentage after E, SP, and CP treatments, with values of 64, 66, and 65% respectively. Seedless fruits were obtained in all treatments except for CP, recovering an average of 5.3 seeds per fruit (Table 1). By taking into account the strictest meaning of obligate and facultative parthenocarpy, it must be considered to be facultative parthenocarpy because ‘Serafines’ is not female-sterile. As for requiring a pollination stimulus for fruit to set, ‘Serafines’ was able to produce seedless fruit from the E flowers with similar percentage values to those obtained from the CP flowers (Table 2). Considering the results herein obtained, we classified ‘Serafines’ as a facultative and vegetative parthenocarpic variety. Vegetative parthenocarpy has been reported in other satsuma varieties generally considered to be male and female sterile (Ollitrault et al., 2007b). However, satsumas have been used in breeding programs as parents in different countries, particularly in Japan (Omura and Shimada, 2016). Extremely interesting new hybrid varieties have been recovered, such as ‘Kiyomi’ tangor (*C. unshiu* x *C. sinensis*) (Nishiura et al., 1983), ‘Queen’ mandarin (*C. unshiu* x unknown) (de Teresa, 2011), ‘Primosole’ mandarin (*C. unshiu* x *C. reticulata*) (Tribulato and La Rosa, 1993), among others. These indicate that the viability of ovules and pollen grains of satsumas is strongly influenced by not only environmental conditions but also by genotype.

Male and female gametophytes (from meiosis to zygote formation) are exposed plant structures, which potentially makes them especially susceptible to environmental conditions (Chasan and Walbot, 1993; Hedhly et al., 2009). Temperature is one of the main environmental conditions that influences the success of plant sexual reproduction (Iizumi et al., 2017; Zhao et al., 2017) and several studies report the impact of temperature on gametophytic generation and the progamic phase (Distefano et al., 2018; Hedhly et al., 2009; Koti et al., 2005; Lora et al., 2011, 2009; Montalt et al., 2019). To the best of our knowledge, influence of environmental conditions in PA is unnoticed in citrus. However, previous research performed under controlled environmental conditions coincided in the influence of temperature in the self-incompatibility reaction, as we have discussed previously in this work.

The results in this work were obtained from a large number of flowers to ensure their robustness and consistency, although it was not been carried out in consecutive years, for which we cannot determine the effect of environmental conditions over data reproducibility. However, in a preliminary study carried out during 2013 and 2014 within this framework, we evaluated the seed production in SP flowers and the fruit setting from E, SP, and CP flowers (Montalt et al., n.d.). A summary of the results obtained in our preliminary study is shown in Supplementary Table S5. Both the number of seeds per fruit obtained from SP treatment and fruit setting percentage comparison between E and CP treatments are in line with those obtained in the present work suggesting that SI and PA in the studied varieties are stable under our Mediterranean field conditions.

Regarding the methodology used in our study, we identified the PA by testing the ability of each genotype to produce seedless fruits. In addition, we assessed the PA degree by comparing the percentages of fruit setting between emasculated (E treatment) and cross-pollinated (CP treatment) flowers (Table 2). In order to ensure the robustness of the results presented in Table 2, a large number of treated flowers was needed (Supplementary Table S1) and we used four trees per variety. Supplementary Table S6 displays the number of treated flowers, recovered fruits, and percentage of fruit setting from E and CP treatments in each of the four trees used per variety. Data shown from the E treatment (Supplementary Table S6) suggest that PA can be identified in most of the varieties by performing E treatment in 50 flowers. Regarding the assessment of the PA degree, the comparison between E-CP treatments displayed the same differences in each replicate separately (Supplementary Table S6) and in merged data (Table 2). ‘Serafines’ displayed similar fruit set percentage from E and CP treatments in each of the four replicates (Supplementary Table S6) and also in merged data (Table 2). In the rest of varieties with PA, E treatment produced lower fruit set than CP treatment in all four replicates (Supplementary Table S6) and in merged data (Table 2). With these results, we suggest that PA can be assessed by utilizing 50 flowers in E and CP treatments, making this protocol more feasible to identify those varieties with higher PA.

Seedlessness is a major characteristic in citrus and a lot of breeding work has been accomplished to develop new seedless cultivars (Ollitrault et al., 2007b). Diploid and triploid breeding programs and mutation breeding are different approaches followed to produce new seedless varieties. Increasing knowledge about self-incompatibility and parthenocarpy is relevant for improving the selection of parents that will be used in sexual hybridizations or mutagenesis.

Mutation breeding by gamma irradiation has been used in citrus to reduce seed production in diploid seedy hybrids (Mikeal L Roose and Williams, 2007). Notwithstanding, the recovery of complete seedlessness or very low seedy genotypes is

a very difficult issue but helpful to point out the importance of having strong parthenocarpic traits in breeding progenies (Caruso et al., 2020). Therefore, it is a key step to acquire prior knowledge of the parthenocarpic aptitude and the parthenocarpy type of the candidate diploid seedy varieties for irradiation since only those with these characteristics, and therefore capable to produce seedless fruits, will be suitable for being irradiated. Mutant lines from low or no PA varieties might require cultural practices such as girdling or GA treatments to stimulate fruit set and seedless fruit production (Garmendia et al., 2019).

4. Conclusions

Parthenocarpic ability is a key reproductive biology component because it enables seedless fruit production when combined with other reproductive features such as male and female sterility or self-incompatibility. We developed an efficient protocol to characterize the self-incompatibility and different types of parthenocarpy. It is based on emasculation, hand self-pollination, and hand cross-pollination and the analysis of fruit setting, seed production, and histological observations of pollen performance. We applied this protocol to analyze the reproductive behavior of nine important citrus varieties used as parents for seedless mandarin breeding. Of the four parthenocarpy types previously described for citrus, we found that ‘Clemenules’ and ‘Moncada’ were strictly self-incompatible with facultative and vegetative parthenocarpy, ‘Imperial’ and ‘Ellendale’ displayed no strict self-incompatibility associated with facultative and vegetative parthenocarpy, ‘Fortune’ was self-incompatible with facultative and stimulative parthenocarpy, and ‘Campeona’ and ‘Salteñita’ were self-compatible but with vegetative PA. ‘Serafines’ associated male sterility with facultative and vegetative parthenocarpy, while ‘Monreal’ clementine was not parthenocarpic. Our protocol can be applied for screening particular parents with previously identified interesting horticultural traits (e.g., production of nonapomictic seeds, resistance to Alternaria fungus, production of red fruit color, organoleptical qualities, etc.) and candidate-selected seedy diploid varieties with the objective to remove seeds by irradiation. A good reproductive behavior knowledge is important for optimizing seedless mandarin breeding programs.

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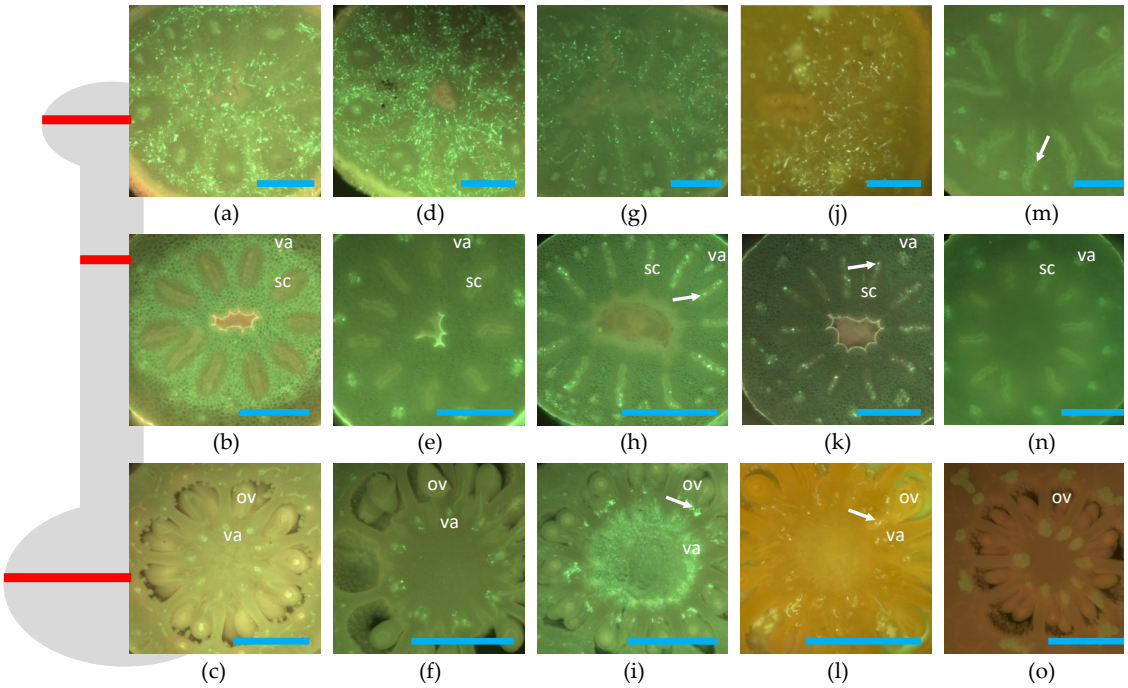
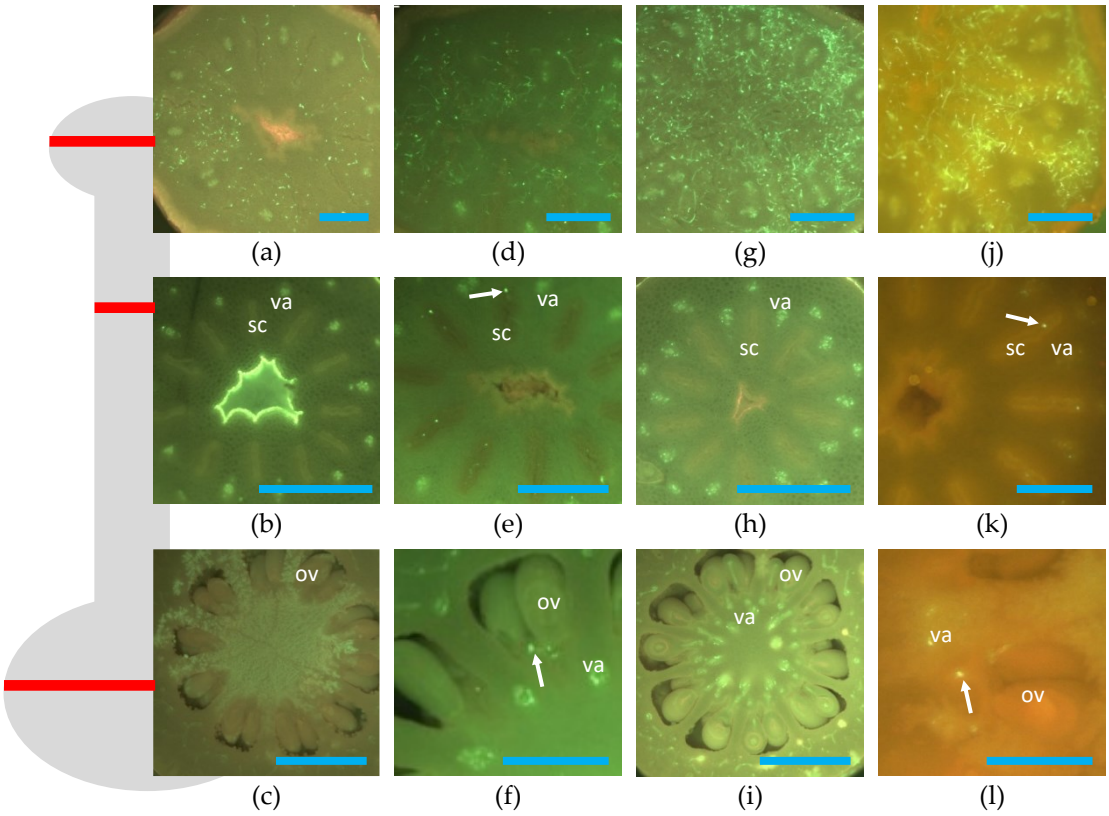
Supplementary information



Supplementary Figure 1. Histological sections of pistils after 10 days of self-pollination. Self-incompatible varieties: (a-c) `Clemenules´ clementine and (d-f) `Moncada´ mandarin; Self-compatible varieties: (g-i) `Campeona´ mandarin and (j-l) `Salteñita´ mandarin; Male sterile `Serafines´ satsuma (m-o) with no pollen tuber growing through the middle stigma. Left side of the figure represent a pistil with red lines indicating the transversal sections observed that corresponds with middle stigma (a), (d), (g), (j) (d), upper style (b), (e), (h), (k) (d), and ovary (c), (f), (i), (l), (o). Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule. Scale bars are depicted by blue lines: (a), (d), (g), (j) and (m) 0.5 mm; (b), (e), (h), (k) and (n) 0.25mm; (c), (f), (i), (l) and (o) 1 mm.



Supplementary Figure 2. Histological sections of pistils after 10 days of self-pollination. (a-f) `Imperial´ mandarin: (a-c) showing selfincompatibility reaction with no pollen tubes growing into the upper style and ovary. (d-f) `Imperial´ mandarin displaying pollen tubes into the style and ovary indicating partial self-incompatibility. (g-l) `Ellendale´ tangor: (g-i) showing selfincompatibility reaction with no pollen tubes growing into the upper style and ovary. (j-l) `Ellendale´ tangor displaying pollen tubes into the style and ovary indicating partial self-incompatibility. Left side of the figure represent a pistil with red lines indicating the transversal sections observed that corresponds with middle stigma (a), (d), (g), (j), upper style (b), (e), (h), (k) and ovary (c), (f), (i), (l). Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule. Scale bars are depicted by blue lines: (a), (b), (d), (e), (g), (h), (j) and (k) 0.25mm; (f) and (l) 0.5mm; (c) and (i) 1 mm.

Supplementary Table 1. Number of the treated flowers and recovered fruits in the performed treatments (E, SP and CP) in each variety.

	E		SP		CP	
	Treated flowers	Recovered fruits	Treated flowers	Recovered fruits	Treated flowers	Recovered fruits
`Clemenules´	250	38	250	39	200	167
`Monreal´	575	0	220	150	220	163
`Campeona´	200	17	200	84	200	112
`Imperial´	210	40	200	65	200	133
`Salteñita´	220	10	220	127	220	155
`Fortune´	310	0	255	23	237	149
`Moncada´	225	77	220	73	220	159
`Ellendale´	305	16	295	16	275	165
`Serafines´	195	125	145	96	205	133

E: Emasculation; SP: hand self-pollination; CP: hand cross-pollination.

Supplementary Table 2. 95% confidence interval in the comparison by pairs of fruit setting obtained in the performed treatments (E, SP and CP) in each variety.

	E-SP	E-CP	SP-CP
`Clemenules´	(-0,05; 0,06)	(0,61; 0,75)	(0,61; 0,74)
`Monreal´	(0,62; 0,74)	(0,68; 0,80)	(-0,02; 0,13)
`Campeona´	(0,25; 0,41)	(0,39; 0,55)	(0,06; 0,21)
`Imperial´	(0,05; 0,21)	(0,39; 0,55)	(0,25; 0,42)
`Salteñita´	(0,46; 0,60)	(0,59; 0,72)	(0,06; 0,19)
`Fortune´	(0,05; 0,12)	(0,56; 0,69)	(0,47; 0,60)
`Moncada´	(-0,10; 0,07)	(0,29; 0,46)	(0,30; 0,47)
`Ellendale´	(-0,03; 0,03)	(0,48; 0,61)	(0,48; 0,60)
`Serafines´	(-0,08; 0,12)	(-0,08; 0,10)	(-0,10; 0,08)

E: Emasculation; SP: hand self-pollination; CP: hand cross-pollination. If the confidence interval excludes zero, there are differences in the fruit setting percentages between the compared treatments.

Supplementary Table 3. SSR markers used for the genetic analysis of the recovered plants from the self-pollinated flowers of `Imperial´ mandarin and `Ellendale´ tangor.

Locus	Noted alleles in Imperial	Noted alleles in Ellendale	LG	Pos	AT	Bibliographic reference
JK-TAA15	188-191	164-204	1	120	55	[1]
Ci02G12	240-250	248-250			55	[2]
JK-TAA41	148-154	135-158	2	161	55	[1]
MEST86	113-120		8	15	55	[3]
MEST192	222-230		6	65	55	[4]
Ci04H06	188-190		2	28	55	[5]
mCrCIR03G05	222-228		4	75	50	[5]
mCrCi05A05	144-152		2	153	50	[5]
mCrCIR02D09	236-240		2	13	55	[5]
Ci02D04b		201-229	4	86	50	[6]
mCrCIR07F11		160-162	9	49	50	[6]
MEST15		174-189			55	[3]
MEST104		236-242	5	35	55	[3]
mCrCIR01C06		127-129	6	89	50	[5]

Numbers indicate the size of alleles in nucleotides for SSR markers. (LG) Linkage group; (Pos) Marker position in the reference Clementine genetic map (Ollitrault et al. 2012), centimorgans (cM); (AT) Annealing Temperature ºC.

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Supplementary Table 4. Observed alleles with the SSR markers used for the genetic analysis of the diploid hybrids recovered from the self-pollination of `Imperial' mandarin and `Ellendale´ tangor.

	JK-TAA41	Ci02G12	Ci04H06	mCrCi05A05	mCrCIR03G05	mCrCIR02D09	MEST86	MEST192
Imperial	148-154	240-250	188-190	144-152	222-228	236-240	113-120	222-230
Imperial SP-1	148-154	240-250	188-188	152-152	222-228	236-240	113-113	230-230
Imperial SP-2	148-154	240-250	188-188	144-152	222-228	236-240	113-120	230-230
Imperial SP-3	154-154	240-240	188-188	152-152	222-228	236-240	113-113	222-230
Imperial SP-4	148-154	240-250	188-188	144-152	222-228	236-236	113-120	222-230
Imperial SP-5	154-154	250-250	188-188	152-152	222-228	236-240	113-113	222-222
Imperial SP-6	148-154	240-250	188-188	144-152	222-222	236-240	113-120	230-230

	JK-TAA15	JK-TAA41	Ci02G12	Ci02D04b	mCrCIR01C06	mCrCIR07F11	MEST15	MEST104
Ellendale	164-204	138-158	248-250	201-229	131-133	160-162	174-189	236-242
Ellendale SP-1	204-204	138-138	248-248	201-229	131-133	162-162	189-189	236-242
Ellendale SP-2	164-204	138-158	248-250	201-229	133-133	160-162	174-189	236-242
Ellendale SP-3	164-164	138-158	248-248	201-229	133-133	162-162	189-189	242-242
Ellendale SP-4	164-164	138-158	250-250	229-229	133-133	160-162	174-189	236-242
Ellendale SP-5	164-164	158-158	250-250	201-229	133-133	160-162	174-189	242-242
Ellendale SP-6	164-204	158-158	248-248	201-229	131-133	160-162	189-189	242-242
Ellendale SP-7	204-204	138-158	248-248	201-201	133-133	160-162	174-189	236-242
Ellendale SP-8	164-164	138-138	248-250	201-229	131-133	160-160	174-189	242-242
Ellendale SP-9	164-204	138-158	248-250	201-229	131-133	160-162	174-189	236-236
Ellendale SP-10	164-164	138-158	248-250	201-229	131-131	160-162	174-189	236-242
Ellendale SP-11	164-164	138-158	248-250	201-229	131-131	160-162	174-189	236-242

Numbers indicate the size of alleles in nucleotides for SSR markers.

Supplementary Table 5. Number of seeds per fruit (nsp) from SP treatment and fruit setting percentage (fsp) from E, SP and CP treatments obtained in preliminary study.

	SP (nsp)	E (fsp)	SP (fsp)	CP (fsp)
`Clemenules´	0	19 (a)	20 (a)	85 (b)
`Monreal´	14.5	0	75 (a)	80 (a)
`Campeona´	5.4	6 (a)	21 (b)	36 (c)
`Imperial´	0	2 (a)	6 (a)	36 (b)
`Salteñita´	10.6	3 (a)	62 (b)	67 (b)
`Fortune´	0	0	13 (a)	74 (b)
`Moncada´	0	38 (a)	31 (a)	88 (b)
`Ellendale´	1.1 *	4 (a)	11 (b)	63 (c)
`Serafines´	Np	60 (a)	Np	60 (a)

SP: hand self-pollination; E: Emasculation; CP: hand cross-pollination. For each variety, significant differences in fsp (95% confidence interval for fruit setting proportions) between treatments are indicated by different letters in brackets. Np: Not performed. *Ellendale produced 57% of seedless fruits and 43% of fruits with an average of 2.6 seeds per fruit.

Supplementary Table 6. Number of treated flowers, recovered fruits and fruit set percentage from treatments E and CP in each of the four replicates R1, R2, R3 and R4, and for each studied variety.

		E			CP		
		Treated flowers	**Recovered fruits**	**Fruit Set (%)**	**Treated flowers**	**Recovered fruits**	**Fruit Set (%)**
`Clemenules´	R1	75	18	24 (a)	50	43	86 (b)
	R2	60	4	7 (a)	50	38	76 (b)
	R3	45	12	27 (a)	50	40	80 (b)
	R4	70	4	6 (a)	50	46	92 (b)
`Monreal´	R1	75	0	0 (a)	60	48	80 (b)
	R2	390	0	0 (a)	50	35	70 (b)
	R3	60	0	0 (a)	60	49	82 (b)
	R4	50	0	0 (a)	50	31	62 (b)
`Campeona´	R1	50	1	2 (a)	50	23	46 (b)
	R2	50	5	10 (a)	50	25	50 (b)
	R3	50	10	20 (a)	50	30	60 (b)
	R4	50	1	2 (a)	50	34	68 (b)
`Imperial´	R1	50	1	2 (a)	50	18	36 (b)
	R2	50	19	38 (a)	50	39	78 (b)
	R3	60	5	8 (a)	50	35	70 (b)
	R4	50	15	30 (a)	50	41	82 (b)
`Salteñita´	R1	70	2	3 (a)	70	47	67 (b)
	R2	50	2	4 (a)	50	38	76 (b)
	R3	50	1	2 (a)	50	29	58 (b)
	R4	50	5	10 (a)	50	41	82 (b)
`Fortune´	R1	60	0	0 (a)	25	18	72 (b)
	R2	100	0	0 (a)	62	45	73 (b)
	R3	50	0	0 (a)	50	28	56 (b)
	R4	100	0	0 (a)	100	58	58 (b)
`Moncada´	R1	75	30	40 (a)	75	52	69 (b)
	R2	20	9	45 (a)	20	20	100 (b)
	R3	80	28	35 (a)	75	57	76 (b)
	R4	50	10	20 (a)	50	30	60 (b)
`Ellendale´	R1	70	0	0 (a)	60	32	53 (b)
	R2	35	5	14 (a)	25	18	72 (b)
	R3	100	4	4 (a)	100	61	61 (b)
	R4	100	7	7 (a)	90	54	60 (b)
`Serafines´	R1	45	27	60 (a)	45	27	60 (a)
	R2	40	25	63 (a)	50	29	58 (a)
	R3	50	30	60 (a)	50	35	70 (a)
	R4	60	43	72 (a)	60	42	70 (a)

E: Emasculation; CP: hand cross-pollination. Four trees of each variety were used to perform the treatments. Thus, four replicates R1, R2, R3 and R4 per treatment were performed in each variety. For each replicate, significant differences in fruit set percentage (95% confidence interval for fruit setting proportions) between treatments are indicated by different letters in brackets.

Chapter 3. Breakdown of self-incompatibility in citrus by temperature stress, bud pollination and polyploidization

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Abstract

Self-incompatibility (SI) is present in around half of all species of flowering plants. SI limits endogamy and contributes to increased genetic diversity. SI is a very important trait in citrus because, when coupled with parthenocarpy, it allows seedless fruit production. Otherwise, SI is an im-pediment to genetic studies and breeding programs. Temperature stress, bud pollination and polyploidization can induce the breakdown of the SI mechanism in several species. In this work, we investigated how the SI mechanism can be broken down in two self-incompatible diploid citrus genotypes: 'Fortune' mandarin and 'Clemenules' clementine. The influence of temperature stress on the SI mechanism was assessed in self-pollinated flowers of 'Fortune' mandarins subjected to 2 temperature regimes (10 °C and 30 °C), whereas the bud pollination effect was investigated in the same genotype and in 'Clemenules' clementines cultivated under field conditions. The tetraploid 'Clemenules' clementine cultivated under field conditions was used to study if tetraploidization can bypass the SI reaction. Histological observations of pollen tube growth and seed production in self-pollinated flowers were used to evaluate the breakdown of SI, while the genetic analysis with SSR and SNP markers confirmed that all recovered plants were zygotic and had been originated by selfing. Our results confirm that the SI reaction can be surpassed by temperature stress, bud pol-lination and tetraploidy. To our knowledge, this is the first report in citrus in which the SI reaction breakdown by these three different strategies is demonstrated by molecular markers.

Keywords: mandarin; flower developmental stage; anthesis; self-pollination; pollen tube; tetraploid; SSR and SNP markers; breeding.

1. Introduction

Self-incompatibility (SI), also called self-sterility, is defined as "*the prevention of self-fertilization by otherwise normal and viable gametes, due to their genetic similarity*" (Franklin-Tong, 2008) and was described by Darwin in 1876. SI has been studied thoroughly and has been the subject of a number of important reviews (Allen and Hiscock, 2008; Barrett, 2013; Charlesworth et al., 2005; Gibbs, 2014; Raduski et al., 2011; Takayama and Isogai, 2005). The Brassicaceae and Solanaceae families have been important models for elucidating the molecular mechanism of SI (McClure, 2004; Sehgal and Singh, 2018; Yamamoto and Nishio, 2014). In woody crops, SI has been described in most of the species included in the Rosaceae (Abdallah et al., 2019; Brancher et al., 2020; Claessen et al., 2019; Maliepaard et al., 1998), Malvaceae (Knight and Rogers, 1953), Oleaceae (Alagna et al., 2019) and Rutaceae families (Zhang et al., 2018). SI avoids endogamy by favoring cross-pollination and contributes to increasing genetic diversity. Thus, it is crucial for species' adaptation and evolution (Abdallah et al., 2020; Goldberg et al., 2010; Lin et al., 2015; Surridge, 2015). SI is widespread in angiosperm species, and it is estimated that 40–60% of all species of flowering plants present SI (Barrett, 2013; de Nettancourt, 1997; Ferrer and Good, 2012; Gibbs, 2014; Igic et al., 2006; Raduski et al., 2011; Sawada et al., 2014; Tovar-Mendez and McClure, 2016).

Based on floral morphology, SI systems are classified as homomorphic and heteromorphic. In heteromorphic SI systems, incompatible phenotypes correlate with distinct floral morphological differences, most notably long (pin) or short (thrum) styles that are characteristic of distyly. In contrast, homomorphic SI systems show no distinct floral morphologies in association with the incompatible phenotype, and they comprise all gametophytic SI (GSI) systems and all multi-allelic sporophytic SI (SSI) systems (de Nettancourt, 1997; Gibbs, 2014). In SSI, the pollen-pistil interaction is determined by the diploid genotype of the parent, whereas in GSI, the compatibility pollen phenotype is determined by the haploid pollen (Ollitrault et al., 2021; Zhang et al., 2018). In the Solanaceae, Scrophulariaceae and Rosaceae families, GSI is controlled by a single multiallelic locus, named the S-locus, which is considered to contain at least two linked genes: one encodes glycoproteins with ribonuclease (S-RNase) activity in pistils, while the other is an F-box pollen-expressed gene (Vilanova et al., 2006). In citrus, Soost (1969, 1965) suggested a gametophytic system and estimated a codominant SI gene (S). This author put forward the notion that the SI gene consisted of one self-fertility allele (Sf) that resulted in self-compatibility and at least eight S alleles (Sn) that resulted in SI. Since then, many studies have been undertaken on the citrus SI system. Based on the (SC:SI) ratio obtained in offspring from different crosses, Vardi et al. (2000) confirmed the gametophytic SI and proposed different alleles in mandarins. Later novel genes associated with self-pollen rejection in citrus have been identified (Caruso et al., 2012). Recent studies (Honsho et al., 2021; Liang et al., 2020) indicate that GSI is based on S-RNase, which acts as a pistil S determinant by inhibiting pollen in an S-specific manner. Both studies analyzed the segregation of markers of S-RNase genes in controlled progenies and found segregations in agreement with this system for some. Based on segregation distortion from reciprocal crosses on high-density genetic maps, Ollitrault et al. (2021) confirmed the location of the SI locus at the beginning of chromosome 7 of the clementine reference genome.

Seedlessness is one of the most important characteristics for citrus fresh-fruit markets because consumers do not accept seedy fruit. Several mechanisms have been described in citrus that, when coupled with parthenocarpy, produce seedless fruit; these include for example, ovule sterility in flowers with no functional pistils (Wilms et al., 1983), degeneration (Osawa, 1912) and chromosome aberrations in the embryo sac (Wong, 1939), stenospermocarpy (Yamasaki et al., 2009, 2007), gene-cytoplasmic interaction (Goto et al., 2016), gamma irradiation (Bermejo et al., 2011; Goldenberg et al., 2014) and triploidy (Navarro et al., 2015; Ollitrault et al., 2007b, 2007a). Besides all these mechanisms, SI is also an efficient way to produce seedless genotypes (Montalt et al., 2021; Ollitrault et al., 2007b). The most important self-incompatible horticultural citrus groups are pummelos (*Citrus maxima* (Burm.) Merr.), clementines (*C. clementina* Hort. ex Tan.), some mandarins, and several natural or artificial hybrids (Kim et al., 2020; Yamamoto et al., 2006; Zhang et al., 2018). By taking advantage of histological techniques to observe pollen tube growth inside pistils, different authors have demonstrated that the SI reaction takes place between the stigma and the ovary (Ngo et al., 2001; Yamamoto et al., 2006). For mandarin hybrids, including the ‘Fortune’ mandarin (*C. clementina* x *C. tangerina*), and the ‘Clemenules’ clementine, several authors agree that the SI reaction originates in the upper part of the style (Distefano et al., 2009b, 2009a; Eti and Stosser, 1992; Montalt et al., 2021; Yamamoto et al., 2006).

Different causes that induce SI breakdown have been described in several species. At the beginning of the last century, the breakdown of SI by bud pollination was indicated in *Nicotiana* (East, 1923). It was later described in other species like *Petunia* (Herrero

and Dickinson, 1980) and *Lilium* (Kim and Niimi, 2002) and in different tribes of the Brassicaceae family (Hiscock and Dickinson, 1993). Temperature stress can also bypass the SI reaction, which has been described in *Lilium* (Ascher and Peloquin, 1966; Campbell and Linskens, 1984), *Trifolium* (Townsend, 1968) and *Arabidopsis* (Yamamoto et al., 2019). The polyploidy and self-compatibility association was reported many years ago. As early as 1923, Crane reported SI in the diploid *Prunus avium*, but SC in the tetraploid *P. cerasus*. Similar behavior has been found between the natural diploid and tetraploid species of *Campanula persicifolia*, *Allium shoenoprasum* and *Tulipa* spp (reviewed in (Lewis, 1947)). However, a strict comparison between a diploid and its autotetraploid was made thanks to the spontaneous chromosome doubling that occurred in the variety 'Fertility' *Pyrus communis,* and also when artificial tetraploids in other species were produced by colchicine treatments (Lewis, 1947)

Previous studies in citrus have demonstrated that temperature affects not only pollen grain germination but also pollen tube growth dynamics and kinetics (Distefano et al., 2018, 2012; Montalt et al., 2019). In self-incompatible genotypes like clementines, pollen tube growth is arrested in the first third of the style at high temperatures (25–30 °C), whereas very few pollen tubes reach ovaries at low temperatures (15–20 °C) (Distefano et al., 2018). Later, Aloisi et al. (2020) indicated the effect of different transglutaminase features and the polyamine pattern on the self-incompatible reaction in clementines. Wakana et al. (2004) reported the breakdown of the SI reaction into six different self-incompatible genotypes, including one clementine, two pummelos ('Banpeiyu' and 'Hirado Buntan') and three Japanese varieties, 'Hassaku' (*C. hassaku* Hort. ex Tan.), 'Hyuganatsu' (*C. tamurana* Hort. ex Tan) and 'Shishiyuzu' (*C. pseudogulgul* Hort. ex Shirai). In all these genotypes, these authors recovered seeds from self-pollinated flowers in various development stages, and they concluded that: (i) an appropriate flower bud size for self-pollination is about half the length of flower buds before anthesis; (ii) no SI reaction takes place in this flower bud development stage. Then, Distefano et al. (2009b) reported that the SI reaction is broken down in two self-incompatible genotypes of mandarin when self-pollinated one day before anthesis. However, these authors did not recover seeds.

Most cultivated citrus plants are diploids (2n = 2x = 18), although aneuploids and euploids can be occasionally found in seedlings (Aleza et al., 2011) or by spontaneous mutations (Yamashita et al., 1990). The above-cited authors identified a tetraploid limb mutation from a diploid self-incompatible 'Hyuganatsu' ancestral Japanese variety. In the reciprocal crosses between diploid and tetraploid 'Hyuganatsu' plants, well-developed and imperfectly developed seeds were recovered in 2x × 4x and 4x × 4x hybridizations, whereas no seeds were obtained in 4x × 2x hybridizations. These results suggest the effect of polyploidy breaking down the SI reaction in citrus.

Despite the above-indicated studies and the importance of SI in citrus, no complete work has demonstrated the breakdown of SI by temperature stress, bud pollination and polyploidization by recovering plants and analyzing their genetic origin with molecular markers. The SI reaction breakdown is a key step to obtain new populations from selfing of self-incompatible parents, which will allow more basic knowledge about SI to be acquired and to expand cross possibilities in breeding programs. The aim of this research was to: (i) evaluate the efficiency for breaking down the SI reaction in citrus by three different methods: temperature stress, bud pollination, and tetraploidy; (ii) recover plants from the three different approaches; (iii) analyze the ploidy level by flow cytometry and

determine the genetic origin of the recovered plants using simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) molecular markers.

2. Material and Methods

2.1. Plant Material

Two self-incompatible and non-apomictic citrus varieties were used to conduct this study: 'Clemenules' Clementine, which is the main mandarin variety cultivated in Spain for its exceptional fruit quality; and 'Fortune' mandarin, which produces fruits with excellent organoleptical qualities and a very late harvesting period. These varieties are grown in plots in the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Moncada, Valencia (Spain). They have the following accession numbers: 'Fortune' mandarin (IVIA-080) and 'Clemenules' clementine (IVIA-022). Tetraploid 'Clemenules' clementine was obtained by shoot tip grafting combined with colchicine treatment (Aleza et al., 2009) and was grafted onto 'Carrizo' citrange (*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.) in the IVIA experimental plot many years ago. Additionally, 2 6-year-old 'Fortune' mandarin plants cultivated in 50-L containers were used and were grown under field conditions until placed in growth chambers to assess the influence of temperature on the SI reaction. These conditions are described in 2.3 Experimental procedure, Subsection 2.3.1, Experiment 1.

2.2. Pollination Procedure, Sample Storage and Seed Germination

In order to perform hand pollinations, anthers were removed from the flowers of the donors randomly harvested in the balloon stage and were dried in Petri dishes on silica gel in a desiccator at room temperature until dehiscence (24–48 h). The dehiscent anthers were used for pollination. The hand-pollinated flowers were emasculated and bagged to avoid undesired pollination. Depending on the experiment conditions, the flowers in the different developmental stages were pollinated at different times spanning from pollination to sample storage. These differences are detailed below. Flowers were fixed in FAA solution (formalin, glacial acetic acid, 70% ethanol, 1:1:18, *v*/*v*) (Johansen, 1940) and stored at 4 °C pending histological observations. The other flowers were left for fruit set. Fruits were collected when ripe, and the number of seeds per fruit was recorded. Normal developed seeds were germinated under standard greenhouse conditions to produce seedlings for ploidy and genetic analyses.

2.3. Experimental Procedure

Three independent self-pollination experiments were carried out under different conditions indicated below as Experiments 1, 2 and 3. Additionally, self- and cross-pollination under field conditions and cross-pollination in the temperature regimes studied in Experiment 1 were performed to confirm the normal behavior of pollen performance in the flowers utilized in our experiments. The average temperature under field conditions within the experimental time frame was 18.5 °C, with a typically gradual increase from less than 10 °C at night to up to 30°C in the daytime. The temperature data was acquired by an automatic weather station located at IVIA (Moncada, Valencia,

Spain). To pollinate ‘Fortune’ mandarin flowers, we used the pollen of the ‘Clemenules’ clementine and vice versa. We employed these genotypes for their high pollination aptitude and the compatibility between them.

2.3.1. Experiment 1

The influence of temperature stress on the SI reaction was assessed in adult trees of the ‘Fortune’ mandarin cultivated in 50-L containers. Two temperature regimes were used: a low-temperature regime with an average of 10 °C and a warm temperature regime with an average of 30 °C. For this purpose, during the flowering period, one adult tree was placed in each of the growth chambers for both temperature regimes and was exposed to 80 µE m^{-2} s^{-1} illumination for 16 h daily. In the immediate following days (1 to 2 days at 30 °C and 1 to 5 days at 10 °C), 25 flowers at anthesis for each temperature regime were used to perform hand-pollination; ten flowers were used for histological observations (Supplementary Table S1), and 15 flowers were left for fruit setting. According to our previous results about the influence of temperature on the citrus progamic phase [64], the time between pollination and FAA fixation differed depending on the temperature regime under study. At 10 °C, flowers were fixed 20 days after pollination, whereas at 30 °C, flowers were fixed 4 days after pollination. Once self-pollinated flowers had been collected for histological observations, trees were left under field conditions.

2.3.2. Experiment 2

The influence of bud pollination on the SI reaction was assessed in adult ‘Fortune’ mandarin and ‘Clemenules’ clementine trees cultivated under field conditions. In each genotype, 40 flower buds of different sizes were selected and measured using a digital caliper. Based on the measured length, flower buds were classified and tagged in four developmental stages (A–D, see Figure 1). The flower bud length used for this experiment spanned from 8.6 to 15.8 mm in ‘Fortune’ mandarin (Supplementary Table S2) and from 8.2 to 16.0 mm in ‘Clemenules’ clementine (Supplementary Table S3). The tagged flower buds were self-pollinated and bagged to avoid undesired cross-pollination. A total of 5 pollinated pistils that corresponded to each developmental stage were harvested 10 days after pollination, fixed in FAA solution and kept at 4 °C until histological observations were performed. The remaining five self-pollinated flowers in each developmental stage were left for fruit setting. In order to categorize developmental stages (A–D, see Figure 1), five more flowers at each stage were labeled and monitored until anthesis occurred. The number of days before anthesis was five to seven for stage A; two to four for stage B; and one to two for stage C. D refers to flowers at the balloon stage (flowers very close to anthesis that opened in one or less than one day after labeling).

2.3.3. Experiment 3

The influence of polyploidization on the SI reaction was assessed in one adult tree of the tetraploid ‘Clemenules’ clementine cultivated under field conditions in the IVIA plots. Twenty-five flowers at anthesis were manually self-pollinated and bagged. Eight of them were fixed in FAA ten days after pollination (Supplementary Table S4), and the rest were left until fruit set.

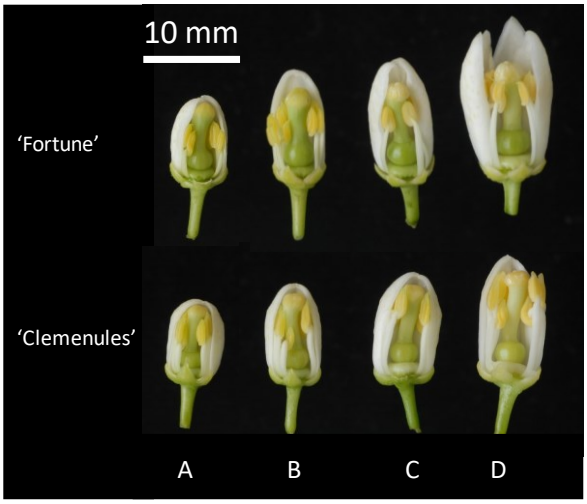


Figure 1. Developmental stages of the self-pollinated flowers used to evaluate SI breakdown by bud pollination in 'Fortune' mandarin and 'Clemenules' clementine.

2.4. Histological Observations

The pistils fixed in FAA were submerged 3 times in water for 1 h. Then, pistils were sliced into 14 cross-sections using a sharp blade. Stigmas were sliced into two sections, styles into eight sections and ovaries into four sections, following the methodology described by Montalt et al. (2019). Sections were stained with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens FH, Esser, 1957). Pollen tube growth was identified by its callose plugs fluorescence (Adhikari et al., 2020) and was visualized under a Leica MZ16FA epifluorescence stereomicroscope using a GFP1 excitation filter 395–455 nm and barrier filter 480 LP. In each analyzed pistil, pollen tube growth was assessed based on the percentage of the pistil reached by pollen tubes and the number of pollen tubes that had reached the ovary (Supplementary Tables S1–S4). In some samples, the number of pollen tubes reaching the ovaries emitted high fluorescence, which did not allow pollen tubes to be individually counted (see Figure 3k), and a decision was made to classify them as ">10". For the easy view and systematic presentation of the results, the number of pollen tubes that reached ovaries appears in brackets; less than five pollen tubes (<5), between five and ten pollen tubes (5–10) and more than ten pollen tubes (>10).

2.5. Ploidy Level Analysis by Flow Cytometry

Ploidy level was determined by flow cytometry according to the methodology described by Aleza et al. (2009). Each sample consisted of a small piece of leaf (~0.5 mm^2) collected from each plant with a similar leaf piece taken from a diploid control plant. Samples were chopped together using a razor blade in the presence of a nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec, Münster, DE). Nuclei were filtered through a 30-µm nylon filter and stained with DAPI (4,6-diamine-2-phenylindol) (High Resolution DNA Kit Type P, solution B; Partec) solution. After a 5-min incubation period, the stained samples were run in a CyFlow Ploidy Analyzer (Partec) flow cytometer equipped with optical parameters to detect DAPI fluorescence. DNA fluorochrome DAPI was excited by the UV-LED at 365 nm. Histograms were analyzed with the CyView software (Partec), which determines the peak position, coefficient of variation (CV), arithmetic mean and median of samples.

2.6. Genetic Analysis with Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) Markers.

The plants recovered from the three independent experiments were genotyped using SSR and SNP markers located along the nine linkage groups of the reference clementine genetic map (Ollitrault et al., 2012a). In all, four SSR and seven SNP heterozygous markers for 'Fortune' mandarin and three SSR and nine SNP heterozygous markers for 'Clemenules' clementine were selected to analyze the genetic origin of the recovered plants. Detailed information and bibliographic references (Cuenca et al., 2011; Froelicher et al., 2008; Garcia-Lor et al., 2013b; Kijas et al., 1997; Ollitrault et al., 2012b) about all the used markers are provided in Supplementary Table S5.

Genomic DNA was extracted from leaves using the Plant DNeasy kit (Quiagen, Hilden, Germany) following the manufacturer's protocol and was measured using a spectrophotometer (NanoDrop 2000C, Thermo Fisher. Waltham, MA, USA). Samples were diluted with sterile water (Sigma-Aldrich, Co. Burlington, MA, USA) at a concentration of 10 ng/μL and stored at 20 °C until used.

Polymerase chain reactions (PCRs) were performed using the SSR markers with a Mastercycler ep gradient S (Eppendorf©. Hamburg, Germany) according to the following protocol: reaction volume 15 μL containing 0.5 μL of 1 U/μL of Taq DNA polymerase (Fermentas©. Waltham, MA, USA), 3 μL of the citrus template DNA (10 ng/μL), 1.5 μL of 2 μM welled (Sigma©. Burlington, MA, USA) dye-labeled forward primer, 1.5 μL of 2 μM non-dye-labeled reverse primer, 0.2 μM of each dNTP, 1.5 μL of PCR reaction buffer 10X and 0.45 μL of 50 mM $MgCl_2$. The cycling program was set as follows: denaturation for 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C or 55 °C, 30 s at 72 °C; and a final elongation step of 8 min at 72 °C. Separation was carried out by capillary gel electrophoresis in a Genetic Analysis System 8000 (Beckman Coulter Inc., Brea, CA, USA). PCR products were initially denatured at 90 °C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were size-based on a DNA size standard (400 bp). The GenomeLab v.10.0 (Beckman Coulter Inc., Brea, CA, USA) genetic analysis software was used for data collection.

The recovered plants were also genotyped with the SNP markers following the KASPar technology (LGC Genomics, Ipswich, UK). The KASPar Genotyping System is a competitive allele-specific dual Förster resonance energy transfer (FRET)-based assay for SNP genotyping. Primers were directly designed by the LGC Genomics Company based on the SNP locus-flanking sequence (~50 nt on each side of the SNP). SNP genotyping was performed by the KASPar technique. A detailed description of the specific conditions and reagents can be found in Cuppen (2007).

2.7. Population Diversity Analysis

The population diversity organization between the diploid and tetraploid plants recovered from the self-pollination of 'Fortune' mandarin and tetraploid 'Clemenules' clementine, respectively, was examined with the DARwin6 software (Perrier and Jacquemoud-Collet, 2006). A neighbor-joining analysis was performed using the simple matching dissimilarity index (d_{i-j}) between pairs of markers (units):

$$d_{i-j} = 1 - \frac{1}{L}\sum_{l=1}^{L}\frac{m_l}{\pi}$$

where d_{i-j} is the dissimilarity between units *i* and *j*, *L* is the number of markers, m_l is the number of matching alleles for marker l, and π is the ploidy level. From the obtained dissimilarity matrix, a weighted neighbor-joining tree (Saitou and Nei, 1987) was computed.

3. Results

Figure 2 shows examples of the performed control pollinations. As expected: (i) in the self-pollinated flowers of 'Clemenules' clementine and 'Fortune' mandarin under field conditions, no pollen tubes reached the ovaries in any analyzed pistil; (ii) in the cross-pollinations between 'Clemenules' × 'Fortune' and 'Fortune' × 'Clemenules' under field conditions, all the analyzed pistils displayed pollen tubes that reached the ovaries with more than 10 pollen tubes in them; (iii) in the cross-pollinations between 'Fortune' × 'Clemenules' at 10 °C and 30 °C, all the analyzed pistils displayed pollen tubes that reached the ovaries with more than 10 pollen tubes in them.

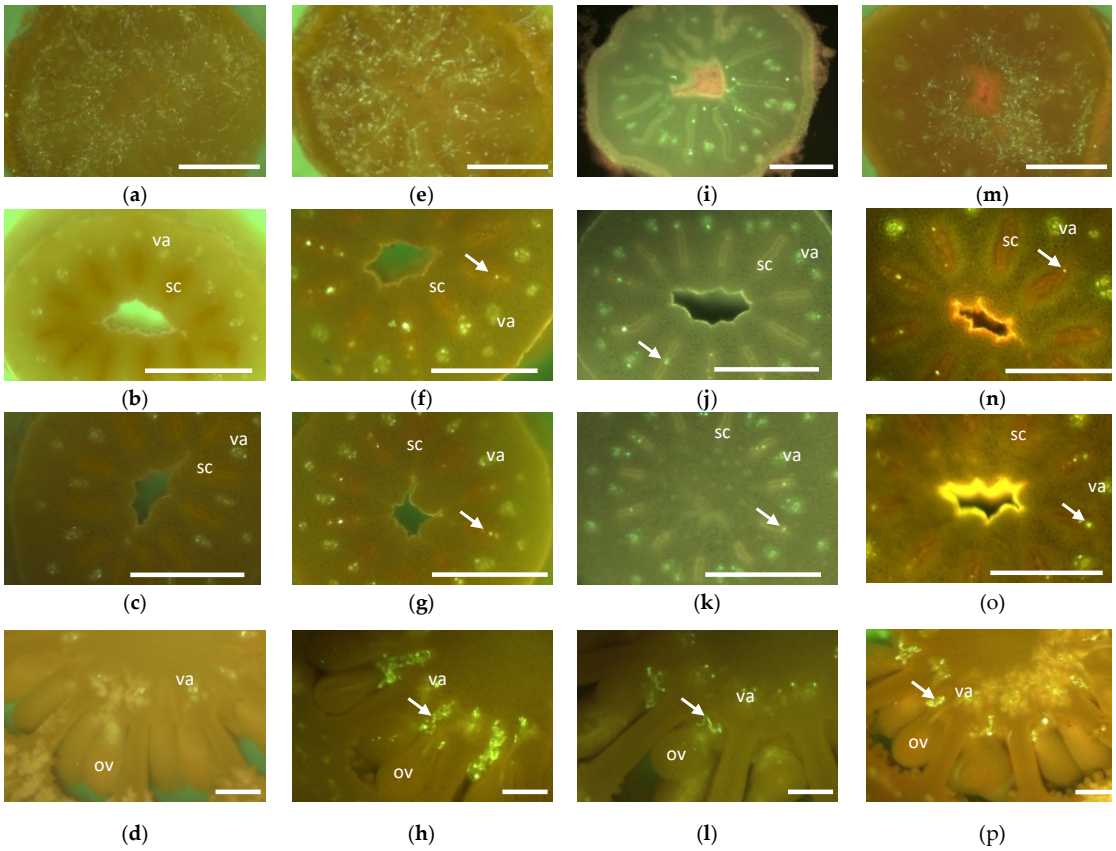


Figure 2. Histological sections of 'Fortune' mandarin pistils. (**a**–**d**) Self-pollinated pistils under field conditions. (**e**–**h**) Cross-pollinated pistils under field conditions. (**i**–**l**) Cross-pollinated pistils at 10 °C. (**m**–**p**) Cross-pollinated pistils at 30 °C. The pollen from 'Clemenules' clementine was used for cross-pollinations. Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule. Scale bars are depicted by white lines: (**a**,**e**,**i**,**m**) 1 mm; (**b**,**c**,**f**,**g**,**j**,**k**,**n**,**o**) 0.5 mm; (**d**,**h**,**l**,**p**) 0.2 mm.

3.1. SI Breakdown by Temperature Stress (Experiment 1)

At 10 °C, we noted that fewer than 5 pollen tubes reached the ovaries (Figure 3c,d) in 80% of the self-pollinated flowers; fruits harvested from the self-pollinated flowers had an average of 1.4 seeds per fruit (Table 1). By considering both histological observations and seed production from the self-pollinated flowers at 10 °C, we demonstrated that pollen tubes were able to reach the ovaries, fertilize ovules and produce seeds. All this indicates that SI in the self-incompatible genotype 'Fortune' mandarin breaks down at 10 °C. At 30 °C, we observed some pollen tubes growing below the upper half of the style in 20% of the self-pollinated flowers. However, no pollen tubes were observed reaching the ovaries in any of them (Figure 3g,h). Moreover, no fruits were obtained, and consequently, seed presence could not be assessed at 30 °C (Table 1).

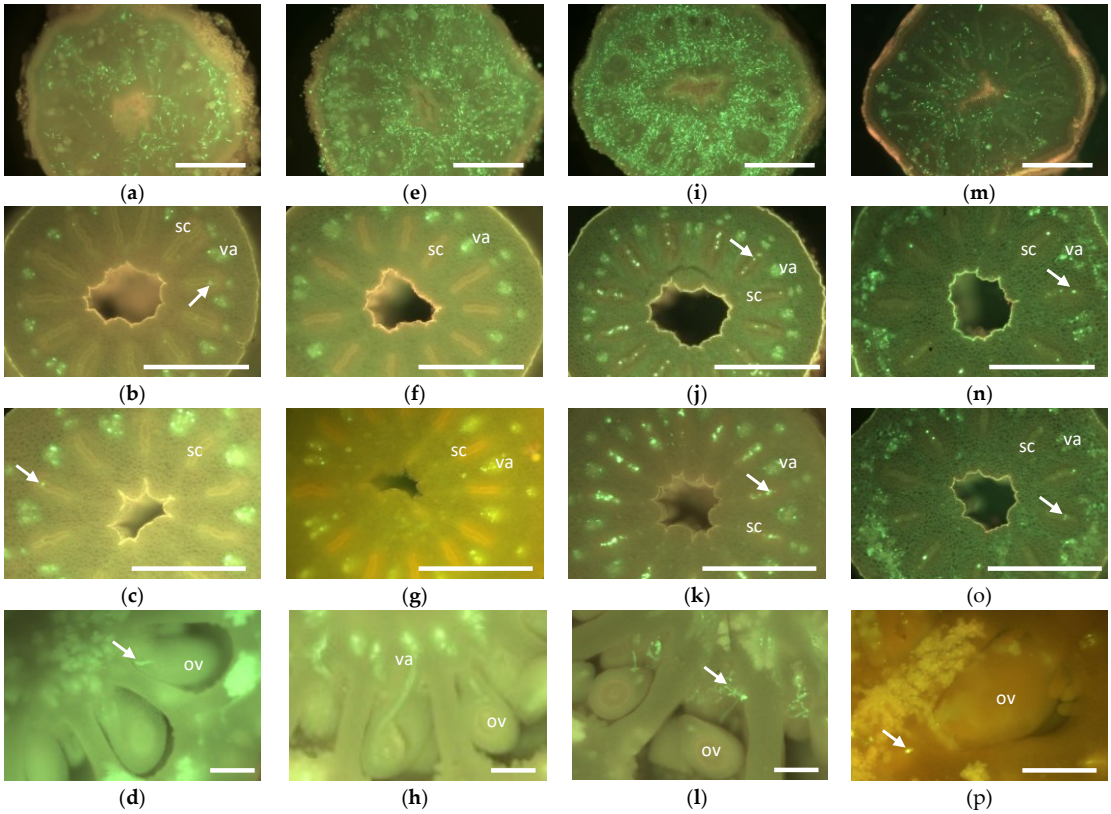


Figure 3. Histological sections of the self-pollinated pistils analyzed in the three experiments. **(a–d)** Self-pollinated pistil of 'Fortune' mandarin at 10 °C. **(e–h)** Self-pollinated pistil of 'Fortune' mandarin at 30 °C. **(i–l)** Self-pollinated buds of 'Fortune' mandarin. **(m–p)**. Self-pollinated pistil of the tetraploid 'Clemenules' clementine. Pollen tubes are marked by an arrow; va: vascular axis; sc: stylar canal; ov: ovule. Scale bars are depicted by white lines: **(a,e,i,m)** 1 mm; **(b,c,f,g,i,k,n,o)** 0.5 mm; **(d,h,l,p)** 0.2 mm.

Table 1. Pollen tube growth (PTG) and seed production obtained from the self-pollinated flowers in each experiment.

Experiment	Genotype	Temp.	Flower Stage	PTG (%)	PTG (No.)	Seed Production (No. Per Fruit)
Exp. 1 Temperature stress	'Fortune'	10 °C	Anthesis	80	<5	1.4 ± 0.8
	'Fortune'	30 °C		0	0	-
Exp. 2 Bud Pollination	'Fortune'	FC	Bud (14.7 ± 1.0) (D)	0	0	0
	'Fortune'	FC	Bud (12.5 ± 0.7) (C)	60	<5	-
	'Fortune'	FC	Bud (9.9 ± 0.9) (A-B)	100	>10	22.3 ± 2.5
	'Clemenules'	FC	Bud (14.9 ± 0.8) (D)	0	0	0
	'Clemenules'	FC	Bud (12.1 ± 0.5) (C)	40	<5	-
	'Clemenules'	FC	Bud (9.4 ± 0.9) (A-B)	100	>10	17.5 ± 7.7
Exp. 3 Polyploidization	'Clemenules' 4x	FC	Anthesis	100	5–10	5 ± 1.4

FC. Field conditions. Flower stage is indicated as 'Anthesis' or 'Bud'. In parenthesis after 'Bud', the following information is included: 1) bud length (mm) expressed as the mean ± SD (n = 5); 2) capital letters (A to D) indicating flower developmental stages, as shown in Figure 1. PTG is expressed as the percentage (%) of pistils in which pollen tubes reached ovaries and as the number (No.) of pollen tubes that reached ovaries. PTG (No.) is given as the maximum interval in Supplementary Tables S1–S4. Seed production (number of seeds per fruit) is expressed as the mean ± SD (n = 5 to 13 depending on the number of fruit obtained in the treatment).

3.2. SI Breakdown by Bud Pollination (Experiment 2)

Both 'Fortune' mandarin and 'Clemenules' clementine showed similar results. In all the self-pollinated buds with average lengths of 14.7 mm in 'Fortune' mandarin and of 14.9 mm in 'Clemenules' clementine (D in Figure 1), no pollen tubes reached the ovaries, and all the obtained fruits were seedless (Table 1). In the self-pollinated buds with an average length of 12.5 mm in 'Fortune' mandarin and 12.1 mm in 'Clemenules' clementine (C in Figure 1), 60% and 40% of pistils showed fewer than 5 pollen tubes reaching the ovaries in 'Fortune' mandarin and 'Clemenules' clementine, respectively, while no pollen tubes were observed in the rest. However, more than 10 pollen tubes (Figure 3k,l) were observed in all the ovaries of the self-pollinated buds at early developmental stages with an average length of 9.9 mm in 'Fortune' mandarin and of 9.4 mm in 'Clemenules' clementine (A and B in Figure 1). All the obtained fruits were seedy and contained an average of 22.3 and 17.5 seeds per fruit in 'Fortune' mandarin and 'Clemenules' clementine, respectively (Table 1). The absence of stigmatic exudation should be noted in stigmas in early developmental stages (A and B in Figure 1) when successful pollen performance occurred. This fact indicates that the presence of stigmatic exudation does not reflect stigmatic receptivity.

3.3. SI Breakdown by Polyploidization (Experiment 3)

The histological observations (Figure 3o,p) showed that pollen tubes reached the ovaries in all the self-pollinated flowers, and fruits harvested from the self-pollinated flowers had an average of five seeds per fruit (Table 1). By taking into account both histological observations and seed production from the self-pollinated flowers of the tetraploid 'Clemenules' clementine, we conclude that SI breaks down by polyploidization in this genotype.

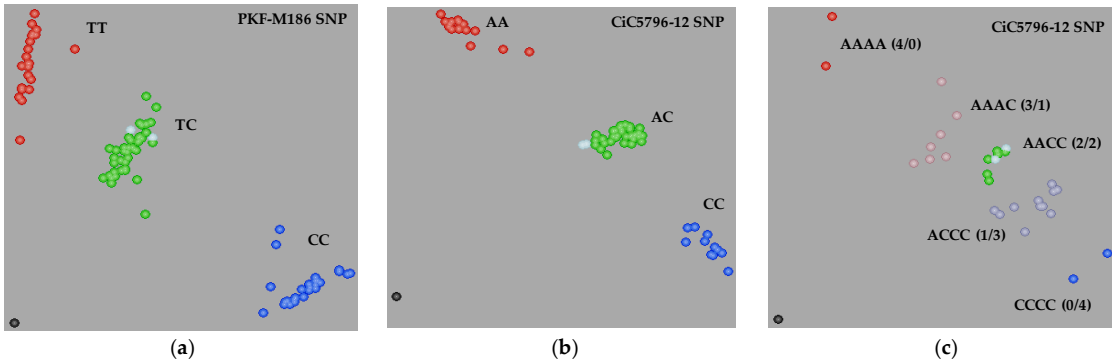
3.4. Ploidy Level Analysis by Flow Cytometry

The number of plants recovered from the germination of the normally developed seeds obtained in each experiment was: (i) 7 plants by self-pollination of the 'Fortune' mandarin at 10 °C (Experiment 1); 81 and 61 plants by bud self-pollination of the 'Fortune' mandarin and 'Clemenules' clementine, respectively (Experiment 2); 29 plants by the self-pollination of the tetraploid 'Clemenules' clementine (Experiment 3). The analysis of flow cytometry showed that all the plants recovered from Experiments 1 and 2 were diploids, and all the plants recovered from Experiment 3 were tetraploids. These results agree with the selfing hypothesis.

3.5. Genetic Analysis with the SSR and SNP Markers

Genetic analyses of recovered plants were performed to rule out any uncontrolled pollination hypothesis and to evaluate segregation distortion. The plants recovered from the self-pollination of the 'Fortune' mandarin at 10 °C (Experiment 1) and from the bud self-pollination of the 'Fortune' mandarin (Experiment 2) were analyzed with four SSR markers heterozygous for 'Fortune' mandarin (Supplementary Table S5). The results for the SSR markers showed that all the plants recovered from Experiment 1 (Supplementary Table S6) and from Experiment 2 (Supplementary Table S7) displayed unambiguous 'Fortune' mandarin alleles.

The plants recovered from the bud self-pollination of the 'Fortune' mandarin (Experiment 2) were also analyzed with seven SNP markers heterozygous for 'Fortune' mandarin (Supplementary Table S5). By way of example, Figure 4a shows the results obtained for the PKF-M186 SNP marker. The analyzed hybrids are clustered in three different groups corresponding to the expected segregation according to the self-pollination hypothesis, with two groups in homozygosity (TT and CC) and one group in heterozygosity (TC), in which the 'Fortune' mandarin is included.



(a) (b) (c)

Figure 4. (a) Plot of the T and C allele signals of the PKF-M186 SNP marker from the cluster analysis of 80 diploid hybrids recovered by the self-pollination of the 'Fortune' mandarin by bud pollination. Diploid homozygous (TT and CC) and heterozygous hybrids (TC) originated from the heterozygous 'Fortune' mandarin indicated by white spots. (**b**) Plot of the A and C allele signals of the CiC5796-12 SNP marker from the cluster analysis of 61 diploid hybrids recovered by the self-pollination of the diploid 'Clemenules' clementine by bud pollination. Diploid homozygous (AA and CC) and heterozygous hybrids (AC) originated from the heterozygous 2x clementine indicated by white spots. (**c**) Plot of the A and C allele signals of the CiC5796-12 SNP marker from the cluster analysis of 29 4x hybrids recovered by the self-pollination of 4x 'Clemenules' clementine. Tetraploid homozygous (AAAA and CCCC, with 4/0 and 0/4 allele dosage, respectively) and tetraploid heterozygous hybrids (AAAC, AACC and ACCC with 3/1, 2/2 and 1/3 allele dosage, respectively) originated from heterozygous 4x clementine indicated by white spots.

None of the plants recovered by Experiments 1 and 2 were identical to the diploid 'Fortune' mandarin (Figure 5a), as they displayed different allelic configurations (Supplementary Tables S6 and S7). Moreover, all the plants displayed some markers in heterozygosity, which allowed the doubled haploid origin hypothesis to be ruled out.

Potential distortions toward Mendelian allelic segregations were tested in the population obtained from bud pollination for each marker using the Chi-squared test (0.05 probability threshold) with Bonferroni correction for multiple testing. Only the NADK2-M285 SNP marker showed a significant segregation distortion (*p*-value = 0.001) (Table 2). Considering that flowers were bagged after self-pollination, flow cytometry and the observed segregation, we conclude that we obtained diploid zygotic plants that resulted from the self-pollination of 'Fortune' mandarin.

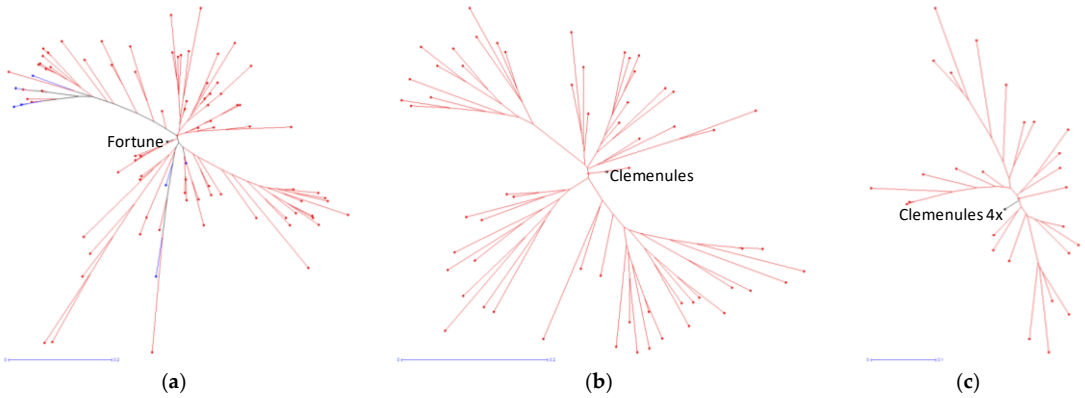


Figure 5. (a) Neighbor-joining tree obtained from the allelic data of the diploid hybrid population recovered from the self-pollination of the 'Fortune' mandarin by temperature stress (blue) and bud pollination (red). (**b**) Neighbor-joining tree obtained from the allelic data of the diploid hybrid population recovered from the self-pollination of the 'Clemenules' clementine by bud pollination. (**c**) Neighbor-joining tree obtained from the allelic data of the tetraploid hybrids population recovered from the self-pollination of the tetraploid 'Clemenules' clementine. The scale for genetic distances over edge is indicated by the blue line length; 0.2 for (**a**) and (**b**), and 0.1 for (**c**).

Table 2. Analysis of Mendelian allelic segregation (Chi-squared test with Bonferroni correction for multiple testing) of the recovered plants from the self-pollinated buds of the 'Fortune' mandarin.

Marker	aa	AA	Aa	Chi-square	P value
SOS1-M50	18	26	36	1.455	0.228
mCrCIR05A05	17	16	47	0.030	0.862
JK-TAA41	16	18	43	0.118	0.732
PKF-M186	22	21	37	0.023	0.879
mCrCIR06B07	17	17	43	0.000	1.000
Ci07C07	17	15	48	0.125	0.724
NADK2-M285	31	9	40	12.100	0.001
LAPX-M238	17	26	36	1.884	0.170
MDH-MP69	18	24	33	0.857	0.355
FLS-M400	18	14	48	0.500	0.480
HYB-M62	10	17	53	1.815	0.178

Detailed information on the markers is provided in Supplementary Table S5. The data under aa, AA and Aa columns indicate the number of individuals with that allelic configuration.

The diploid plants recovered from the bud self-pollination of ‘Clemenules’ clementine (Experiment 2), and the tetraploid plants obtained from the self-pollination of tetraploid ‘Clemenules’ clementine (Experiment 3) were analyzed with three SSR markers heterozygous for ‘Clemenules’ clementine (Supplementary Table S5). The results for the SSR markers showed that all the plants recovered from Experiment 2 (Supplementary Table S8) and from Experiment 3 (Supplementary Table S9) displayed unambiguous alleles of ‘Clemenules’ clementine. By considering these results and the fact that flowers were bagged after self-pollination, we discarded any undesired cross-pollination.

The plants recovered from the bud self-pollination of ‘Clemenules’ clementine (Experiment 2) were also analyzed with nine SNP markers heterozygous for ‘Clemenules’ clementine (Supplementary Table S5). By way of example, Figure 4b shows the results obtained for the CiC5796-12 SNP marker by identifying three clusters, of which two correspond to the homozygous allelic configurations (AA and CC) and one to heterozygosity. None of these plants were identical to the diploid ‘Clemenules’ clementine (Figure 5b) as they displayed different allelic configurations (Supplementary Table S8). Potential distortions from Mendelian allelic segregations were tested in the population obtained from the bud pollination for each marker by the Chi-squared test (0.05 probability threshold) with Bonferroni correction for multiple testing (Table 3). The genetic analysis agreed with the self-pollination hypothesis, as all the diploid hybrids displayed unambiguous alleles of ‘Clemenules’ clementine in a Mendelian segregation manner. According to the observed segregation, the flow cytometry and histological data, we conclude that the diploid zygotic plants were produced from the self-pollination of ‘Clemenules’ clementine.

Table 3. Analysis of Mendelian allelic segregation (Chi-squared test with Bonferroni correction for multiple testing) of the recovered plants from the self-pollinated buds of the ‘Clemenules’ clementine.

Marker	aa	AA	Aa	Chi-square	P value
CiC2110-01	15	16	30	0.032	0.857
CiC5950-02	11	21	29	3.125	0.077
CiC6278-01	10	17	32	1.815	0.178
CiC3712-01	13	21	27	1.882	0.170
JK-TAA41	22	28	14	1.778	0.182
mCrCIR06B07	14	31	19	0.758	0.384
CiC5796-12	16	11	34	0.926	0.336
CiC1380-05	18	11	32	1.690	0.194
Ci07C07	14	30	20	1.059	0.303
CiC5164-03	17	14	29	0.290	0.590
CiC1749-05	15	12	34	0.333	0.564
CiC5087-01	13	18	30	0.806	0.369

Detailed information on the markers is provided in Supplementary Table S5. The data under the aa, AA and Aa columns indicate the number of individuals with that allelic configuration.

Allele dosage of the tetraploid plants obtained from the self-pollination of the tetraploid ‘Clemenules’ clementine (Experiment 3) was verified based on the relative allele signals. For the SSR markers, tetraploid allelic configurations were assigned by the microsatellite allele counting-peak ratios method (MAC-PR; (Esselink et al., 2004)). For the SNP markers, KASPar technology is an efficient way to estimate allele dosage in citrus tetraploid plants (Aleza et al., 2012; Cuenca et al., 2013) (Figure 4c). All the obtained

tetraploid plants differed from the tetraploid 'Clemenules' clementine (Figure 5c) due to the distinct allelic configuration and the several allele doses (0/4; 3/1; 1/3 or 4/0) for the heterozygous SNPs markers in clementine (the 2/2 dose in the tetraploid) (Supplementary Table S9).

Allelic segregation distortion was tested under random tetrasomic segregation (Muller, 1914) for each marker by the Chi-squared test (0.05 probability threshold) with Bonferroni correction for multiple testing. Three SNPs showed significant segregation distortion: CiC5796-12 (*p*-value = 0.030); CiC1380-05 (*p*-value = 0.024) and CiC1749-05 (*p*-value = 0) (Table 4). From the histological, flow cytometry and marker results, we conclude that the self-pollination of tetraploid 'Clemenules' clementine surpassed the SI reaction and was able to produce tetraploid zygotic plants.

Table 4. Analysis of tetrasomic random allelic segregation of the tetraploid recovered plants from the self-pollinated flowers of the tetraploid 'Clemenules' clementine.

Marker	aaaa	aaaA	AAaa	AAAa	AAAA	Chi-square	P value
CiC2110-01	0	7	11	9	2	4.483	0.345
CiC5950-02	2	5	16	3	0	5.115	0.276
CiC6278-01	1	3	12	9	1	3.423	0.490
CiC3712-01	0	7	11	8	3	8.052	0.090
JK-TAA41	1	5	16	3	1	2.346	0.672
mCrCIR06B07	1	5	13	5	2	2.577	0.631
CiC5796-12	2	7	7	11	2	10.690	0.030
CiC1380-05	3	10	13	2	1	11.207	0.024
Ci07C07	1	4	17	5	1	1.804	0.772
CiC5164-03	0	5	10	9	1	3.520	0.475
CiC1749-05	8	7	12	2	0	68.603	0.000
CiC5087-01	0	2	18	8	1	5.138	0.273

Detailed information on the markers is provided in Supplementary Table S5. The data under the aaaa, aaaA, AAaa, AAAa and AAAA columns indicate the number of individuals with that allelic configuration.

4. Discussion

SI is a relevant trait in citrus because, when coupled with parthenocarpy, it enables seedless fruit production. However, SI can be an obstacle for genetic studies and plant breeding programs. Our results show that the breakdown of SI in citrus can be caused by temperature stress, bud pollination and polyploidization. All the plants obtained by means of temperature stress and bud pollination were diploids, whereas the self-pollination of the tetraploid 'Clemenules' clementine produced, as expected, tetraploid plants. Unfortunately, no 'Clemenules' clementine trees cultivated in containers were available to be cultivated inside growth chambers at 10 °C and 30 °C. As a result, the comparison in terms of effectiveness between temperature stress (Experiment 1) and polyploidization (Experiment 3) was not possible. However, according to our results, bud pollination (Experiment 2) appears to be a more effective way to break down SI than temperature stress in the 'Fortune' mandarin (Experiment 1) as well as more effective than polyploidization in the 'Clemenules' clementine (Experiment 3).

In Experiment 1, histological observations showed SI breakdown in the 'Fortune' mandarin when the progamic phase took place at a constant temperature of 10 °C.

However, very few pollen tubes reached the ovaries in 80% of the self-pollinated flowers, and no pollen tubes were observed reaching ovaries in 20% of the self-pollinated flowers, which could indicate that the SI reaction breakdown at 10 °C is unstable and partial. Distefano et al. (2018) suggested that low temperatures delay pistil maturation, and immature tissues allow pollen tube growth until the ovaries. This hypothesis agrees with the results obtained in our work. Recently, Aloisi et al. (2020) indicated that temperature contributed to a different activation of the SI reaction in *C. clementina*, which occurred at an optimal temperature of 25 °C and was by-passed at 15 °C. For the first time in citrus, we recovered zygotic plants that resulted from self-pollination at 10 °C of a self-incompatible genotype.

Although we observed very few pollen tubes reaching the basal pistil sections in 2 of the 10 self-pollinated flowers of 'Fortune' mandarin at high temperatures (30 °C), we did not recover fruit, and consequently, seed presence could not be assessed. In a previous research work about the influence of high temperatures on the SI reaction in citrus, Kawano et al. (2016) reported that pollen tubes reached the base of styles in self-pollinated flowers at 30 °C in a self-incompatible 'Hyuganatsu' Japanese variety. In contrast, Distefano et al. (2018, 2012) indicated that constant temperatures at 30 °C did not affect the SI reaction in clementines. These discrepant results may suggest that the SI reaction breakdown in citrus is genotype-dependent. Nevertheless, more research is needed to shed some light on molecular mechanisms. Yamamoto et al. (2019) indicated that alterations in the plasma membrane localization of *S-locus receptor kinase* genes are responsible for SI breakdown at high temperature in *Arabidopsis thaliana*. What is clear is that temperature plays a very important role in the SI reaction breakdown in citrus, which could have implications for gametophytic selection (reviewed by Hedhly et al. (2009)).

SI breakdown by bud self-pollination is probably related to the fact that SI machinery has not yet been synthesized (Cabin et al., 1996). This has been known in *Petunia* since 1934 (Yasuda, 1934). Based on histological observations, Distefano et al. (2009b) reported the SI breakdown by bud pollination in 'Fortune' mandarin, whereas Wakana et al. (2004) reported it in clementine based on seeded fruit production from bud self-pollinations. Our results consistently confirmed these previous studies because they are based on histological observations, seed production, and also on demonstrating the zygotic origin by selfing of the recovered plants with the SSR and SNP markers, which had not yet been demonstrated in citrus.

Despite the global agreement of our results with the above-mentioned research, we encountered some differences in the bud developmental stage when SI was surpassed. Distefano et al. (2009b) reported 11 pollen tubes reaching the style base in 100% of self-pollinated buds of 'Fortune' mandarin 1 day before anthesis. However, we observed very few pollen tubes (<5) reaching the ovaries in 60% of self-pollinated buds 1 to 2 days before anthesis (12.5 mm average length), and no pollen tubes were observed in the remaining 40%. With clementine, Wakana et al. (2004) obtained an average of 20.5 seeds in the fruit from the buds pollinated between 6 and 8 mm in length, which suggests that a half-sized flower bud is the optimum stage to produce self-fertilized seeds, and the SI reaction grew beyond this size. This suggestion is supported by our results, although we obtained seedy fruit from self-pollinated buds (9.4 mm average length), which were 60% of the size compared to those upon anthesis, and the number of seeds per fruit was almost the same as those produced from cross-pollination under field conditions.

Inducing self-compatibility in self-incompatible genotypes can result from either physiological or genetic changes (Claessen et al., 2019; de Nettancourt, 1997). Physiological changes may change over time and can induce different responses to the SI reaction in some genotypes. For example, in a previous work (Montalt et al., 2021), we classified 'Imperial' mandarin and 'Ellendale' tangor as non-strict self-incompatible genotypes because they produced both seedless and low-seeded fruit from self-pollinated flowers, which suggests an impact of environmental conditions (Sykes, 2008). Similarly, Claessen et al. (2019) indicated in pear and apple that the strength of the SI reaction differed depending on distinct intrinsic and extrinsic factors, including flower age and quality, temperature and application of plant hormones.

Chromosome doubling is one of the genetic changes that can result in self-compatibility for self-incompatible diploid genotypes (Claessen et al., 2019; de Nettancourt, 1997). This ploidy effect has been studied since the beginning of the last century. Diploid plants are usually associated with SI, whereas their derived tetraploid plants are usually self-compatible (Golz et al., 2000). By considering the gametophytic self-incompatibility and tetraploid plants recovered from self-incompatible diploid plants (S1S2) by either chemical treatments or spontaneous chromosome duplication, the genetic configuration for the S locus would be S1S1S2S2, thus producing diploid pollen with either two identical S alleles or two different S alleles. It has been proposed in the *Petunia* (Golz et al., 2000), *Pyrus* (Crane and Lewis, 1942) and *Malus* genera (Adachi et al., 2009) that pollen tube growth stops when pollen grain is homozygous for one S allele (S1S1 or S2S2), whereas heteroallelic (S1S2) pollen can grow through the pistil. For instance, the tetraploid progeny recovered from a self-pollinated autotetraploid plant is expected to be self-compatible and heterozygous at the S locus with the following genetic configuration: S1S1S1S2, S1S1S2S2 or S1S2S2S2.

In citrus, information about this phenomenon is scarce, as there is only one previous research work. Yamashita et al. (1990) reported SI reaction loss in the tetraploid 'Hyuganatsu' genotype (recovered by spontaneous limb mutation) and obtained 1.14 ± 1 well-developed seeds per fruit from selfing. In our work, we obtained the same results using a tetraploid plant of the 'Clemenules' clementine obtained by in vitro shoot-tip grafting combined with colchicine treatment (Aleza et al., 2009). Self-pollination of tetraploid flowers in anthesis allowed us to recover 29 tetraploid plants, and we demonstrated their zygotic origin with the SSR and SNP markers. This is the first report in citrus for which this phenomenon is formally demonstrated. According to Kim et al. (2020), clementines contain S3S11 SI alleles and, in line with the above-indicated hypothesis, only heteroallelic pollen grains can grow through the pistil to fertilize ovules producing heterozygous tetraploid hybrids. This hypothesis should be tested in larger tetraploid progenies obtained by selfing and by the analysis of molecular marker segregation, taking advantage of the SNP markers recently identified in the close vicinity of the citrus S locus located at the beginning of chromosome 7 of the clementine reference genome (Ollitrault et al., 2021). As part of our breeding program (Navarro et al., 2015), tetraploid plants of the self-incompatible genotypes 'Chandler' pummelo and 'Moncada' mandarin have been obtained using the same above-described colchicine treatment. The self-pollination of the tetraploid flowers of these genotypes at anthesis allowed us to recover seeds and tetraploid hybrids originated from selfing (data nor shown). This result may indicate that this phenomenon does not depend on the genotype and could be an intrinsic characteristic of citrus reproductive biology or even of the gametophytic self-incompatible system in doubled diploids of self-incompatible diploid genotypes.

Marker segregation distortion is a natural phenomenon (Zuo et al., 2019). In the genetic analysis of tetraploid plants obtained from self-pollination of the tetraploid ‘Clemenules’ clementine, we observed segregation distortion in three SNPs markers: CiC1380-05, CiC5796-12 and CiC1749-05. Ollitrault et al. (2012a) established the reference clementine genetic map and reported segregation distortion in CiC1380-05 SNP marker in the male and female clementine maps. However, no segregation distortion was observed for CiC5796-12 and CiC1749-05 SNP markers (Ollitrault et al., 2012a). Regarding these two markers, the segregation distortion observed in our analysis may be associated with the vicinity of genes involved in reproductive biology. CiC5796-12 (LG3, position 41,554,598) is located near the Ciclev10023991m.g gene (LG3; position 42,587,792 to 42,588,197), which is involved with the plant self-incompatibility protein S1. CiC1749-05 (LG8; position 24,429,013) is close to the Ciclev10030173m.g gene (LG8; position 24,433,990 to 24,437,012), which is associated with aberrant pollen development protein, according to the *C. clementina* v1.0 reference genome, available at Phytozome platform (“Phytozome 13.,” n.d.). In the genetic analysis of plants obtained from bud self-pollination of ‘Fortune’ mandarin, we observed segregation distortion in the NADK2-M285 SNP marker (LG5; position 37,772,763). This marker was not included in the reference clementine map. However, the segregation distortion observed in our study might be explained by the high segregation distortion in most parts of the LG5 reported by Ollitrault et al. (2012a).

5. Conclusions

We analyzed the influence of three potential approaches to induce the breakdown of the SI system in mandarins and clementines: temperature stress, bud pollination and chromosome doubling. The SI phenotype was characterized by a histological study of pollen tube growth and ovule fertilization. The ploidy of the plants obtained in the selfing experiments was characterized by flow cytometry, and their genotyping was performed with SNP and SSR markers. This molecular marker analysis allowed us to demonstrate that all the obtained plants were zygotic from selfing. The three methods were successful in recovering selfed plants, and bud pollination was the most efficient approach. Chromosome doubling was also efficient, but involved developing tetraploid plants, which is only interesting within the framework of triploid variety or in tetraploid rootstock breeding programs. Cold temperature stress allowed us to obtain a few diploid selfed plants. However, this method proved much more complex to apply than bud pollination in specific breeding programs. The recent new insight into the molecular determinants of SI in citrus and our current results would allow the efficiency of producing selfing progenies from self-incompatible genotypes. Furthermore, it would also enable their use for genetic studies and breeding programs.

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Supplementary information

Supplementary Table 1. Pollen tube (PT) growth observed in each self-pollinated flower of the 'Fortune' mandarin at anthesis in the constant temperature regimes of 10ºC and 30ºC evaluated in Experiment 1.

Flower	Temp. (ºC)	% Pistil length	Number of PT reaching the ovaries
1	10	100	<5 (3)
2	10	100	<5 (4)
3	10	100	<5 (1)
4	10	100	<5 (2)
5	10	70	0
6	10	70	0
7	10	100	<5 (2)
8	10	100	<5 (1)
9	10	100	<5 (3)
10	10	100	<5 (2)
1	30	30	0
2	30	30	0
3	30	30	0
4	30	20	0
5	30	20	0
6	30	20	0
7	30	40	0
8	30	20	0
9	30	60	0
10	30	70	0

Pollen tube growth is expressed as the percentage of pistil length reached by pollen tubes and the number of pollen tubes observed reaching ovaries, classified into intervals as explained in Section 2.4 of Material and Methods. The number of counted pollen tubes appears in brackets.

Supplementary Table 2. Pollen tube (PT) growth observed in each self-pollinated flower bud of the 'Fortune' mandarin at field conditions in the different flower bud lengths evaluated in Experiment 2.

Flower	Length (mm)	% Pistil length	Number of PT reaching the ovaries
1	15.8	20	0
2	15.4	20	0
3	14.7	30	0
4	13.4	20	0
5	13.3	10	0
6	13.3	30	0
7	13.1	20	0
8	12.1	100	<5 (3)
9	11.9	100	<5 (2)
10	11.9	50	0
11	11.3	100	>10
12	10.8	100	5-10 (8)
13	10.7	100	>10
14	10.5	100	>10
15	10.0	100	5-10 (6)
16	9.6	100	>10
17	9.5	100	>10
18	9.1	100	>10
19	8.7	100	>10
20	8.6	100	>10

Pollen tube growth is expressed as the percentage of pistil length reached by pollen tubes and the number of pollen tubes observed reaching ovaries, classified into intervals as explained in Section 2.4 of Material and Methods. The number of counted pollen tubes appears in brackets.

Supplementary Table 3. Pollen tube (PT) growth observed in each self-pollinated flower bud of the 'Clemenules' clementine at field conditions in the different flower bud lengths evaluated in Experiment 2.

Flower	Length (mm)	% Pistil length	Number of PT reaching the ovaries
1	16,0	20	0
2	15.3	10	0
3	15,0	20	0
4	14.1	10	0
5	13.1	40	0
6	12.6	20	0
7	12.4	20	0
8	12.3	100	<5 (1)
9	11.9	30	0
10	11.4	100	5-10 (7)
11	10.6	100	>10
12	10.5	100	<5 (4)
13	10.3	100	>10
14	9.8	100	>10
15	9.8	100	>10
16	8.9	100	>10
17	8.8	100	>10
18	8.7	100	>10
19	8.5	100	>10
20	8.2	100	>10

Pollen tube growth is expressed as the percentage of pistil length reached by pollen tubes and the number of pollen tubes observed reaching ovaries, classified into intervals as explained in Section 2.4 of Material and Methods. The number of counted pollen tubes appears in brackets.

Supplementary Table 4. Pollen tube (PT) growth observed in each of self-pollinated flower at anthesis of the tetraploid 'Clemenules' clementine cultivated under field conditions evaluated in Experiment 3.

Flower	% Pistil length	Number of PT reaching the ovaries
1	100	<5 (1)
2	100	<5 (3)
3	100	<5 (1)
4	100	<5 (2)
5	100	<5 (2)
6	100	<5 (1)
7	100	5-10 (5)
8	100	5-10 (8)

Pollen tube growth is expressed as the percentage of pistil length reached by pollen tubes and the number of pollen tubes observed reaching ovaries, classified into intervals as explained in Section 2.4 of Material and Methods. The number of counted pollen tubes appears in brackets.

Supplementary Table 5. Molecular markers used in the genetic analysis with their location in the C. clementine V1.0 reference genome, noted alleles and bibliographic reference.

Marker	Type	Chromosome	Physical location (kb)	Noted alleles[1] in Fortune	Noted alleles[1] in Clemenules	Bibliographic reference
SOS1-M50	SNP	2	26306845	GA		(Garcia-Lor et al., 2013a)
mCrCIR05A05	SSR	2	34232586	144 - 162		(Cuenca et al., 2011)
JK-TAA41	SSR	2	35861169	138 - 148		(Kijas et al., 1997)
PKF-M186	SNP	2	35686592	TC		(Garcia-Lor et al., 2013a)
mCrCIR06B07	SSR	2	35138894	106 - 108		(Froelicher et al., 2008)
MDH-MP69	SNP	3	7266623	AC		(Garcia-Lor et al., 2013a)
NADK2-M285	SNP	5	37772763	TC		(Garcia-Lor et al., 2013a)
LAPX-M238	SNP	6	11706007	CG		(Garcia-Lor et al., 2013a)
Ci07C07	SSR	7	15803823	242 - 258		(Froelicher et al., 2008)
FLS-M400	SNP	7	6013417	TC		(Garcia-Lor et al., 2013a)
HYB-M62	SNP	9	29490826	AC		(Garcia-Lor et al., 2013a)
CiC2110-01	SNP	1	3241022		AC	(P. Ollitrault et al., 2012b)
CiC5950-02	SNP	1	25122291		AG	(P. Ollitrault et al., 2012b)
CiC6278-01	SNP	2	20807016		AC	(P. Ollitrault et al., 2012b)
CiC3712-01	SNP	2	27936802		AC	(P. Ollitrault et al., 2012b)
JK-TAA41	SSR	2	35861169		148 - 154	(Kijas et al., 1997)
mCrCIR06B07	SSR	2	35138894		104 - 106	(Froelicher et al., 2008)
CiC5796-12	SNP	3	41554598		AC	(P. Ollitrault et al., 2012b)
CiC1380-05	SNP	5	10987419		TC	(P. Ollitrault et al., 2012b)
Ci07C07	SSR	7	15803823		242 - 280	(Froelicher et al., 2008)
CiC5164-02	SNP	8	4872921		TC	(P. Ollitrault et al., 2012b)
CiC1749-05	SNP	8	24429013		TG	(P. Ollitrault et al., 2012b)
CiC5087-01	SNP	9	1955754		AT	(P. Ollitrault et al., 2012b)

[1]Noted alleles. Numbers indicate the size of the alleles in nucleotides for the SSR markers. Letters correspond to the SNP markers alleles. For SNPs, the position is the exact one in the Clementine reference genome (Phytozome; Citrus Clementina V1.0). For SSRs, it is the position of the first base of the gene, where the SSRs was mined. For TAA41 it is the position of the last base of the reverse primer.

Supplementary Table 6.: Genotyping of 7 recovered plants obtained from self-pollination of `Fortune´ mandarin (F) under temperature stress (10ºC) with SSR and SNP markers.

	TAA41	Ci01C07	Ci06B07	Ci05A05	SOS1-M50	PKF-M186	NADK2-M285	LAPX-M238	MDH-MP69	FLS-M400	HYB-M62
F	138/148	242/258	106/108	144/162	GA	TC	TC	CG	AC	TC	AC
1	138/148	242/258	106/108	144/162	-	TC	TC	CG	AC	TC	AC
2	138/148	242/258	106/108	144/162	-	TC	CC	-	-	TC	AC
3	148/148	242/242	106/106	144/144	AA	CC	TC	GG	CC	TC	CC
4	148/148	242/242	106/106	144/144	AA	CC	CC	CG	AC	CC	AC
5	148/148	242/242	106/106	144/144	AA	CC	CC	CG	CC	CC	AC
6	138/148	242/258	106/108	144/162	GA	TC	TC	GG	AC	TC	AC
7	148/148	242/242	106/106	144/144	AA	CC	TC	CC	CC	TC	AA

Supplementary Table 7. Genotyping of 81 recovered plants obtained from self-pollination of `Fortune´ mandarin (F) by bud pollination with SSR and SNP markers.

	TAA41	Ci01C07	Ci06B07	Ci05A05	SOS1-M50	PKF-M186	NADK2-M285	LAPX-M238	MDH-MP69	FLS-M400	HYB-M62
F	138/148	242/258	106/108	144/162	GA	TC	TC	CG	AC	TC	AC
1	148/148	242/242	106/106	144/144	AA	CC	TT	CG	AC	TC	AA
2	138/148	242/258	106/108	144/162	GA	TC	TT	CG	AA	TC	AC
3	148/148	242/258	106/106	144/162	GA	TC	TC	GG	AC	TC	AA
4	148/148	242/242	106/106	144/144	AA	CC	TT	GG	AC	TC	AC
5	148/148	242/242	106/106	144/144	GA	CC	TT	CG	AC	TC	AA
6	138/148	242/258	106/108	144/162	AA	TC	TC	GG	CC	TT	CC
7	138/148	242/258	106/108	144/162	GA	TC	TC	CC	CC	TC	AC
8	138/148	242/258	106/108	144/162	GA	TC	TC	CG	AA	CC	AC
9	138/148	-	106/108	-	GG	TT	CC	-	-	TC	AC
10	138/148	242/258	106/108	144/162	AA	TC	TC	CG	AC	TC	AC
11	138/138	258/258	108/108	144/144	GA	TT	TC	CG	CC	TT	AC
12	148/148	242/258	106/108	144/162	GA	TC	TT	CG	AC	TT	AC
13	148/148	242/242	106/106	144/144	GA	CC	TC	GG	AA	TT	AC
14	138/138	258/258	108/108	144/144	GG	TT	TC	CG	AC	TC	AC
15	138/138	258/258	108/108	162/162	GG	TT	TT	CG	AC	TC	AC
16	-	242/242	-	162/162	AA	CC	CC	CC	-	TT	AC
17	-	242/258	-	144/162	GG	TC	TC	GG	AC	TC	CC
18	138/148	242/242	106/108	144/144	AA	TC	TT	CG	CC	TT	CC
19	138/148	242/258	106/108	144/162	AA	TC	TT	CG	AC	TT	AC
20	138/138	258/258	108/108	162/162	GA	TT	TT	CC	AA	TC	CC
21	138/148	242/258	106/108	144/162	GG	TC	TC	GG	AC	TC	CC
22	148/148	242/258	106/106	144/162	GG	CC	TT	GG	CC	TC	CC
23	138/148	242/242	106/108	162/162	GA	TC	TT	CC	AC	CC	CC
24	138/148	242/258	106/108	144/162	GG	TC	CC	CC	AC	TT	AC
25	148/148	242/242	106/106	144/144	AA	CC	TT	CG	CC	TT	AC
26	138/148	242/258	106/108	144/162	AA	TC	TT	CG	AC	TC	AA
27	138/138	258/258	108/108	162/162	AA	TT	TC	GG	AC	CC	AC
28	138/148	242/258	106/108	144/162	AA	-	-	GG	CC	TC	AC
29	138/138	242/258	108/108	144/162	AA	TT	TC	CC	AA	TC	AC
30	-	258/258	-	162/162	AA	TT	CC	CG	-	TC	AA

	TAA41	Ci01C07	Ci06B07	Ci05A05	SOS1-M50	PKF-M186	NADK2-M285	LAPX-M238	MDH-MP69	FLS-M400	HYB-M62
31	138/148	242/258	106/108	144/162	GA	TC	TC	GG	AC	TT	AA
32	138/138	258/258	108/108	162/162	GG	TT	TC	GG	CC	CC	AC
33	138/148	242/258	106/108	144/162	-	TC	TC	-	CC	TC	AC
34	138/148	242/258	106/108	144/162	GA	TC	TT	CG	CC	TC	AC
35	138/148	242/258	106/108	144/162	GG	TC	TC	CG	AC	CC	AC
36	148/148	242/242	106/106	144/144	AA	CC	TC	CC	AC	TC	AA
37	148/148	242/258	106/108	144/162	AA	CC	TC	CC	AA	TC	CC
38	148/148	242/242	106/106	144/144	GA	CC	TT	GG	AC	TC	AA
39	148/148	242/242	106/106	144/144	GA	CC	TT	GG	AA	TT	AC
40	138/148	242/258	106/108	144/162	AA	TC	TC	CG	AC	CC	AC
41	138/138	258/258	108/108	162/162	GA	TT	TT	CC	AC	CC	AC
42	138/138	258/258	108/108	162/162	GA	TT	CC	CG	AC	TC	AC
43	138/138	258/258	108/108	162/162	GA	TT	TC	GG	AC	TC	AC
44	138/148	242/258	106/108	144/162	GA	TC	CC	GG	AA	TC	AC
45	138/148	242/258	106/108	144/162	AA	TC	TC	CG	AA	CC	CC
46	138/148	242/242	106/106	144/144	GA	CC	TT	CC	AC	TC	AC
47	138/148	242/258	106/108	144/162	AA	TC	TC	CG	CC	TC	AC
48	138/138	258/258	106/106	162/162	AA	TT	CC	GG	AA	TT	AC
49	138/148	242/258	106/108	144/162	GA	TC	TT	CG	AC	TT	AC
50	138/138	258/258	108/108	162/162	GA	TT	CC	GG	CC	TT	AA
51	148/148	242/242	106/106	144/144	GG	CC	TC	CG	CC	CC	AC
52	148/148	242/258	106/108	144/162	GA	CC	TT	CC	AC	TC	AC
53	138/148	242/258	106/108	144/162	GG	TC	TT	CG	CC	TC	AA
54	148/148	242/242	108/108	144/144	AA	CC	TC	GG	AA	CC	CC
55	148/148	242/242	106/106	144/144	AA	CC	TC	CC	AC	TC	AC
56	138/148	242/258	106/108	144/162	AA	TC	TT	CG	AA	TC	-
57	138/148	242/258	106/108	144/162	GA	TT	TT	CG	AA	TC	AC
58	138/138	258/258	108/108	162/162	GA	TT	TT	CC	AC	TT	AC
59	138/148	242/258	106/108	144/162	GA	TC	TT	CC	CC	TT	CC
60	138/148	242/258	106/108	144/162	GA	TC	TC	GG	AA	TC	CC
61	138/148	242/258	106/108	144/162	GA	TC	TC	CC	CC	CC	CC
62	138/148	258/258	106/108	162/162	GG	TC	TT	GG	-	TC	CC
63	138/148	242/258	106/108	144/162	GA	TC	TC	CG	CC	TC	AC

	TAA41	Ci01C07	Ci06B07	Ci05A05	SOS1-M50	PKF-M186	NADK2-M285	LAPX-M238	MDH-MP69	FLS-M400	HYB-M62
64	138/138	242/258	106/108	144/162	GA	TT	TC	GG	AC	TC	AC
65	138/148	242/258	106/108	144/162	AA	CC	TT	GG	AA	TC	AC
66	148/148	242/242	106/106	144/144	AA	CC	TC	CG	CC	CC	AC
67	138/138	242/242	106/106	144/144	GG	CC	TC	GG	CC	TC	AC
68	-	242/258	-	144/162	GA	TC	TC	GG	-	TT	AC
69	138/148	242/258	106/108	144/162	GA	TC	TC	CC	CC	TC	AC
70	148/148	242/258	106/106	144/162	GG	CC	TC	CG	CC	TC	AC
71	138/138	258/258	108/108	162/162	GG	TT	TC	CG	AA	CC	AC
72	138/148	242/258	106/108	144/162	GG	TC	TC	CG	CC	TC	CC
73	138/148	242/258	106/108	144/162	GA	TC	TC	GG	AA	TC	AC
74	138/148	242/258	106/108	144/162	GG	TT	TT	CG	CC	TC	AC
75	138/148	242/258	106/108	144/162	GA	TT	TC	GG	AC	TC	CC
76	138/148	242/258	108/108	144/162	AA	TC	TT	CG	CC	TC	CC
77	138/148	242/258	108/108	144/162	GA	TT	TC	CG	AC	CC	AC
78	138/148	242/258	106/108	144/162	GA	CC	TC	CG	AC	TC	AC
79	138/148	242/258	108/108	162/162	GA	TT	TT	CG	AA	TC	AC
80	138/138	242/258	106/108	144/162	GG	TC	CC	CC	AC	TT	AC
81	138/148	242/258	106/108	144/162	AA	TC	TT	CG	-	-	AC

Supplementary Table 8. Genotyping of 61 recovered plants obtained from self-pollination of `Clemenules´ clementine (Cl) by bud pollination with SSR and SNP markers.

	TAA41	Ci06B07	Ci07C07	CiC1380-05	CiC5087-01	CiC6278-01	CiC2110-01	CiC5950-02	CiC3712-01	CiC5164-03	CiC5796-12	CiC1749-05
Cl	148/154	104/106	242/280	TC	AT	AC	AC	AG	AC	TC	AC	TG
1	148/154	104/106	242/280	TT	TT	AA	AA	AG	AA	CC	AC	TG
2	148/154	104/106	242/280	TT	AT	AC	AA	GG	AC	TC	AA	GG
3	154/154	104/104	280/280	TT	AA	CC	AC	GG	AA	TT	AC	TG
4	148/154	104/106	242/280	TC	TT	CC	CC	AG	AC	TC	AC	TG
5	148/148	106/106	242/242	TC	AT	CC	CC	AG	CC	TC	AC	GG
6	148/154	104/106	242/280	TT	AT	AA	CC	AG	AC	TT	CC	TG
7	148/154	104/106	242/280	TT	AT	AC	AA	GG	CC	TT	AC	TG
8	154/154	104/104	280/280	TC	AA	AC	AC	AG	AC	CC	AC	TG
9	148/148	104/106	242/280	CC	AA	CC	AA	GG	CC	TC	CC	TG
10	148/154	104/106	242/280	TC	AT	CC	CC	AG	CC	TC	AC	TG
11	148/154	104/106	242/280	CC	AA	AC	AC	GG	AC	CC	AC	GG
12	148/148	106/106	242/242	TC	AT	AC	AA	GG	CC	TC	AA	TG
13	148/154	104/106	242/280	TT	AT	AC	CC	GG	AC	TT	AA	TG
14	148/154	104/106	242/280	CC	TT	CC	AC	AG	AC	CC	AA	TT
15	148/154	104/106	242/280	TC	AA	CC	CC	AG	AC	CC	AC	TT
16	154/154	104/104	280/280	TC	AT	-	AC	AG	AC	-	AC	TG
17	154/154	104/104	280/280	TT	TT	AC	CC	AA	AC	TC	AC	TG
18	148/148	106/106	280/280	CC	AT	AA	AC	AG	AA	TC	AA	TG
19	148/154	104/106	242/280	TC	AT	AC	AA	AG	AC	CC	AC	TG
20	148/148	106/106	242/242	TT	AT	AC	AC	AA	AC	TC	AC	GG
21	148/148	106/106	242/242	TC	AT	AC	CC	AA	CC	TC	CC	GG
22	154/154	104/106	242/242	TT	TT	CC	AC	GG	CC	TC	AC	TG
23	154/154	104/104	280/280	TC	AT	AC	AC	GG	AA	TT	AC	TT
24	148/154	104/106	242/280	TT	AT	AA	AA	GG	AC	TT	AC	TG
25	148/154	104/106	242/280	TC	AA	AC	AA	AG	AA	TC	AC	TT
26	148/148	106/106	242/242	CC	TT	AC	AA	GG	AC	CC	AC	TG
27	154/154	104/104	280/280	TC	AT	AC	AC	AA	AA	TT	AA	GG
28	148/154	104/106	242/280	CC	AA	AA	CC	AG	AA	TT	CC	TG
29	148/148	106/106	242/242	TC	TT	CC	AC	AG	AC	TC	AA	TG
30	148/148	104/106	242/280	CC	AT	AC	AC	AG	CC	TC	AC	GG

	TAA41	Ci06B07	Ci07C07	CiC1380-05	CiC5087-01	CiC6278-01	CiC2110-01	CiC5950-02	CiC3712-01	CiC5164-03	CiC5796-12	CiC1749-05
31	154/154	104/104	280/280	CC	AT	CC	AA	AG	AC	CC	AC	TG
32	148/148	106/106	242/242	TT	TT	AC	AC	GG	CC	TC	AA	TT
33	148/148	106/106	242/242	CC	TT	AC	AC	AG	CC	TT	AC	GG
34	154/154	104/104	280/280	TC	AT	AC	AC	AG	AC	CC	AA	TT
35	148/154	104/106	242/242	TC	AA	AC	AC	GG	AC	TC	AA	TG
36	148/148	106/106	242/242	TT	AT	AC	CC	AG	CC	CC	CC	TG
37	148/154	104/106	242/280	TC	TT	CC	AC	AA	CC	TT	CC	TT
38	148/148	104/106	242/242	TC	AT	AC	AC	AG	CC	TC	AC	TT
39	148/148	104/104	242/242	CC	AT	CC	AC	AA	CC	CC	CC	TG
40	148/154	104/106	242/280	TC	TT	AA	AC	AG	AC	TC	AC	GG
41	154/154	104/104	280/280	TT	AA	AC	AA	GG	AA	TC	AC	TG
42	148/148	106/106	242/242	TC	AA	AA	CC	GG	AA	TT	AA	TT
43	148/154	104/106	242/280	TT	TT	CC	CC	AG	CC	TC	AA	TG
44	154/154	104/104	280/280	TC	TT	AA	AC	GG	AA	TC	AC	TG
45	148/154	104/106	242/280	CC	AA	CC	AA	AG	CC	TT	AA	TT
46	154/154	104/104	280/280	TC	TT	AC	AC	GG	AC	TC	CC	TG
47	154/154	104/104	280/280	TC	TT	AA	CC	AA	AA	TC	AC	TG
48	148/148	106/106	242/242	TC	AA	AC	CC	GG	CC	TC	AC	TT
49	148/148	106/106	242/242	TT	TT	CC	AA	AG	CC	TT	AC	TG
50	148/154	104/106	242/280	TC	AT	AC	AC	GG	AC	TT	AC	GG
51	148/154	104/106	242/280	TC	AT	AC	AC	AA	AA	CC	AC	GG
52	148/154	104/106	242/280	TC	AT	AC	AA	GG	AC	TC	AC	TG
53	148/154	104/106	242/280	TT	AT	CC	AA	AA	CC	CC	AA	TG
54	148/154	104/106	242/280	TC	AT	AC	AC	AG	AC	TT	AC	TT
55	148/148	106/106	242/242	TC	AA	-	AC	AA	AC	TC	CC	TT
56	148/154	104/106	242/280	TC	AT	AC	AC	AG	CC	TC	AC	TG
57	154/154	104/104	280/280	TT	TT	AC	CC	AA	AC	TC	AC	TG
58	148/148	106/106	242/242	TC	AT	AC	AC	AG	AC	TT	AA	GG
59	148/148	106/106	242/242	TT	AT	AA	AC	AG	AA	CC	CC	TG
60	148/148	106/106	242/242	TC	TT	CC	AC	AG	AC	TT	AA	TT
61	148/148	106/106	242/242	TC	AA	AC	CC	GG	CC	TC	CC	TT

Supplementary Table 9. Genotyping of 29 tetraploid recovered plants obtained from self-pollination of tetraploid `Clemenules´ clementine (Cl4x) with SSR and SNP markers.

	TAA41	Ci06B07	Ci07C07	CiC1380-05	CiC5087-01	CiC6278-01	CiC2110-01	CiC5950-02	CiC3712-01	CiC5164-03	CiC5796-12	CiC1749-05
Cl4x	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	AATT	AACC	AACC	AAGG	AACC	TTCC	AACC	TTGG
1	148/148/148/148	106/106/106/106	242/242/242/280	TTCC	AATT	AAAC	ACCC	AAGG	CCCC	TTCC	CCCC	TTTG
2	148/154/154/154	104/104/104/106	242/280/280/280	TTCC	AATT	AACC	AAAC	AGGG	AAAC	TTTC	ACCC	TTTT
3	148/154/154/154	104/104/104/106	242/280/280/280	TTCC	AATT	ACCC	AAAC	AGGG	AAAC	TTTC	ACCC	TTTT
4	148/148/154/154	104/104/106/106	242/242/280/280	TTTT	TTTT	-	AACC	AAGG	AACC	TTCC	AACC	TTTT
5	148/148/154/154	104/106/106/106	242/242/280/280	TTTC	AATT	AACC	AACC	-	AACC	-	AACC	TTGG
6	148/148/148/154	104/106/106/106	242/242/280/280	TTTC	AATT	AACC	AAAC	AAGG	AACC	TCCC	ACCC	TTGG
7	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	ATTT	ACCC	ACCC	AAGG	ACCC	TCCC	AAAC	TTGG
8	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	ATTT	ACCC	ACCC	AAGG	ACCC	TCCC	AAAC	TTGG
9	148/148/154/154	104/104/104/106	280/280/280/280	TTCC	AATT	AAAC	AACC	AAGG	AAAC	CCCC	ACCC	TTTG
10	148/148/148/154	104/106/106/106	242/242/242/280	TTCC	ATTT	ACCC	ACCC	AAGG	ACCC	TTTC	AAAC	TTTG
11	148/148/154/154	104/104/106/106	242/242/242/280	CCCC	AATT	AACC	AACC	AAGG	ACCC	TCCC	ACCC	TTGG
12	148/148/154/154	104/104/106/106	242/242/280/280	TCCC	AATT	ACCC	ACCC	AAAA	ACCC	TTCC	AAAC	TTGG
13	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	ATTT	AACC	AACC	AAGG	AACC	TTCC	ACCC	TTTT
14	148/148/154/154	104/104/104/106	242/280/280/280	TTTC	AATT	ACCC	AACC	AAAG	AACC	TCCC	AAAC	TTTT
15	148/148/148/148	104/106/106/106	242/242/242/280	TTTC	AAAT	AACC	ACCC	AAGG	ACCC	TTTC	AAAC	TTGG
16	148/148/154/154	104/104/106/106	242/242/280/280	TTTC	AATT	AACC	ACCC	AAAG	AACC	TTCC	ACCC	TGGG
17	154/154/154/154	104/104/104/104	242/280/280/280	TTTC	AATT	AAAA	AAAC	AGGG	AACC	TTCC	CCCC	TTTG
18	148/148/154/154	104/104/106/106	242/242/280/280	TTTC	AATT	AACC	ACCC	AAAG	AACC	TTCC	ACCC	TGGG
19	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	AATT	ACCC	AACC	AAAA	AACC	TCCC	ACCC	TTGG
20	148/148/148/154	104/106/106/106	242/242/280/280	TTCC	AATT	-	CCCC	-	ACCC	-	ACCC	TTGG
21	148/148/154/154	104/104/106/106	242/242/280/280	TCCC	ATTT	ACCC	AACC	AAGG	AAAC	TCCC	AACC	TTGG
22	148/148/154/154	106/106/106/106	242/242/242/242	TTTT	AAAT	ACCC	ACCC	AAGG	CCCC	TTTC	AACC	TTTT
23	148/148/154/154	104/104/106/106	242/242/280/280	TTTC	AATT	CCCC	AACC	AAGG	CCCC	TCCC	AACC	TTTT
24	148/148/154/154	104/104/106/106	242/242/280/280	TTCC	AATT	AAAC	AAAC	AAGG	AACC	TTCC	AAAA	TTGG
25	148/148/148/154	104/104/106/106	242/242/280/280	TTTC	ATTT	AACC	AAAC	AAAG	AAAC	TTCC	AAAA	TTTG
26	148/154/154/154	104/104/104/106	242/280/280/280	TTTC	AATT	-	AACC	AAGG	AACC	-	AAAC	TTTG
27	148/148/154/154	104/104/106/106	242/242/242/280	TTCC	ATTT	AACC	CCCC	AAAG	ACCC	TCCC	AACC	TTGG
28	148/148/154/154	104/104/106/106	242/242/280/280	TTTT	AATT	AACC	AAAC	AAGG	AAAC	TTCC	ACCC	TTTT
29	148/148/148/154	104/106/106/106	242/242/280/280	TTCC	ATTT	AACC	AACC	-	AAAC	-	AACC	TTTG

Chapter 4. Genotyping by sequencing for SNP-based linkage analysis and the development of KASPar markers for male sterility and polyembryony in citrus

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Abstract

Polyembryony and male sterility (MS) are essential characters for citrus breeding. MS, coupled with parthenocarpy, allows for addressing the diversification of diploid seedless mandarin varieties, and nucleocytoplasmic MS is the most prevalent system. Polyembryony limits the use of seed parents in scion breeding programs, and the recovery of monoembryonic hybrids to be used as female parents is a crucial pre-breeding component. The objectives of this work were the identification of SNPs closely linked with the genes implied in these traits for marker-assisted selection. Genotyping by sequencing was used to genotype 61 diploid hybrids from an F1 progeny recovered from crossing 'Kiyomi' and 'Murcott' tangors. A total of 6444 segregating markers were identified and used to establish the two parental genetic maps. They consisted of 1374 and 697 markers encompassing 1416.287 and 1339.735 cM for 'Kiyomi' and 'Murcott', respectively. Phenotyping for MS and polyembryony was performed. The genotype–trait association study identified a genomic region on LG8 which was significantly associated with MS, and a genomic region on LG1 which was significantly associated with polyembryony. Annotation of the identified region for MS revealed 19 candidate genes. One SNP KASPar marker was developed and fully validated for each trait.

Keywords: mandarin; pollen grain; apomixis; association study; marker-assisted selection

1. Introduction

Among the different traits that characterize the complexity of reproductive biology in plants, male sterility and polyembryony are found within the *Citrus* genera and represent important features of citrus breeding programs.

Male sterility is an important trait because, when coupled with parthenocarpy (Montalt et al., 2021), it allows to partially address the diversification of seedless mandarin varieties at the diploid level. Male sterility has been reported in *Citrus aurantifolia* (Nakamura, 1943), *C. limon* hybrids (Frost and Soost, 1968), *C. medica* (Oppenheim and Frankel, 1929), *C. sinensis* (Nakamura, 1943; Osawa, 1912), *C. yatsushiro* (Nakamura, 1943), *C. unshiu* (Osawa, 1912), and its hybrids (Iwamasa, 1966). Several levels and mechanisms of male sterility have been identified in citrus. Chromosomal aberrations, such as asynapsis, reciprocal translocation, and failure of spindle formation, are important phenomena causing pollen sterility. For example, reciprocal translocation is found to cause pollen sterility in the 'Valencia' sweet orange (*C. sinensis*) (Iwamasa, 1966), inversion is the cause of partial pollen sterility in the 'Mexican' lime (*C. aurantifolia*) (Iwamasa, 1966), and asynapsis with a genetic determinant has been identified in the 'Mukaku Yuzu' (*C. junos*), while this is induced by low temperature in the 'Eureka' lemon (*C. limon*) and the 'Mexican' lime (Iwamasa, 1966; Ollitrault et al., 2007). Besides chromosomal aberration, nucleocytoplasmic male sterility (CMS) is the most prevalent system in citrus, and it has been proposed that satsuma (*C. unshiu*) and progenies derived from satsuma (as female parent) display CMS caused by the cooperative action of both cytoplasmic and nuclear genes. Several studies have been performed to decipher the genetic control of male sterility derived from satsuma. Yamamoto et al. (1997) demonstrate the interaction between nuclear and cytoplasmic genes by reciprocal

hybridizations. Subsequent research has pointed to the involvement of nuclear genes in male sterility (Chae et al., 2011; Dewi et al., 2013b, 2013a; Goto et al., 2018, 2016; Nakano et al., 2001; Zheng et al., 2012). DNA marker analysis for nuclear and cytoplasmic genomes and genome-wide SNP marker analysis showed that CMS in the satsuma was derived from its seed parent, the 'Kishu' mandarin (*C. kinokuni*), and that the nuclear genes come from the male parent, 'Kunenbo' mandarin (*C. nobilis*) (Goto et al., 2018; Shimizu et al., 2017, 2016). Goto et al. (2016) posited that male sterility was associated with failed pollen grain development and scant viability. These authors compute the index of male sterility in a population derived from satsuma using two parameters: (i) the number of pollen grains per anther (NPGA), and (ii) the apparent pollen fertility (APF). Both parameters are inherited by their progeny, suggesting the involvement of a nuclear factor. Recently, two QTLs related to male sterility have been reported: MS-P1, which is a major QTL for reducing the number of pollen grains per anther; and MS-F1, related to lower apparent pollen fertility (Goto et al., 2018). However, the resolution of the genetic map was too low to develop efficient markers for early selection. For instance, one objective of this work is the development of new markers associated with male sterility trait for seedless breeding.

Apomixis (asexual embryo formation) has been observed in more than 400 plant species (Zhang et al., 2018); however, apomixis is not particularly common in agriculturally important woody crops, with the exception of apple, mango, and citrus (Dwivedi et al., 2010; Koltunow, 1993). In citrus, apomixis is sporophytic (also referred to as adventitious embryony from nucellar cells) (Frost and Soost, 1968; Koltunow, 1993), and it is present in most genotypes, with the exception of citron, pummelo, clementines, and some mandarin hybrids. The seeds of non-apomictic genotypes, also called monoembryonic genotypes, contain only one sexual embryo, whereas in apomictic genotypes (polyembryonic), there is one sexual embryo and multiple nucellar embryos genetically identical to the mother plant. In the seeds of polyembryonic citrus genotypes, the formation of the nucellar embryos can be initiated before fertilization (Wakana and Uemoto, 1987), and competition between the zygotic and nucellar embryos generally results in the failure of the development of the zygotic embryo (Frost and Soost, 1968; Koltunow, 1993). This characteristic is a strong limitation for using polyembryonic genotypes as female parents in sexual hybridizations, since it hampers the recovery of large hybrid populations. In programs aiming to introgress specific traits over several cycles of hybridization, the recovery and selection of monoembryonic hybrids to be used as female parents for further breeding is crucial due to the low number of parents available. Considering the very long juvenile phase in citrus, the development of molecular markers for marker-assisted selection (MAS) appears particularly important for this trait. At the opposite end of the scale, polyembryony is very advantageous for rootstock production, since plants obtained from polyembryonic seeds are identical to the mother plant. That is why rootstock breeding programs look for polyembryonic hybrids to ensure clonal propagation by seedlings of the newly selected rootstock. Therefore, the development of markers associated with monoembryony and polyembryony will be very useful for MAS in both varieties and rootstock breeding programs.

On the basis of genomic analyses of primitive, wild, and cultivated citrus, Wang et al. (2017) highlighted the emergence of apomixis during citrus domestication. These authors narrowed down the genetic locus responsible for citrus polyembryony to an 80 kb region located on chromosome 4 of the Chinese pummelo genome assembly (MKYQ00000000.1), containing 11 candidate genes. Among these genes, a candidate

gene, *CitRWP,* was identified for the single dominant allele responsible for polyembryony, and a miniature inverted-repeat TE (MITE) insertion in the promoter region of the *CitRWP* gene cosegregated with the polyembryonic phenotype (Wang et al., 2017). Later, Shimada et al. (2018) reported the candidate gene, *CitRKD1*, at the polyembryonic locus, which plays a principal role in regulating somatic embryogenesis. These authors suggested that a MITE insertion in the upstream region might be involved in regulating the *CitRKD1* transcription. For instance, the *CitRKD1* gene comprises two alleles, polyembryonic allele with a MITE insertion, and monoembryonic without a MITE insertion. Recently, Catalano et al. (2022) confirmed the allelic configuration for *CitRKD1* in different lemon genotypes using MITE primers. Alongside that, based on this MITE insertion, our research group developed an InDel marker, which has been evaluated in segregation progenies and germplasm genetic diversity, obtaining good results with the genotypes analyzed. However, InDel analysis is time-consuming (PCR products must be resolved by electrophoresis in long-agarose gel), as well as expensive for studying a large number of progenies. Therefore, it is important to develop alternatives, such as SNP (Single nucleotide polymorphism) markers based on KASPar technology, to simplify and make the analysis faster and cheaper.

SNP genotyping by the KBiosciences Competitive Allele Specific PCR SNP genotyping (KASPar) technology is simple and cost-effective for genotyping a limited number of markers in large populations, as compared with other SNP genotyping assays. Highly efficient protocols have been adapted to work with citrus by Cuenca et al. (2013) and Garcia-Lor et al. (2013). It therefore appears to be a very well-adapted methodology for MAS. The efficiency of MAS is directly linked to the vicinity of the used markers with the genes or factors directly implied in the expression of the targeted trait. The ability to identify candidate genes associated with useful traits has progressed significantly with the development of next-generation sequencing (NGS) technologies, thereby facilitating the massive identification of SNP markers in large populations, as well as working on reduced genome representations. Examples include restriction-site-associated DNA sequencing (RADseq) (Davey et al., 2011; Davey and Blaxter, 2010), diversity array technology sequencing (DArTseq) (Sansaloni et al., 2011), and genotyping by sequencing (GBS) (Elshire et al., 2011; Glaubitz et al., 2014). In citrus GBS, RAD sequencing and DARTSeq have been successfully developed and used to study germplasm diversity and decipher related phylogenomic structures (Ahmed et al., 2019; Oueslati et al., 2017; Penjor et al., 2014), high density genome mapping (Curtolo et al., 2017b; Guo et al., 2015; Huang et al., 2018; Ollitrault et al., 2021), as well as QTL analyses (Curtolo et al., 2017a) and genome-wide association studies (GWAS) (Imai et al., 2018).

The aims of this study were: (i) the identification of SNPs closely linked with male sterility and polyembryony. This was carried out by combining GBS data and analyzing the number of pollen grains per anther (NPG), apparent pollen fertility (APF), and polyembryony in a segregant progeny recovered from a cross between the male sterile and monoembryonic ‘Kiyomi’ tangor (*C. unshiu* × *C. sinensis*) as the female parent, and the male fertile and polyembryonic ‘Murcott’ tangor (*C. sinensis* × unknown mandarin) as the male parent); (ii) the identification of candidate genes associated with male sterility; and (iii) the development and assessment of SNP markers based on KASPar technology for MAS in citrus breeding programs for these two important traits.

2. Material and Methods

2.1. Plant Material

A diploid hybrid population, derived from the cross between the diploids 'Kiyomi' tangor (IVIA-405) as the female parent and 'Murcott' tangor (IVIA-196) as the male parent, was used. The 'Kiyomi' tangor is a male sterile and monoembryonic hybrid between the 'Miyagawa-wase' satsuma and the 'Trovita' sweet orange (Nishiura et al., 1983), while the 'Murcott' tangor is a male fertile and polyembryonic genotype presumed F1 hybrid of sweet orange and an unknown mandarin. Both parents, belong to the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (IVIA), located in Moncada, Valencia (Spain). Sixty-two hybrids were recovered and all of them were analyzed by flow cytometry, according to Aleza et al. (2010). Sixty-one were diploids and one triploid hybrid was obtained from female unreduced gamete, which was not included in the genetic analysis. The progeny was grafted in June 2011 onto C. macrophylla Wester rootstock at the IVIA experimental orchard for genetic analysis and further studies related with fruit quality.

2.2. Plant Genotyping

A total of 61 diploid hybrids from the 'Kiyomi' × 'Murcott' cross and the two parents were subjected to genotyping by sequencing (GBS), as described by Ollitrault et al. (2021). Genomic DNA was isolated using the Plant DNAeasy® kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration of genomic DNA was adjusted to 20 ng/µL, and the ApekI GBS libraries were prepared following the protocol described by Elshire et al. (2011). The DNA of each sample (200 ng) was digested with the ApekI enzyme (New England Biolabs, Hitchin, UK). Digestion took place at 75 ºC for 2 h, and then at 65 ºC for 20 min to inactivate the enzyme. The ligation reaction was completed in the same plate as the digestion, again using T4 DNA ligase enzyme (New England Biolabs, Hitchin, UK) at 22 ºC for 1 h; the ligase was inactivated prior to pooling the samples by holding it at 65 ºC for 20 min. For each library, ligated samples were pooled (i.e., 2 multiplex libraries of 96 samples) and PCR-amplified in a single tube. Complexity was further reduced using PCR primers with one selective base (A), as described by Sonah et al. (2013). Single-end sequencing was performed on a single lane of an Illumina HiSeq4000. Keygene N.V. (Keygene, Wageningen, The Netherlands) owns the patents and patent applications protecting its sequence-based genotyping technologies. SNP genotype calling was performed using data from the DNA sequence reads with the TASSEL 5 GBS v2 pipeline (Glaubitz et al., 2014) (available at https://bitbucket.org/tasseladmin/tassel-5-source/wiki/Tassel5GBSv2Pipeline, accessed on 7 June 2022) with default parameters, to identify good quality, unique, sequence reads with barcodes. These sequences were aligned on the *C. clementina* 1.0 reference genome (available at https://phytozome.jgi.doe.gov, accessed 07 June 2022) using Bowtie v2/2.3.2. For genotype calling, positions with less than five reads were considered as missing data. Next, polymorphic positions were filtered for diallelic SNPs and minor allele frequencies (MAF) over 0.05.

2.3. Linkage Analysis and Genetic Mapping

The two-way pseudo-testcross mapping strategy implemented for genetic mapping from progenies, resulting from crosses between two heterozygous parents (Ritter et al., 1990) and used in previous high-density mapping studies in citrus (Curtolo et al., 2017a; Guo et al., 2015; Huang et al., 2018; Ollitrault et al., 2021, 2012), was applied to establish the 'Kiyomi' and 'Murcott' genetic maps. For each map, SNP markers were selected according to their respective heterozygosity for the mapped parent and homozygosity for the other one. Each set of data for the 61 hybrids was filtered to retain markers and hybrids with less than 15% of missing data. Linkage analysis and genetic mapping were then performed using JoinMap5 (https://www.kyazma.nl/index.php/JoinMap/; accessed 7 June 2022). Linkage mapping was performed in the «Hap» option for both 'Kiyomi' and 'Murcott' tangors. Markers were grouped using the independence LOD score. The phases (coupling and repulsion) of the linked marker loci were automatically detected by the software. Map distances were estimated in cM, using the regression mapping algorithm. After a first mapping round, singletons, i.e., an individual genotype that suggested recombination with its two flanking markers, were identified and replaced by missing data, as recommended by van Os et al. (2005) for high density genetic maps. At the same time, a number of individuals displaying an aberrant number of recombination, set by examining the global recombination distribution, were removed, as we considered their genotype calling quality to be insufficient. The synteny and collinearity of both the 'Kiyomi' and 'Murcott' genetic maps, with the reference clementine genome, were visualized using Circos v0.69-9 (Krzywinski et al., 2009); (http://circos.ca; accessed on 07 June 2022) in Galaxy (Rasche and Hiltemann, 2020). Marey maps were drawn using Microsoft® Excel® 365 MSO (16.0.15601.20526) to visualize changes in the recombination rate along the genome.

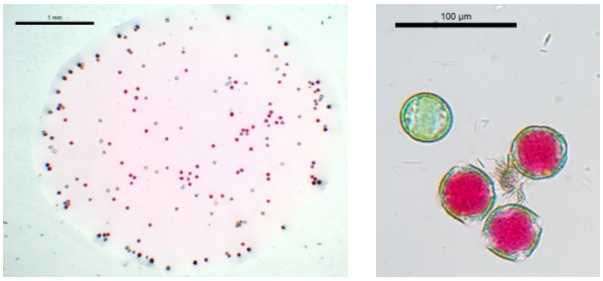
2.4. Histological Observations

The male sterility phenotyping was based on the number of pollen grains per anther (NPGA) and the apparent pollen fertility (APF) of hybrids which flowered on each blossom. For this, three flowers per genotype were collected on the day of anthesis. A total of 10 anthers per flower were removed with forceps and placed into 3 different 1.5 mL Eppendorf tubes, with 3 tubes per genotype. Following this, opened Eppendorf tubes were left in a desiccator with silica gel at room temperature, until dehiscence. Dehiscent anthers were confirmed under stereomicroscope, and samples were sorted into three levels, according to pollen grain quantity visually observed: high (Figure 1a–c), moderate-to-low (Figure 1d–f), and very low-to-null (Figure 1g–i). Phenotyping was performed during three flowering periods belonging to the following years: 2019, 2020, and 2021.

Figure 1. Different types of flower phenotypes observed in the segregation progeny recovered from the cross between 'Kiyomi' and 'Murcott'. **(a-c)** High number of pollen grains; **(d-f)** Moderate to low; **(g-i)** Very low to null pollen grain quantity. This quantification was observed in flowers at anthesis **(a, d, g)**, fresh anthers **(b, e, h)**, and dehiscent anthers **(c, f, i)**.

Samples were stored at -20ºC until quantification. For pollen grain suspension a staining solution (Peterson et al., 2010), based on Alexander staining (Alexander, 1969), was added into the 1.5 mL Eppendorf tube containing the dehiscent anthers. The volume of staining added depended on scored visual observations of dehiscent anthers: 25 µL was added to those samples scored as having null-to-very low quantity of pollen grains, 50 µL to low and moderate samples, and 100 µL to samples scored as moderate-to-high. Eppendorf tubes containing the dehiscent anthers with the staining solution were placed at 70 °C for 30 min. Next, a spin of one hour at 10,000 rpm was performed to separate pollen grains from the theca.

The pollen grain dispersion was shaken with a vortex and, immediately, 15 drops of 0.3 µL were placed onto a slide. Drops were photographed (Figure 2) with a Leica DMLS microscope, and the number of pollen grains per drop was counted with the ImageJ 2.0.0-rc-61/1.52n software (Schindelin et al., 2015).



(**a**) (**b**)

Figure 2. (a) Example of a 0.3 µL drop with pollen grains dispersed in the staining solution. **(b)** Detail of viable pollen grains stained magenta-red, and non-viable pollen grains stained blue-green.

The number of pollen grains per anther (NPGA) was calculated as follows:

NPGA = (sum of NPG/ (15×0.3)) × (Vol/10). Where the sum of NPG is the total number of pollen grains counted in the 15 drops of 0.3 µL; 10 is the number of anthers; and Vol is the volume (25, 50 o 100 µL) of staining solution added for pollen grain dispersion.

Stain solution colored non-viable pollen grains as blue-green, and viable pollen grains as magenta-red. Since staining solution was used as a liquid medium to disperse the pollen grains, APF and NPGA values were evaluated simultaneously (Figure 2). To determine if there were significant differences between NPGA and APF variables of phenotyped hybrids, a one-way ANOVA test was performed using the Statgraphics Centurion XVI statistical software package, v16.1.03. A *p*-value of less than 0.05 was classified as statistically significant between the averages of NPGA and APF of phenotyped hybrids.

2.5. Seeds phenotyping for polyembryony

Fruits from the hybrids between 'Kiyomi' and 'Murcott' tangors were harvested when ripe, and seeds were then extracted. Each seed was peeled, eliminating the outer and inner seed coats with forceps. Seeds with only one embryo were classified as monoembryonic, whereas seeds with more than one embryo were recorded as polyembryonic.

2.6. Genotype-phenotype association

TASSEL v5.2.87 (Bradbury et al., 2007) was used to perform the genotype–phenotype association study. Genotype and phenotype data sets were joined by the union of taxa using the Union Join command. Then, the genotype–phenotype association was evaluated using the GLM (general linear model) procedure under the default settings, and the results were displayed using the Manhattan plot graph.

2.7. SNP genotyping

SNP markers were genotyped using KASPar™ technology by KBioscience® (https://www.biosearchtech.com/ (accessed on 7 June 2022). KASPar™ technology uses allele-specific amplification, followed by fluorescence detection. Sample DNA is amplified with a thermal cycler using allele-specific primers based on the SNP locus-

flanking sequence (approx. 50 nucleotides on each side of the SNP). The KASPar™ system uses two Förster resonance energy transfer (FRET) cassettes, where fluorometric dye is conjugated to the primer, but quenched via resonance energy transfer when the FRET cassette primer is hybridized with DNA (Cuppen, 2007).

3. Results and Discussion

3.1. SNP calling

According to the initial parameters indicated in the Materials and Methods section, the TASSEL software identified 22,326 di-allelic SNPs. We then filtered the positions where all replicates of the parents were identical, with at least one of the parents being heterozygous; other filters included those with less than 15% missing data, and with at least 10% minor allele frequency. This resulted in the selection of 6444 SNPs.

3.2. Genetic linkage maps of 'Kiyomi' and 'Murcott' tangors; synteny and collinearity with the reference genome of Clementine.

The SNP matrix, containing 6444 segregating markers and 61 individuals, was used to construct the genetic maps of the 'Kiyomi' and 'Murcott' tangors. Markers which were heterozygous for the 'Kiyomi' tangor and homozygous for the 'Murcott' tangor were filtered for the linkage mapping of the 'Kiyomi' tangor. Markers which were heterozygous for the 'Murcott' tangor and homozygous for the 'Kiyomi' tangor were filtered for the linkage mapping of the 'Murcott' tangor. Markers with unexpected segregation according to the parents were eliminated. By the end of this process, in order to optimize the quality of genotyping data, only the SNPs within genes were selected, and only one marker per gene was conserved to limit the redundancy of the markers. The number of discarded markers in these filters is provided in Supplementary Table S1.

Linkage mapping of the 'Kiyomi' tangor was performed using a matrix of 1396 segregating SNPs and 61 individuals. A total of 1374 SNPs were assigned to one of the nine resulting linkage groups (LGs), which corresponds to the number of haploid chromosomes in citrus (Table 1 and Supplementary Table S2). The number of markers was unequally distributed among the LGs. LG6 included only 53 SNPs, while 324 SNPs were attributed to LG3. The small number of markers found in LG6 was due to the high homozygosity of the 'Kiyomi' tangor in a large part of the corresponding chromosome. LG1 displayed the lowest genetic size (96.8 cM). LG3, comprising 324 SNPs, displayed the largest genetic size (237.7 cM) (Table 1). The entire map spanned 1416.3 cM, with an average interlocus distance of 1.04 cM. A total of 87% of SNPs had an interlocus gap of less than 3 cM, 12.6% of SNPs had an interlocus gap between 3 and 10 cM, and only 0.4% had a gap measuring more than 10 cM. Most of the LGs were composed of SNPs mapped onto the syntenic pseudo-chromosomes (Sc) of the clementine reference genome. The Circos representation and the Marey map plot between the genetic and physical locations over the clementine reference genome are provided in Supplementary Figure S1. The genetic map displayed high global synteny (98%). LG1, LG4, and LG9 displayed full synteny with the reference genome. LG2 (one in Sc5), LG3 (one in Sc1 and one in Sc2), LG5 (one in Sc4), and LG6 (one in Sc3 and one in Sc8) displayed almost full synteny. LG7 and LG8 stood out. Two, one, and six markers physically located

on chromosomes 1, 4, and 5, respectively, were genetically mapped on LG7. One, six, and five markers physically located on chromosomes 1, 3, and 9, respectively, were mapped on LG8.

Linkage mapping of the 'Murcott' tangor was performed using a matrix of 737 segregating SNP markers and 61 individuals. A total of 697 were assigned to one of the nine resulting LGs, and the number of markers ranged from 40 for LG2 to 168 for LG8 (Table 1 and Supplementary Table S3). The total size of the genetic map was 1339.7 cM, with an average interlocus distance of 1.95 cM. The smallest LG was LG9, measuring 103.4 cM, while LG3 was the largest (231.3 cM). The interlocus gap of 74.9% of the SNPs was less than 3 cM, 23.3% of SNPs had an interlocus gap between 3 and 10 cM, while the genetic distance was more than 10 cM in only 1.9% of SNPs. Overall, synteny was high (96.1%) between the 'Murcott' tangor genetic map and the clementine reference genome (Supplementary Figure S2). LG2, LG5, and LG9 displayed full synteny with the reference genome. LG1 (one in Sc6), LG3 (one in Sc4, one in Sc8, and one in Sc9), LG4 (one in Sc2 and one in Sc3), LG6 (one in Sc3 and two in Sc8), and LG7 (one in Sc4 and two in Sc5) displayed almost full synteny. As already observed in the 'Kiyomi' tangor, LG8 had more SNPs that were not mapped on the corresponding pseudo-chromosome, with counts of four and 11 SNPs (out of a total of 168) located on the physical assembly of pseudo-chromosomes 3 and 9, respectively.

The 'Kiyomi' genetic map displayed high collinearity with the clementine reference genome, although incongruency between the genetic map and the physical positions over the reference genome was observed in a cluster of 14 markers between 30 and 34 cM on LG3. This misplaced genomic region was also shown for 'Murcott'. However, only two markers were concerned due to the low number of heterozygous markers in this genomic region for 'Murcott'. Additional shared discrepancies between the 'Kiyomi' and 'Murcott' genetic maps, and the *C. clementina* v1.0 assembly were observed for markers of chromosomes 3, 5 and 9 located, respectively, on LGs 8, 7, and 9. Similar discrepancies for the same genomic regions of the *C. clementina* v1.0 assembly were also identified in the high-density genetic maps of sweet orange and trifoliate orange (Huang et al., 2018), as well as in the reference genetic map of clementine (Ollitrault et al., 2012) and 'Fortune' (*C. clementina* × *C. tangerina*) and 'Ellendale' (*C. reticulata* × *C. sinensis*) (Ollitrault et al., 2021). In this regard, Ollitrault et al. (2021) suggested that most of the apparent non-syntenic or non-colinear markers were rather due to minor errors in the clementine genome assembly. Overall, the high synteny and collinearity with the clementine reference genome shown in the two genetic maps is consistent with previous studies concluding high synteny and collinearity between *Citrus* species (Bernet et al., 2010; Ollitrault et al., 2021, 2012; Yu et al., 2016). 'Murcott' and 'Kiyomi' tangors are interesting parents, widely used for mandarin breeding, and the high-density genetic maps presented here can prove useful for optimizing their use in breeding programs.

Table 1. Summary of 'Kiyomi' and 'Murcott' tangors mapping data

	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9	Total
Kiyomi										
No. of SNPs	118	189	324	128	214	53	180	80	88	1,374
Size (cM)	96.8	141.3	237.7	112.3	219.4	107.0	152.3	189.7	159.8	1,416.3
Murcott										
No. of SNPs	76	40	75	97	57	81	60	168	43	697
Size (cM)	154.5	138.6	231.3	154.6	145.4	133.2	133.9	144.8	103.4	1,339.7

LG: Linkage group.

3.3. Phenotypes and marker-trait association studies

Phenotypes

Among the 61 genotyped hybrids, 53 flowered during the three-year experiment, 52 of them produced fruits, and in 32 of these, the fruits contained seeds. Data obtained for the phenotyped traits NPGA and APF, as well as the polyembryony of each hybrid, are displayed in Supplementary Table S4.

Male sterility phenotyping was performed based on NPGA and APF. The ANOVA analysis showed significant differences between genotypes for NPGA, while no differences were observed between genotypes for APF (Table 2).

Table 2. ANOVA for number of pollen grains per anther and apparent pollen fertility

	Sum of squares	Df	Mean squares	F ratio	P-value
No. of pollen grains per anther					
Between hybrids	5.16×10^8	52	9.92×10^6	7.6	0
Within hybrids	1.62×10^8	124	1.30×10^6		
Total	6.78×10^8	176			
Apparent pollen fertility					
Between hybrids	3.58060	52	0.0688576	1.18	0.2369
Within hybrids	5.40762	93	0.0581465		
Total	8.98822	145			

Statistical differences for $p<0.05$; Df: Degrees of freedom

As shown in Figure 3, great differences, particularly for APF, were observed within several genotypes, while the data obtained for NPGA were more homogeneous within genotypes. Taking both parameters together, some genotypes showed high APF, but very low NPGA values. For example, for KM-1 and KM-2 with similar APF averages of 73% and 82%, respectively, the average NPGA values were 1950 and 81, respectively. Therefore, KM-1 is a male fertile hybrid, while KM-2 is practically a male sterile hybrid (Figure 3 and Supplementary Table S4).

None of the hybrids with high NPGA values showed APF values low enough to cause male sterility, suggesting that NPGA is the key factor in male sterility in the ’Kiyomi‘ × ‘Murcott’ offspring. In this line, Goto et al. (2016) evaluated NPGA and APF in a satsuma progeny, and reported that male sterility is primarily caused by decreased NPGA. Although satsuma is generally described as male sterile, several studies have pointed out that male sterility in satsuma is partial and influenced by both environmental conditions and genotype. In fact, new varieties have been obtained using pollen from satsuma (Yamamoto, 2014). In this regard, Yang and Nakagawa (1970, 1969) reported that temperature treatments at 15 °C and 20 °C during flower bud growth and development are favorable in the recovery of male fertility in satsuma. In addition to this, a low degree of male fertility has also been achieved under field conditions, as has been shown by Vithanage (1991), who reported two seeds per fruit when ‘Ellendale’ tangor was pollinated with satsuma, and by Goto et al. (2016), who reported an average of 389 NPGA in ‘Okitsu wase’ satsuma. In the same paper, Goto et al. (2016) reported that pollen grains in ‘Kiyomi’ (satsuma × sweet orange) were not detected, suggesting that male sterility in ‘Kiyomi’ is stricter than in satsuma. In this study, we have observed an average of one NPGA in ‘Kiyomi’. This very low value points to strict male sterility in

'Kiyomi', and the fact that it is not complete. Beyond the differences in NPGA values between hybrids producing low numbers of pollen grains, Goto et al. (2018) suggested that the release of pollen grains from anthers occurs when a certain NPGA value is exceeded. They also assumed that the presence of less than approximately 1300 NPGA was a crucial criterion for male sterility. In this study, we have observed pollen grain release in those anthers with more than 1000 NPGA. Thus, we have established 1000 NPGA as the criterion of male sterility.

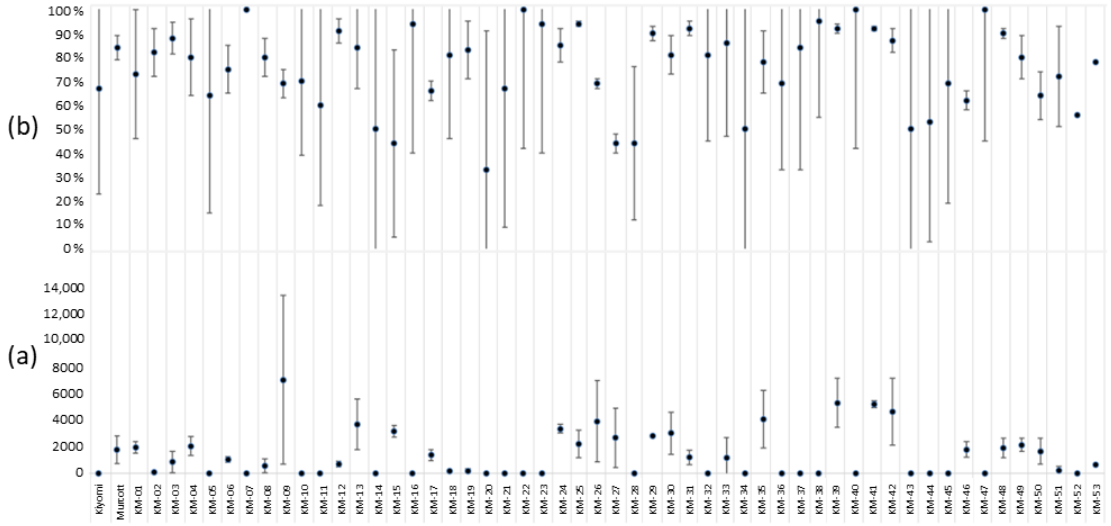


Figure 3. Average and standard deviation representation of **(a)** the number of pollen grains per anther and **(b)** the percentage of apparent pollen fertility in the 'Kiyomi' × 'Murcott' offspring phenotyped for male sterility. The x-axis indicates the name of the parents and hybrids.

In Figure 4, we display the histogram obtained for NPGA. A total of 49% of the hybrids plus 'Kiyomi' produced less than 250 NPGA, and 6% produced between 500 and 1000 NPGA; 36% of the hybrids plus 'Murcott' produced between 1000 and 4000 NPGA, and 9% produced more than 4000 NPGA (Figure 4 and Supplementary Table S4).

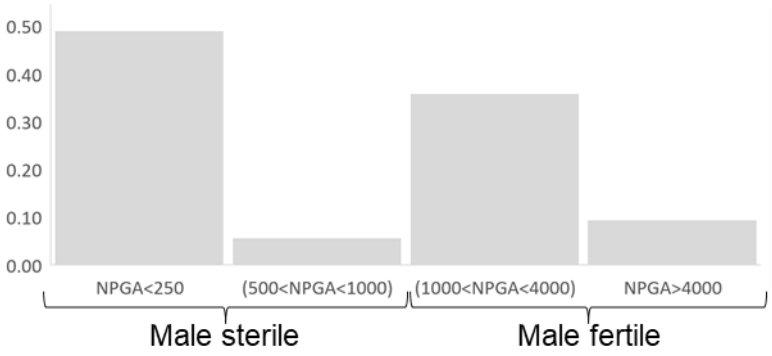


Figure 4. Histogram displaying the number of pollen grain per anther (NPGA) distribution in the diploid hybrids analyzed.

As expected, all seeds of the 'Kiyomi' tangor were monoembryonic, and all seeds of the 'Murcott' tangor were polyembryonic. Of the 32 hybrids that produced fruits with seeds, 12 of them produced monoembryonic seeds (37.5%), and the other 20 (62.5%) hybrids

produced polyembryonic seeds. In the last group, we found nine hybrids with solely polyembryonic seeds, and 11 hybrids with percentages of polyembryonic seeds ranging between 14 and 92% (Supplementary Table S4).

Marker-Trait Association

Through a general linear model (GLM) using default parameters in the TASSEL 5 software, polyembryony and male sterility marker–trait association studies were separately evaluated in both 'Kiyomi' and 'Murcott' tangors maps. The statistical significance of the genetic and phenotypic associations was calculated with a 0.05 probability threshold, as well as applying the Bonferroni correction for multiple testing. For the `Murcott' tangor, the probability threshold was $p \leq 7.5 \times 10^{-5}$ (0.05/670) or −log(p) >= 4.01, whereas for `Kiyomi´, it was $p \leq 3.7 \times 10^{-5}$ (0.05/1346) or −log(p) >= 4.43.

The TASSEL software was designed to evaluate trait associations, evolutionary patterns, and linkage disequilibrium using GWAS. It has been successfully used for marker–trait association studies using bi-parental progenies. Applying GLM, Sumitomo et al. (2019) tagged SNP markers onto the flower color genes in autohexaploid *Chrysanthemum*, and Shibaya et al. (2022) identified QTLs for root color and carotenoid contents in carrot. In Japanese plum, Salazar et al. (2017) identified QTLs linked to fruit quality traits using three F1 progenies with a common female parent.

Here, a genotype–phenotype association study for male sterility was performed for the NPGA trait. APF was not used for association studies, since no significant differences were observed between the hybrids. In the 'Murcott' gamete map, the GLM identified 68 SNP markers with statistical significance (Supplementary Table S5). All of them were located on 68 different genes on LG8. A total of 49 of these markers were clustered in a region of 4.79 Mb (between the positions 2107212 and 6899421 bp), corresponding to a genetic region of 26.735 cM (between 26.771 and 53.506 cM) (Figure 5a and Supplementary Table S5). The most significant marker identified was S08_4417545, with a *p*-value of 1.02E-10 (LOD = 9.99) and a genetic position of 46.436 cM (Supplementary Table S5).

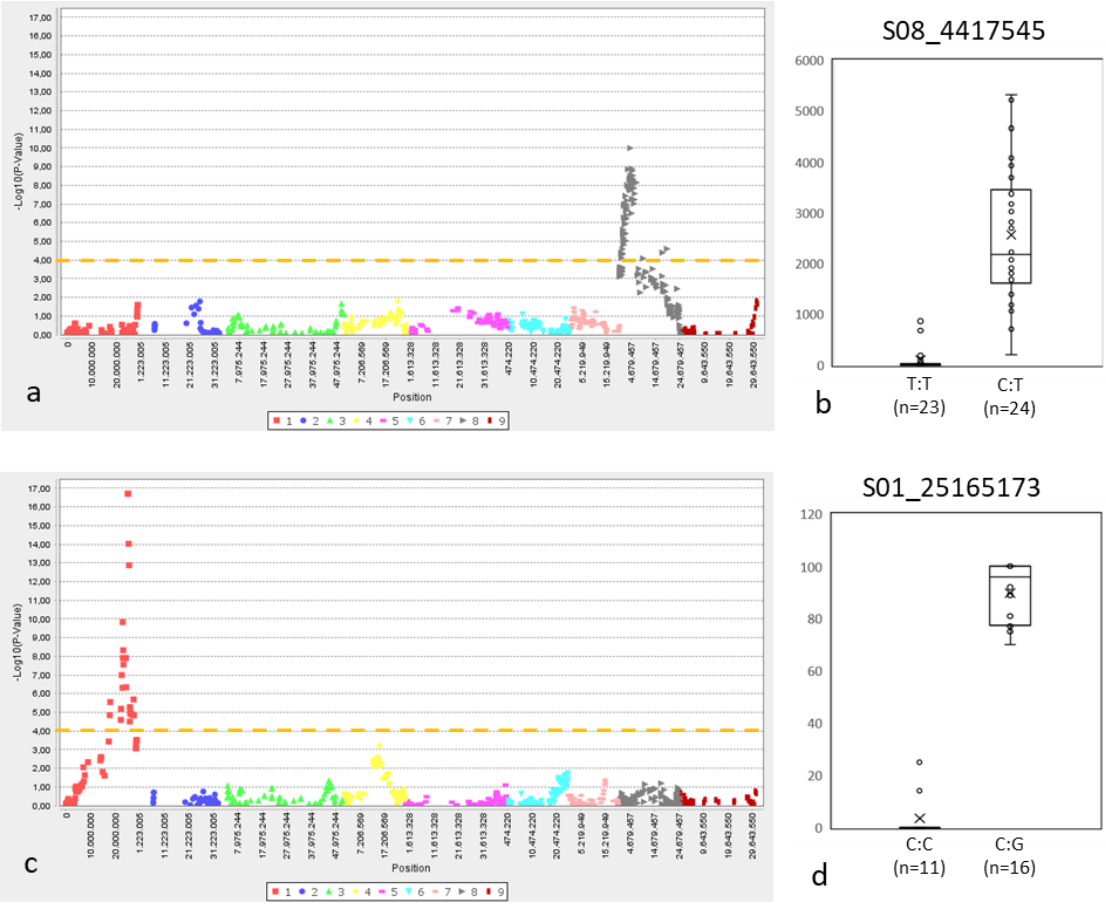


Figure 5. Association study for Murcott gametes; **(a-b)** Number of pollen grains per anther; **(a)** Manhattan plot; **(b)** box plot representation for the most significant marker, SNP S08_4417545; **(c-d)** Polyembryony; **(c)** Manhattan plot; **(d)** box plot representation for the most significant marker, SNP S01_25165173. The orange line is the threshold for significants p values.

All hybrids with the TT allelic configuration for the SNP S08_4417545 were male-sterile. The vast majority of the hybrids with the CT allelic configuration produced more than 1000 NPGA, none of them showing very low values for NPGA (Figure 5b). It should be noted that some significative SNPs were identified outside of the clustered region (between 26.771 and 53.506 cM) (Figure 5a and Supplementary Table S5), therefore it is likely that other genomic regions on LG8 can be involved in male sterility. Through QTL mapping of a population derived from two satsuma hybrids, Goto et al. (2018) identified three QTLs (*MS-P1*, *MS-P2* and *MS-P3*) associated with NPGA. The most associated *MS-P1*, located on LG8 (genetic position 37.5 cM), may correspond to the association genomic region for NPGA identified in our GLM analysis. In addition, the two other QTLs with lower associations, *MS-P2* and *MS-P3*, both located on LG6b and separated by a genetic distance of 29 cM, suggest that other genomic regions can also be involved in this trait.

For polyembryony, through the genetic association study performed in the 'Murcott' gamete map, GLM identified 25 SNPs markers with statistical significance, all of them located on 25 different genes on chromosome 1. These markers were clustered in a region of 9.85 Mb (located between positions 17831378 and 27687717 bp), corresponding with a genetic region of 64.127 cM (between 79.356 and 143.483 cM) (Figure 5c and Supplementary Table S5). For the most significant marker (S01_25165173), all hybrids with the C:G allele produced polyembryonic seeds, the total

of which exceeded 25% (Figure 5d). Averages of polyembryonic seeds between 69.8 and 91.4% have been reported in apomictic genotypes by Kishore et al. (2012). We performed a BLASTn search of the sequence of the *CitRWP* gene of the pummelo genome in the genome assembly *of C. clementina* v1.0. Wang et al. (2017) demonstrated that the insertion of a MITE in close vicinity of this gene was responsible for polyembryony in mandarins. The BLAST analysis identified the annotated gene Ciclev10010497m as the homologue of *CitRWP* with a high-scoring segment pair (HSP), with a positive identity of 99.89%. This gene is located on chromosome 1 at position 25480488-25482037 bp of the *C. clementina* assembly. The genomic regions of 25165173-25690547 bp on chromosome 1, defined by the markers included within a 5 cM interval each side of the higher signal marker in our association study, include the Ciclev10010497m location. Therefore, our results are in full agreement with previous conclusions regarding the importance of *CitRWP* for polyembryony in mandarin.

3.4. Gene annotations of the genomic region associated with male sterility

The 4.79 Mb genomic region identified in our GWAS was examined for gene annotations in the clementine genome (https://phytozome-next.jgi.doe.gov (“Phytozome 13.,” n.d.) (accessed on 7 June 2022). A genomic region (between 5913054 and 6901468 bp) containing 67 annotated genes, 19 of which are involved in different biological pathways that may affect pollen formation or development, draws our attention. These genes include papain-like cysteine protease enzymes, pentatricopeptide repeat, ATP binding, plant homeodomain-finger family protein, WD40 repeat-like, and 3-oxo-5-alpha-steroid 4-dehydrogenase. Gene annotations are provided in Supplementary Table S6.

The annotation functions of two genes (Ciclev10028670m.g and Ciclev10029917m.g) are associated with papain-like cysteine protease enzymes. Cysteine protease plays a critical regulatory role in programmed cell death (PCD), and its regulation is influenced by temperature stress (Testillano, 2019). Tapetum, a layer of cells surrounding microspores, is key in pollen development, providing nutritive proteins, enzymes, and sporopollenin precursors for pollen maturation (Liu et al., 2018). During the late stages of pollen development, tapetum undergoes PCD, and either premature (Ku et al., 2003) or inhibition (Kawanabe et al., 2006) PCD of tapetal cells will result in male sterility. The importance of cysteine proteases in pollen formation has been reported in tobacco (Shukla et al., 2016, 2014; Zhang et al., 2009), *Arabidopsis* (Yang et al., 2014; Zhang et al., 2014), soybean (Li et al., 2016), tomato (Omidvar et al., 2017), rice (Rao et al., 2018), cabbage (Xing et al., 2018), and brassica (Gautam et al., 2019).

In total, five genes around SNP08_6142645 (Ciclev10030265m.g, Ciclev10029744m.g, Ciclev10028481m.g, Ciclev10030279m.g, and Ciclev10029914m.g) are annotated as Pentatricopeptide repeat (PPR). The PPR superfamily protein represents the most frequent protein class identified as restorers of fertility (Rf). Examples of PPRs characterized as Rf and confirmed through transgenic analysis include *Rf-PPR592* in petunia, *Rfo* in radish, and *Rf1A*, *Rf1B*, *Rf3*, *Rf4*, *Rf5*, *Rf6*, *GRP162,* and *PPR762* in rice (reviewed in Gaborieau et al. 2016).

Another nine genes around SNP08_6142645 (Ciclev10029967m.g, Ciclev10028124m.g, Ciclev10030242m.g, Ciclev10028233m.g. Ciclev10030082m.g, Ciclev10029947m.g, Ciclev10030361m.g, Ciclev10028181m.g, and Ciclev10030145m.g) are annotated as related to ATP binding, a transport protein

involved in sporopollenin (the material that forms the durable, chemically stable outer layer on pollen grains) export and/or shuttling from the tapetum. Chang et al. (2018) reported that the *OsABCG3* gene (an ATP binding cassette) is essential for pollen development in rice. Other genes in this genomic region include Ciclev10029260m.g, which encodes a plant homeodomain (PHD)-finger family protein. In *Arabidopsis*, the male meiocyte death1 gene encodes a PHD-finger protein which is required for male meiosis (Yang et al., 2003); Ciclev10028263m.g encodes a WD repeat protein, which regulates pollen growth and viability in Flax (*Linum usitatissium* L.) (Kumar et al., 2013); and Ciclev10028796m.g with the 3-oxo-5-alpha-steroid 4-dehydrogenase domain localized on the C-terminal part of Polyprenol reductase2, of which the deficiency causes male sterility in *Arabidopsis* (Jozwiak et al., 2015).

Pollen grain number in angiosperms is a key reproductive trait that has been studied extensively for decades. Despite its agricultural and evolutionary importance, the genetic basis of the pollen grain number has remained elusive, primarily due to its quantitative nature (Kakui et al., 2021). The information generated from gene annotations allows us to focus our efforts on 19 genes related to male sterility over the 67 genes annotated in the genomic region identified by the QTL analysis. This limited number will now allow for the development of affordable, albeit time-consuming, approaches to determine whether these genes are actually involved in the male sterility and citrus interaction. Further experiments will be necessary to shed light on this complex trait of citrus reproductive biology.

3.5. Development and validation of SNPs markers associated to male sterility and polyembryony

According to our GWAS analysis, we developed one KASPar SNP marker for each trait. For male sterility, the candidate region, between 5,913,054 and 6,901,468 Kb, contained three SNPs: S08_6026790 in Ciclev10027952m.g, S08_6050573 in Ciclev10027768m.g, and S08_6142645 in Ciclev10028670m.g (Supplementary Table S6). Since Ciclev10028670m.g is annotated to encode papain-like cysteine protease enzymes (of which the importance in pollen formation has been widely reported), we chose S08_6142645 (hereinafter SNP8) for the development of the male sterility KASPar SNP marker. SNP8 is located on the physical position 6,142,645 on chromosome 8 of the *C. clementina* v1.0 genome assembly, corresponding to the genetical position 53.506 cM on the 'Murcott' map.

In the framework of our breeding program, a progeny of 20 diploid hybrids obtained from open pollinated 'Kiyomi' tangor was phenotyped for male sterility and analyzed thorough KASPar with the S08 SNP marker (Table 3).

A total of eight of the 20 hybrids produced flowers with pale yellow or off-white anthers and low-to-null pollen quantity. All of these hybrids displayed CC allelic configuration for SNP8. On the other hand, 12 hybrids displayed flowers with a fertile phenotype (yellow anthers and a high quantity of pollen grains) associated with CT allelic configuration for this SNP. In addition, 11 different commercial mandarin cultivars were analyzed (Supplementary Table S7). 'Okitsu' satsuma, 'Kiyomi' tangor, and 'Queen' mandarin were classified as male sterile (CC), whereas the other mandarins were genotyped as fertile, with CT allelic configuration for 'Nadorcott', 'Murcott', 'Kara', and 'Encore' mandarins, and TT allelic configuration for 'Clemenules', 'Campeona', 'Fortune', and 'Ellendale'. It is expected that when crossing a male sterile (CC) cultivar as the seed

parent with pollen from a fertile (CT) cultivar, 50% of the hybrids will be male sterile and the other 50% will be fertile, meanwhile 100% of the hybrids will be male fertile when using pollen from a (TT) fertile cultivar. Nevertheless, this SNP marker has been tested in these two populations with very good results and it would be of interest to test in others genetic contexts, that we do not have at this moment.

Table 3. Genetic analysis of 20 hybrids recovered with 'Kiyomi' as a female parent and an unknown male parent, with the S08_6142645 SNP marker associated to male sterility.

Individual	Phenotype	Genotype
Kiyomi × Unknown-1	Fertile	C:T
Kiyomi × Unknown-2	Sterile	C:C
Kiyomi × Unknown-3	Sterile	C:C
Kiyomi × Unknown-4	Fertile	C:T
Kiyomi × Unknown-5	Sterile	C:C
Kiyomi × Unknown-6	Fertile	C:T
Kiyomi × Unknown-7	Fertile	C:T
Kiyomi × Unknown-8	Sterile	C:C
Kiyomi × Unknown-9	Fertile	C:T
Kiyomi × Unknown-10	Sterile	C:C
Kiyomi × Unknown-11	Sterile	C:C
Kiyomi × Unknown-12	Fertile	C:T
Kiyomi × Unknown-13	Fertile	C:T
Kiyomi × Unknown-14	Sterile	C:C
Kiyomi × Unknown-15	Fertile	C:T
Kiyomi × Unknown-16	Fertile	C:T
Kiyomi × Unknown-17	Fertile	C:T
Kiyomi × Unknown-18	Fertile	C:T
Kiyomi × Unknown-19	Fertile	C:T
Kiyomi × Unknown-20	Sterile	C:C

Male fertile or sterile phenotype is based on the observations of both the fresh anthers color and the quantity of pollen grains in dehiscent anthers (see Figure 1). KASPar plot obtained with the S08_6142645 SNP marker is provided in Supplementary Figure S3.

Regarding polyembryony, among the most significant SNPs identified in our association study for polyembryony, S01_25497528 was the most closely positioned to the blasted sequence of the *CitRWP* gene (Wang et al., 2017) in the *C. clementina* v1.0 reference. Thus, we used this SNP to develop the hereinafter SNP1, located on chromosome 1 of the *C. clementina* v1.0 genome assembly at position 25497528, corresponding to the genetic position of 126.64 cM on the 'Murcott' genetic map. This marker was analyzed in 83 citrus genotypes, including 53 polyembryonic, and 30 monoembryonic cultivars. A summary of the results from phenotyping and SNP1 genotyping is indicated in Table 4. SNP1 marker genotype was in agreement with the mono/polyembryony phenotype in most horticultural groups, except Citrumelo (*C. paradisi* × *P. trifoliata*), *Fortunella* spp., and *Poncirus* spp. (Table 4 and Supplementary Table S8). For *Fortunella* and *Poncirus* spp., these mismatches are in accordance with the hypothesis reported by Wang et al. (2022), who suggest that the parallel evolution of *Fortunella* and *Citrus* has driven the evolution of apomixis in these genera in a differentiated way, resulting in the

heterogeneity of genes causing polyembryony in *Citrinae*, a subtribe comprising *Fortunella*, *Poncirus* and *Citrus* genera, among others. Polyembryonic *Poncirus* genotypes do not have MITE insertions in the promoter region of the *CitRWP* gene, and it is also not expressed in nucellar ovule cells, suggesting another causal gene (Wang et al., 2022; Xu et al., 2021). Therefore, the SNP1 marker is fully validated for apomixis characterization of germplasm and hybrids of breeding projects derived from admixture between *C. reticulata* and *C. maxima* or/and *C. medica*, where the polyembryonic trait was inherited from the *C. reticulata* ancestor.

The SNP1 marker will be very useful for the selection of new monoembryonic parents aimed at obtaining new varieties. That is particularly relevant in view of the limited number of monoembryonic female parents available today for the use in breeding programs. In fact, we are routinely using the SNP1 marker in our breeding program to select monoembryonic parents. This, together with the selection for resistance to Alternaria brown spot fungus disease (Cuenca et al., 2016), allows us to be more efficient in the selection of parents with improved characteristics. The selected parents are subsequently induced for early flowering by a viral vector based on the *Citrus leaf blotch virus* (Velázquez et al., 2016). This strategy shortens the time needed to recover new improved genetic combinations. In addition, the SNP1 marker will be very useful for the identification of polyembryonic hybrids in rootstock breeding programs which look for polyembryony, because it allows for ease, expense reduction, and consistency of rootstock propagation in the nursery (Castle, 2010). All these show the high potential of SNP1 for MAS.

Table 4. Horticultural groups with the number of accessions analyzed with the S01_25497528 SNP marker.

Horticultural group	No of accessions	A:A	A:G	G:G	% well assigned
Satsuma mandarin	2	0	2	0	100
Clementine mandarin	5	5	0	0	100
Mandarin	14	3	9	2	100
Sweet Orange	9	0	9	0	100
Sour Orange	2	0	2	0	100
Grapefruit	3	0	3	0	100
Lemon	3	1	2	0	100
Lime	2	0	2	0	100
Citron	3	3	0	0	100
Pummelo	3	3	0	0	100
Bergamot	1	1	0	0	100
Mandarin hybrid	18	9	9	0	100
Tangor	6	3	3	0	100
Tangelo	4	1	3	0	100
Citrange	1	0	1	0	100
Citrumelo	1	1	0	0	0
Fortunella	4	4	0	0	25
Poncirus	2	2	0	0	0

The SNP1 allele linked with polyembryony is G. GG and AG allelic configurations correspond with polyembryonic genotypes, whereas AA allelic configurations are monoembryonic. Detailed information of genotypes and phenotypes is provided in Supplementary Table S8, and the KASPar plot in Supplementary Figure S3.

6. Conclusion

GBS was used to genotype 61 diploid hybrids from an F1 progeny recovered from crossing the male sterile and monoembryonic ‘Kiyomi’ tangor as the female parent with the male fertile and polyembryonic ‘Murcott’ tangor as the male parent. Raw sequences were aligned to the clementine genome and 6444 SNPs were obtained. After filtering for SNPs within genes and heterozygous for only one of the parents, we established the genetic map for each parent with the JoinMap.5 software. The two maps, respectively, include 1374 and 697 markers, and encompass 1416.287 and 1339.735 cM for ‘Kiyomi’ and ‘Murcott’. The two maps were globally highly syntenic and colinear with the *C. clementina* v1.0 assembly; however, they confirmed previous constatations for probable small incongruences of the *C. clementina* genome assembly in chromosomes 3, 5, and 9. The progenies were phenotyped for male sterility based on the number of pollen grains per anther (NPG) and apparent pollen fertility (APF) values, as well as for polyembryony. The genotype–trait association study, using the general linear model (GLM), identified a genomic region on linkage group 8 significantly associated with NPGA; however, no association was observed for APF, indicating that NPGA is the major factor for male sterility in the progeny derived from ‘Kiyomi’ × ‘Murcott’. We also identified a genomic region on linkage group 1 significantly associated with polyembryony. The analysis of gene annotation in the region of chromosome 8 associated with NPGA revealed 19 candidate genes implied in pollen development in other plant species. An SNP marker (S08_6142645) based on KASPar technology was developed in the Ciclev10028670m.g gene, appertaining to the papain cysteine protease family, well known for its importance in pollen development. It was validated on a family of uncontrolled hybrids of ‘Kiyomi’ mother plants. We also developed an SNP marker for polyembryony, choosing the SNP in the ‘Murcott’ genetic map closest to the CitRWP gene involved in mandarin apomixis. This marker was fully validated on a collection of varieties derived from *C. reticulata*, *C. maxima,* and *C. medica* ancestors. However, it was not efficient for polyembryonic accessions derived from *P. trifoliata*, and the *Fortunella* sp. This last result is in agreement with previous hypotheses for multiple origins of polyembryony in the true citrus genera.

Male sterility is a desirable trait for seedless breeding and polyembryony is a crucial reproductive feature to be considered in breeding, for both rootstocks and varieties. Marker-assisted selection (MAS) is key in breeding programs, particularly in tree species with long juvenile period, such as citrus, since the selection of target genotypes can be carried out at the seedling stage. In recent years, molecular tagging techniques have evolved, and SNP markers have emerged as an indispensable tool in genetic applications and breeding programs. To our knowledge, the SNP1_25497528 and SNP8_6142645 developed here are the first available to be successfully used in MAS for polyembryony and male sterility in a wide range of citrus genotypes, and will be very useful for MAS breeding programs for varieties and rootstocks.

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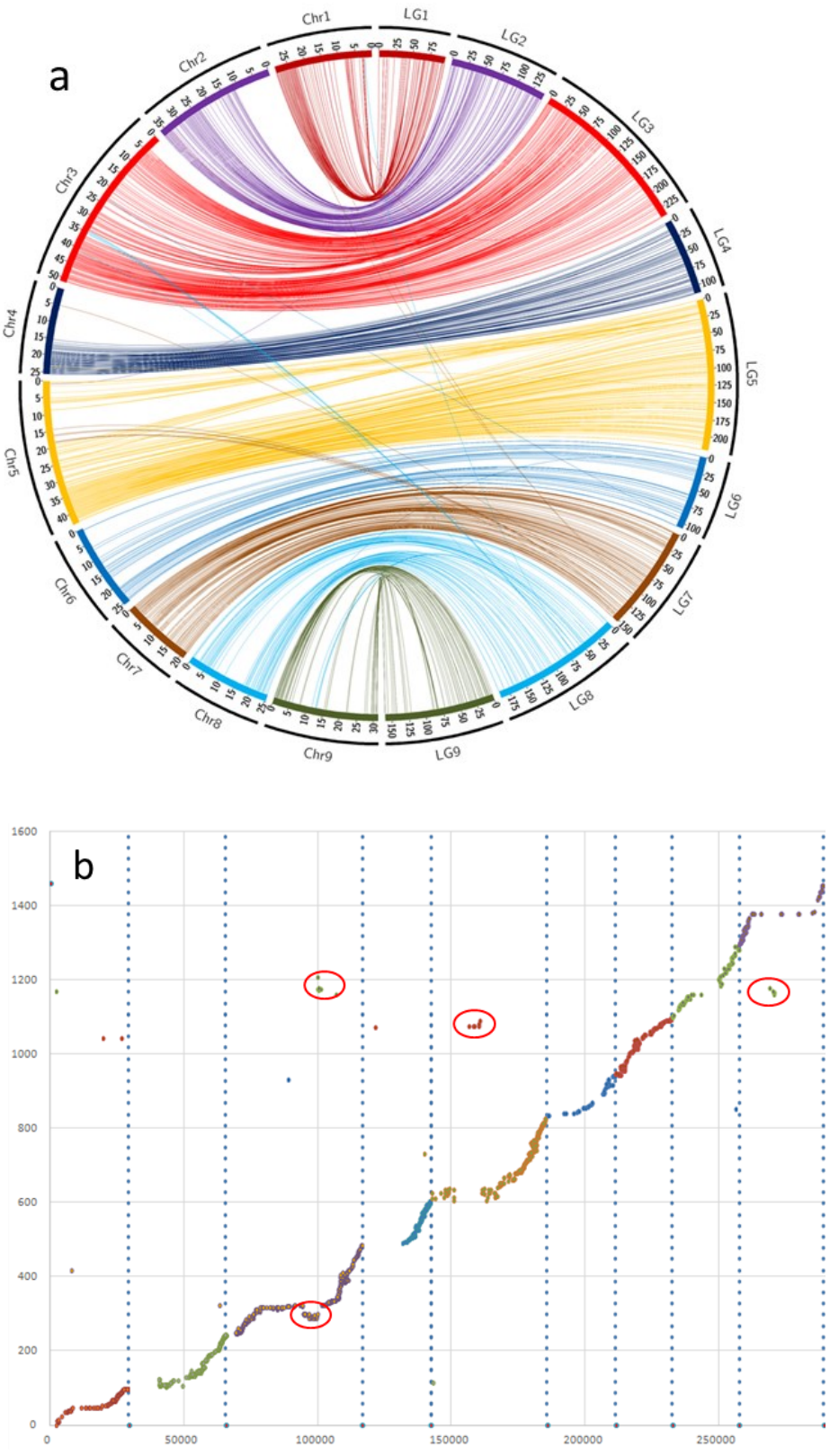
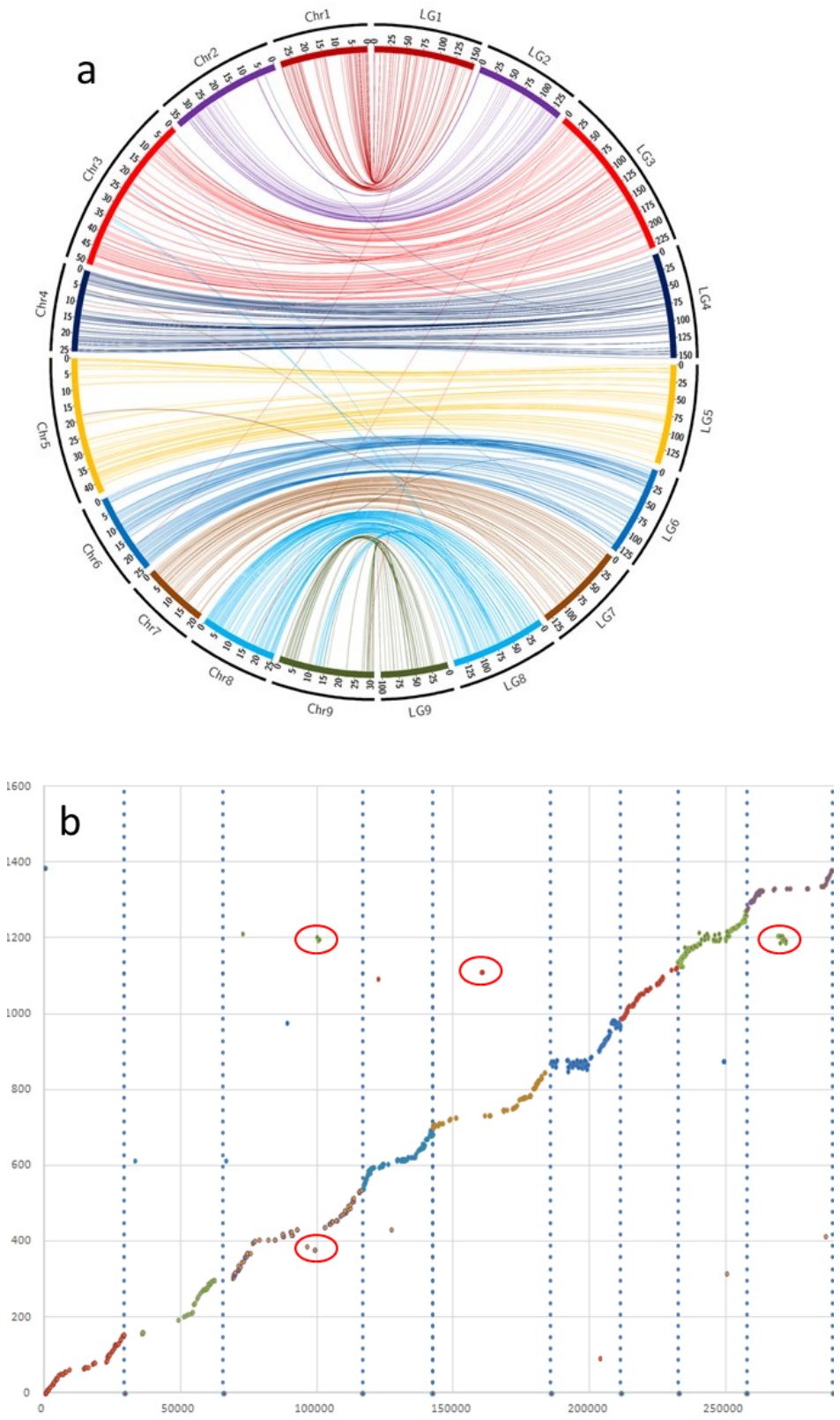
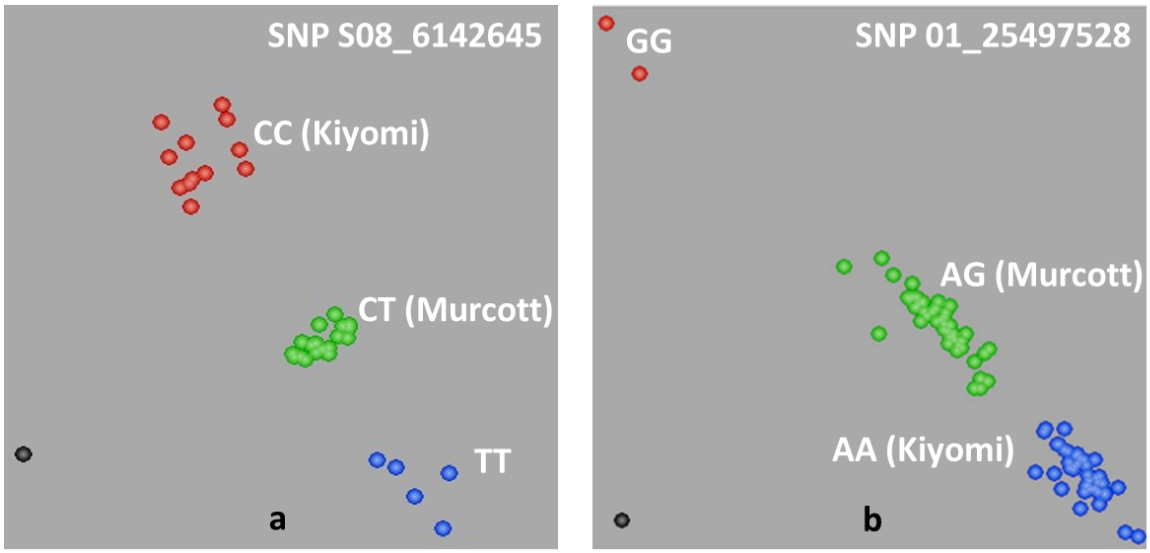
Supplementary information



Supplementary Figure 1. Synteny and collinearity of the 'Kiyomi' tangor genetic map with the reference genome of clementine. **(a)** Circos representation of links between the position of markers on the 'Kiyomi' genetic map (LG1 to LG9) and on the chromosome assembly of clementine genome (Ch1 to Ch9). Scales are in cM for LGs and in Mb for chromosomes. **(b)** Marey map plot. The x-axis represents the physical positions on the clementine reference genome and y-axis represents the position on the 'Kiyomi' tangor genetic map. The red circles indicates the main incongruences between the two genetic maps and the *C. clementina* genome assembly.



Supplementary Figure 2. Synteny and collinearity of the 'Murcott' tangor genetic map with the reference genome of clementine. **(a)** Circos representation of links between the position of markers on the ''Murcott' genetic map (LG1 to LG9) and on the chromosome assembly of clementine genome (Ch1 to Ch9). Scales are in cM for LGs and in Mb for chromosomes. **(b)** Marey map plot. The x-axis represents the physical positions on the clementine reference genome and y-axis represents the position on the 'Murcott' tangor genetic map. The red circles indicates the main incongruences between the two genetic maps and the *C. clementina* V1.0 genome assembly.



Supplementary Figure 3. Plots of the allele signals of KASPar analysis for S08_6142645 and S01_25497528 SNP markers. **(a)** Plot of the C and T allele signals of the S08_6142645 SNP marker from the cluster analysis of 20 diploid hybrids recovered by the cross-pollination of the 'Kiyomi' tangor with unknown pollen genotype and 11 mandarin varieties. **(b)** Plot of the A and G allele signals of the S01_25497528 SNP marker from the cluster analysis of germplasm collection.

Supplementary Table 1. Number of filtered, discarded and used SNPs markers to perform the linkage mapping of `Kiyomi' and 'Murcott' tangors.

Number of SNPs selected after SNP calling filters	**6.444**
Heterozygous for Kiyomi and Homozygous for Murcott	2.912
Discarded due to unexpected segregation	896
Discarded because they are located outside of a gene	503
Discarded because there is another SNP in the same gene	117
Used to perform the linkage mapping of Kiyomi	**1.396**
Heterozygous for Murcott and Homozygous for Kiyomi	1.370
Discarded due to unexpected segregation	302
Discarded because they are located outside of a gene	290
Discarded because there is another SNP in the same gene	41
Used to perform the linkage mapping of Murcott	**737**

Supplementary Table 2. Detail of the 'Kiyomi' genetic map including physical position (clementine reference genome), genetic position and the gene name on which the marker is located (Provided at the end of this document p. 207-234).

Supplementary Table 3. Detail of the 'Murcott' genetic map including physical position (clementine reference genome), genetic position and the gene name on which the marker is located. (Provided at the end of this document p. 235-249).

Supplementary Table 4. Phenotypes of pollen traits and polyembryony for Kiyomi, Murcott and the Kiyomi × Murcott progeny.

	No of Pollen Grains per Anther (NPGA)				**% of Apparent Pollen Fertility (APF)**				**% of PE seeds**
Genotype	**Max.**	**Min.**	**Av.**	**SD**	**Max.**	**Min.**	**Av.**	**SD**	
Kiyomi	5	0	1	1	100	0	67	44	0
Murcott	2700	644	1768	1041	92	76	84	5	100
KM-01	2350	1500	1950	427	95	43	73	27	nd
KM-02	124	42	81	41	93	74	82	10	75
KM-03	1589	4	864	801	93	83	88	7	nd
KM-04	2817	1400	2069	712	94	63	80	16	0
KM-05	23	0	9	10	100	0	64	49	nd
KM-06	1278	850	1059	214	83	63	75	10	70
KM-07	2	0	1	1	100	100	100	0	nd
KM-08	1170	172	568	530	86	71	80	8	50
KM-09	14322	2722	7041	6342	75	63	69	6	nd
KM-10	29	4	13	14	100	38	70	31	0
KM-11	1	0	1	1	60	0	60	42	nd
KM-12	939	516	708	214	94	84	91	5	0
KM-13	5863	2236	3687	1919	98	65	84	17	0
KM-14	1	0	0	0	100	0	50	58	0
KM-15	3674	12	3171	443	78	0	44	39	nd
KM-16	7	0	2	4	100	0	94	54	0
KM-17	1737	942	1393	408	69	63	66	4	nd
KM-18	304	0	192	92	100	0	81	35	100
KM-19	318	24	198	154	94	70	83	12	100
KM-20	17	1	6	9	100	0	33	58	100
KM-21	2	0	1	1	100	0	67	58	nd

	No of Pollen Grains per Anther (NPGA)				% of Apparent Pollen Fertility (APF)				% of PE seeds
Genotype	**Max.**	**Min.**	**Av.**	**SD**	**Max.**	**Min.**	**Av.**	**SD**	
KM-22	0	0	0	0	100	0	100	58	nd
KM-23	9	0	3	5	100	0	94	54	nd
KM-24	3613	3126	3370	345	90	80	85	7	100
KM-25	3272	1161	2223	1056	94	92	94	1	25
KM-26	7413	1622	3930	3069	71	67	69	2	nd
KM-27	4050	3911	2690	2236	47	41	44	4	0
KM-28	116	1	29	50	89	0	44	32	100
KM-29	2818	2805	2812	9	92	88	90	3	100
KM-30	4711	1133	3026	1600	87	69	81	8	0
KM-31	1628	610	1203	530	95	89	92	3	nd
KM-32	26	0	6	9	100	0	81	36	nd
KM-33	2915	314	1187	1496	94	0	86	39	0
KM-34	2	0	1	1	100	0	50	52	nd
KM-35	5985	1728	4071	2161	88	64	78	13	78
KM-36	4	0	1	2	80	0	69	36	nd
KM-37	9	0	4	5	100	0	84	51	nd
KM-38	74	0	21	30	100	0	95	40	89
KM-39	6870	2722	5320	1838	95	90	92	2	100
KM-40	1	0	0	1	100	0	100	58	100
KM-41	5411	5021	5216	276	93	91	92	1	100
KM-42	6600	1828	4661	2509	91	81	87	5	77
KM-43	2	0	1	1	100	0	50	58	nd
KM-44	3	1	2	1	100	0	53	50	nd
KM-45	89	0	30	51	100	0	69	50	81
KM-46	2233	1385	1809	600	65	59	62	4	75
KM-47	1	0	0	0	100	0	100	55	0
KM-48	2585	1119	1904	739	92	89	90	2	0
KM-49	2674	1678	2142	502	90	72	80	9	92
KM-50	2806	1053	1679	978	77	55	64	10	nd
KM-51	559	23	230	288	95	52	72	21	nd
KM-52	3	1	2	2	100	0	56	51	0
KM-53	1439	223	681	661	92	67	78	13	14

Max. Correspond with the maximun number, Min. Minimun number, Av. Average and SD standard desviation; PE: Polyembryonic; nd. No data.

Supplementary Table 5. Markers associated with the Number of pollen grains per anther (NPGA) and with Polyembryony (PE) in the Murcott gametes map.

Trait	Marker	p-value	R2	LOD score	Linkage Group	Phisical position	Genetic position	Gene
NPGA	S08_4417545	1.02E-10	0.61	9.99	8	4417545	46.436	ID=Ciclev10029642m.g
NPGA	S08_4969549	1.29E-09	0.57	8.89	8	4969549	46.436	ID=Ciclev10028120m.g
NPGA	S08_3494245	1.38E-09	0.55	8.86	8	3494245	39.217	ID=Ciclev10028454m.g
NPGA	S08_5326450	1.65E-09	0.56	8.78	8	5326450	46.436	ID=Ciclev10028904m.g
NPGA	S08_4461663	3.02E-09	0.53	8.52	8	4461663	46.436	ID=Ciclev10029365m.g
NPGA	S08_5469542	3.02E-09	0.53	8.52	8	5469542	46.436	ID=Ciclev10030368m.g
NPGA	S08_4889493	4.24E-09	0.53	8.37	8	4889493	46.436	ID=Ciclev10027789m.g
NPGA	S08_4026112	4.74E-09	0.52	8.32	8	4026112	40.974	ID=Ciclev10028201m.g
NPGA	S08_5201102	4.81E-09	0.52	8.32	8	5201102	46.436	ID=Ciclev10029966m.g
NPGA	S08_5491094	4.81E-09	0.52	8.32	8	5491094	46.436	ID=Ciclev10027908m.g
NPGA	S08_4297257	4.86E-09	0.52	8.31	8	4297257	46.436	ID=Ciclev10028264m.g
NPGA	S08_4860794	4.86E-09	0.52	8.31	8	4860794	48.176	ID=Ciclev10027804m.g
NPGA	S08_5153846	4.86E-09	0.52	8.31	8	5153846	46.436	ID=Ciclev10028494m.g
NPGA	S08_5461426	6.12E-09	0.52	8.21	8	5461426	46.436	ID=Ciclev10028890m.g
NPGA	S08_5343601	6.92E-09	0.52	8.16	8	5343601	46.436	ID=Ciclev10029896m.g
NPGA	S08_6050573	7.18E-09	0.51	8.14	8	6050573	53.506	ID=Ciclev10027768m.g
NPGA	S08_6899421	7.18E-09	0.51	8.14	8	6899421	53.506	ID=Ciclev10028796m.g
NPGA	S08_3130127	9.85E-09	0.51	8.01	8	3130127	46.436	ID=Ciclev10029362m.g
NPGA	S08_4555396	9.85E-09	0.51	8.01	8	4555396	47.306	ID=Ciclev10029826m.g
NPGA	S08_3701588	1.16E-08	0.52	7.94	8	3701588	46.436	ID=Ciclev10027659m.g
NPGA	S08_4892310	1.22E-08	0.52	7.91	8	4892310	46.436	ID=Ciclev10028655m.g
NPGA	S08_4075187	1.36E-08	0.52	7.87	8	4075187	46.436	ID=Ciclev10028081m.g
NPGA	S08_3998172	1.41E-08	0.51	7.85	8	3998172	46.436	ID=Ciclev10029698m.g
NPGA	S08_5956341	1.42E-08	0.50	7.85	8	5956341	53.506	ID=Ciclev10027795m.g
NPGA	S08_4140439	1.66E-08	0.52	7.78	8	4140439	46.436	ID=Ciclev10029477m.g
NPGA	S08_4553146	1.81E-08	0.51	7.74	8	4553146	46.436	ID=Ciclev10029521m.g
NPGA	S08_6142645	2.82E-08	0.49	7.55	8	6142645	53.506	ID=Ciclev10028670m.g
NPGA	S08_2438501	3.68E-08	0.49	7.43	8	2438501	48.176	ID=Ciclev10028922m.g
NPGA	S08_5052181	5.48E-08	0.50	7.26	8	5052181	46.436	ID=Ciclev10030203m.g
NPGA	S08_6026790	5.82E-08	0.49	7.24	8	6026790	53.506	ID=Ciclev10027952m.g
NPGA	S08_2971896	6.58E-08	0.47	7.18	8	2971896	37.459	ID=Ciclev10027850m.g
NPGA	S08_5896067	9.65E-08	0.46	7.02	8	5896067	51.75	ID=Ciclev10029285m.g
NPGA	S08_2107212	1.03E-07	0.47	6.99	8	2107212	28.512	ID=Ciclev10028015m.g
NPGA	S08_3026111	1.10E-07	0.48	6.96	8	3026111	32.615	ID=Ciclev10028435m.g
NPGA	S08_1716603	1.22E-07	0.45	6.91	8	1716603	23.197	ID=Ciclev10028052m.g
NPGA	S08_3000113	1.54E-07	0.45	6.81	8	3000113	37.459	ID=Ciclev10027863m.g
NPGA	S08_2818887	2.03E-07	0.44	6.69	8	2818887	30.267	ID=Ciclev10029198m.g
NPGA	S08_2854197	2.11E-07	0.44	6.68	8	2854197	30.267	ID=Ciclev10030127m.g
NPGA	S08_2844093	2.13E-07	0.44	6.67	8	2844093	30.267	ID=Ciclev10027715m.g
NPGA	S08_2635803	2.13E-07	0.44	6.67	8	2635803	30.267	ID=Ciclev10027853m.g
NPGA	S08_2720245	2.13E-07	0.44	6.67	8	2720245	30.267	ID=Ciclev10028040m.g
NPGA	S08_4871092	3.18E-07	0.48	6.50	8	4871092	46.436	ID=Ciclev10029936m.g
NPGA	S08_2788922	3.89E-07	0.43	6.41	8	2788922	30.267	ID=Ciclev10029184m.g
NPGA	S08_1796166	5.26E-07	0.42	6.28	8	1796166	23.197	ID=Ciclev10030194m.g
NPGA	S08_2850376	5.87E-07	0.44	6.23	8	2850376	30.267	ID=Ciclev10027734m.g
NPGA	S08_2188117	6.83E-07	0.40	6.17	8	2188117	28.512	ID=Ciclev10029597m.g
NPGA	S08_2289097	1.12E-06	0.40	5.95	8	2289097	28.512	ID=Ciclev10028343m.g
NPGA	S08_2263871	1.21E-06	0.40	5.92	8	2263871	28.512	ID=Ciclev10028649m.g

Trait	Marker	p-value	R2	LOD score	Linkage Group	Phisical position	Genetic position	Gene
NPGA	S08_2172917	1.21E-06	0.40	5.92	8	2172917	28.512	ID=Ciclev10029274m.g
NPGA	S08_1493068	2.38E-06	0.38	5.62	8	1493068	0	ID=Ciclev10028513m.g
NPGA	S08_1422214	3.21E-06	0.37	5.49	8	1422214	0	ID=Ciclev10028083m.g
NPGA	S08_1359809	3.62E-06	0.38	5.44	8	1359809	0	ID=Ciclev10028967m.g
NPGA	S08_2529040	3.84E-06	0.38	5.42	8	2529040	27.705	ID=Ciclev10029973m.g
NPGA	S08_1411532	3.90E-06	0.36	5.41	8	1411532	21.442	ID=Ciclev10027748m.g
NPGA	S08_1433038	3.90E-06	0.36	5.41	8	1433038	21.442	ID=Ciclev10028868m.g
NPGA	S08_1649129	4.25E-06	0.38	5.37	8	1649129	0	ID=Ciclev10027661m.g
NPGA	S08_1605876	6.60E-06	0.35	5.18	8	1605876	21.442	ID=Ciclev10028366m.g
NPGA	S08_2429542	9.25E-06	0.35	5.03	8	2429542	26.771	ID=Ciclev10028186m.g
NPGA	S08_1640724	1.11E-05	0.35	4.95	8	1640724	21.442	ID=Ciclev10027822m.g
NPGA	S08_1618894	1.12E-05	0.35	4.95	8	1618894	21.442	ID=Ciclev10030142m.g
NPGA	S08_1814724	1.55E-05	0.36	4.81	8	1814724	23.197	ID=Ciclev10029877m.g
NPGA	S08_19394032	2.53E-05	0.35	4.60	8	19394032	93.334	ID=Ciclev10028229m.g
NPGA	S08_1241936	2.63E-05	0.32	4.58	8	1241936	19.688	ID=Ciclev10029765m.g
NPGA	S08_17500054	4.05E-05	0.32	4.39	8	17500054	67.714	ID=Ciclev10028402m.g
NPGA	S08_689245	4.22E-05	0.31	4.37	8	689245	7.215	ID=Ciclev10028093m.g
NPGA	S08_1165513	4.55E-05	0.31	4.34	8	1165513	16.115	ID=Ciclev10028863m.g
NPGA	S08_1214214	4.62E-05	0.31	4.33	8	1214214	19.688	ID=Ciclev10029600m.g
NPGA	S08_864884	5.38E-05	0.29	4.27	8	864884	7.215	ID=Ciclev10029529m.g
PE	S01_25165173	1.97E-17	0.95	16.71	1	25165173	119.322	ID=Ciclev10007884m.g
PE	S01_25331390	1.97E-17	0.95	16.71	1	25331390	122.99	ID=Ciclev10010155m.g
PE	S01_25497528	9.56E-15	0.92	14.02	1	25497528	126.64	ID=Ciclev10008781m.g
PE	S01_25690547	1.34E-13	0.91	12.87	1	25690547	126.64	ID=Ciclev10007867m.g
PE	S01_23110907	1.51E-10	0.81	9.82	1	23110907	101.361	ID=Ciclev10009869m.g
PE	S01_23259161	4.73E-09	0.71	8.33	1	23259161	101.361	ID=Ciclev10010406m.g
PE	S01_23162602	1.23E-08	0.71	7.91	1	23162602	101.361	ID=Ciclev10010287m.g
PE	S01_24116702	1.23E-08	0.71	7.91	1	24116702	109.636	ID=Ciclev10007802m.g
PE	S01_24329530	1.29E-08	0.68	7.89	1	24329530	109.636	ID=Ciclev10007904m.g
PE	S01_23431732	2.80E-08	0.70	7.55	1	23431732	101.361	ID=Ciclev10007691m.g
PE	S01_22694645	1.01E-07	0.63	7.00	1	22694645	95.277	ID=Ciclev10010199m.g
PE	S01_24525941	4.53E-07	0.65	6.34	1	24525941	111.35	ID=Ciclev10008687m.g
PE	S01_22975873	4.93E-07	0.61	6.31	1	22975873	95.277	ID=Ciclev10007536m.g
PE	S01_27512849	2.09E-06	0.60	5.68	1	27512849	139.953	ID=Ciclev10009728m.g
PE	S01_18009707	2.84E-06	0.59	5.55	1	18009707	79.356	ID=Ciclev10007869m.g
PE	S01_26030989	5.30E-06	0.56	5.28	1	26030989	129.05	ID=Ciclev10007933m.g
PE	S01_26060166	6.18E-06	0.51	5.21	1	26060166	129.05	ID=Ciclev10007734m.g
PE	S01_22449826	6.47E-06	0.56	5.19	1	22449826	84.55	ID=Ciclev10009601m.g
PE	S01_26102810	1.16E-05	0.50	4.93	1	26102810	129.05	ID=Ciclev10007497m.g
PE	S01_26796432	1.32E-05	0.49	4.88	1	26796432	130.746	ID=Ciclev10010390m.g
PE	S01_26815936	1.32E-05	0.49	4.88	1	26815936	130.746	ID=Ciclev10008911m.g
PE	S01_17831378	1.44E-05	0.51	4.84	1	17831378	79.356	ID=Ciclev10009061m.g
PE	S01_27687717	1.47E-05	0.52	4.83	1	27687717	143.483	ID=Ciclev10007320m.g
PE	S01_22281840	2.55E-05	0.50	4.59	1	22281840	82.836	ID=Ciclev10007873m.g
PE	S01_25990600	3.10E-05	0.52	4.51	1	25990600	129.05	ID=Ciclev10007524m.g

Supplementary Table 6. Gene annotations in the assembled sequence of the genomic region of chromosome 8 associated with male sterility. Annotations related to pollen development are indicated in red letters. (https://phytozome-next.jgi.doe.gov). Number in brackets close to the gene identifier indicates that it contains a SNP marker. (1) S08_6026790; (2) S08_6050573 and (3) S08_6142645.

Gene identifier	Initial position	Final position	Size	Sense	Functional anotations in Phytozome
Ciclev10029967m.g	5913054	5914658	1604	forward	ATP binding;nucleic acid binding;helicases
Ciclev10030261m.g	5923981	5925498	1517	forward	Malectin/receptor-like protein kinase family protein
Ciclev10028169m.g	5939929	5946922	6993	reverse	SUMO-activating enzyme 2
Ciclev10027795m.g	5950969	5956596	5627	forward	SNF2 domain-containing protein / helicase domain-containing protein
Ciclev10030207m.g	5957670	5958563	893	reverse	Tetratricopeptide repeat (TPR)-like superfamily protein
Ciclev10030265m.g	5969535	5970491	956	reverse	Pentatricopeptide repeat (PPR) superfamily protein
Ciclev10028132m.g	5985063	5987794	2731	reverse	Tetratricopeptide repeat (TPR)-like superfamily protein
Ciclev10028124m.g	5991003	5994224	3221	reverse	ATP binding;nucleic acid binding;helicases
Ciclev10029744m.g	6024237	6025801	1564	reverse	Pentatricopeptide repeat (PPR) superfamily protein
Ciclev10027952m.g (1)	6025802	6031497	5695	reverse	BEL1-like homeodomain 6
Ciclev10028838m.g	6041184	6043615	2431	reverse	alpha/beta-Hydrolases superfamily protein
Ciclev10027768m.g (2)	6047429	6054970	7541	forward	RNA-binding (RRM/RBD/RNP motifs) family protein
Ciclev10030257m.g	6057618	6062060	4442	forward	beta-galactosidase 3
Ciclev10029260m.g	6065092	6068908	3816	forward	PHD finger family protein / bromo-adjacent homology domain-cont.protein
Ciclev10028263m.g	6068944	6075685	6741	reverse	Transducin/WD40 repeat-like superfamily protein
Ciclev10028320m.g	6076663	6079985	3322	reverse	purple acid phosphatase 10
Ciclev10029917m.g	6109131	6109451	320	forward	Papain family cysteine protease
Ciclev10028369m.g	6124675	6128024	3349	reverse	purple acid phosphatase 10
Ciclev10028670m.g (3)	6141140	6143433	2293	forward	Papain family cysteine protease
Ciclev10027828m.g	6146653	6154380	7727	reverse	vacuolar proton ATPase A3
Ciclev10027807m.g	6157357	6162759	5402	reverse	Minichromosome maintenance (MCM2/3/5) family protein
Ciclev10029805m.g	6178781	6179651	870	reverse	Lateral root primordium (LRP) protein-related

Gene identifier	Initial position	Final position	Size	Sense	Functional anotations in Phytozome
Ciclev10027697m.g	6193646	6199421	5775	reverse	Coatomer, alpha subunit
Ciclev10029294m.g	6253006	6254421	1415	forward	B-box zinc finger family protein
Ciclev10027716m.g	6255916	6268429	12513	reverse	Kinesin motor family protein
Ciclev10028302m.g	6276551	6280582	4031	forward	xylulose kinase-1
Ciclev10028627m.g	6289477	6292850	3373	forward	RNA-binding CRS1 / YhbY (CRM) domain protein
Ciclev10028123m.g	6293020	6297887	4867	reverse	MATE efflux family protein
Ciclev10028133m.g	6298166	6304194	6028	reverse	MATE efflux family protein
Ciclev10028537m.g	6315013	6322458	7445	forward	squalene synthase 1
Ciclev10029611m.g	6322767	6323840	1073	reverse	SH3 domain-containing protein
Ciclev10029446m.g	6326415	6328971	2556	reverse	SH3 domain-containing protein
Ciclev10030242m.g	6355458	6359023	3565	forward	ATP binding;nucleic acid binding;helicases
Ciclev10028481m.g	6373589	6374942	1353	forward	Pentatricopeptide repeat (PPR) superfamily protein
Ciclev10028233m.g	6402840	6404443	1603	forward	ATP binding;nucleic acid binding;helicases
Ciclev10030082m.g	6411573	6413114	1541	forward	ATP binding;nucleic acid binding;helicases
Ciclev10030363m.g	6432107	6432927	820	forward	S-adenosyl-L-homocysteine hydrolase
Ciclev10030279m.g	6434300	6435849	1549	forward	Pentatricopeptide repeat (PPR) superfamily protein
Ciclev10029914m.g	6451978	6453577	1599	forward	Pentatricopeptide repeat (PPR) superfamily protein
Ciclev10030131m.g	6457393	6459217	1824	forward	Subtilisin-like serine endopeptidase family protein
Ciclev10029947m.g	6460563	6461664	1101	forward	ATP binding;nucleic acid binding;helicases
Ciclev10027857m.g	6463695	6468742	5047	forward	Subtilase family protein
Ciclev10028860m.g	6474195	6475882	1687	forward	GATA transcription factor 5
Ciclev10029925m.g	6480797	6481843	1046	reverse	homolog of human DNA ligase iv-binding protein XRCC4
Ciclev10030000m.g	6520094	6520453	359	forward	myb domain protein 10
Ciclev10028122m.g	6535912	6540574	4662	forward	ferulic acid 5-hydroxylase 1
Ciclev10029230m.g	6554902	6560226	5324	reverse	Protein of unknown function (DUF1640)

Gene identifier	Initial position	Final position	Size	Sense	Functional anotations in Phytozome
Ciclev10029948m.g	6608855	6610058	1203	forward	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Ciclev10027673m.g	6632695	6643448	10753	reverse	nucleic acid binding;zinc ion binding;DNA binding
Ciclev10029089m.g	6651051	6656927	5876	reverse	zinc knuckle (CCHC-type) family protein
Ciclev10027856m.g	6659340	6662205	2865	reverse	Glycosyl hydrolase family protein
Ciclev10028301m.g	6681476	6683886	2410	forward	glycosyl hydrolase 9B18
Ciclev10028594m.g	6689819	6692205	2386	forward	fructose-bisphosphate aldolase 2
Ciclev10029284m.g	6700441	6703748	3307	reverse	B-box type zinc finger family protein
Ciclev10027758m.g	6720459	6728359	7900	forward	ATP binding microtubule motor family protein
Ciclev10029601m.g	6735308	6738952	3644	forward	LYR family of Fe/S cluster biogenesis protein
Ciclev10028728m.g	6744257	6749656	5399	forward	NAD(P)-binding Rossmann-fold superfamily protein
Ciclev10028844m.g	6750503	6754741	4238	reverse	ubiquitin fusion degradation 1
Ciclev10030361m.g	6757857	6759743	1886	reverse	ATP binding;nucleic acid binding;helicases
Ciclev10029872m.g	6770440	6772359	1919	reverse	TTF-type zinc finger protein with HAT dimerisation domain
Ciclev10028181m.g	6790357	6792194	1837	reverse	ATP binding;nucleic acid binding;helicases
Ciclev10030145m.g	6805621	6806699	1078	reverse	ATP binding;nucleic acid binding;helicases
Ciclev10027873m.g	6834487	6837762	3275	reverse	arginine decarboxylase 1
Ciclev10029417m.g	6861446	6864004	2558	reverse	ATPase, F0/V0 complex, subunit C protein
Ciclev10030192m.g	6887388	6887976	588	forward	NAD(P)-linked oxidoreductase superfamily protein
Ciclev10028892m.g	6893732	6897309	3577	forward	NAD(P)-linked oxidoreductase superfamily protein
Ciclev10028796m.g	6897536	6901468	3932	reverse	3-oxo-5-alpha-steroid 4-dehydrogenase family protein

Supplementary Table 7. Genotypes for S08_6142645 SNP in different commercial mandarin cultivars.

Group		Variety	Reference	Male sterile cytoplasm	Phenotype	S08_6142645
Satsuma mandarin	*Citrus unshiu* (Mak.) Marc.	Okitsu	IVIA-195	yes	sterile	C:C
Clementine mandarin	*C. clementina* Hort. ex Tan.	Clemenules	IVIA-022	no	fertile	T:T
Mandarin	*C. nobilis* Lour.	Campeona	IVIA-193	no	fertile	T:T
Mandarin hybrid	C. clementina × C. tangerina	Fortune	IVIA-080	no	fertile	T:T
Mandarin hybrid	C. nobilis × C. deliciosa	Encore	IVIA-155	no	fertile	C:T
Mandarin hybrid	C. unshiu × C. nobilis	Kara	IVIA-218	yes	fertile	C:T
Mandarin hybrid	C. unshiu × Unkwown	Queen	IVIA-579	yes	sterile	C:C
Tangor	C. reticulata × C. sinensis	Ellendale	IVIA-194	no	fertile	T:T
Tangor	C. unshiu × C. sinensis	Kiyomi	IVIA-405	yes	sterile	C:C
Tangor	C. sinensis × Unkwown mandarin	Murcott	IVIA-196	no	fertile	C:T
Tangor	C. reticulata × C. sinensis	Nadorcott	IVIA-388	no	fertile	C:T

Supplementary Table 8. Phenotypes for polyembryony and genotypes for S01_25497528 SNP (SNP1) in a germplasm collection.

Group		Variety	Reference	Phenotype	SNP1
Satsuma mandarin	Citrus unshiu (Mak.) Marc.	Clausellina	IVIA-019	Poly	A:G
Satsuma mandarin	C. unshiu (Mak.) Marc.	Okitsu	IVIA-195	Poly	A:G
Clementine mandarin	C. clementina Hort. ex Tan.	Clemenules	IVIA-022	Mono	A:A
Clementine mandarin	C. clementina Hort. ex Tan.	Fina	IVIA-039	Mono	A:A
Clementine mandarin	C. clementina Hort. ex Tan.	Arrufatina	IVIA-058	Mono	A:A
Clementine mandarin	C. clementina Hort. ex Tan.	Oronules	IVIA-132	Mono	A:A
Clementine mandarin	C. clementina Hort. ex Tan.	Monreal	IVIA-459	Mono	A:A
Mandarin	C. temple Hort.	Temple	IVIA-081	Mono	A:A
Mandarin	C. tangerina Hort. ex Tan.	Parson's Special	IVIA-168	Poly	A:G
Mandarin	C. tangerina Hort. ex Tan.	Dancy	IVIA-434	Poly	A:G
Mandarin	C. reticulata Blanco	Anana	IVIA-390	Poly	A:G
Mandarin	C. reticulata Blanco	Emperor	IVIA-394	Poly	A:G
Mandarin	C. reticulata Blanco	Scarlet	IVIA-411	Poly	G:G
Mandarin	C. reticulata Blanco	Ponkan	IVIA-482	Poly	A:G
Mandarin	C. reticulata Blanco	Temple Hybrid	IVIA-634	Mono	A:A
Mandarin	C. nobilis Lour.	Campeona	IVIA-193	Mono	A:A
Mandarin	C. nobilis Lour.	King	IVIA-477	Poly	G:G
Mandarin	C. deliciosa Ten.	Común	IVIA-154	Poly	A:G
Mandarin	C. deliciosa Ten.	Tardivo Ciaculli	IVIA-186	Poly	A:G
Mandarin	C. deliciosa Ten.	Salteñita	IVIA-361	Poly	A:G
Mandarin	C. deliciosa Ten.	Común sin hueso	IVIA-383	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Sucreña	IVIA-032	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Sanguinelli	IVIA-034	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Newhall	IVIA-055	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Salustiana Gil	IVIA-125	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Lane Late	IVIA-198	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Shamouti	IVIA-270	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Tarocco	IVIA-271	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Valencia Late Delta	IVIA-363	Poly	A:G
Sweet Orange	C. sinensis (L.) Osb.	Maltaise Blonde	IVIA-391	Poly	A:G
Sweet Orange	C. aurantifum L.	Sevillano	IVIA-117	Poly	A:G
Sweet Orange	C. aurantifum L.	Bouquet de fleurs	IVIA-139	Poly	A:G
Grapefruit	C. paradisi Macf.	Star Ruby	IVIA-197	Poly	A:G
Grapefruit	C. paradisi Macf.	Duncan	IVIA-274	Poly	A:G
Grapefruit	C. paradisi Macf.	Rio Red	IVIA-289	Poly	A:G
Lemon	C. meyeri Y. Tan.	Meyer	IVIA-145	Mono	A:A
Lemon	C. limon (L.) Burm f.	Eureka Frost	IVIA-297	Poly	A:G
Lemon	C. jambhiri Lush.	Rugoso	IVIA-333	Poly	A:G
Lime	C. limonia Osb.	Rangpur	IVIA-334	Poly	A:G
Lime	C. limettioides Tan.	Dulce Palestina	IVIA-305	Poly	A:G
Citron	C. medica L.	Arizona	IVIA-169	Mono	A:A
Citron	C. medica L.	Diamante	IVIA-560	Mono	A:A
Citron	C. medica L.	Córcega	IVIA-567	Mono	A:A

Group		Variety	Reference	Phenotype	SNP1
Pummelo	C. grandis × C. grandis	Chandler	IVIA-207	Mono	A:A
Pummelo	C. grandis (L.) Osb.	Pink	IVIA-275	Mono	A:A
Pummelo	C. grandis (L.) Osb.	Azimboa	IVIA-420	Mono	A:A
Bergamot	C. bergamia Risso and Poit.	Calabria	IVIA-254	Mono	A:A
Mandarin hybrid	C. unshiu × C. nobilis	Kara	IVIA-218	Poly	A:G
Mandarin hybrid	C. unshiu × C. deliciosa	Simeto	IVIA-413	Poly	A:G
Mandarin hybrid	C. unshiu × C. deliciosa	Primosole	IVIA-414	Mono	A:A
Mandarin hybrid	C. nobilis × C. deliciosa	Wilking	IVIA-028	Mono	A:A
Mandarin hybrid	C. nobilis × C. deliciosa	Kinnow	IVIA-033	Poly	A:G
Mandarin hybrid	C. nobilis × C. deliciosa	Encore	IVIA-155	Mono	A:A
Mandarin hybrid	C. nobilis × C. deliciosa	Honey	IVIA-209	Mono	A:A
Mandarin hybrid	C. clementina × C. tangerina	Fortune	IVIA-080	Mono	A:A
Mandarin hybrid	C. clementina × C. reticulata	Fremont	IVIA-082	Poly	A:G
Mandarin hybrid	C. clementina × C. nobilis	Palazzelli	IVIA-188	Mono	A:A
Mandarin hybrid	C. clementina × (C. unshiu × C. nobilis)	Moncada	IVIA-421	Mono	A:A
Mandarin hybrid	C. clementina × (C. unshiu × C. nobilis)	N-27	IVIA-423	Poly	A:G
Mandarin hybrid	C. clementina × (C. paradisi × C. tangerina)	Fairchild	IVIA-083	Poly	A:G
Mandarin hybrid	C. clementina × (C. paradisi × C. tangerina)	Nova	IVIA-074	Poly	A:G
Mandarin hybrid	[C. clementina × (C. paradisi × C. tangerina)] × C. temple	Fallglo	IVIA-466	Mono	A:A
Mandarin hybrid	[C. clementina × (C. paradisi × C. tangerina)] × [C. clementina × (C. paradisi × C. tangerina)]	Sunburst	IVIA-200	Poly	A:G
Mandarin hybrid	(C. paradisi × C. tangerina) × C. clementina	Page	IVIA-079	Poly	A:G
Mandarin hybrid	(C. clementina × C. tangerina) × (C. clementina × C. reticulata)	Daisy	IVIA-362	Mono	A:A
Tangor	C. unshiu × C. sinensis	Umatilla	IVIA-100	Mono	A:A
Tangor	C. unshiu × C. sinensis	Kiyomi	IVIA-405	Mono	A:A
Tangor	C. tangerina × C. sinensis	Dweet	IVIA-165	Poly	A:G
Tangor	C. sinensis × Unkwown mandarin	Murcott	IVIA-196	Poly	A:G
Tangor	C. reticulata × C. sinensis	Ellendale	IVIA-194	Mono	A:A
Tangor	C. reticulata × C. sinensis	Ortanique	IVIA-276	Poly	A:G
Tangelo	C. paradisi × C. tangerina	Minneola	IVIA-084	Poly	A:G
Tangelo	C. reticulata × C. paradisi	Ugli	IVIA-689	Mono	A:A
Tangelo	C. paradisi × C. tangerina	Orlando	IVIA-101	Poly	A:G
Tangelo	C. paradisi × C. tangerina	Seminole	IVIA-348	Poly	A:G
Citrange	C. sinensis × P. trifoliata	Troyer	IVIA-386	Poly	A:G
Citrumelo	C. paradisi × P. trifoliata	CPB 4475	IVIA-208	Poly	A:A
Fortunella	F. polyandra (Ridl.) Tan.	Malayan Kumquat	IVIA-375	Poly	A:A
Fortunella	F. japonica (Thumb.) Swing.	Round Kumquat	IVIA-381	Poly	A:A
Fortunella	F. hindsii (Champ.) Swing.	Hong Kong Kumquat	IVIA-281	Mono	A:A
Fortunella	F. crassifolia Swing.	Meiwa Kumquat	IVIA-280	Poly	A:A
Poncirus	Poncirus trifoliata (L.) Raf.	Rubidoux	IVIA-217	Poly	A:A
Poncirus	P. trifoliata (L.) Raf.	Flying dragon	IVIA-537	Poly	A:A

n: number of seeds evaluated; PE (%): percentage of polyembryonic seeds; Phenotype: Mono (monoembryonic) or Poly (polyembryonic).

General discussion

In this general discussion we mainly draw on the specific discussions of the papers presented in the central part of the PhD dissertation. Thus, our aim in this section is discussing the links between them and synthesising the main findings and their significance in the research context established in the Introduction.

Background

Citrus reproductive biology is complex and often involves female and male sterility, self-incompatibility (SI) and apomixis in different degrees. During the reproductive cycle, a key phase in achieving successful mating is the progamic phase which elapses from pollination to fertilization. In this period, specific interactions between the male gametophyte and the pistil occur and is extremely vulnerable to prevailing environmental conditions (Hedhly, 2011). Seedlessness is one of the main objectives of mandarin breeding and parthenocarpic ability (PA) coupled with SI is one of the main mechanisms for seedless mandarin production. A thorough review of the research devoted to the PA and SI in some mandarins evidences that the information available now is still insufficient and, in some cases, contradictory. Beyond the critical importance of SI for seedless fruit production, SI is an obstacle for breeding programs based on hybridization as it reduces crossing possibilities. In other genera, the breakdown of SI has been reported to be caused by temperature stress, bud pollination and polyploidization. However, very few studies on the breakdown of the SI reaction have been carried out in citrus and some of them present partial results. Male sterility is a desirable trait for seedless breeding and polyembryony is crucial reproductive feature to take on board in breeding for both rootstocks and varieties. In recent years SNP markers have emerged as an indispensable tool in genetic applications and breeding programs. However, no SNPs are available for male-sterility and polyembryony in citrus which would be very useful for marker-assisted selection (MAS) in breeding programs.

As many of the results presented in this PhD dissertation are based on histological observations, the first step was to develop a method that allows a complete and representative study of the specific interactions between the male and female counterparts occurred during the progamic phase. It permitted (i) to acquire accurate knowledge about the influence of temperature in important events that take place during the progamic phase including pollen performance –pollen grain germination (PGG) and pollen tube growth (PTG)–, pistil degeneration –stigmatic receptivity (SR) and ovule degeneration (OD)– and its interaction. We (ii) developed an efficient protocol based on emasculation, hand self-pollination and hand cross-pollination and applied it to study the PA and SI in nine mandarin varieties. We also (iii) compared the efficiency of the SI reaction breakdown by temperature stress, bud pollination and tetraploidy. Finally, (iv) we have developed two SNPs markers –based on KASPar technology– associated to polyembryony and male sterility.

Temperature strongly affects citrus progamic phase

To assess the impact of temperature in the programic phase, we used three constant temperature regimes –10ºC, 20ºC and 30ºC– representing cool to warm Mediterranean temperatures during citrus flowering period. The results obtained in chapter 1 show how each process in the progamic phase –stigmatic receptivity (SR), ovule degeneration (OD), pollen grain germination (PGG) and pollen tube growth (PTG)– is affected by

temperature. In chapter 3, our results show how temperature stress causes the breakdown of the SI reaction. These interesting findings points to the potential effects that current global climate change may cause on the reproductive biology of plants.

In chapter 1, we have shown that the 'Fortune' stigma is receptive at anthesis. This is of great practical value for citrus breeding programs based on sexual hybridization, as effective pollination can take place when the flowers are at anthesis, which facilitates the process. This also occurs in other woody species such as peach, sweet cherry and kiwi (Sanzol and Herrero, 2001), while post-anthesis maturation is necessary for optimal SR in almond (YI et al., 2006). In all temperature regimes used in this study, pistil senescence began with loss of SR, followed by OD and finally by ovary style abscission. We performed the evaluation of the SR *in vivo*, observing both the PGG on the stigmatic surface and the PTG inside the stigma. This methodology provides better understanding of the effects of the stigmatic degeneration in citrus. The percentage of germinated pollen grains progressively decreased as flowers were pollinated in the days following anthesis. Similarly, we observed that the number of pollen tubes growing in the stigma decreased. In all temperature regimes of this study, the ability of pollen tubes to grow is lost before the ability of pollen grains to germinate. Similar results have been found in sweet cherry (Hedhly et al., 2003) and peach (Hedhly et al., 2005). Taken together, our results show that temperature has a clear effect on pistil degeneration. Warm temperature regimes shorten the SR period and ovule life span, and anticipate ovary style abscission, whereas the cold temperature regime has the opposite effect.

Regarding the male counterpart, we observed PGG at 10ºC for all three pollen genotypes studied, which disagrees with previous studies in citrus. This discrepancy may be because PGG analyses were carried out directly on the stigmatic surface *in planta*, rather than previous methods that tested for PGG *in vitro*. Differences in the PGG rate have also been shown between *in vitro* and *in vivo* tests on tobacco (Shivanna et al., 1991) and sweet cherry (Hormaza and Herrero, 1999). This suggests that *in vitro* germination media do not provide the optimal conditions afforded by the stigma. In contrast, analysis of *in planta* pollen grain germination seems to be a more representative method.

The evaluation of three pollen genotypes allowed the influence of temperature, genotype, as well as their interaction, to be assessed. Moreover, as the samples were fixed every day since pollination, the daily progression of the distance traveled by pollen tubes from the stigma surface through the pistil could be observed. Thus, our results describe both dynamics and kinetics of PTG.

In all three genotypes, warm temperatures of 30ºC accelerate the rate of PTG, reducing the time required by pollen tubes to reach the ovules, whereas low temperature of 10ºC produced the opposite effect. PTG kinetics under field conditions and 20ºC differed significantly in the three genotypes, which may be unexpected since the average temperature under field conditions (18.5ºC) was close to 20ºC in the growth chamber. However, this could be due to a temperature drop around 10ºC for several hours per day in the FC regime, which slowed down the PTG kinetics.

Our results show differences in the optimal temperature for PGG and PTG kinetics, consistent with the previously reported independence of these processes (Distefano et al., 2012; Kakani et al., 2002; Mckee and Richards, 1998). Among the 12 pollen-temperature combinations analysed, the largest differences in both PGG and PTG were observed between 'Pineapple' and 'Ichang' at 10ºC. The poorer pollen performance observed in 'Pineapple', in contrast to the best performance observed in 'Ichang', may

be associated with previously reported high frost sensitivity of ‘Pineapple’ and the cold resistance of ‘Ichang’ (Hodgson, 1967). However, no significant differences were observed between genotypes at the warmest temperature in our study (30°C).

Under temperature stress, male-female synchrony is achieved for successful mating

For the reproductive process to be successful, pollen grains must germinate, and pollen tubes must grow to transport the male gametophytes to the ovules and fertilize them. Loss of SR, abscission of the style and OD are processes related to the pistil senescence. This senescence does not depend on pollen performance but is strongly influenced by temperature as reported here. The effective pollination period (EPP) determines the number of days in which pollination can produce seed-bearing fruits. Mesejo et al. (2007) determined the EPP in ‘Owari’, ‘Clemenules’ and ‘Valencia’. These authors reported that OD is the major factor limiting EPP for ‘Owari’ while SR is the major factor limiting EPP for ‘Clemenules’ and ‘Valencia’. Genotype-dependent differences were also described in other species by Sanzol and Herrero (2001).

Here we show for ‘Fortune’ that SR is the first to degenerate in all temperature regimes studied, being the main limiting factor of the EPP. However, EPP was apt because pollen tubes were able to reach the ovules in all combinations evaluated in the 10 experimental days, except for the cross ‘Fortune’ x ‘Pineapple’ at 10ºC. But considering as neither OD nor pistil abscission was observed at this temperature, it could be possible that the ‘Pineapple’ pollen tubes reached the ‘Fortune’ ovaries after the 10 days of the experiment.

Under the temperature span studied, our results show that performing pollinations at anthesis, the crosses evaluated were able to maintain the male-female synchrony described as necessary for mating success (Herrero, 2003). This plasticity is reflected in the fact that citrus plants are grown in 147 countries worldwide (FAOSTAT, 2020), and approximately between latitudes of 40°N and 40°S comprising tropical, subtropical and cooler zones. This adaptation provides an opportunity for additional selection pressure to occur during this reproductive phase, as has been reported for other species, in which the PGG and PTG response to temperature stress have been used to select genotypes tolerant to both high and low temperatures and also to transfer this tolerance to the offspring (Zamir et al. 1982; Kakani et al. 2002; Domínguez et al. 2005; Liu et al. 2006).

An efficient protocol to evaluate the parthenocarpic ability and the self-incompatibility has been implemented.

SI or self-compatibility (SC), has been determined based on the presence or absence of pollen tubes reaching the ovaries or based on fruit production with or without seeds from self-pollinated flowers. Considering our previous results for PTG under field conditions, we performed the histological observations in pistils fixed ten days after pollination. In addition, the evaluation of the number of seeds per fruit allows not only to confirm the histological observations, but also to recover plants and analyse their genetic origin by SSRs markers.

For PA identification, we tested the ability of each genotype to produce seedless fruits or not. Under natural conditions, PA in self-incompatible genotypes is easily identified by

avoiding cross-pollination. However, in self-compatible genotypes, PA can only be determined by emasculating and bagging. In addition, a high or low degree of PA was evaluated by comparing the percentages of fruit set between cross-pollinated flowers that produced only seeded fruit and emasculated flowers that produced only seedless fruits. Since emasculating and bagging prevent pollination stimulus, the fruit set obtained from emasculated flowers (which are mandatorily seedless) was evaluated to test the requirement of pollination stimulus for fruit set.

In citrus, competition between flowers results in a marked drop of flowers and fruitlets (Agustí et al., 1982). Along with several factors that affect fruit set, such as flower load, inflorescence type and flower position (Garcia-Papi and Garcia-Martinez, 1984), more research is needed using a large number of flowers on different trees to evaluate reliable PA data. The results presented here are supported by the large number of flowers treated and therefore provide consistent PA data. We propose a protocol based on emasculation, hand self-pollination and hand cross-pollination to analyze fruit setting and seed production, and to observe pollen performance. We also suggest that PA can be assessed using 50 flowers in the emasculation and cross-pollination treatments, making this protocol more feasible to identify those varieties with higher PA.

A great variability of parthenocarpic behaviour was revealed among nine mandarins.

By applying the protocol above, we classified nine mandarin varieties according to the four types of parthenocarpy described in citrus by Vardi et al. (2008). These authors make a distinction between obligatory parthenocarpy –for those varieties that always produce seedless fruit–, and facultative parthenocarpy, in which seedless fruits are produced when cross-pollination with compatible pollen sources is prevented. In addition, a distinction is made between vegetative parthenocarpy (also called autonomous parthenocarpy) to refer to seedless fruits that develop without requiring any external stimulus, and stimulative parthenocarpy, which requires the pollination stimulus for seedless fruit set. Among the nine mandarins studied, only ‘Monreal’ was unable to produce seedless fruits, suggesting its lack of PA.

We classified `Clemenules´ and `Moncada´ both as vegetative and facultative parthenocarpic varieties. In the case of `Clemenules´, this result agrees with the pollination-independent facultative parthenocarpy proposed by Mesejo et al. (2013), while it questions its classification as stimulative parthenocarpic variety reported by Vardi et al. (2000).

In self-pollinated flowers of `Ellendale´ and `Imperial´, pollen tubes were observed throughout the stigma, and the growth of most of them stopped at the upper style, although some pollen tubes reached the ovaries in small percentages. In addition, these varieties produced both seedless and low-seeded fruits from self-pollinated flowers. Since the average number of seeds per fruit was 0.8 and 0.7 for ‘Imperial’ and ‘Ellendale’ respectively, we suggest that they are facultative parthenocarpic, but note that low-seeded fruits can be produced even if cross-pollination is avoided. Thus, we classified ‘Ellendale’ and ‘Imperial’ as vegetative and facultative parthenocarpic varieties without strict SI. In the case of `Ellendale´, our results for self-pollinated flowers are in line with the low-seeded fruits from self-pollinated flowers obtained by Vithanage (1991), although this author considered `Ellendale´ as a self-compatible and non-parthenocarpic genotype. However, other authors reached opposite conclusions, such as the stimulative

parthenocarpy reported by Vardi et al. (2000) and the vegetative (autonomous) parthenocarpy reported by Sykes (2008b).

The varieties ‘Campeona’ and ‘Salteñita’ are self-compatible, thus producing seeded fruits from all self-pollinated flowers. However, they were able to produce seedless fruits when self-pollination was avoided by emasculation. This means that they have PA and do not need a pollination stimulus to set seedless fruits. As the term parthenocarpy is used to refer to seedless fruit production, self-compatible varieties can be classified as non-parthenocarpic. However, since ‘Campeona’ and ‘Salteñita’ possess PA, classifying these varieties as non-parthenocarpic can be confusing. Under natural conditions, self-compatible genotypes produce seeded fruits even if cross pollination is avoided and seedless fruit can be recovered only from emasculation, which must be done by hand. This explains why the scientific literature only reports them as seed-bearing varieties (Hodgson, 1967) and why there is no information available about PA. We classify ‘Campeona’ and ‘Salteñita’ as self-compatible varieties with PA, which provides relevant information on the parthenocarpy of these varieties.

Satsuma varieties are generally considered to be female sterile. However, we report an average of 5.3 seeds per fruit from cross-pollinated ‘Serafines’ flowers. Thus, taking into account the stricter meaning of obligatory and facultative parthenocarpy, it should be considered as facultative parthenocarpy. In fact, satsumas have been used in breeding programs as parents in different countries, especially in Japan (Omura and Shimada, 2016).

In addition to the parthenocarpy classification performed, the high or low degree of PA was evaluated by comparing fruit setting percentages between emasculation and cross-pollination. In ‘Clemenules’, ‘Campeona’, ‘Imperial’, ‘Salteñita’, ‘Fortune’, ‘Moncada’, and ‘Ellendale’, fruit set percentages obtained by cross pollination were higher than those obtained by emasculation, indicating that the presence of fertilized ovules strongly influenced fruit set.

In contrast, ‘Serafines’ showed no differences, implying higher PA in this variety. When comparing satsuma and clementine, previous research has associated high levels of endogenous GA in developing ovaries of satsuma with greater PA, while clementine produced lower GA levels and showed lower PA (Mesejo et al., 2016; Talon et al., 1992). Our results showed ‘Serafines’ as the variety with highest PA in which seed production did not seem crucial for fruit set.

The knowledge presented here on SI and PA is of great importance for the selection of parents that will be used in sexual hybridizations and for the selection of candidate-select seeded diploid varieties with the objective to remove seeds by irradiation.

Bud pollination is the most effective strategy to breakdown the SI reaction

In this PhD dissertation, we used two self-incompatible genotypes, ‘Fortune’ and ‘Clemenules’, to compare the effectiveness of the SI reaction breakdown caused by three factors previously identified in other plant species: temperature stress, bud pollination and polyploidization. Our results showed that these three factors were successful in recovering selfed plants, and that bud pollination was the most effective for obtaining a high number of hybrids from self-incompatible crosses. In addition, the selfing origin of the recovered plants was demonstrated with SSR and SNPs markers.

As mentioned above in this discussion, one interesting effect of temperature during progamic phase is the breakdown of the SI reaction. We observed a few pollen tubes reaching the ovaries in self-pollinated flowers of 'Fortune' when the progamic phase took place at a constant temperature of 10°C coinciding with the hypothesis put forward by Distefano et al. (2018) who suggest that low temperatures delay pistil maturation, and immature tissues allow pollen tube growth to the ovaries. In this sense, Aloisi et al. (2020) indicated that temperature contributed to a different activation of the SI reaction in *C. clementina*, which occurred at an optimum temperature of 25 °C and did not occur at 15 °C. For the first time in citrus, we recovered plants from `Fortune´ self-pollinated flowers at 10°C. When the progamic phase took place at a constant temperature of 30°C, we also observed very few pollen tubes (fewer than at 10ºC) reaching the basal pistil sections in some self-pollinated flowers of 'Fortune'. Although our observations suggest that warm temperature may cause SI rupture, we did not recover fruits, and consequently, seed presence could not be assessed. In a previous research work on the influence of high temperatures on SI reaction in citrus, Kawano et al. (2016) reported that pollen tubes reached the base of styles in self-pollinated flowers at 30 °C in a self-incompatible 'Hyuganatsu' mandarin. In contrast, Distefano et al. (2018, 2012) indicated that constant temperatures at 30 °C did not affect the SI reaction in clementines. These conflicting results may suggest that the breakdown of SI reaction in citrus is genotype dependent. However, more research is needed to shed light on the molecular mechanisms that take place.

Chromosome doubling was more effective than temperature stress in the recovery of selfed plants, but it involved the development of tetraploid plants, which is only of interest within the framework of breeding programs for obtaining triploid varieties or tetraploid rootstocks. Considering gametophytic self-incompatibility and tetraploid plants recovered from self-incompatible diploid plants (S1S2), the genetic configuration for the *S* locus should be S1S1S2S2, thus producing diploid pollen with either two identical *S* alleles or two different *S* alleles. It has been proposed in the genera *Petunia* (Golz et al., 2000), *Pyrus* (Crane and Lewis, 1942) and *Malus* (Adachi et al., 2009) that pollen tube growth stops when the pollen grain is homozygous for one *S* allele (S1S1 or S2S2), whereas heteroallelic (S1S2) pollen can grow through the pistil. For example, tetraploid progeny recovered from a self-pollinated autotetraploid plant is expected to be self-compatible and heterozygous at the S locus with the following genetic configuration: S1S1S1S2, S1S1S2S2 or S1S2S2S2.

SI breakdown caused by bud self-pollination was reported in 'Fortune' mandarin by Distefano et al. (2009b). These authors based their results on histological observations, while Wakana et al. (2004) did so in clementine based on seeded fruit production. Our results consistently confirmed these previous studies because they are based on histological observations, seed production, and also on demonstration the genetic origin of the recovered plants. In addition, our results showed differences in the effectiveness of the SI reaction breakdown depending on the developmental stage –expressed as days before anthesis (DBA)– of the pollinated buds. A high number of pollen tubes was observed reaching the ovaries when pollinating buds between 7 and 3 DBA. Fewer than five pollen tubes were observed reaching the ovaries when pollinating buds between 2 and 1 DBA. No pollen tubes were observed in bud flowers very close to anthesis (<1DBA). Our results are consistent with the hypothesis stating that SI machinery is not active in the bud developmental stage (Cabin et al., 1996). Our results also suggest that for mandarins SI machinery is activated between 2 DBA and anthesis. Therefore, self-pollination of buds 3 DBA or earlier appears to be a very effective method to produce

selfing progenies from self-incompatible genotypes, thus increasing the possibilities for crossing in breeding programs.

Selfing progenies can also be of interest for genetic studies. Indeed, in the genetic analysis of tetraploid plants obtained from self-pollination of the tetraploid clementine 'Clemenules', we observed segregation distortion in three SNP markers: CiC1380-05, CiC5796-12 and CiC1749-05. Ollitrault et al. (2012) established the reference clementine genetic map and reported segregation distortion in CiC1380-05 SNP marker in both male and female clementine maps. However, no segregation distortion was observed for CiC5796-12 and CiC1749-05 SNP markers (Ollitrault et al., 2012). For these two markers, the segregation distortion observed in our analysis may be associated with the vicinity of genes involved in reproductive biology. CiC5796-12 (LG3, position 41,554,598) is located close to the Ciclev10023991m.g gene (LG3; position 42,587,792 to 42,588,197), which is involved with the plant SI protein *S*1. CiC1749-05 (LG8; position 24,429,013) is close to the Ciclev10030173m.g gene (LG8; position 24,433,990 to 24,437,012), which is associated with aberrant pollen development protein, according to the *C. clementina* v1.0 reference genome, available on the Phytozome platform ("Phytozome 13.," n.d.). In the genetic analysis of plants obtained from bud self-pollination of 'Fortune' mandarin, we observed segregation distortion in the NADK2-M285 SNP marker (LG5; position 37,772,763). This marker was not included in the clementine reference map. However, the segregation distortion observed in our study could be explained by the high segregation distortion in most of the LG5 reported by Ollitrault et al. (2012).

Efficient KASPar SNP markers have been developed for early selection for polyembryony and male sterility

Male sterility is a desirable trait for seedless breeding and polyembryony is crucial reproductive feature to take on board in breeding for both rootstocks and varieties. Marker-assisted selection (MAS) is a key in breeding programs, particularly in tree species with long juvenile period such citrus, because selection of target genotypes can be carried out at the seedling stage. Mechanisms involved in the biology of reproduction have been tagged with molecular markers including apomixis (García et al., 1999; Kepiro and Roose, 2010; Nakano et al., 2013, 2012; Wang et al., 2017) and male sterility (Goto et al., 2018). In recent years, molecular tagging techniques have evolved and next generation sequencing (NGS) technologies have facilitated the massive identification of SNPs markers in large populations. So, SNPs markers have emerged as an indispensable tool in genetic applications and breeding programs. However, no SNP markers are available to implement MAS for male-sterility and polyembryony.

With the aim of developing SNPs markers associated with male sterility and polyembryony, an offspring of 61 hybrids derived from the 'Kiyomi' x 'Murcott' cross was genotyped by GBS. Linkage analysis and genetic mapping were performed with the 6,444 segregating markers displayed by the GBS. Both 'Kiyomi' and 'Murcott' genetic linkage maps displayed high synteny and collinearity with the *C. clementina* V1.0 assembly which is consistent with the high synteny an collinearity between *Citrus* species reported previously (Bernet et al., 2010; Ollitrault et al., 2021, 2012; Yu et al., 2016). 'Murcott' and 'Kiyomi' tangors are interesting parents widely used for mandarin breeding and the high-density genetic maps presented here can be very useful in breeding programs and genetic studies.

Among the 61 genotyped hybrids, 53 flowered during the three-year experiment —which were phenotyped for male sterility— and 32 of them produced fruits bearing seeds —which were phenotyped for polyembryony—. Following the methodology proposed by Goto et al. (2016), male sterility phenotyping was based in two parameters: number of pollen grains per anther (NPGA) and apparent pollen fertility (APF). NPGA was the key factor causing male sterility in the studied population in line with the results reported by Goto et al. (2016) in a satsuma progeny. The release of pollen grains from anthers occurs when a certain NPGA is exceeded. In this regard Goto et al. (2018) assumed approximately 1300 NPGA as criterion of male sterility. Since we have observed pollen grain release in those anthers with more than 1000 NPGA, we have reduced the criterion of male sterility to 1000 NPGA.

Through genetic association studies, we identified a genomic region on chromosome 1 associated with polyembryony. The genomic region of 25165173-25690547 in chromosome 1 —defined by the markers included in a 5 cM interval each side of the marker with the higher signal of our association study— includes the Ciclev10010497m gene. A BLASTn search identified Ciclev10010497m as the homologue of the *CitRWP* gene reported by Wang et al. (2017) who demonstrated that the insertion of a MITE in close vicinity of *CitRWP* gene is responsible of polyembryony in mandarins. Therefore, our results are in full agreement with previous conclusions regarding the importance of *CitRWP* for polyembryony. We also identified a genomic region on chromosome 8 associated with NPGA. A search in the *C. clementina* v1.0 reference genome, available on the Phytozome platform ("Phytozome 13.," n.d.) showed that several genes identified in our association study are related to different biological pathways that may result in male sterility. The information generated from gene annotations allows to focus our efforts on 19 genes related to male sterility over the 67 genes annotated in the genomic region identified by the QTL analysis. This limited number will now allow to make affordable although time-consuming approaches to determine whether these genes are really involved in the male sterility and citrus interaction. Further experiments will be necessary to shed light on this complex trait of citrus reproductive biology.

Finally, one KASPar SNP marker closely linked with implied genes for each trait —SNP1 for polyembryony and SNP8 for male sterility— was developed and validated. The efficiency of MAS is directly liked to the vicinity of the used markers with the genes or factors directly implied in the expression of the targeted trait. To our knowledge, the SNP1 and SNP8 developed here are the first available to be successfully used for MAS in a wide range of citrus genotypes.

General conclusions and perspectives

1. Temperature has a great influence on both the male and the female counterparts and their interaction during the progamic phase. However, we have shown that the parental combinations tested were capable of responding to environmental changes and ensuring good fertilization levels. The knowledge generated in this PhD dissertation can be useful for improving pollination efficiency and adapting breeding programmes to the temperature forecasts during the pollination period.

2. The results reported in this PhD dissertation suggest that pollen performance-based screening may be a useful strategy to select better adapted citrus genotypes to different environmental conditions, and also to explore gametophytic selection within genotypes. In future research, it would be relevant to investigate the influence of temperature and genotype during gametogenesis. If coupled with our results obtained for the progamic phase, such investigation could be useful for enhancing the efficiency of citrus breeding programmes based on sexual hybridization, and in particular with those programmes aimed to obtain new varieties that can adapt to both colder areas and current areas becoming warmer due to global climate change.

3. We have developed an efficient protocol to characterize the self-incompatibility and different types of parthenocarpy. It is based on emasculation, hand self-pollination, and hand cross-pollination and the analysis of fruit setting, seed production, and histological observations of pollen performance. Our protocol can be applied for screening particular parents with previously identified interesting horticultural traits and candidate-selected seedy diploid varieties with the objective to remove seeds by irradiation.

4. We have applied this protocol to analyze the reproductive behavior of nine important citrus varieties used as parents for seedless mandarin breeding. We found that ‘Clemenules’ and ‘Moncada’ were strictly self-incompatible with facultative and vegetative parthenocarpy, ‘Imperial’ and ‘Ellendale’ displayed no strict self-incompatibility associated with facultative and vegetative parthenocarpy, ‘Fortune’ was self-incompatible with facultative and stimulative parthenocarpy, and ‘Campeona’ and ‘Salteñita’ were self-compatible but with vegetative PA. ‘Serafines’ satsuma was classified as associated male sterileity with facultative and vegetative parthenocarpy, while ‘Monreal’ clementine was not parthenocarpic. Reproductive behavior knowledge is important for optimizing seedless mandarin breeding programs.

5. We have analyzed the influence of three potential approaches to induce the breakdown of the SI system in mandarins and clementines: temperature stress, bud pollination and polyploidization. The three methods were successful in recovering selfed plants, and bud pollination was the most efficient approach. Tetraploidy was also efficient, but involved developing tetraploid plants, which is only interesting within the framework of triploid variety or in tetraploid rootstock breeding programs. Our current results would allow to recover new progenies by selfing from self-incompatible genotypes and pave the way for basic genetic studies related with the SI system in citrus.

6. We have developed and validated efficient KASPar SNP markers for early selection of two essential feature of citrus reproductive biology: apomixis and nucleo-cytoplasmic male sterility. Considering the long juvenile phase of Citrus, the possibility for early marker selection of reproductive characters at the seedling stage constitutes a major advance to optimize breeding programs aimed at creating new varieties of seedless mandarin.

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Kiyomi genetic map

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S01_2232106	1	2232106	1	0	ID=Ciclev10008268m.g
S01_2918735	1	2918735	1	7.407	ID=Ciclev10007989m.g
S01_2698390	1	2698390	1	7.407	ID=Ciclev10007225m.g
S01_2902940	1	2902940	1	7.407	ID=Ciclev10007316m.g
S01_2620817	1	2620817	1	11.105	ID=Ciclev10007235m.g
S01_2570376	1	2570376	1	11.105	ID=Ciclev10007403m.g
S01_2608730	1	2608730	1	11.105	ID=Ciclev10008850m.g
S01_3002166	1	3002166	1	13.896	ID=Ciclev10010565m.g
S01_3863756	1	3863756	1	22.991	ID=Ciclev10009236m.g
S01_5473434	1	5473434	1	34.049	ID=Ciclev10007554m.g
S01_6210592	1	6210592	1	34.054	ID=Ciclev10009547m.g
S01_6158523	1	6158523	1	34.054	ID=Ciclev10007961m.g
S01_6415400	1	6415400	1	37.442	ID=Ciclev10008039m.g
S01_6430810	1	6430810	1	37.442	ID=Ciclev10007326m.g
S01_6422664	1	6422664	1	37.442	ID=Ciclev10007670m.g
S01_7062349	1	7062349	1	39.109	ID=Ciclev10008044m.g
S01_7390266	1	7390266	1	39.109	ID=Ciclev10007331m.g
S01_7281373	1	7281373	1	39.109	ID=Ciclev10008215m.g
S01_7285538	1	7285538	1	39.109	ID=Ciclev10007544m.g
S01_7420146	1	7420146	1	39.109	ID=Ciclev10008865m.g
S01_7793731	1	7793731	1	42.51	ID=Ciclev10008296m.g
S01_8077740	1	8077740	1	45.911	ID=Ciclev10010579m.g
S01_11525469	1	11525469	1	45.911	ID=Ciclev10007325m.g
S01_15742204	1	15742204	1	45.911	ID=Ciclev10010852m.g
S01_17493202	1	17493202	1	45.911	ID=Ciclev10007392m.g
S01_12886276	1	12886276	1	45.911	ID=Ciclev10007297m.g
S01_17534460	1	17534460	1	45.911	ID=Ciclev10007828m.g
S01_16740034	1	16740034	1	45.911	ID=Ciclev10008595m.g
S01_13128413	1	13128413	1	45.911	ID=Ciclev10010588m.g
S01_14412926	1	14412926	1	45.911	ID=Ciclev10007619m.g
S01_14597210	1	14597210	1	45.911	ID=Ciclev10007863m.g
S01_18569368	1	18569368	1	45.911	ID=Ciclev10009573m.g
S01_17577385	1	17577385	1	45.911	ID=Ciclev10007821m.g
S01_19124468	1	19124468	1	52.94	ID=Ciclev10010163m.g
S01_21094771	1	21094771	1	52.94	ID=Ciclev10010479m.g
S01_20442097	1	20442097	1	52.94	ID=Ciclev10007843m.g
S01_21326252	1	21326252	1	54.61	ID=Ciclev10008777m.g
S01_21410202	1	21410202	1	56.281	ID=Ciclev10008564m.g
S01_22168276	1	22168276	1	59.679	ID=Ciclev10008020m.g
S01_22449412	1	22449412	1	59.679	ID=Ciclev10009601m.g
S01_22793349	1	22793349	1	61.349	ID=Ciclev10008176m.g
S01_23692191	1	23692191	1	64.746	ID=Ciclev10007763m.g
S01_23248268	1	23248268	1	64.746	ID=Ciclev10010386m.g
S01_23160445	1	23160445	1	64.746	ID=Ciclev10010297m.g
S01_23505119	1	23505119	1	64.746	ID=Ciclev10010623m.g
S01_23904647	1	23904647	1	64.746	ID=Ciclev10010746m.g
S01_23162531	1	23162531	1	64.746	ID=Ciclev10010287m.g
S01_24006613	1	24006613	1	64.746	ID=Ciclev10010451m.g
S01_23096037	1	23096037	1	64.746	ID=Ciclev10007927m.g
S01_23647496	1	23647496	1	64.746	ID=Ciclev10007701m.g
S01_24177031	1	24177031	1	64.746	ID=Ciclev10007234m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S01_23227492	1	23227492	1	64.746	ID=Ciclev10007266m.g
S01_23582210	1	23582210	1	64.746	ID=Ciclev10007571m.g
S01_23070654	1	23070654	1	65.573	ID=Ciclev10007929m.g
S01_24173645	1	24173645	1	66.4	ID=Ciclev10008211m.g
S01_24162151	1	24162151	1	66.4	ID=Ciclev10008243m.g
S01_23408448	1	23408448	1	67.227	ID=Ciclev10008774m.g
S01_23562557	1	23562557	1	68.053	ID=Ciclev10007585m.g
S01_23523652	1	23523652	1	68.053	ID=Ciclev10008896m.g
S01_23866990	1	23866990	1	68.053	ID=Ciclev10008762m.g
S01_23699596	1	23699596	1	68.053	ID=Ciclev10007552m.g
S01_23701591	1	23701591	1	68.053	ID=Ciclev10007550m.g
S01_22975910	1	22975910	1	68.053	ID=Ciclev10007536m.g
S01_23240974	1	23240974	1	68.053	ID=Ciclev10007245m.g
S01_23708566	1	23708566	1	68.053	ID=Ciclev10010552m.g
S01_23149460	1	23149460	1	68.053	ID=Ciclev10007324m.g
S01_24105561	1	24105561	1	68.053	ID=Ciclev10010631m.g
S01_24401648	1	24401648	1	71.455	ID=Ciclev10009634m.g
S01_24458398	1	24458398	1	73.131	ID=Ciclev10008392m.g
S01_24635064	1	24635064	1	73.131	ID=Ciclev10008437m.g
S01_24559362	1	24559362	1	73.131	ID=Ciclev10007546m.g
S01_24676351	1	24676351	1	73.131	ID=Ciclev10007286m.g
S01_24836083	1	24836083	1	78.317	ID=Ciclev10007377m.g
S01_25313589	1	25313589	1	79.273	ID=Ciclev10007408m.g
S01_24932532	1	24932532	1	79.977	ID=Ciclev10007512m.g
S01_24915332	1	24915332	1	79.977	ID=Ciclev10010149m.g
S01_24943635	1	24943635	1	80.52	ID=Ciclev10007964m.g
S01_25165178	1	25165178	1	81.073	ID=Ciclev10007884m.g
S01_25359037	1	25359037	1	81.626	ID=Ciclev10007999m.g
S01_25342413	1	25342413	1	81.626	ID=Ciclev10007327m.g
S01_25592968	1	25592968	1	81.626	ID=Ciclev10009756m.g
S01_25596644	1	25596644	1	81.626	ID=Ciclev10010180m.g
S01_25076057	1	25076057	1	81.626	ID=Ciclev10009053m.g
S01_25861333	1	25861333	1	85.017	ID=Ciclev10007583m.g
S01_25813500	1	25813500	1	85.017	ID=Ciclev10007427m.g
S01_26030964	1	26030964	1	85.017	ID=Ciclev10007933m.g
S01_25841049	1	25841049	1	85.017	ID=Ciclev10007931m.g
S01_25953136	1	25953136	1	85.017	ID=Ciclev10007858m.g
S01_26577190	1	26577190	1	88.408	ID=Ciclev10009162m.g
S01_26248633	1	26248633	1	88.408	ID=Ciclev10007456m.g
S01_26473578	1	26473578	1	88.408	ID=Ciclev10010203m.g
S01_26632565	1	26632565	1	88.408	ID=Ciclev10007337m.g
S01_26392831	1	26392831	1	88.408	ID=Ciclev10007292m.g
S01_26102811	1	26102811	1	88.408	ID=Ciclev10007497m.g
S01_26430186	1	26430186	1	88.408	ID=Ciclev10007853m.g
S01_27057113	1	27057113	1	91.801	ID=Ciclev10007368m.g
S01_26796388	1	26796388	1	92.554	ID=Ciclev10010390m.g
S01_26814365	1	26814365	1	93.456	ID=Ciclev10008911m.g
S01_26836555	1	26836555	1	93.456	ID=Ciclev10009750m.g
S01_26683868	1	26683868	1	93.456	ID=Ciclev10009285m.g
S01_27877252	1	27877252	1	96.847	ID=Ciclev10010602m.g
S01_27690239	1	27690239	1	96.847	ID=Ciclev10007320m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S01_27780071	1	27780071	1	96.847	ID=Ciclev10008947m.g
S01_28751822	1	28751822	1	96.847	ID=Ciclev10007695m.g
S01_28808018	1	28808018	1	96.847	ID=Ciclev10007895m.g
S01_28483916	1	28483916	1	96.847	ID=Ciclev10008669m.g
S01_27127855	1	27127855	1	96.847	ID=Ciclev10007405m.g
S01_28711802	1	28711802	1	96.847	ID=Ciclev10007366m.g
S01_27810869	1	27810869	1	96.847	ID=Ciclev10008554m.g
S01_28388308	1	28388308	1	96.847	ID=Ciclev10008334m.g
S01_27881940	1	27881940	1	96.847	ID=Ciclev10009180m.g
S01_28776975	1	28776975	1	96.847	ID=Ciclev10010215m.g
S01_28588106	1	28588106	1	96.847	ID=Ciclev10010403m.g
S01_27789129	1	27789129	1	96.847	ID=Ciclev10008052m.g
S01_28860413	1	28860413	1	96.847	ID=Ciclev10009248m.g
S01_27476085	1	27476085	1	96.847	ID=Ciclev10007754m.g
S01_28441149	1	28441149	1	96.847	ID=Ciclev10008754m.g
S01_27461143	1	27461143	1	96.847	ID=Ciclev10007720m.g
S02_13919863	2	13919863	2	0	ID=Ciclev10014358m.g
S02_13865031	2	13865031	2	1.923	ID=Ciclev10017541m.g
S02_13526842	2	13526842	2	3.775	ID=Ciclev10015578m.g
S02_14277094	2	14277094	2	3.775	ID=Ciclev10017154m.g
S02_19958880	2	19958880	2	3.775	ID=Ciclev10017298m.g
S02_11917966	2	11917966	2	3.775	ID=Ciclev10015537m.g
S02_11505615	2	11505615	2	4.111	ID=Ciclev10014134m.g
S02_11496293	2	11496293	2	4.455	ID=Ciclev10014459m.g
S02_11974826	2	11974826	2	4.813	ID=Ciclev10016116m.g
S02_14429648	2	14429648	2	5.166	ID=Ciclev10015755m.g
S02_13941677	2	13941677	2	5.506	ID=Ciclev10014964m.g
S02_13962229	2	13962229	2	5.506	ID=Ciclev10014385m.g
S02_13473358	2	13473358	2	5.506	ID=Ciclev10014073m.g
S02_12309555	2	12309555	2	5.506	ID=Ciclev10015638m.g
S02_11501764	2	11501764	2	5.506	ID=Ciclev10016894m.g
S02_12374769	2	12374769	2	5.506	ID=Ciclev10017128m.g
S02_11654641	2	11654641	2	5.506	ID=Ciclev10017898m.g
S02_13142135	2	13142135	2	5.506	ID=Ciclev10014024m.g
S02_12079062	2	12079062	2	5.506	ID=Ciclev10014123m.g
S02_15062079	2	15062079	2	5.506	ID=Ciclev10015471m.g
S02_11439513	2	11439513	2	6.761	ID=Ciclev10016257m.g
S02_11740976	2	11740976	2	8.123	ID=Ciclev10016552m.g
S02_15834904	2	15834904	2	10.789	ID=Ciclev10015260m.g
S02_15708409	2	15708409	2	10.789	ID=Ciclev10016311m.g
S05_810890	5	810890	2	10.789	ID=Ciclev10000238m.g
S02_15796336	2	15796336	2	10.789	ID=Ciclev10017424m.g
S02_15489574	2	15489574	2	10.789	ID=Ciclev10015280m.g
S02_11676307	2	11676307	2	10.789	ID=Ciclev10016068m.g
S02_16601833	2	16601833	2	17.733	ID=Ciclev10014998m.g
S02_16757342	2	16757342	2	17.733	ID=Ciclev10017547m.g
S02_18311109	2	18311109	2	17.733	ID=Ciclev10018370m.g
S02_11408784	2	11408784	2	19.919	ID=Ciclev10017576m.g
S02_11346390	2	11346390	2	21.676	ID=Ciclev10015375m.g
S02_22346671	2	22346671	2	27.142	ID=Ciclev10015580m.g
S02_22243091	2	22243091	2	27.142	ID=Ciclev10017567m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S02_21380389	2	21380389	2	27.142	ID=Ciclev10018332m.g
S02_23326915	2	23326915	2	30.718	ID=Ciclev10014979m.g
S02_23814366	2	23814366	2	30.718	ID=Ciclev10014335m.g
S02_23764607	2	23764607	2	30.718	ID=Ciclev10015492m.g
S02_24634812	2	24634812	2	30.718	ID=Ciclev10014215m.g
S02_24340423	2	24340423	2	30.718	ID=Ciclev10016448m.g
S02_23744691	2	23744691	2	30.718	ID=Ciclev10015423m.g
S02_23724853	2	23724853	2	30.718	ID=Ciclev10017035m.g
S02_24537212	2	24537212	2	30.718	ID=Ciclev10014451m.g
S02_23000965	2	23000965	2	30.718	ID=Ciclev10014919m.g
S02_23767510	2	23767510	2	31.581	ID=Ciclev10018169m.g
S02_23180705	2	23180705	2	32.458	ID=Ciclev10016668m.g
S02_23311591	2	23311591	2	32.458	ID=Ciclev10014523m.g
S02_22961670	2	22961670	2	33.047	ID=Ciclev10014865m.g
S02_23318610	2	23318610	2	33.623	ID=Ciclev10015116m.g
S02_23827583	2	23827583	2	33.623	ID=Ciclev10015049m.g
S02_24643835	2	24643835	2	34.193	ID=Ciclev10014764m.g
S02_23875278	2	23875278	2	34.193	ID=Ciclev10017917m.g
S02_23099598	2	23099598	2	34.193	ID=Ciclev10014491m.g
S02_24776834	2	24776834	2	34.193	ID=Ciclev10018131m.g
S02_23830690	2	23830690	2	34.193	ID=Ciclev10014845m.g
S02_23926699	2	23926699	2	34.193	ID=Ciclev10014902m.g
S02_23951256	2	23951256	2	34.193	ID=Ciclev10014185m.g
S02_24921581	2	24921581	2	34.193	ID=Ciclev10014351m.g
S02_23670563	2	23670563	2	34.193	ID=Ciclev10014722m.g
S02_25968411	2	25968411	2	37.765	ID=Ciclev10016497m.g
S02_25324635	2	25324635	2	37.765	ID=Ciclev10014088m.g
S02_26016410	2	26016410	2	37.765	ID=Ciclev10016106m.g
S02_26031648	2	26031648	2	37.765	ID=Ciclev10014821m.g
S02_25522536	2	25522536	2	37.765	ID=Ciclev10018269m.g
S02_25994513	2	25994513	2	38.636	ID=Ciclev10018355m.g
S02_25939194	2	25939194	2	39.506	ID=Ciclev10017221m.g
S02_26867044	2	26867044	2	39.506	ID=Ciclev10017583m.g
S02_26194484	2	26194484	2	39.506	ID=Ciclev10017924m.g
S02_25205376	2	25205376	2	40.092	ID=Ciclev10015195m.g
S02_26662822	2	26662822	2	40.678	ID=Ciclev10014296m.g
S02_25246598	2	25246598	2	41.241	ID=Ciclev10014131m.g
S02_25229549	2	25229549	2	41.241	ID=Ciclev10014094m.g
S02_25204110	2	25204110	2	42.111	ID=Ciclev10016771m.g
S02_26187437	2	26187437	2	42.981	ID=Ciclev10016207m.g
S02_25731110	2	25731110	2	42.981	ID=Ciclev10016721m.g
S02_26751710	2	26751710	2	42.981	ID=Ciclev10015253m.g
S02_26746419	2	26746419	2	42.981	ID=Ciclev10015599m.g
S02_26607799	2	26607799	2	42.981	ID=Ciclev10014588m.g
S02_26079650	2	26079650	2	42.981	ID=Ciclev10018196m.g
S02_23331182	2	23331182	2	44.506	ID=Ciclev10018308m.g
S02_27019458	2	27019458	2	44.729	ID=Ciclev10016899m.g
S02_27021612	2	27021612	2	44.729	ID=Ciclev10015171m.g
S02_27076702	2	27076702	2	46.484	ID=Ciclev10014563m.g
S02_27248185	2	27248185	2	46.537	ID=Ciclev10016265m.g
S02_27512483	2	27512483	2	48.237	ID=Ciclev10014119m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S02_27311016	2	27311016	2	48.237	ID=Ciclev10014030m.g
S02_27777899	2	27777899	2	49.899	ID=Ciclev10018252m.g
S02_27960995	2	27960995	2	51.75	ID=Ciclev10014737m.g
S02_28122331	2	28122331	2	52.494	ID=Ciclev10015390m.g
S02_28132267	2	28132267	2	53.491	ID=Ciclev10014287m.g
S02_28193997	2	28193997	2	53.491	ID=Ciclev10015744m.g
S02_28238175	2	28238175	2	53.491	ID=Ciclev10014079m.g
S02_27937911	2	27937911	2	57.076	ID=Ciclev10014584m.g
S02_27547541	2	27547541	2	60.66	ID=Ciclev10014686m.g
S02_27536057	2	27536057	2	60.66	ID=Ciclev10014242m.g
S02_27587556	2	27587556	2	64.233	ID=Ciclev10017324m.g
S02_27617318	2	27617318	2	64.233	ID=Ciclev10017056m.g
S02_27580871	2	27580871	2	64.233	ID=Ciclev10017466m.g
S02_27780105	2	27780105	2	64.233	ID=Ciclev10016184m.g
S02_28847004	2	28847004	2	71.657	ID=Ciclev10015330m.g
S02_28829110	2	28829110	2	71.657	ID=Ciclev10014492m.g
S02_28684016	2	28684016	2	73.416	ID=Ciclev10017575m.g
S02_28649783	2	28649783	2	73.416	ID=Ciclev10014969m.g
S02_29339277	2	29339277	2	77.01	ID=Ciclev10014294m.g
S02_29650322	2	29650322	2	82.498	ID=Ciclev10014061m.g
S02_29941930	2	29941930	2	84.261	ID=Ciclev10017803m.g
S02_30156565	2	30156565	2	84.261	ID=Ciclev10018102m.g
S02_30049717	2	30049717	2	84.261	ID=Ciclev10016394m.g
S02_29998890	2	29998890	2	84.261	ID=Ciclev10014052m.g
S02_30409168	2	30409168	2	86.017	ID=Ciclev10014844m.g
S02_30532751	2	30532751	2	86.017	ID=Ciclev10014153m.g
S02_30880581	2	30880581	2	87.772	ID=Ciclev10014116m.g
S02_30849954	2	30849954	2	87.772	ID=Ciclev10015984m.g
S02_30925228	2	30925228	2	87.772	ID=Ciclev10016127m.g
S02_32002159	2	32002159	2	95.201	ID=Ciclev10014983m.g
S02_31894488	2	31894488	2	95.201	ID=Ciclev10017760m.g
S02_32407943	2	32407943	2	96.963	ID=Ciclev10017888m.g
S02_32403306	2	32403306	2	96.963	ID=Ciclev10014789m.g
S02_33698992	2	33698992	2	100.536	ID=Ciclev10016529m.g
S02_33717023	2	33717023	2	100.536	ID=Ciclev10016200m.g
S02_33028594	2	33028594	2	100.536	ID=Ciclev10014112m.g
S02_33660810	2	33660810	2	101.324	ID=Ciclev10017171m.g
S02_33462551	2	33462551	2	102.117	ID=Ciclev10014447m.g
S02_33390529	2	33390529	2	102.273	ID=Ciclev10016152m.g
S02_33421461	2	33421461	2	102.273	ID=Ciclev10014282m.g
S02_33550301	2	33550301	2	102.605	ID=Ciclev10014136m.g
S02_33806216	2	33806216	2	105.827	ID=Ciclev10014391m.g
S02_33730114	2	33730114	2	105.827	ID=Ciclev10014023m.g
S02_33981815	2	33981815	2	108.485	ID=Ciclev10016080m.g
S02_33956055	2	33956055	2	108.485	ID=Ciclev10018204m.g
S02_33934204	2	33934204	2	108.485	ID=Ciclev10017786m.g
S02_33840055	2	33840055	2	111.14	ID=Ciclev10015064m.g
S02_33864813	2	33864813	2	111.14	ID=Ciclev10017211m.g
S02_33960945	2	33960945	2	111.14	ID=Ciclev10014227m.g
S02_34077715	2	34077715	2	116.603	ID=Ciclev10015687m.g
S02_34289652	2	34289652	2	116.603	ID=Ciclev10014476m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S02_34604487	2	34604487	2	118.361	ID=Ciclev10018023m.g
S02_34365146	2	34365146	2	118.361	ID=Ciclev10014155m.g
S02_34350260	2	34350260	2	118.361	ID=Ciclev10016384m.g
S02_34337528	2	34337528	2	118.361	ID=Ciclev10014603m.g
S02_34517558	2	34517558	2	119.232	ID=Ciclev10015472m.g
S02_34503018	2	34503018	2	120.101	ID=Ciclev10014430m.g
S02_34535203	2	34535203	2	120.101	ID=Ciclev10014889m.g
S02_34450478	2	34450478	2	120.101	ID=Ciclev10017841m.g
S02_34664615	2	34664615	2	121.857	ID=Ciclev10014347m.g
S02_34824839	2	34824839	2	121.857	ID=Ciclev10014753m.g
S02_34861899	2	34861899	2	123.612	ID=Ciclev10018230m.g
S02_34926170	2	34926170	2	123.612	ID=Ciclev10017980m.g
S02_34925116	2	34925116	2	123.612	ID=Ciclev10017622m.g
S02_34957343	2	34957343	2	127.186	ID=Ciclev10014315m.g
S02_35108374	2	35108374	2	128.942	ID=Ciclev10015527m.g
S02_35163647	2	35163647	2	128.942	ID=Ciclev10015817m.g
S02_35100970	2	35100970	2	128.942	ID=Ciclev10015602m.g
S02_35097221	2	35097221	2	128.942	ID=Ciclev10017950m.g
S02_35303569	2	35303569	2	130.697	ID=Ciclev10017731m.g
S02_35289407	2	35289407	2	130.697	ID=Ciclev10015200m.g
S02_35282987	2	35282987	2	130.697	ID=Ciclev10014968m.g
S02_35332869	2	35332869	2	132.46	ID=Ciclev10017854m.g
S02_35551951	2	35551951	2	134.222	ID=Ciclev10015649m.g
S02_35670206	2	35670206	2	134.222	ID=Ciclev10015792m.g
S02_36142192	2	36142192	2	134.222	ID=Ciclev10017002m.g
S02_35693839	2	35693839	2	134.222	ID=Ciclev10015934m.g
S02_35531499	2	35531499	2	134.222	ID=Ciclev10017994m.g
S02_35830901	2	35830901	2	134.222	ID=Ciclev10018181m.g
S02_35667952	2	35667952	2	134.222	ID=Ciclev10017034m.g
S02_35498766	2	35498766	2	134.222	ID=Ciclev10016599m.g
S02_35717353	2	35717353	2	134.222	ID=Ciclev10014348m.g
S02_35995975	2	35995975	2	134.222	ID=Ciclev10016754m.g
S02_35944678	2	35944678	2	134.222	ID=Ciclev10018303m.g
S02_35616512	2	35616512	2	134.77	ID=Ciclev10014418m.g
S02_35983734	2	35983734	2	135.364	ID=Ciclev10017072m.g
S02_35754781	2	35754781	2	135.959	ID=Ciclev10014814m.g
S02_35759893	2	35759893	2	135.959	ID=Ciclev10016358m.g
S02_35799976	2	35799976	2	135.959	ID=Ciclev10016018m.g
S02_35474336	2	35474336	2	136.828	ID=Ciclev10015237m.g
S02_36072477	2	36072477	2	137.699	ID=Ciclev10016401m.g
S02_36145932	2	36145932	2	137.699	ID=Ciclev10016440m.g
S02_35971444	2	35971444	2	137.699	ID=Ciclev10014811m.g
S02_35485622	2	35485622	2	137.699	ID=Ciclev10017564m.g
S02_35896884	2	35896884	2	137.699	ID=Ciclev10014770m.g
S02_36282174	2	36282174	2	139.453	ID=Ciclev10018000m.g
S02_36262971	2	36262971	2	139.453	ID=Ciclev10017669m.g
S02_36270473	2	36270473	2	139.453	ID=Ciclev10014046m.g
S02_36312793	2	36312793	2	139.453	ID=Ciclev10014935m.g
S02_36154966	2	36154966	2	139.453	ID=Ciclev10014746m.g
S02_36308023	2	36308023	2	139.471	ID=Ciclev10015031m.g
S02_36180728	2	36180728	2	141.304	ID=Ciclev10015350m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S02_36156903	2	36156903	2	141.304	ID=Ciclev10015898m.g
S03_4155519	3	4155519	3	0	ID=Ciclev10024278m.g
S03_4195718	3	4195718	3	0	ID=Ciclev10019499m.g
S03_4248266	3	4248266	3	0	ID=Ciclev10023901m.g
S03_4355684	3	4355684	3	0	ID=Ciclev10019793m.g
S03_3909279	3	3909279	3	0	ID=Ciclev10023569m.g
S03_4215379	3	4215379	3	0	ID=Ciclev10019547m.g
S03_4349753	3	4349753	3	0	ID=Ciclev10024401m.g
S03_4385466	3	4385466	3	0	ID=Ciclev10020324m.g
S03_4265549	3	4265549	3	0	ID=Ciclev10019526m.g
S03_4169677	3	4169677	3	0.233	ID=Ciclev10018939m.g
S03_4052980	3	4052980	3	0.469	ID=Ciclev10018616m.g
S03_4260930	3	4260930	3	0.704	ID=Ciclev10021896m.g
S03_4120128	3	4120128	3	0.94	ID=Ciclev10021889m.g
S03_4232453	3	4232453	3	1.176	ID=Ciclev10022969m.g
S03_3999541	3	3999541	3	1.411	ID=Ciclev10018456m.g
S03_3746954	3	3746954	3	1.644	ID=Ciclev10023549m.g
S03_3882107	3	3882107	3	1.644	ID=Ciclev10020762m.g
S03_5509989	3	5509989	3	3.311	ID=Ciclev10019315m.g
S03_4958204	3	4958204	3	3.311	ID=Ciclev10019492m.g
S03_5173517	3	5173517	3	3.311	ID=Ciclev10019636m.g
S03_6000973	3	6000973	3	4.138	ID=Ciclev10021834m.g
S03_4874306	3	4874306	3	4.964	ID=Ciclev10023329m.g
S03_5472652	3	5472652	3	4.964	ID=Ciclev10020835m.g
S03_5362039	3	5362039	3	4.964	ID=Ciclev10021067m.g
S03_5432747	3	5432747	3	4.964	ID=Ciclev10018818m.g
S03_4802426	3	4802426	3	4.964	ID=Ciclev10024446m.g
S03_5291501	3	5291501	3	5.783	ID=Ciclev10020964m.g
S03_5476746	3	5476746	3	6.618	ID=Ciclev10020521m.g
S03_5373803	3	5373803	3	7.453	ID=Ciclev10019196m.g
S03_5797850	3	5797850	3	8.272	ID=Ciclev10019412m.g
S03_5629524	3	5629524	3	8.272	ID=Ciclev10022924m.g
S03_5746873	3	5746873	3	8.272	ID=Ciclev10022042m.g
S03_5948446	3	5948446	3	8.823	ID=Ciclev10020462m.g
S03_5717760	3	5717760	3	9.376	ID=Ciclev10018916m.g
S03_4911827	3	4911827	3	9.922	ID=Ciclev10018937m.g
S03_5866515	3	5866515	3	9.922	ID=Ciclev10021633m.g
S03_4916014	3	4916014	3	9.922	ID=Ciclev10022085m.g
S03_5977557	3	5977557	3	9.922	ID=Ciclev10020501m.g
S03_4623429	3	4623429	3	9.922	ID=Ciclev10018424m.g
S03_5521588	3	5521588	3	9.922	ID=Ciclev10018443m.g
S03_4645064	3	4645064	3	15.099	ID=Ciclev10021747m.g
S03_6075290	3	6075290	3	15.099	ID=Ciclev10019462m.g
S03_6236749	3	6236749	3	15.099	ID=Ciclev10021953m.g
S03_6401636	3	6401636	3	18.49	ID=Ciclev10018779m.g
S03_6425353	3	6425353	3	20.158	ID=Ciclev10020346m.g
S03_6802911	3	6802911	3	21.826	ID=Ciclev10024228m.g
S03_6683775	3	6683775	3	21.826	ID=Ciclev10018726m.g
S03_6828939	3	6828939	3	21.826	ID=Ciclev10021923m.g
S03_6773376	3	6773376	3	21.826	ID=Ciclev10018489m.g
S03_6516671	3	6516671	3	21.826	ID=Ciclev10024346m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_6469216	3	6469216	3	23.486	ID=Ciclev10019738m.g
S03_7447201	3	7447201	3	25.165	ID=Ciclev10019899m.g
S03_7273659	3	7273659	3	26.832	ID=Ciclev10023301m.g
S03_7197845	3	7197845	3	26.832	ID=Ciclev10020967m.g
S03_6967330	3	6967330	3	30.223	ID=Ciclev10023269m.g
S03_6942060	3	6942060	3	30.223	ID=Ciclev10019482m.g
S03_7166302	3	7166302	3	30.223	ID=Ciclev10018833m.g
S03_7173657	3	7173657	3	30.223	ID=Ciclev10021645m.g
S03_6971017	3	6971017	3	30.223	ID=Ciclev10019148m.g
S03_7793041	3	7793041	3	31.051	ID=Ciclev10019841m.g
S03_7678721	3	7678721	3	31.879	ID=Ciclev10018667m.g
S03_7659223	3	7659223	3	31.879	ID=Ciclev10024165m.g
S03_7690588	3	7690588	3	31.879	ID=Ciclev10020924m.g
S03_7707543	3	7707543	3	31.879	ID=Ciclev10020323m.g
S03_7761724	3	7761724	3	32.291	ID=Ciclev10022528m.g
S03_7951357	3	7951357	3	32.704	ID=Ciclev10018461m.g
S03_8411647	3	8411647	3	33.112	ID=Ciclev10020292m.g
S03_8591846	3	8591846	3	33.526	ID=Ciclev10020920m.g
S03_7772462	3	7772462	3	34.353	ID=Ciclev10020317m.g
S03_8192525	3	8192525	3	35.179	ID=Ciclev10023292m.g
S03_8159176	3	8159176	3	35.179	ID=Ciclev10023312m.g
S03_8528700	3	8528700	3	35.179	ID=Ciclev10022680m.g
S03_8245409	3	8245409	3	37.709	ID=Ciclev10023536m.g
S03_7455551	3	7455551	3	37.709	ID=Ciclev10019755m.g
S03_8231667	3	8231667	3	38.96	ID=Ciclev10020540m.g
S03_10020250	3	10020250	3	40.193	ID=Ciclev10020493m.g
S03_33033456	3	33033456	3	40.193	ID=Ciclev10020160m.g
S03_31309690	3	31309690	3	40.193	ID=Ciclev10019972m.g
S03_33158488	3	33158488	3	40.193	ID=Ciclev10019496m.g
S03_33709983	3	33709983	3	40.193	ID=Ciclev10023366m.g
S03_10151394	3	10151394	3	40.193	ID=Ciclev10023109m.g
S03_9717684	3	9717684	3	40.193	ID=Ciclev10019473m.g
S03_32752310	3	32752310	3	40.193	ID=Ciclev10019208m.g
S03_32545358	3	32545358	3	40.193	ID=Ciclev10023690m.g
S03_10022579	3	10022579	3	41.02	ID=Ciclev10018859m.g
S03_8843388	3	8843388	3	41.847	ID=Ciclev10019201m.g
S03_9028425	3	9028425	3	41.847	ID=Ciclev10018692m.g
S03_31352902	3	31352902	3	41.847	ID=Ciclev10021151m.g
S03_31166382	3	31166382	3	41.847	ID=Ciclev10018721m.g
S03_9370860	3	9370860	3	41.847	ID=Ciclev10018539m.g
S03_32715305	3	32715305	3	45.238	ID=Ciclev10019454m.g
S03_33746645	3	33746645	3	45.238	ID=Ciclev10023053m.g
S03_34002061	3	34002061	3	45.238	ID=Ciclev10023387m.g
S03_31486568	3	31486568	3	46.065	ID=Ciclev10024261m.g
S03_9719851	3	9719851	3	46.892	ID=Ciclev10019417m.g
S03_32798242	3	32798242	3	46.892	ID=Ciclev10020490m.g
S03_8642675	3	8642675	3	46.892	ID=Ciclev10019790m.g
S03_9744114	3	9744114	3	46.892	ID=Ciclev10022919m.g
S03_9747315	3	9747315	3	46.892	ID=Ciclev10022419m.g
S03_32989072	3	32989072	3	48.558	ID=Ciclev10020396m.g
S03_10472142	3	10472142	3	50.229	ID=Ciclev10022203m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_30875349	3	30875349	3	50.229	ID=Ciclev10021488m.g
S03_30508384	3	30508384	3	50.229	ID=Ciclev10023422m.g
S03_30580994	3	30580994	3	50.229	ID=Ciclev10020825m.g
S03_29541940	3	29541940	3	50.229	ID=Ciclev10019766m.g
S03_30520145	3	30520145	3	50.229	ID=Ciclev10019362m.g
S03_30955763	3	30955763	3	51.056	ID=Ciclev10018908m.g
S03_34183984	3	34183984	3	51.883	ID=Ciclev10023261m.g
S03_10604769	3	10604769	3	51.883	ID=Ciclev10023629m.g
S03_29175158	3	29175158	3	51.883	ID=Ciclev10020632m.g
S03_10252645	3	10252645	3	51.883	ID=Ciclev10018793m.g
S03_29135792	3	29135792	3	51.883	ID=Ciclev10022266m.g
S03_29879990	3	29879990	3	52.709	ID=Ciclev10023702m.g
S03_29559508	3	29559508	3	53.536	ID=Ciclev10018666m.g
S03_29382932	3	29382932	3	53.536	ID=Ciclev10019003m.g
S03_10431700	3	10431700	3	53.536	ID=Ciclev10020181m.g
S03_11635322	3	11635322	3	58.713	ID=Ciclev10019884m.g
S03_12793287	3	12793287	3	58.713	ID=Ciclev10020083m.g
S03_11501031	3	11501031	3	58.713	ID=Ciclev10018464m.g
S03_11273448	3	11273448	3	58.713	ID=Ciclev10018785m.g
S03_11399770	3	11399770	3	58.713	ID=Ciclev10019376m.g
S03_12415501	3	12415501	3	62.106	ID=Ciclev10021983m.g
S03_12221833	3	12221833	3	62.106	ID=Ciclev10020013m.g
S03_12835728	3	12835728	3	62.87	ID=Ciclev10023517m.g
S03_12823367	3	12823367	3	63.76	ID=Ciclev10021946m.g
S03_11663181	3	11663181	3	63.76	ID=Ciclev10020388m.g
S03_11178819	3	11178819	3	63.76	ID=Ciclev10021271m.g
S03_11412158	3	11412158	3	63.76	ID=Ciclev10018947m.g
S03_19267026	3	19267026	3	63.76	ID=Ciclev10021647m.g
S03_20743439	3	20743439	3	67.151	ID=Ciclev10020750m.g
S03_19330786	3	19330786	3	67.151	ID=Ciclev10023970m.g
S03_19321766	3	19321766	3	67.151	ID=Ciclev10021509m.g
S03_20917155	3	20917155	3	67.705	ID=Ciclev10018931m.g
S03_17749069	3	17749069	3	68.254	ID=Ciclev10018888m.g
S03_13227497	3	13227497	3	68.801	ID=Ciclev10018879m.g
S03_18731963	3	18731963	3	68.801	ID=Ciclev10021799m.g
S03_12708054	3	12708054	3	68.801	ID=Ciclev10019244m.g
S03_15178172	3	15178172	3	68.801	ID=Ciclev10019405m.g
S03_19286641	3	19286641	3	68.801	ID=Ciclev10023501m.g
S03_19310760	3	19310760	3	68.801	ID=Ciclev10019695m.g
S03_13582782	3	13582782	3	68.801	ID=Ciclev10019177m.g
S03_20672495	3	20672495	3	68.801	ID=Ciclev10020938m.g
S03_20688460	3	20688460	3	68.801	ID=Ciclev10021347m.g
S03_13812636	3	13812636	3	68.801	ID=Ciclev10022215m.g
S03_19793836	3	19793836	3	68.801	ID=Ciclev10020303m.g
S03_20872644	3	20872644	3	68.801	ID=Ciclev10020602m.g
S03_17457150	3	17457150	3	68.801	ID=Ciclev10023627m.g
S03_16916833	3	16916833	3	68.801	ID=Ciclev10024534m.g
S03_16436685	3	16436685	3	68.801	ID=Ciclev10018592m.g
S03_13829187	3	13829187	3	68.801	ID=Ciclev10018632m.g
S03_21814685	3	21814685	3	70.469	ID=Ciclev10024358m.g
S03_23735428	3	23735428	3	72.137	ID=Ciclev10021154m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_23760820	3	23760820	3	72.137	ID=Ciclev10024319m.g
S03_23034923	3	23034923	3	72.137	ID=Ciclev10021219m.g
S03_22789302	3	22789302	3	72.137	ID=Ciclev10018754m.g
S03_23316081	3	23316081	3	72.137	ID=Ciclev10022438m.g
S03_25543237	3	25543237	3	73.805	ID=Ciclev10018540m.g
S03_25499142	3	25499142	3	73.805	ID=Ciclev10023260m.g
S03_28407513	3	28407513	3	74.25	ID=Ciclev10020481m.g
S03_36097117	3	36097117	3	75.462	ID=Ciclev10022339m.g
S03_27953094	3	27953094	3	75.462	ID=Ciclev10024591m.g
S03_27799994	3	27799994	3	75.462	ID=Ciclev10023071m.g
S03_28021795	3	28021795	3	75.462	ID=Ciclev10024300m.g
S03_25635398	3	25635398	3	75.462	ID=Ciclev10018897m.g
S03_28332755	3	28332755	3	75.462	ID=Ciclev10018900m.g
S02_34045417	2	34045417	3	75.462	ID=Ciclev10014703m.g
S03_25660242	3	25660242	3	75.462	ID=Ciclev10024293m.g
S03_28686570	3	28686570	3	75.462	ID=Ciclev10018758m.g
S03_36008533	3	36008533	3	75.462	ID=Ciclev10020253m.g
S03_36263160	3	36263160	3	75.462	ID=Ciclev10024276m.g
S03_36834839	3	36834839	3	77.129	ID=Ciclev10022050m.g
S03_36705043	3	36705043	3	77.129	ID=Ciclev10018957m.g
S03_37489477	3	37489477	3	78.797	ID=Ciclev10023730m.g
S03_37466354	3	37466354	3	78.797	ID=Ciclev10023352m.g
S03_37846161	3	37846161	3	80.465	ID=Ciclev10023535m.g
S03_38260648	3	38260648	3	80.739	ID=Ciclev10024419m.g
S03_38308043	3	38308043	3	81.432	ID=Ciclev10022579m.g
S03_38117144	3	38117144	3	82.116	ID=Ciclev10019615m.g
S03_38331144	3	38331144	3	82.116	ID=Ciclev10022280m.g
S03_37899482	3	37899482	3	82.116	ID=Ciclev10021850m.g
S03_38604290	3	38604290	3	83.049	ID=Ciclev10018680m.g
S03_39038033	3	39038033	3	83.77	ID=Ciclev10023340m.g
S03_38526495	3	38526495	3	83.77	ID=Ciclev10019579m.g
S03_38471674	3	38471674	3	83.77	ID=Ciclev10018556m.g
S03_39076102	3	39076102	3	83.77	ID=Ciclev10020116m.g
S03_38552912	3	38552912	3	83.77	ID=Ciclev10018533m.g
S03_39352413	3	39352413	3	85.437	ID=Ciclev10020134m.g
S03_39658597	3	39658597	3	86.267	ID=Ciclev10021187m.g
S03_39761024	3	39761024	3	87.09	ID=Ciclev10022381m.g
S03_39706281	3	39706281	3	87.09	ID=Ciclev10018740m.g
S03_39622153	3	39622153	3	87.09	ID=Ciclev10021532m.g
S03_39644380	3	39644380	3	87.09	ID=Ciclev10019670m.g
S03_39402780	3	39402780	3	87.09	ID=Ciclev10024181m.g
S03_39407544	3	39407544	3	87.09	ID=Ciclev10019508m.g
S03_39772257	3	39772257	3	87.09	ID=Ciclev10018764m.g
S03_40116521	3	40116521	3	87.09	ID=Ciclev10021522m.g
S03_39475113	3	39475113	3	87.09	ID=Ciclev10018676m.g
S03_39756508	3	39756508	3	87.09	ID=Ciclev10018854m.g
S03_40110323	3	40110323	3	87.09	ID=Ciclev10021732m.g
S03_40965053	3	40965053	3	88.759	ID=Ciclev10020717m.g
S03_40254614	3	40254614	3	88.759	ID=Ciclev10019808m.g
S03_40758400	3	40758400	3	88.759	ID=Ciclev10020331m.g
S03_40747514	3	40747514	3	88.759	ID=Ciclev10022368m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_40505014	3	40505014	3	88.759	ID=Ciclev10018711m.g
S03_40242542	3	40242542	3	88.759	ID=Ciclev10020928m.g
S03_41291892	3	41291892	3	91.285	ID=Ciclev10021582m.g
S03_41570370	3	41570370	3	93.811	ID=Ciclev10018636m.g
S03_41858577	3	41858577	3	93.811	ID=Ciclev10019408m.g
S03_41819607	3	41819607	3	93.811	ID=Ciclev10020854m.g
S03_41482920	3	41482920	3	93.811	ID=Ciclev10023255m.g
S03_41871325	3	41871325	3	94.638	ID=Ciclev10020145m.g
S03_41793995	3	41793995	3	95.464	ID=Ciclev10019589m.g
S03_41893771	3	41893771	3	95.464	ID=Ciclev10019321m.g
S03_41976275	3	41976275	3	97.131	ID=Ciclev10021118m.g
S03_41923940	3	41923940	3	97.131	ID=Ciclev10019872m.g
S03_41990192	3	41990192	3	97.956	ID=Ciclev10021072m.g
S03_41884276	3	41884276	3	98.785	ID=Ciclev10020438m.g
S03_42073495	3	42073495	3	99.615	ID=Ciclev10021457m.g
S03_41768131	3	41768131	3	100.44	ID=Ciclev10021843m.g
S03_41787380	3	41787380	3	100.44	ID=Ciclev10020215m.g
S03_41785313	3	41785313	3	100.44	ID=Ciclev10019878m.g
S03_42552591	3	42552591	3	108.569	ID=Ciclev10019039m.g
S03_42152970	3	42152970	3	108.571	ID=Ciclev10020691m.g
S03_42129496	3	42129496	3	108.571	ID=Ciclev10021891m.g
S03_42373441	3	42373441	3	114.522	ID=Ciclev10022827m.g
S03_42499788	3	42499788	3	114.522	ID=Ciclev10024546m.g
S03_42476371	3	42476371	3	114.522	ID=Ciclev10018522m.g
S03_42271689	3	42271689	3	114.522	ID=Ciclev10018446m.g
S03_42163215	3	42163215	3	116.939	ID=Ciclev10019411m.g
S03_42626530	3	42626530	3	120.068	ID=Ciclev10018498m.g
S03_42641966	3	42641966	3	122.699	ID=Ciclev10020728m.g
S03_42637792	3	42637792	3	122.699	ID=Ciclev10019392m.g
S03_42802167	3	42802167	3	128.179	ID=Ciclev10018935m.g
S03_42725294	3	42725294	3	128.179	ID=Ciclev10018480m.g
S03_42781107	3	42781107	3	128.179	ID=Ciclev10018910m.g
S03_42742998	3	42742998	3	128.179	ID=Ciclev10024404m.g
S03_42760478	3	42760478	3	128.179	ID=Ciclev10020620m.g
S03_43055475	3	43055475	3	133.356	ID=Ciclev10020365m.g
S03_42841000	3	42841000	3	133.356	ID=Ciclev10019299m.g
S03_42817208	3	42817208	3	133.356	ID=Ciclev10019675m.g
S03_43436155	3	43436155	3	136.749	ID=Ciclev10023734m.g
S03_44498996	3	44498996	3	138.417	ID=Ciclev10018523m.g
S03_44413589	3	44413589	3	138.417	ID=Ciclev10022807m.g
S03_43377174	3	43377174	3	140.085	ID=Ciclev10023554m.g
S03_44984204	3	44984204	3	140.085	ID=Ciclev10018445m.g
S03_42959920	3	42959920	3	140.085	ID=Ciclev10021437m.g
S03_43207597	3	43207597	3	140.085	ID=Ciclev10024489m.g
S03_44609642	3	44609642	3	140.085	ID=Ciclev10023667m.g
S03_44961280	3	44961280	3	140.085	ID=Ciclev10019732m.g
S03_43421033	3	43421033	3	140.085	ID=Ciclev10021699m.g
S03_43001220	3	43001220	3	140.085	ID=Ciclev10018639m.g
S03_44891319	3	44891319	3	143.476	ID=Ciclev10020955m.g
S03_43156285	3	43156285	3	143.476	ID=Ciclev10024202m.g
S03_45721553	3	45721553	3	143.476	ID=Ciclev10021143m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_45217646	3	45217646	3	143.476	ID=Ciclev10021328m.g
S03_45139752	3	45139752	3	143.476	ID=Ciclev10018791m.g
S03_45639281	3	45639281	3	143.476	ID=Ciclev10018516m.g
S03_45426454	3	45426454	3	143.476	ID=Ciclev10018729m.g
S03_42979085	3	42979085	3	143.476	ID=Ciclev10021908m.g
S03_43544007	3	43544007	3	146.867	ID=Ciclev10024363m.g
S03_44915196	3	44915196	3	146.867	ID=Ciclev10019581m.g
S03_44090696	3	44090696	3	148.538	ID=Ciclev10018426m.g
S03_44273052	3	44273052	3	150.209	ID=Ciclev10018988m.g
S03_42968609	3	42968609	3	150.209	ID=Ciclev10018896m.g
S03_44318983	3	44318983	3	150.209	ID=Ciclev10022764m.g
S03_43130500	3	43130500	3	153.602	ID=Ciclev10018992m.g
S03_42884244	3	42884244	3	153.602	ID=Ciclev10023136m.g
S03_44932539	3	44932539	3	153.602	ID=Ciclev10022724m.g
S03_43699487	3	43699487	3	153.602	ID=Ciclev10023822m.g
S03_42929666	3	42929666	3	153.602	ID=Ciclev10019502m.g
S03_44125885	3	44125885	3	153.602	ID=Ciclev10019959m.g
S03_43491541	3	43491541	3	153.602	ID=Ciclev10023393m.g
S03_44943432	3	44943432	3	154.408	ID=Ciclev10022630m.g
S03_44632014	3	44632014	3	155.235	ID=Ciclev10019118m.g
S03_44388628	3	44388628	3	156.082	ID=Ciclev10023775m.g
S03_43628658	3	43628658	3	157.343	ID=Ciclev10023342m.g
S03_45026377	3	45026377	3	158.578	ID=Ciclev10019923m.g
S03_44166201	3	44166201	3	158.578	ID=Ciclev10023714m.g
S03_44853658	3	44853658	3	158.578	ID=Ciclev10018517m.g
S03_44006569	3	44006569	3	158.578	ID=Ciclev10020513m.g
S03_42951144	3	42951144	3	158.578	ID=Ciclev10018866m.g
S03_45000524	3	45000524	3	158.578	ID=Ciclev10019778m.g
S03_44604997	3	44604997	3	159.792	ID=Ciclev10019771m.g
S03_45477567	3	45477567	3	161.063	ID=Ciclev10023420m.g
S03_45418716	3	45418716	3	163.594	ID=Ciclev10020375m.g
S03_45299812	3	45299812	3	163.594	ID=Ciclev10022800m.g
S03_45317791	3	45317791	3	163.594	ID=Ciclev10023989m.g
S03_43635432	3	43635432	3	163.594	ID=Ciclev10024066m.g
S03_45244789	3	45244789	3	163.594	ID=Ciclev10022531m.g
S03_45888488	3	45888488	3	168.771	ID=Ciclev10021907m.g
S03_46168747	3	46168747	3	168.771	ID=Ciclev10022679m.g
S03_46006205	3	46006205	3	168.771	ID=Ciclev10019602m.g
S03_45895468	3	45895468	3	168.771	ID=Ciclev10023029m.g
S01_7850625	1	7850625	3	168.771	ID=Ciclev10008287m.g
S03_45912922	3	45912922	3	168.771	ID=Ciclev10024146m.g
S03_46729276	3	46729276	3	175.783	ID=Ciclev10019038m.g
S03_46959072	3	46959072	3	177.992	ID=Ciclev10018861m.g
S03_47087968	3	47087968	3	180.195	ID=Ciclev10018812m.g
S03_47293831	3	47293831	3	182.032	ID=Ciclev10020859m.g
S03_47151560	3	47151560	3	182.032	ID=Ciclev10019510m.g
S03_47321454	3	47321454	3	187.772	ID=Ciclev10020349m.g
S03_47477496	3	47477496	3	187.772	ID=Ciclev10018964m.g
S03_47667620	3	47667620	3	195.805	ID=Ciclev10019596m.g
S03_48013739	3	48013739	3	199.467	ID=Ciclev10019982m.g
S03_47676344	3	47676344	3	199.467	ID=Ciclev10019421m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_48587204	3	48587204	3	205.251	ID=Ciclev10020434m.g
S03_48519181	3	48519181	3	205.251	ID=Ciclev10018596m.g
S03_48928324	3	48928324	3	208.265	ID=Ciclev10019660m.g
S03_49009134	3	49009134	3	208.265	ID=Ciclev10018441m.g
S03_48841205	3	48841205	3	208.265	ID=Ciclev10022320m.g
S03_49207236	3	49207236	3	211.283	ID=Ciclev10021613m.g
S03_49221085	3	49221085	3	212.225	ID=Ciclev10023577m.g
S03_49242176	3	49242176	3	213.254	ID=Ciclev10019380m.g
S03_49363546	3	49363546	3	215.844	ID=Ciclev10022007m.g
S03_49369084	3	49369084	3	220.088	ID=Ciclev10019530m.g
S03_49597261	3	49597261	3	222.162	ID=Ciclev10023337m.g
S03_49634764	3	49634764	3	222.162	ID=Ciclev10022618m.g
S03_49660532	3	49660532	3	222.162	ID=Ciclev10018796m.g
S03_49664864	3	49664864	3	222.162	ID=Ciclev10024070m.g
S03_49863684	3	49863684	3	226.074	ID=Ciclev10023651m.g
S03_50039719	3	50039719	3	230.006	ID=Ciclev10020384m.g
S03_50499940	3	50499940	3	236.002	ID=Ciclev10024143m.g
S03_50643021	3	50643021	3	237.675	ID=Ciclev10019814m.g
S03_50919459	3	50919459	3	237.675	ID=Ciclev10022762m.g
S04_15280165	4	15280165	4	0	ID=Ciclev10031282m.g
S04_16048577	4	16048577	4	3.51	ID=Ciclev10032012m.g
S04_16752887	4	16752887	4	3.51	ID=Ciclev10031946m.g
S04_17043690	4	17043690	4	10.665	ID=Ciclev10030490m.g
S04_17190472	4	17190472	4	10.665	ID=Ciclev10030475m.g
S04_17683563	4	17683563	4	10.665	ID=Ciclev10031558m.g
S04_17795051	4	17795051	4	12.335	ID=Ciclev10033437m.g
S04_17965387	4	17965387	4	14.007	ID=Ciclev10031531m.g
S04_18195910	4	18195910	4	14.007	ID=Ciclev10030747m.g
S04_18659002	4	18659002	4	16.534	ID=Ciclev10033525m.g
S04_18620098	4	18620098	4	16.534	ID=Ciclev10030817m.g
S04_19555531	4	19555531	4	19.057	ID=Ciclev10031364m.g
S04_19057075	4	19057075	4	19.057	ID=Ciclev10030859m.g
S04_19587416	4	19587416	4	19.057	ID=Ciclev10030710m.g
S04_19461327	4	19461327	4	19.057	ID=Ciclev10031654m.g
S04_19140157	4	19140157	4	19.057	ID=Ciclev10033734m.g
S04_19546195	4	19546195	4	19.057	ID=Ciclev10031322m.g
S04_19415304	4	19415304	4	24.234	ID=Ciclev10033991m.g
S04_19432872	4	19432872	4	24.234	ID=Ciclev10030866m.g
S04_18962402	4	18962402	4	24.234	ID=Ciclev10033897m.g
S04_18956001	4	18956001	4	24.234	ID=Ciclev10032094m.g
S04_18782360	4	18782360	4	24.234	ID=Ciclev10031672m.g
S04_19092661	4	19092661	4	27.614	ID=Ciclev10032493m.g
S04_19410098	4	19410098	4	27.614	ID=Ciclev10031060m.g
S04_19693252	4	19693252	4	31.038	ID=Ciclev10033721m.g
S04_20723525	4	20723525	4	36.238	ID=Ciclev10033781m.g
S04_20664534	4	20664534	4	36.238	ID=Ciclev10033488m.g
S04_20765827	4	20765827	4	36.238	ID=Ciclev10032483m.g
S04_21173506	4	21173506	4	36.238	ID=Ciclev10031391m.g
S04_20856866	4	20856866	4	36.238	ID=Ciclev10031472m.g
S04_20906255	4	20906255	4	36.238	ID=Ciclev10030493m.g
S04_20943371	4	20943371	4	36.238	ID=Ciclev10032794m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S04_19819083	4	19819083	4	41.415	ID=Ciclev10030538m.g
S04_20500576	4	20500576	4	41.415	ID=Ciclev10030646m.g
S04_20253520	4	20253520	4	41.415	ID=Ciclev10031372m.g
S04_19762691	4	19762691	4	43.082	ID=Ciclev10032187m.g
S04_20155595	4	20155595	4	43.082	ID=Ciclev10031053m.g
S04_20591816	4	20591816	4	43.65	ID=Ciclev10033741m.g
S04_20124471	4	20124471	4	44.824	ID=Ciclev10032464m.g
S04_21166240	4	21166240	4	46.407	ID=Ciclev10031133m.g
S04_20675734	4	20675734	4	47.193	ID=Ciclev10031132m.g
S04_19785933	4	19785933	4	48.061	ID=Ciclev10031108m.g
S04_20686880	4	20686880	4	48.861	ID=Ciclev10033727m.g
S04_21196858	4	21196858	4	49.716	ID=Ciclev10030951m.g
S04_21288594	4	21288594	4	51.385	ID=Ciclev10031713m.g
S04_21362395	4	21362395	4	53.055	ID=Ciclev10031347m.g
S04_21629627	4	21629627	4	56.45	ID=Ciclev10031257m.g
S04_21423112	4	21423112	4	56.45	ID=Ciclev10030531m.g
S04_21438313	4	21438313	4	56.45	ID=Ciclev10030842m.g
S04_21502932	4	21502932	4	56.45	ID=Ciclev10030770m.g
S04_21605197	4	21605197	4	56.45	ID=Ciclev10033456m.g
S04_21717379	4	21717379	4	56.45	ID=Ciclev10031490m.g
S04_21562748	4	21562748	4	56.45	ID=Ciclev10030774m.g
S04_21572140	4	21572140	4	56.45	ID=Ciclev10030830m.g
S04_21521355	4	21521355	4	56.45	ID=Ciclev10031330m.g
S04_21647757	4	21647757	4	56.45	ID=Ciclev10030504m.g
S04_22142420	4	22142420	4	60.726	ID=Ciclev10033962m.g
S04_22134733	4	22134733	4	60.726	ID=Ciclev10031753m.g
S04_22151820	4	22151820	4	65.003	ID=Ciclev10030964m.g
S04_22120298	4	22120298	4	65.003	ID=Ciclev10031235m.g
S04_21836804	4	21836804	4	65.003	ID=Ciclev10031171m.g
S04_22147167	4	22147167	4	65.003	ID=Ciclev10033904m.g
S04_22409195	4	22409195	4	65.003	ID=Ciclev10030499m.g
S04_21807466	4	21807466	4	65.829	ID=Ciclev10033895m.g
S04_21791171	4	21791171	4	66.656	ID=Ciclev10030479m.g
S04_21925669	4	21925669	4	66.656	ID=Ciclev10031545m.g
S04_22025865	4	22025865	4	66.656	ID=Ciclev10033591m.g
S04_21798490	4	21798490	4	68.347	ID=Ciclev10032277m.g
S04_22369695	4	22369695	4	69.993	ID=Ciclev10033774m.g
S04_22347681	4	22347681	4	69.993	ID=Ciclev10030794m.g
S04_22264059	4	22264059	4	69.993	ID=Ciclev10031735m.g
S04_21832101	4	21832101	4	72.525	ID=Ciclev10032789m.g
S04_23150759	4	23150759	4	75.058	ID=Ciclev10032093m.g
S04_23053737	4	23053737	4	75.058	ID=Ciclev10032870m.g
S04_22770095	4	22770095	4	80.235	ID=Ciclev10033161m.g
S04_22501366	4	22501366	4	80.235	ID=Ciclev10031561m.g
S04_22803505	4	22803505	4	80.235	ID=Ciclev10031155m.g
S04_22842390	4	22842390	4	80.235	ID=Ciclev10030755m.g
S04_22636864	4	22636864	4	80.235	ID=Ciclev10032958m.g
S04_22487380	4	22487380	4	80.235	ID=Ciclev10030967m.g
S04_22720944	4	22720944	4	80.235	ID=Ciclev10033010m.g
S04_22642756	4	22642756	4	80.235	ID=Ciclev10033635m.g
S04_22416886	4	22416886	4	83.626	ID=Ciclev10032926m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S04_23209047	4	23209047	4	83.626	ID=Ciclev10030507m.g
S04_23205172	4	23205172	4	83.626	ID=Ciclev10030551m.g
S04_22566610	4	22566610	4	83.626	ID=Ciclev10030577m.g
S04_22543073	4	22543073	4	83.626	ID=Ciclev10033714m.g
S04_22628126	4	22628126	4	83.626	ID=Ciclev10031097m.g
S04_23251177	4	23251177	4	85.295	ID=Ciclev10032274m.g
S04_23612800	4	23612800	4	88.688	ID=Ciclev10033863m.g
S04_23572907	4	23572907	4	88.688	ID=Ciclev10032366m.g
S04_23631318	4	23631318	4	88.688	ID=Ciclev10031221m.g
S04_23541904	4	23541904	4	88.688	ID=Ciclev10031752m.g
S04_23505719	4	23505719	4	88.688	ID=Ciclev10032229m.g
S04_23610591	4	23610591	4	88.688	ID=Ciclev10033729m.g
S04_23528878	4	23528878	4	89.166	ID=Ciclev10031067m.g
S04_23452413	4	23452413	4	89.751	ID=Ciclev10031519m.g
S04_23311348	4	23311348	4	90.338	ID=Ciclev10030537m.g
S04_23375972	4	23375972	4	90.338	ID=Ciclev10033337m.g
S04_23636292	4	23636292	4	90.338	ID=Ciclev10033589m.g
S04_23784806	4	23784806	4	92.006	ID=Ciclev10033698m.g
S04_23883742	4	23883742	4	92.006	ID=Ciclev10030768m.g
S04_23845947	4	23845947	4	92.006	ID=Ciclev10030989m.g
S04_23657593	4	23657593	4	92.006	ID=Ciclev10033827m.g
S04_23804558	4	23804558	4	92.006	ID=Ciclev10032771m.g
S04_23791107	4	23791107	4	92.006	ID=Ciclev10031757m.g
S04_24484268	4	24484268	4	97.188	ID=Ciclev10033431m.g
S04_24616043	4	24616043	4	97.188	ID=Ciclev10032244m.g
S04_24280396	4	24280396	4	100.237	ID=Ciclev10030574m.g
S04_24752171	4	24752171	4	100.566	ID=Ciclev10031481m.g
S04_24688316	4	24688316	4	100.566	ID=Ciclev10031389m.g
S04_24151110	4	24151110	4	101.395	ID=Ciclev10031585m.g
S04_24653776	4	24653776	4	102.222	ID=Ciclev10030698m.g
S04_24067578	4	24067578	4	103.209	ID=Ciclev10030675m.g
S04_24780990	4	24780990	4	103.878	ID=Ciclev10030586m.g
S04_24380432	4	24380432	4	103.878	ID=Ciclev10033760m.g
S04_24789899	4	24789899	4	103.878	ID=Ciclev10030728m.g
S04_24196444	4	24196444	4	104.36	ID=Ciclev10030593m.g
S04_24205637	4	24205637	4	104.872	ID=Ciclev10032989m.g
S04_24646836	4	24646836	4	105.529	ID=Ciclev10033890m.g
S04_25075183	4	25075183	4	107.199	ID=Ciclev10033914m.g
S04_25548282	4	25548282	4	110.005	ID=Ciclev10033633m.g
S04_25431316	4	25431316	4	110.621	ID=Ciclev10030505m.g
S04_25271083	4	25271083	4	111.728	ID=Ciclev10030862m.g
S04_25359904	4	25359904	4	111.952	ID=Ciclev10031826m.g
S04_25356236	4	25356236	4	112.128	ID=Ciclev10031320m.g
S04_25232278	4	25232278	4	112.302	ID=Ciclev10033853m.g
S04_25171733	4	25171733	4	112.302	ID=Ciclev10033110m.g
S05_8576511	5	8576511	5	0	ID=Ciclev10000273m.g
S05_20933393	5	20933393	5	0	ID=Ciclev10002295m.g
S05_1475226	5	1475226	5	5.268	ID=Ciclev10002114m.g
S05_598285	5	598285	5	5.268	ID=Ciclev10000109m.g
S05_5089919	5	5089919	5	6.936	ID=Ciclev10000637m.g
S05_21186048	5	21186048	5	8.604	ID=Ciclev10003744m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_8532614	5	8532614	5	8.604	ID=Ciclev10000069m.g
S05_24048789	5	24048789	5	8.604	ID=Ciclev10001893m.g
S05_24071319	5	24071319	5	8.604	ID=Ciclev10000705m.g
S05_24375523	5	24375523	5	10.276	ID=Ciclev10003896m.g
S05_24807109	5	24807109	5	11.95	ID=Ciclev10002116m.g
S05_6001789	5	6001789	5	14.486	ID=Ciclev10000233m.g
S05_5838380	5	5838380	5	17.017	ID=Ciclev10003600m.g
S05_20084654	5	20084654	5	17.533	ID=Ciclev10000307m.g
S05_6483028	5	6483028	5	18.673	ID=Ciclev10000702m.g
S05_5333222	5	5333222	5	18.673	ID=Ciclev10000672m.g
S05_21811056	5	21811056	5	18.673	ID=Ciclev10000710m.g
S05_19186786	5	19186786	5	18.673	ID=Ciclev10000289m.g
S05_263109	5	263109	5	18.673	ID=Ciclev10002464m.g
S05_19118969	5	19118969	5	18.673	ID=Ciclev10001436m.g
S05_19514582	5	19514582	5	18.673	ID=Ciclev10002548m.g
S05_3514231	5	3514231	5	19.5	ID=Ciclev10003972m.g
S05_24110819	5	24110819	5	20.327	ID=Ciclev10000617m.g
S05_21524934	5	21524934	5	20.327	ID=Ciclev10001212m.g
S05_23725104	5	23725104	5	20.327	ID=Ciclev10000349m.g
S05_6727126	5	6727126	5	22.55	ID=Ciclev10001528m.g
S05_19107264	5	19107264	5	27.137	ID=Ciclev10000577m.g
S05_5288458	5	5288458	5	27.137	ID=Ciclev10000066m.g
S05_22272118	5	22272118	5	27.137	ID=Ciclev10000572m.g
S05_8643917	5	8643917	5	27.137	ID=Ciclev10000024m.g
S05_6075042	5	6075042	5	28.104	ID=Ciclev10000976m.g
S05_21581252	5	21581252	5	28.791	ID=Ciclev10002554m.g
S05_6736415	5	6736415	5	30.459	ID=Ciclev10003667m.g
S05_19785467	5	19785467	5	32.126	ID=Ciclev10002513m.g
S05_24797361	5	24797361	5	33.795	ID=Ciclev10001613m.g
S05_25798577	5	25798577	5	37.195	ID=Ciclev10003116m.g
S05_25966816	5	25966816	5	40.596	ID=Ciclev10000735m.g
S05_26336326	5	26336326	5	42.266	ID=Ciclev10003287m.g
S05_25862618	5	25862618	5	43.935	ID=Ciclev10003885m.g
S05_25843160	5	25843160	5	44.595	ID=Ciclev10000944m.g
S05_26489602	5	26489602	5	44.926	ID=Ciclev10003268m.g
S05_26006828	5	26006828	5	45.586	ID=Ciclev10000690m.g
S05_26991841	5	26991841	5	47.253	ID=Ciclev10001973m.g
S05_27123907	5	27123907	5	47.253	ID=Ciclev10003996m.g
S05_28856925	5	28856925	5	51.841	ID=Ciclev10000274m.g
S05_30642977	5	30642977	5	54.067	ID=Ciclev10000168m.g
S05_30769892	5	30769892	5	54.067	ID=Ciclev10000353m.g
S05_30628139	5	30628139	5	54.067	ID=Ciclev10000082m.g
S05_30803372	5	30803372	5	57.458	ID=Ciclev10001724m.g
S05_31431890	5	31431890	5	57.458	ID=Ciclev10000394m.g
S05_30818937	5	30818937	5	57.458	ID=Ciclev10000367m.g
S05_27376509	5	27376509	5	60.85	ID=Ciclev10003137m.g
S05_27147402	5	27147402	5	60.85	ID=Ciclev10002550m.g
S05_29429471	5	29429471	5	62.518	ID=Ciclev10000014m.g
S05_28836044	5	28836044	5	65.909	ID=Ciclev10003490m.g
S05_28882827	5	28882827	5	65.909	ID=Ciclev10002930m.g
S05_29303781	5	29303781	5	65.909	ID=Ciclev10002780m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_29126569	5	29126569	5	70.909	ID=Ciclev10000893m.g
S05_29484586	5	29484586	5	70.909	ID=Ciclev10001524m.g
S05_31340973	5	31340973	5	71.069	ID=Ciclev10003196m.g
S05_31870737	5	31870737	5	71.069	ID=Ciclev10000365m.g
S05_31883435	5	31883435	5	71.069	ID=Ciclev10003335m.g
S05_32270852	5	32270852	5	71.069	ID=Ciclev10003811m.g
S05_32320460	5	32320460	5	71.069	ID=Ciclev10001418m.g
S05_33693180	5	33693180	5	76.246	ID=Ciclev10003641m.g
S05_33247279	5	33247279	5	76.246	ID=Ciclev10001783m.g
S05_32721476	5	32721476	5	76.246	ID=Ciclev10000732m.g
S05_33264535	5	33264535	5	76.246	ID=Ciclev10000234m.g
S05_33399695	5	33399695	5	76.246	ID=Ciclev10002947m.g
S05_33348819	5	33348819	5	76.266	ID=Ciclev10003399m.g
S05_33244543	5	33244543	5	77.913	ID=Ciclev10000070m.g
S05_32629464	5	32629464	5	78.742	ID=Ciclev10001088m.g
S05_33850758	5	33850758	5	79.567	ID=Ciclev10002418m.g
S05_33699294	5	33699294	5	79.567	ID=Ciclev10000558m.g
S05_33028994	5	33028994	5	79.567	ID=Ciclev10000984m.g
S05_33288621	5	33288621	5	79.567	ID=Ciclev10000227m.g
S05_33758296	5	33758296	5	80.394	ID=Ciclev10000089m.g
S05_33888576	5	33888576	5	81.221	ID=Ciclev10000704m.g
S05_32908697	5	32908697	5	81.221	ID=Ciclev10003592m.g
S05_34333646	5	34333646	5	82.888	ID=Ciclev10001730m.g
S05_34166737	5	34166737	5	82.888	ID=Ciclev10003144m.g
S05_34179407	5	34179407	5	82.888	ID=Ciclev10003854m.g
S05_34025509	5	34025509	5	82.888	ID=Ciclev10002200m.g
S05_34401451	5	34401451	5	82.888	ID=Ciclev10000565m.g
S05_34050282	5	34050282	5	83.869	ID=Ciclev10001988m.g
S05_34143844	5	34143844	5	84.542	ID=Ciclev10003403m.g
S05_34077426	5	34077426	5	84.542	ID=Ciclev10003914m.g
S05_33985291	5	33985291	5	84.542	ID=Ciclev10003420m.g
S05_34414822	5	34414822	5	87.933	ID=Ciclev10001042m.g
S05_34480328	5	34480328	5	87.933	ID=Ciclev10000363m.g
S05_34454808	5	34454808	5	87.933	ID=Ciclev10001445m.g
S05_34491312	5	34491312	5	87.933	ID=Ciclev10001990m.g
S05_34662048	5	34662048	5	90.454	ID=Ciclev10001733m.g
S05_34572304	5	34572304	5	90.454	ID=Ciclev10003450m.g
S05_34659442	5	34659442	5	92.977	ID=Ciclev10003023m.g
S05_34698173	5	34698173	5	92.977	ID=Ciclev10001488m.g
S05_35193227	5	35193227	5	94.646	ID=Ciclev10003118m.g
S05_34915829	5	34915829	5	96.315	ID=Ciclev10001508m.g
S05_34932398	5	34932398	5	97.985	ID=Ciclev10000171m.g
S05_35016554	5	35016554	5	97.985	ID=Ciclev10000361m.g
S05_35119778	5	35119778	5	99.652	ID=Ciclev10000073m.g
S05_35139896	5	35139896	5	99.652	ID=Ciclev10000765m.g
S05_35521981	5	35521981	5	101.321	ID=Ciclev10001318m.g
S05_35481020	5	35481020	5	101.321	ID=Ciclev10000002m.g
S05_35975188	5	35975188	5	104.716	ID=Ciclev10001677m.g
S05_35901593	5	35901593	5	105.471	ID=Ciclev10001050m.g
S05_35897627	5	35897627	5	106.373	ID=Ciclev10003510m.g
S05_35811843	5	35811843	5	106.373	ID=Ciclev10003720m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_35825120	5	35825120	5	106.373	ID=Ciclev10000370m.g
S05_36014384	5	36014384	5	106.373	ID=Ciclev10001098m.g
S05_36021776	5	36021776	5	106.373	ID=Ciclev10001605m.g
S05_35812531	5	35812531	5	106.373	ID=Ciclev10003472m.g
S05_36108329	5	36108329	5	108.04	ID=Ciclev10002573m.g
S05_36092538	5	36092538	5	108.04	ID=Ciclev10000706m.g
S05_36251891	5	36251891	5	109.708	ID=Ciclev10000925m.g
S05_37091596	5	37091596	5	111.376	ID=Ciclev10000652m.g
S05_36456697	5	36456697	5	111.376	ID=Ciclev10000337m.g
S05_36825383	5	36825383	5	111.376	ID=Ciclev10004036m.g
S05_36952857	5	36952857	5	116.566	ID=Ciclev10001827m.g
S05_37817382	5	37817382	5	119.979	ID=Ciclev10000005m.g
S05_36879692	5	36879692	5	123.379	ID=Ciclev10001364m.g
S05_36753850	5	36753850	5	123.379	ID=Ciclev10003301m.g
S04_23428140	4	23428140	5	125.048	ID=Ciclev10030927m.g
S05_36799437	5	36799437	5	125.048	ID=Ciclev10001268m.g
S05_36673311	5	36673311	5	125.048	ID=Ciclev10000007m.g
S05_36699324	5	36699324	5	125.048	ID=Ciclev10000521m.g
S05_36647034	5	36647034	5	125.048	ID=Ciclev10002627m.g
S05_37150390	5	37150390	5	126.715	ID=Ciclev10000093m.g
S05_37383324	5	37383324	5	126.715	ID=Ciclev10001712m.g
S05_37303944	5	37303944	5	126.715	ID=Ciclev10001739m.g
S05_37211938	5	37211938	5	126.715	ID=Ciclev10003221m.g
S05_37181214	5	37181214	5	127.541	ID=Ciclev10000940m.g
S05_37409865	5	37409865	5	128.367	ID=Ciclev10000438m.g
S05_37437637	5	37437637	5	128.367	ID=Ciclev10001829m.g
S05_37241107	5	37241107	5	128.367	ID=Ciclev10002717m.g
S05_37256604	5	37256604	5	128.368	ID=Ciclev10001460m.g
S05_38579459	5	38579459	5	130.89	ID=Ciclev10002398m.g
S05_38132186	5	38132186	5	133.414	ID=Ciclev10000341m.g
S05_39359301	5	39359301	5	135.083	ID=Ciclev10000691m.g
S05_38750649	5	38750649	5	135.083	ID=Ciclev10003841m.g
S05_38421950	5	38421950	5	138.476	ID=Ciclev10002261m.g
S05_38603855	5	38603855	5	139.289	ID=Ciclev10002923m.g
S05_38926830	5	38926830	5	140.133	ID=Ciclev10003802m.g
S05_39161302	5	39161302	5	143.526	ID=Ciclev10003530m.g
S05_38342371	5	38342371	5	143.526	ID=Ciclev10001382m.g
S05_37750945	5	37750945	5	145.196	ID=Ciclev10000954m.g
S05_39301578	5	39301578	5	147.72	ID=Ciclev10002068m.g
S05_39314291	5	39314291	5	150.244	ID=Ciclev10000033m.g
S05_39091840	5	39091840	5	150.244	ID=Ciclev10003681m.g
S05_37544652	5	37544652	5	151.915	ID=Ciclev10003265m.g
S05_37534273	5	37534273	5	153.587	ID=Ciclev10000949m.g
S05_37559697	5	37559697	5	153.587	ID=Ciclev10000080m.g
S05_37597799	5	37597799	5	153.587	ID=Ciclev10003678m.g
S05_37570919	5	37570919	5	153.587	ID=Ciclev10000010m.g
S05_37671282	5	37671282	5	155.255	ID=Ciclev10000425m.g
S05_38516353	5	38516353	5	156.923	ID=Ciclev10002251m.g
S05_39224893	5	39224893	5	156.923	ID=Ciclev10000749m.g
S05_38669745	5	38669745	5	160.314	ID=Ciclev10000627m.g
S05_39283025	5	39283025	5	160.314	ID=Ciclev10004050m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_38813295	5	38813295	5	160.314	ID=Ciclev10002431m.g
S05_39111772	5	39111772	5	160.314	ID=Ciclev10001743m.g
S05_39296648	5	39296648	5	160.314	ID=Ciclev10000533m.g
S05_38774965	5	38774965	5	160.314	ID=Ciclev10000601m.g
S05_38124867	5	38124867	5	161.14	ID=Ciclev10003893m.g
S05_38566755	5	38566755	5	161.968	ID=Ciclev10001715m.g
S05_38235074	5	38235074	5	161.968	ID=Ciclev10000185m.g
S05_39145728	5	39145728	5	161.968	ID=Ciclev10003908m.g
S05_38093846	5	38093846	5	161.968	ID=Ciclev10001030m.g
S05_39252307	5	39252307	5	161.968	ID=Ciclev10000771m.g
S05_39600601	5	39600601	5	165.37	ID=Ciclev10000021m.g
S05_39511206	5	39511206	5	167.046	ID=Ciclev10002730m.g
S05_40386303	5	40386303	5	170.444	ID=Ciclev10001214m.g
S05_39694366	5	39694366	5	170.444	ID=Ciclev10000804m.g
S05_39893984	5	39893984	5	170.444	ID=Ciclev10002344m.g
S05_40035854	5	40035854	5	173.835	ID=Ciclev10003898m.g
S05_39702778	5	39702778	5	173.835	ID=Ciclev10000682m.g
S05_40091538	5	40091538	5	175.502	ID=Ciclev10003815m.g
S05_39741425	5	39741425	5	175.502	ID=Ciclev10000723m.g
S05_40349357	5	40349357	5	177.171	ID=Ciclev10000175m.g
S05_40010125	5	40010125	5	178.839	ID=Ciclev10000459m.g
S05_40141399	5	40141399	5	178.839	ID=Ciclev10001159m.g
S05_40503604	5	40503604	5	180.506	ID=Ciclev10000779m.g
S05_40042391	5	40042391	5	180.506	ID=Ciclev10000364m.g
S05_40429150	5	40429150	5	183.03	ID=Ciclev10001332m.g
S05_39929044	5	39929044	5	185.552	ID=Ciclev10001636m.g
S05_40633018	5	40633018	5	185.552	ID=Ciclev10002047m.g
S05_40676947	5	40676947	5	187.219	ID=Ciclev10003605m.g
S05_40636398	5	40636398	5	187.219	ID=Ciclev10003036m.g
S05_40876462	5	40876462	5	188.886	ID=Ciclev10000910m.g
S05_40749195	5	40749195	5	188.886	ID=Ciclev10001493m.g
S05_40961328	5	40961328	5	192.28	ID=Ciclev10000013m.g
S05_40921442	5	40921442	5	193.735	ID=Ciclev10003473m.g
S05_41064133	5	41064133	5	193.943	ID=Ciclev10000217m.g
S05_41100584	5	41100584	5	193.943	ID=Ciclev10000579m.g
S05_41243971	5	41243971	5	194.76	ID=Ciclev10001170m.g
S05_41286196	5	41286196	5	195.599	ID=Ciclev10002738m.g
S05_41441028	5	41441028	5	197.269	ID=Ciclev10002101m.g
S05_41481628	5	41481628	5	198.938	ID=Ciclev10001438m.g
S05_41720208	5	41720208	5	200.609	ID=Ciclev10001297m.g
S05_41595676	5	41595676	5	202.277	ID=Ciclev10000097m.g
S05_41654822	5	41654822	5	202.277	ID=Ciclev10002853m.g
S05_41590255	5	41590255	5	202.277	ID=Ciclev10000322m.g
S05_41722950	5	41722950	5	202.277	ID=Ciclev10000183m.g
S05_41568189	5	41568189	5	203.946	ID=Ciclev10002195m.g
S05_42220767	5	42220767	5	205.616	ID=Ciclev10000767m.g
S05_41881666	5	41881666	5	207.283	ID=Ciclev10001255m.g
S05_42235610	5	42235610	5	207.283	ID=Ciclev10003990m.g
S05_42103002	5	42103002	5	207.283	ID=Ciclev10000054m.g
S05_41790595	5	41790595	5	207.283	ID=Ciclev10001007m.g
S05_41819674	5	41819674	5	208.951	ID=Ciclev10000087m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_42638044	5	42638044	5	212.343	ID=Ciclev10003823m.g
S05_42842551	5	42842551	5	219.372	ID=Ciclev10000306m.g
S05_42900985	5	42900985	5	219.372	ID=Ciclev10000611m.g
S05_42876899	5	42876899	5	219.372	ID=Ciclev10001114m.g
S06_552062	6	552062	6	0	ID=Ciclev10011820m.g
S06_498493	6	498493	6	0	ID=Ciclev10011912m.g
S06_564189	6	564189	6	0	ID=Ciclev10013309m.g
S06_10092115	6	10092115	6	7.034	ID=Ciclev10011188m.g
S06_6304063	6	6304063	6	7.034	ID=Ciclev10011125m.g
S06_7125949	6	7125949	6	7.034	ID=Ciclev10012732m.g
S06_11102965	6	11102965	6	10.435	ID=Ciclev10012548m.g
S06_11462163	6	11462163	6	12.109	ID=Ciclev10011862m.g
S06_11938538	6	11938538	6	12.109	ID=Ciclev10011108m.g
S08_23623480	8	23623480	6	17.94	ID=Ciclev10027837m.g
S06_14230074	6	14230074	6	20.716	ID=Ciclev10011102m.g
S06_13371589	6	13371589	6	20.716	ID=Ciclev10011092m.g
S06_13631934	6	13631934	6	20.716	ID=Ciclev10012740m.g
S06_13449082	6	13449082	6	20.716	ID=Ciclev10011745m.g
S06_14373074	6	14373074	6	20.716	ID=Ciclev10011358m.g
S06_15341827	6	15341827	6	24.107	ID=Ciclev10012857m.g
S06_15221853	6	15221853	6	24.107	ID=Ciclev10011305m.g
S06_15072197	6	15072197	6	24.107	ID=Ciclev10011268m.g
S06_15679040	6	15679040	6	27.5	ID=Ciclev10013588m.g
S06_16235415	6	16235415	6	29.177	ID=Ciclev10013484m.g
S06_16841629	6	16841629	6	34.386	ID=Ciclev10010996m.g
S06_17007902	6	17007902	6	36.229	ID=Ciclev10013125m.g
S06_20855147	6	20855147	6	58.485	ID=Ciclev10011131m.g
S06_20894784	6	20894784	6	58.485	ID=Ciclev10010903m.g
S06_21037367	6	21037367	6	60.151	ID=Ciclev10012049m.g
S06_21108317	6	21108317	6	60.151	ID=Ciclev10010892m.g
S06_21066818	6	21066818	6	60.151	ID=Ciclev10010952m.g
S06_21086189	6	21086189	6	60.151	ID=Ciclev10013782m.g
S06_21133611	6	21133611	6	63.551	ID=Ciclev10012216m.g
S06_21165534	6	21165534	6	63.551	ID=Ciclev10013017m.g
S06_21300943	6	21300943	6	63.551	ID=Ciclev10011036m.g
S06_21481949	6	21481949	6	67.372	ID=Ciclev10011065m.g
S06_21786425	6	21786425	6	75.359	ID=Ciclev10013336m.g
S06_21983625	6	21983625	6	77.233	ID=Ciclev10011580m.g
S06_22828282	6	22828282	6	79.805	ID=Ciclev10013371m.g
S06_23147627	6	23147627	6	82.025	ID=Ciclev10012575m.g
S06_22544691	6	22544691	6	84.218	ID=Ciclev10012974m.g
S06_23579311	6	23579311	6	84.218	ID=Ciclev10011302m.g
S06_24380310	6	24380310	6	84.218	ID=Ciclev10013340m.g
S06_22307870	6	22307870	6	87.619	ID=Ciclev10012265m.g
S06_22095421	6	22095421	6	87.619	ID=Ciclev10012626m.g
S06_23068554	6	23068554	6	92.827	ID=Ciclev10011430m.g
S06_22986901	6	22986901	6	98.002	ID=Ciclev10011795m.g
S03_23488114	3	23488114	6	98.002	ID=Ciclev10024661m.g
S06_23028307	6	23028307	6	98.002	ID=Ciclev10013264m.g
S06_23041125	6	23041125	6	98.002	ID=Ciclev10013755m.g
S06_24753054	6	24753054	6	106.954	ID=Ciclev10010889m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S06_25150352	6	25150352	6	106.954	ID=Ciclev10013796m.g
S06_24686898	6	24686898	6	106.954	ID=Ciclev10012004m.g
S06_25320333	6	25320333	6	106.954	ID=Ciclev10013417m.g
S06_24739514	6	24739514	6	106.954	ID=Ciclev10011009m.g
S06_25021089	6	25021089	6	106.954	ID=Ciclev10011967m.g
S06_25225315	6	25225315	6	106.954	ID=Ciclev10011242m.g
S07_1739845	7	1739845	7	0	ID=Ciclev10025354m.g
S07_1820689	7	1820689	7	0	ID=Ciclev10025352m.g
S07_1843281	7	1843281	7	0	ID=Ciclev10026346m.g
S07_1908962	7	1908962	7	0	ID=Ciclev10024987m.g
S07_1760308	7	1760308	7	0	ID=Ciclev10027167m.g
S07_1875032	7	1875032	7	0	ID=Ciclev10025287m.g
S07_1942753	7	1942753	7	0	ID=Ciclev10025926m.g
S07_1737900	7	1737900	7	0	ID=Ciclev10025528m.g
S07_1826961	7	1826961	7	0	ID=Ciclev10024708m.g
S07_1363133	7	1363133	7	1.786	ID=Ciclev10027574m.g
S07_273717	7	273717	7	1.786	ID=Ciclev10025215m.g
S07_930529	7	930529	7	1.786	ID=Ciclev10025092m.g
S07_902040	7	902040	7	1.786	ID=Ciclev10026139m.g
S07_371237	7	371237	7	1.786	ID=Ciclev10026057m.g
S07_882690	7	882690	7	1.786	ID=Ciclev10025798m.g
S07_290288	7	290288	7	1.786	ID=Ciclev10025307m.g
S07_740721	7	740721	7	1.786	ID=Ciclev10026205m.g
S07_1395950	7	1395950	7	1.786	ID=Ciclev10025467m.g
S07_1180913	7	1180913	7	1.786	ID=Ciclev10024677m.g
S07_492485	7	492485	7	1.786	ID=Ciclev10025620m.g
S07_926251	7	926251	7	1.786	ID=Ciclev10027108m.g
S07_1433907	7	1433907	7	1.786	ID=Ciclev10024691m.g
S07_877465	7	877465	7	1.786	ID=Ciclev10026691m.g
S07_915534	7	915534	7	1.786	ID=Ciclev10026181m.g
S07_401258	7	401258	7	1.786	ID=Ciclev10026997m.g
S07_192032	7	192032	7	1.786	ID=Ciclev10025677m.g
S07_1349137	7	1349137	7	1.786	ID=Ciclev10027500m.g
S07_205615	7	205615	7	1.786	ID=Ciclev10025598m.g
S07_681344	7	681344	7	1.786	ID=Ciclev10025900m.g
S07_1680444	7	1680444	7	1.786	ID=Ciclev10025725m.g
S07_861106	7	861106	7	1.786	ID=Ciclev10024800m.g
S07_180153	7	180153	7	1.786	ID=Ciclev10024825m.g
S07_185479	7	185479	7	1.786	ID=Ciclev10026688m.g
S07_731138	7	731138	7	1.786	ID=Ciclev10025182m.g
S07_871172	7	871172	7	1.786	ID=Ciclev10025325m.g
S07_1266727	7	1266727	7	1.786	ID=Ciclev10026308m.g
S07_175901	7	175901	7	5.428	ID=Ciclev10027191m.g
S07_3294596	7	3294596	7	10.993	ID=Ciclev10024929m.g
S07_3447086	7	3447086	7	10.993	ID=Ciclev10025109m.g
S07_3385070	7	3385070	7	10.993	ID=Ciclev10024826m.g
S07_2228843	7	2228843	7	12.78	ID=Ciclev10025048m.g
S07_2895844	7	2895844	7	14.567	ID=Ciclev10027275m.g
S07_2688435	7	2688435	7	14.567	ID=Ciclev10024810m.g
S07_2939174	7	2939174	7	14.567	ID=Ciclev10025791m.g
S07_3390623	7	3390623	7	18.197	ID=Ciclev10027323m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S07_3227521	7	3227521	7	18.204	ID=Ciclev10025089m.g
S07_2139155	7	2139155	7	18.204	ID=Ciclev10025746m.g
S07_3401126	7	3401126	7	18.204	ID=Ciclev10025986m.g
S07_2682404	7	2682404	7	18.227	ID=Ciclev10024926m.g
S07_2206656	7	2206656	7	21.841	ID=Ciclev10027059m.g
S07_2593033	7	2593033	7	21.841	ID=Ciclev10026511m.g
S07_2074917	7	2074917	7	21.841	ID=Ciclev10025149m.g
S07_2637371	7	2637371	7	21.841	ID=Ciclev10024678m.g
S07_2954250	7	2954250	7	21.841	ID=Ciclev10025192m.g
S07_3040010	7	3040010	7	21.841	ID=Ciclev10025861m.g
S07_2715037	7	2715037	7	22.445	ID=Ciclev10025487m.g
S07_2608244	7	2608244	7	23.048	ID=Ciclev10026303m.g
S07_2014970	7	2014970	7	23.607	ID=Ciclev10025293m.g
S07_3406608	7	3406608	7	23.607	ID=Ciclev10024767m.g
S07_3236795	7	3236795	7	23.607	ID=Ciclev10025943m.g
S07_3149605	7	3149605	7	23.607	ID=Ciclev10024681m.g
S07_2985143	7	2985143	7	25.394	ID=Ciclev10025216m.g
S07_3571967	7	3571967	7	27.185	ID=Ciclev10025336m.g
S07_4058524	7	4058524	7	33.967	ID=Ciclev10027450m.g
S07_3917346	7	3917346	7	33.967	ID=Ciclev10025596m.g
S07_3859099	7	3859099	7	36.108	ID=Ciclev10024760m.g
S07_4128191	7	4128191	7	38.256	ID=Ciclev10026545m.g
S07_4278714	7	4278714	7	41.902	ID=Ciclev10025483m.g
S07_4822912	7	4822912	7	49.495	ID=Ciclev10024875m.g
S07_5146366	7	5146366	7	53.161	ID=Ciclev10024755m.g
S07_5410789	7	5410789	7	56.827	ID=Ciclev10027273m.g
S07_5704418	7	5704418	7	60.476	ID=Ciclev10025496m.g
S07_6370885	7	6370885	7	62.269	ID=Ciclev10024781m.g
S07_6440586	7	6440586	7	64.06	ID=Ciclev10025448m.g
S07_6387837	7	6387837	7	64.06	ID=Ciclev10024961m.g
S07_6838252	7	6838252	7	65.848	ID=Ciclev10024676m.g
S07_6905350	7	6905350	7	67.636	ID=Ciclev10025070m.g
S07_6929895	7	6929895	7	67.636	ID=Ciclev10027473m.g
S07_8395132	7	8395132	7	71.279	ID=Ciclev10024743m.g
S07_7845654	7	7845654	7	71.279	ID=Ciclev10027008m.g
S07_8146614	7	8146614	7	73.067	ID=Ciclev10026930m.g
S07_7959180	7	7959180	7	74.854	ID=Ciclev10026808m.g
S07_7945015	7	7945015	7	74.854	ID=Ciclev10026383m.g
S07_8211816	7	8211816	7	76.641	ID=Ciclev10025938m.g
S07_7827620	7	7827620	7	76.641	ID=Ciclev10024934m.g
S07_8513898	7	8513898	7	76.641	ID=Ciclev10024891m.g
S07_7400719	7	7400719	7	77.527	ID=Ciclev10024725m.g
S07_7023456	7	7023456	7	78.411	ID=Ciclev10025601m.g
S07_8246376	7	8246376	7	78.411	ID=Ciclev10025419m.g
S07_8327760	7	8327760	7	81.115	ID=Ciclev10025716m.g
S07_7324809	7	7324809	7	81.115	ID=Ciclev10025154m.g
S07_7265514	7	7265514	7	83.82	ID=Ciclev10025411m.g
S07_8249936	7	8249936	7	83.82	ID=Ciclev10025103m.g
S07_7962481	7	7962481	7	83.82	ID=Ciclev10025233m.g
S07_8506617	7	8506617	7	83.82	ID=Ciclev10026283m.g
S07_7797843	7	7797843	7	83.82	ID=Ciclev10025816m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S07_8440906	7	8440906	7	83.82	ID=Ciclev10025950m.g
S07_8373422	7	8373422	7	83.82	ID=Ciclev10025091m.g
S07_8340332	7	8340332	7	83.82	ID=Ciclev10025452m.g
S07_8764184	7	8764184	7	85.606	ID=Ciclev10025189m.g
S07_9042069	7	9042069	7	85.606	ID=Ciclev10026140m.g
S07_8858370	7	8858370	7	85.606	ID=Ciclev10024803m.g
S07_8909779	7	8909779	7	87.241	ID=Ciclev10027173m.g
S07_8774196	7	8774196	7	87.387	ID=Ciclev10025368m.g
S07_9021397	7	9021397	7	87.387	ID=Ciclev10024822m.g
S07_7965306	7	7965306	7	91.025	ID=Ciclev10025289m.g
S07_7642042	7	7642042	7	91.025	ID=Ciclev10025604m.g
S07_8640065	7	8640065	7	92.812	ID=Ciclev10027587m.g
S07_7876325	7	7876325	7	93.903	ID=Ciclev10027324m.g
S07_7064298	7	7064298	7	94.585	ID=Ciclev10025181m.g
S07_7350256	7	7350256	7	94.585	ID=Ciclev10025673m.g
S07_7898863	7	7898863	7	96.372	ID=Ciclev10025232m.g
S01_26623341	1	26623341	7	98.159	ID=Ciclev10010842m.g
S07_10271486	7	10271486	7	98.159	ID=Ciclev10027224m.g
S01_19804022	1	19804022	7	99.945	ID=Ciclev10010060m.g
S07_10470218	7	10470218	7	101.731	ID=Ciclev10024977m.g
S07_10414972	7	10414972	7	101.731	ID=Ciclev10024707m.g
S07_10011844	7	10011844	7	103.519	ID=Ciclev10025498m.g
S07_11245034	7	11245034	7	107.16	ID=Ciclev10027305m.g
S07_11307349	7	11307349	7	107.16	ID=Ciclev10026390m.g
S07_11028023	7	11028023	7	107.16	ID=Ciclev10024858m.g
S07_10762519	7	10762519	7	107.16	ID=Ciclev10026079m.g
S07_10807651	7	10807651	7	108.946	ID=Ciclev10025851m.g
S07_10866226	7	10866226	7	108.946	ID=Ciclev10026275m.g
S07_13322380	7	13322380	7	112.584	ID=Ciclev10026525m.g
S07_13173748	7	13173748	7	112.584	ID=Ciclev10025584m.g
S07_13003362	7	13003362	7	112.584	ID=Ciclev10024946m.g
S07_13393945	7	13393945	7	112.584	ID=Ciclev10024717m.g
S07_13027708	7	13027708	7	116.229	ID=Ciclev10026249m.g
S07_14151723	7	14151723	7	119.875	ID=Ciclev10024905m.g
S07_14137109	7	14137109	7	119.875	ID=Ciclev10024841m.g
S07_14284271	7	14284271	7	121.661	ID=Ciclev10026151m.g
S07_14385697	7	14385697	7	121.661	ID=Ciclev10024814m.g
S07_14584086	7	14584086	7	125.297	ID=Ciclev10025544m.g
S07_14681491	7	14681491	7	125.298	ID=Ciclev10027257m.g
S07_15212800	7	15212800	7	125.298	ID=Ciclev10027095m.g
S07_15091784	7	15091784	7	125.298	ID=Ciclev10026430m.g
S07_15877339	7	15877339	7	127.681	ID=Ciclev10027033m.g
S04_5032444	4	5032444	7	127.681	ID=Ciclev10031631m.g
S05_14344710	5	14344710	7	132.607	ID=Ciclev10000225m.g
S05_18108480	5	18108480	7	132.607	ID=Ciclev10004053m.g
S07_16630111	7	16630111	7	132.607	ID=Ciclev10025084m.g
S07_16844040	7	16844040	7	132.607	ID=Ciclev10027040m.g
S07_16872462	7	16872462	7	132.607	ID=Ciclev10027387m.g
S05_16132934	5	16132934	7	132.607	ID=Ciclev10000587m.g
S07_16552175	7	16552175	7	132.607	ID=Ciclev10025271m.g
S05_16528497	5	16528497	7	132.607	ID=Ciclev10001203m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S07_16948186	7	16948186	7	132.607	ID=Ciclev10026982m.g
S07_16691411	7	16691411	7	132.607	ID=Ciclev10024723m.g
S07_16615255	7	16615255	7	136.245	ID=Ciclev10026434m.g
S05_18135395	5	18135395	7	136.245	ID=Ciclev10000018m.g
S07_17255984	7	17255984	7	138.032	ID=Ciclev10025939m.g
S07_17487643	7	17487643	7	139.819	ID=Ciclev10027028m.g
S07_17901828	7	17901828	7	141.606	ID=Ciclev10024737m.g
S07_17534295	7	17534295	7	141.606	ID=Ciclev10026032m.g
S07_17676692	7	17676692	7	141.606	ID=Ciclev10027405m.g
S07_17807020	7	17807020	7	141.606	ID=Ciclev10025253m.g
S07_18311797	7	18311797	7	143.392	ID=Ciclev10024679m.g
S07_18343520	7	18343520	7	143.392	ID=Ciclev10027573m.g
S07_18351742	7	18351742	7	143.536	ID=Ciclev10027395m.g
S07_18992617	7	18992617	7	145.173	ID=Ciclev10024954m.g
S07_19114004	7	19114004	7	145.173	ID=Ciclev10025197m.g
S05_18394450	5	18394450	7	145.173	ID=Ciclev10000834m.g
S07_19119673	7	19119673	7	145.173	ID=Ciclev10024728m.g
S07_19529441	7	19529441	7	145.173	ID=Ciclev10027269m.g
S07_19596607	7	19596607	7	145.173	ID=Ciclev10027547m.g
S07_19618556	7	19618556	7	145.173	ID=Ciclev10026971m.g
S07_20115273	7	20115273	7	146.959	ID=Ciclev10025730m.g
S07_20091557	7	20091557	7	146.959	ID=Ciclev10024812m.g
S07_20105781	7	20105781	7	146.959	ID=Ciclev10024684m.g
S07_20041368	7	20041368	7	146.959	ID=Ciclev10027075m.g
S07_20526014	7	20526014	7	148.746	ID=Ciclev10027553m.g
S07_20540518	7	20540518	7	148.746	ID=Ciclev10027510m.g
S07_20555593	7	20555593	7	150.532	ID=Ciclev10025052m.g
S07_20900267	7	20900267	7	150.533	ID=Ciclev10025648m.g
S07_20847549	7	20847549	7	152.318	ID=Ciclev10025269m.g
S07_20867391	7	20867391	7	152.318	ID=Ciclev10025805m.g
S07_21025170	7	21025170	7	152.318	ID=Ciclev10027400m.g
S07_21004059	7	21004059	7	152.318	ID=Ciclev10025447m.g
S07_21079556	7	21079556	7	152.318	ID=Ciclev10026951m.g
S08_184086	8	184086	8	0	ID=Ciclev10028098m.g
S08_36471	8	36471	8	1.695	ID=Ciclev10027745m.g
S08_265031	8	265031	8	1.695	ID=Ciclev10027787m.g
S08_289006	8	289006	8	1.695	ID=Ciclev10028003m.g
S08_536935	8	536935	8	5.11	ID=Ciclev10028131m.g
S08_2173659	8	2173659	8	18.497	ID=Ciclev10029274m.g
S08_2513786	8	2513786	8	22.513	ID=Ciclev10027846m.g
S08_2720265	8	2720265	8	25.487	ID=Ciclev10028040m.g
S08_2635854	8	2635854	8	28.457	ID=Ciclev10027853m.g
S08_2814622	8	2814622	8	28.457	ID=Ciclev10028334m.g
S08_2850191	8	2850191	8	28.457	ID=Ciclev10027734m.g
S08_2947677	8	2947677	8	33.634	ID=Ciclev10030049m.g
S08_3701645	8	3701645	8	33.634	ID=Ciclev10027659m.g
S08_3494242	8	3494242	8	33.634	ID=Ciclev10028454m.g
S08_4417547	8	4417547	8	33.634	ID=Ciclev10029642m.g
S08_4555434	8	4555434	8	38.822	ID=Ciclev10029826m.g
S08_4046868	8	4046868	8	40.499	ID=Ciclev10027657m.g
S08_5052209	8	5052209	8	45.686	ID=Ciclev10030203m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S08_4892311	8	4892311	8	45.686	ID=Ciclev10028655m.g
S08_6050577	8	6050577	8	47.353	ID=Ciclev10027768m.g
S08_5719271	8	5719271	8	47.91	ID=Ciclev10029834m.g
S08_5326467	8	5326467	8	48.459	ID=Ciclev10028904m.g
S08_5491130	8	5491130	8	49.002	ID=Ciclev10027908m.g
S08_6643244	8	6643244	8	49.002	ID=Ciclev10027673m.g
S08_5315216	8	5315216	8	49.002	ID=Ciclev10028526m.g
S08_5941212	8	5941212	8	54.179	ID=Ciclev10028169m.g
S08_6197486	8	6197486	8	54.179	ID=Ciclev10027697m.g
S08_5583583	8	5583583	8	54.179	ID=Ciclev10028780m.g
S08_5909537	8	5909537	8	54.179	ID=Ciclev10028779m.g
S08_8140547	8	8140547	8	61.209	ID=Ciclev10029494m.g
S08_6946355	8	6946355	8	61.209	ID=Ciclev10028076m.g
S09_13043876	9	13043876	8	61.209	ID=Ciclev10005064m.g
S08_10889123	8	10889123	8	61.209	ID=Ciclev10030244m.g
S03_41034561	3	41034561	8	61.209	ID=Ciclev10018706m.g
S09_12840779	9	12840779	8	66.385	ID=Ciclev10005352m.g
S09_12849941	9	12849941	8	66.386	ID=Ciclev10004300m.g
S01_2135444	1	2135444	8	68.055	ID=Ciclev10010816m.g
S09_12572791	9	12572791	8	68.055	ID=Ciclev10005234m.g
S03_34691029	3	34691029	8	73.24	ID=Ciclev10020798m.g
S03_34306466	3	34306466	8	74.914	ID=Ciclev10019185m.g
S03_35326046	3	35326046	8	75.703	ID=Ciclev10022222m.g
S03_34838072	3	34838072	8	76.574	ID=Ciclev10020693m.g
S09_11452840	9	11452840	8	78.258	ID=Ciclev10004119m.g
S08_18020390	8	18020390	8	85.326	ID=Ciclev10029485m.g
S08_18277250	8	18277250	8	88.736	ID=Ciclev10027742m.g
S08_18364649	8	18364649	8	88.736	ID=Ciclev10027955m.g
S08_17837136	8	17837136	8	94.706	ID=Ciclev10029424m.g
S08_17845290	8	17845290	8	102.854	ID=Ciclev10027695m.g
S08_17432786	8	17432786	8	102.854	ID=Ciclev10029749m.g
S08_17785227	8	17785227	8	106.245	ID=Ciclev10027700m.g
S03_34322627	3	34322627	8	106.245	ID=Ciclev10024330m.g
S08_18015469	8	18015469	8	109.639	ID=Ciclev10030024m.g
S08_18863594	8	18863594	8	113.704	ID=Ciclev10029347m.g
S08_18745498	8	18745498	8	113.704	ID=Ciclev10027754m.g
S08_19741035	8	19741035	8	117.769	ID=Ciclev10027891m.g
S08_20271848	8	20271848	8	120.332	ID=Ciclev10028244m.g
S08_19627873	8	19627873	8	120.332	ID=Ciclev10027692m.g
S08_20481799	8	20481799	8	126.83	ID=Ciclev10028932m.g
S08_20149720	8	20149720	8	126.83	ID=Ciclev10028028m.g
S08_21418800	8	21418800	8	130.221	ID=Ciclev10028138m.g
S08_19503894	8	19503894	8	130.221	ID=Ciclev10029136m.g
S08_21155896	8	21155896	8	130.221	ID=Ciclev10029196m.g
S08_21141580	8	21141580	8	138.808	ID=Ciclev10028853m.g
S08_21560963	8	21560963	8	140.8	ID=Ciclev10027892m.g
S08_21848703	8	21848703	8	142.471	ID=Ciclev10028754m.g
S08_22014383	8	22014383	8	144.141	ID=Ciclev10030432m.g
S08_22483886	8	22483886	8	146.294	ID=Ciclev10029532m.g
S08_22307772	8	22307772	8	151.719	ID=Ciclev10029783m.g
S08_22303922	8	22303922	8	157.75	ID=Ciclev10028524m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S08_22466220	8	22466220	8	159.662	ID=Ciclev10028995m.g
S08_22191888	8	22191888	8	159.662	ID=Ciclev10028294m.g
S08_22576575	8	22576575	8	159.662	ID=Ciclev10030198m.g
S08_23203911	8	23203911	8	165.337	ID=Ciclev10030119m.g
S08_23159685	8	23159685	8	165.337	ID=Ciclev10029160m.g
S08_23366188	8	23366188	8	167.159	ID=Ciclev10028459m.g
S08_23591407	8	23591407	8	172.855	ID=Ciclev10027848m.g
S08_24701637	8	24701637	8	180.609	ID=Ciclev10030370m.g
S08_24921859	8	24921859	8	180.609	ID=Ciclev10028037m.g
S08_24319549	8	24319549	8	189.725	ID=Ciclev10029884m.g
S08_23601478	8	23601478	8	189.725	ID=Ciclev10027940m.g
S09_112649	9	112649	9	0	ID=Ciclev10006637m.g
S09_122335	9	122335	9	3.45	ID=Ciclev10006990m.g
S09_154694	9	154694	9	3.45	ID=Ciclev10005815m.g
S09_231864	9	231864	9	3.45	ID=Ciclev10004227m.g
S09_238948	9	238948	9	3.45	ID=Ciclev10006430m.g
S09_392520	9	392520	9	5.116	ID=Ciclev10004428m.g
S09_458321	9	458321	9	5.116	ID=Ciclev10004114m.g
S09_355832	9	355832	9	5.116	ID=Ciclev10004217m.g
S09_379789	9	379789	9	5.116	ID=Ciclev10004458m.g
S09_326306	9	326306	9	5.116	ID=Ciclev10004234m.g
S09_330099	9	330099	9	6.516	ID=Ciclev10004126m.g
S09_546517	9	546517	9	6.778	ID=Ciclev10004474m.g
S09_570128	9	570128	9	8.447	ID=Ciclev10004507m.g
S09_694272	9	694272	9	8.447	ID=Ciclev10006924m.g
S09_595862	9	595862	9	10.118	ID=Ciclev10005460m.g
S09_575039	9	575039	9	10.118	ID=Ciclev10005014m.g
S09_1043853	9	1043853	9	13.514	ID=Ciclev10004649m.g
S09_906725	9	906725	9	13.514	ID=Ciclev10004963m.g
S09_863897	9	863897	9	13.514	ID=Ciclev10006996m.g
S09_901031	9	901031	9	13.514	ID=Ciclev10006081m.g
S09_1023497	9	1023497	9	13.514	ID=Ciclev10004645m.g
S09_1059657	9	1059657	9	13.514	ID=Ciclev10004369m.g
S09_1211326	9	1211326	9	13.514	ID=Ciclev10006099m.g
S09_744853	9	744853	9	13.514	ID=Ciclev10006076m.g
S09_1314521	9	1314521	9	13.514	ID=Ciclev10005026m.g
S09_823567	9	823567	9	16.906	ID=Ciclev10004362m.g
S09_1196251	9	1196251	9	16.906	ID=Ciclev10006107m.g
S09_1198878	9	1198878	9	16.906	ID=Ciclev10006713m.g
S09_855025	9	855025	9	16.906	ID=Ciclev10004295m.g
S09_1148619	9	1148619	9	20.303	ID=Ciclev10004470m.g
S09_1104654	9	1104654	9	20.303	ID=Ciclev10004258m.g
S09_1014938	9	1014938	9	23.701	ID=Ciclev10005421m.g
S09_1001350	9	1001350	9	23.701	ID=Ciclev10004273m.g
S09_947743	9	947743	9	25.369	ID=Ciclev10004131m.g
S09_992087	9	992087	9	25.369	ID=Ciclev10004547m.g
S09_1645472	9	1645472	9	28.771	ID=Ciclev10004153m.g
S09_2111472	9	2111472	9	31.145	ID=Ciclev10004654m.g
S09_2054499	9	2054499	9	31.145	ID=Ciclev10004220m.g
S09_2366464	9	2366464	9	34.593	ID=Ciclev10005625m.g
S09_2501713	9	2501713	9	34.593	ID=Ciclev10004135m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S09_2274543	9	2274543	9	34.593	ID=Ciclev10004155m.g
S09_2200396	9	2200396	9	38.976	ID=Ciclev10006777m.g
S09_1823561	9	1823561	9	38.976	ID=Ciclev10007035m.g
S09_1798855	9	1798855	9	38.976	ID=Ciclev10004171m.g
S09_1733229	9	1733229	9	38.976	ID=Ciclev10004172m.g
S09_1896629	9	1896629	9	38.976	ID=Ciclev10006926m.g
S09_1678335	9	1678335	9	38.976	ID=Ciclev10004985m.g
S09_2147287	9	2147287	9	44.559	ID=Ciclev10006861m.g
S09_2136064	9	2136064	9	44.559	ID=Ciclev10004482m.g
S09_2284625	9	2284625	9	47.229	ID=Ciclev10004115m.g
S09_2340949	9	2340949	9	47.229	ID=Ciclev10007121m.g
S09_2325150	9	2325150	9	47.229	ID=Ciclev10004940m.g
S09_3091420	9	3091420	9	49.23	ID=Ciclev10006689m.g
S09_3119487	9	3119487	9	50.907	ID=Ciclev10005119m.g
S09_3180804	9	3180804	9	55.303	ID=Ciclev10006596m.g
S09_3239681	9	3239681	9	58.73	ID=Ciclev10004648m.g
S09_3331801	9	3331801	9	61.642	ID=Ciclev10005615m.g
S09_3362889	9	3362889	9	64.569	ID=Ciclev10004460m.g
S09_3696693	9	3696693	9	71.563	ID=Ciclev10005268m.g
S09_3756438	9	3756438	9	71.563	ID=Ciclev10006772m.g
S09_4656771	9	4656771	9	82.516	ID=Ciclev10004701m.g
S09_4660005	9	4660005	9	82.516	ID=Ciclev10007173m.g
S09_4612936	9	4612936	9	82.516	ID=Ciclev10006407m.g
S09_4606153	9	4606153	9	82.516	ID=Ciclev10007078m.g
S09_4735638	9	4735638	9	82.516	ID=Ciclev10005003m.g
S09_5562927	9	5562927	9	82.516	ID=Ciclev10005975m.g
S09_4624533	9	4624533	9	82.516	ID=Ciclev10006968m.g
S09_8109229	9	8109229	9	84.183	ID=Ciclev10006937m.g
S09_15918065	9	15918065	9	84.183	ID=Ciclev10006793m.g
S09_21960674	9	21960674	9	84.183	ID=Ciclev10004681m.g
S09_7877643	9	7877643	9	84.183	ID=Ciclev10006816m.g
S09_22211744	9	22211744	9	84.183	ID=Ciclev10004496m.g
S09_15761271	9	15761271	9	84.183	ID=Ciclev10006510m.g
S09_15324651	9	15324651	9	84.183	ID=Ciclev10006215m.g
S09_27290546	9	27290546	9	87.574	ID=Ciclev10004476m.g
S09_28043135	9	28043135	9	89.249	ID=Ciclev10004838m.g
S09_29368872	9	29368872	9	121.465	ID=Ciclev10006470m.g
S09_29563517	9	29563517	9	127.673	ID=Ciclev10004809m.g
S09_29881108	9	29881108	9	130.153	ID=Ciclev10005522m.g
S09_29643142	9	29643142	9	130.153	ID=Ciclev10006482m.g
S09_30182174	9	30182174	9	142.523	ID=Ciclev10004367m.g
S09_30389968	9	30389968	9	142.523	ID=Ciclev10004679m.g
S09_30806073	9	30806073	9	146.337	ID=Ciclev10004260m.g
S09_30848557	9	30848557	9	149.237	ID=Ciclev10005140m.g
S09_30876685	9	30876685	9	155.345	ID=Ciclev10005306m.g
S09_31244755	9	31244755	9	159.79	ID=Ciclev10004357m.g
S09_30997030	9	30997030	9	159.79	ID=Ciclev10005524m.g
S09_31192410	9	31192410	9	159.79	ID=Ciclev10006542m.g

Murcott genetic map

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Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S01_52926	1	52926	1	0	ID=Ciclev10007306m.g
S01_70274	1	70274	1	0	ID=Ciclev10009318m.g
S01_96161	1	96161	1	1.724	ID=Ciclev10008079m.g
S01_112805	1	112805	1	1.724	ID=Ciclev10007230m.g
S01_150394	1	150394	1	1.724	ID=Ciclev10010213m.g
S01_512857	1	512857	1	9.01	ID=Ciclev10009036m.g
S01_564236	1	564236	1	9.01	ID=Ciclev10010585m.g
S01_875719	1	875719	1	9.01	ID=Ciclev10010730m.g
S01_879162	1	879162	1	9.01	ID=Ciclev10008089m.g
S01_1552102	1	1552102	1	15.609	ID=Ciclev10007910m.g
S01_1673316	1	1673316	1	15.609	ID=Ciclev10009428m.g
S01_2014067	1	2014067	1	18.332	ID=Ciclev10009882m.g
S01_2498133	1	2498133	1	24.935	ID=Ciclev10009234m.g
S01_2517446	1	2517446	1	24.935	ID=Ciclev10008356m.g
S01_2523698	1	2523698	1	24.935	ID=Ciclev10008018m.g
S01_2608734	1	2608734	1	24.935	ID=Ciclev10008850m.g
S01_2661745	1	2661745	1	24.935	ID=Ciclev10008063m.g
S01_2778358	1	2778358	1	24.935	ID=Ciclev10007228m.g
S01_2830529	1	2830529	1	24.935	ID=Ciclev10007447m.g
S01_3059913	1	3059913	1	28.898	ID=Ciclev10008414m.g
S01_3246088	1	3246088	1	28.898	ID=Ciclev10008071m.g
S01_3266244	1	3266244	1	28.898	ID=Ciclev10008690m.g
S01_3731194	1	3731194	1	33.914	ID=Ciclev10009901m.g
S01_3824062	1	3824062	1	38.925	ID=Ciclev10009011m.g
S01_4031477	1	4031477	1	38.925	ID=Ciclev10008860m.g
S01_4099866	1	4099866	1	38.925	ID=Ciclev10007603m.g
S01_4235975	1	4235975	1	38.925	ID=Ciclev10007747m.g
S01_4753466	1	4753466	1	46.875	ID=Ciclev10009893m.g
S01_5392696	1	5392696	1	49.336	ID=Ciclev10007575m.g
S01_5688188	1	5688188	1	51.312	ID=Ciclev10008775m.g
S01_6168773	1	6168773	1	51.312	ID=Ciclev10010574m.g
S01_6429918	1	6429918	1	51.312	ID=Ciclev10009505m.g
S01_6451641	1	6451641	1	51.312	ID=Ciclev10007776m.g
S01_6452853	1	6452853	1	51.312	ID=Ciclev10008857m.g
S01_7045839	1	7045839	1	54.769	ID=Ciclev10008049m.g
S01_7227854	1	7227854	1	54.769	ID=Ciclev10007513m.g
S01_7681251	1	7681251	1	56.471	ID=Ciclev10007269m.g
S01_8876030	1	8876030	1	63.187	ID=Ciclev10008074m.g
S01_14009139	1	14009139	1	65.311	ID=Ciclev10009291m.g
S01_14115823	1	14115823	1	65.311	ID=Ciclev10007317m.g
S01_14391203	1	14391203	1	66.921	ID=Ciclev10007788m.g
S01_14866103	1	14866103	1	67.008	ID=Ciclev10008533m.g
S01_15742231	1	15742231	1	69.136	ID=Ciclev10010852m.g
S01_17415203	1	17415203	1	75.878	ID=Ciclev10009308m.g
S01_17831378	1	17831378	1	79.356	ID=Ciclev10009061m.g
S01_18009707	1	18009707	1	79.356	ID=Ciclev10007869m.g
S01_22281840	1	22281840	1	82.836	ID=Ciclev10007873m.g
S01_22449826	1	22449826	1	84.55	ID=Ciclev10009601m.g
S06_17873935	6	17873935	1	91.78	ID=Ciclev10011668m.g
S01_22694645	1	22694645	1	95.277	ID=Ciclev10010199m.g
S01_22975873	1	22975873	1	95.277	ID=Ciclev10007536m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S01_23110907	1	23110907	1	101.361	ID=Ciclev10009869m.g
S01_23162602	1	23162602	1	101.361	ID=Ciclev10010287m.g
S01_23259161	1	23259161	1	101.361	ID=Ciclev10010406m.g
S01_23431732	1	23431732	1	101.361	ID=Ciclev10007691m.g
S01_24116702	1	24116702	1	109.636	ID=Ciclev10007802m.g
S01_24329530	1	24329530	1	109.636	ID=Ciclev10007904m.g
S01_24525941	1	24525941	1	111.35	ID=Ciclev10008687m.g
S01_25165173	1	25165173	1	119.322	ID=Ciclev10007884m.g
S01_25331390	1	25331390	1	122.99	ID=Ciclev10010155m.g
S01_25497528	1	25497528	1	126.64	ID=Ciclev10008781m.g
S01_25690547	1	25690547	1	126.64	ID=Ciclev10007867m.g
S01_25990600	1	25990600	1	129.05	ID=Ciclev10007524m.g
S01_26030989	1	26030989	1	129.05	ID=Ciclev10007933m.g
S01_26060166	1	26060166	1	129.05	ID=Ciclev10007734m.g
S01_26102810	1	26102810	1	129.05	ID=Ciclev10007497m.g
S01_26796432	1	26796432	1	130.746	ID=Ciclev10010390m.g
S01_26815936	1	26815936	1	130.746	ID=Ciclev10008911m.g
S01_27512849	1	27512849	1	139.953	ID=Ciclev10009728m.g
S01_27687717	1	27687717	1	143.483	ID=Ciclev10007320m.g
S01_28492455	1	28492455	1	152.664	ID=Ciclev10009068m.g
S01_28511164	1	28511164	1	152.664	ID=Ciclev10010826m.g
S01_28532754	1	28532754	1	152.664	ID=Ciclev10010075m.g
S01_28588113	1	28588113	1	152.664	ID=Ciclev10010403m.g
S01_28751816	1	28751816	1	154.516	ID=Ciclev10007695m.g
S01_28776995	1	28776995	1	154.516	ID=Ciclev10010215m.g
S02_6774090	2	6774090	2	0	ID=Ciclev10015576m.g
S02_6778380	2	6778380	2	0	ID=Ciclev10014192m.g
S02_6885306	2	6885306	2	1.667	ID=Ciclev10016322m.g
S02_6903316	2	6903316	2	1.667	ID=Ciclev10015808m.g
S02_6941172	2	6941172	2	1.667	ID=Ciclev10014939m.g
S02_7012425	2	7012425	2	1.667	ID=Ciclev10017500m.g
S02_19842211	2	19842211	2	33.548	ID=Ciclev10014668m.g
S02_21917848	2	21917848	2	41.924	ID=Ciclev10014373m.g
S02_22965328	2	22965328	2	44.989	ID=Ciclev10015493m.g
S02_23764644	2	23764644	2	49.13	ID=Ciclev10015492m.g
S02_24776837	2	24776837	2	53.392	ID=Ciclev10018131m.g
S02_25324691	2	25324691	2	55.934	ID=Ciclev10014088m.g
S02_25522543	2	25522543	2	75.237	ID=Ciclev10018269m.g
S02_25633834	2	25633834	2	75.237	ID=Ciclev10018242m.g
S02_26031705	2	26031705	2	78.903	ID=Ciclev10014821m.g
S02_27021606	2	27021606	2	89.887	ID=Ciclev10015171m.g
S02_27195773	2	27195773	2	96.968	ID=Ciclev10018125m.g
S02_28082828	2	28082828	2	105.982	ID=Ciclev10016251m.g
S02_28939419	2	28939419	2	113.056	ID=Ciclev10014065m.g
S02_28969067	2	28969067	2	113.056	ID=Ciclev10014843m.g
S02_29437209	2	29437209	2	114.723	ID=Ciclev10014085m.g
S02_29538344	2	29538344	2	114.723	ID=Ciclev10015668m.g
S02_29650379	2	29650379	2	114.723	ID=Ciclev10014061m.g
S02_29760006	2	29760006	2	114.723	ID=Ciclev10016366m.g
S02_30000162	2	30000162	2	114.723	ID=Ciclev10014052m.g
S02_30049719	2	30049719	2	118.12	ID=Ciclev10016394m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S02_30598362	2	30598362	2	118.12	ID=Ciclev10016196m.g
S02_30849953	2	30849953	2	123.314	ID=Ciclev10015984m.g
S02_30904080	2	30904080	2	123.314	ID=Ciclev10018122m.g
S02_30942547	2	30942547	2	128.507	ID=Ciclev10014133m.g
S02_31894526	2	31894526	2	130.183	ID=Ciclev10017760m.g
S02_31927860	2	31927860	2	131.856	ID=Ciclev10016904m.g
S02_32002184	2	32002184	2	133.528	ID=Ciclev10014983m.g
S02_32075451	2	32075451	2	135.201	ID=Ciclev10016130m.g
S02_32283983	2	32283983	2	136.873	ID=Ciclev10016643m.g
S02_32308358	2	32308358	2	136.873	ID=Ciclev10017157m.g
S02_32843659	2	32843659	2	138.569	ID=Ciclev10015592m.g
S02_32926265	2	32926265	2	138.569	ID=Ciclev10014187m.g
S02_33026060	2	33026060	2	138.569	ID=Ciclev10014112m.g
S02_33247761	2	33247761	2	138.569	ID=Ciclev10015933m.g
S03_3687034	3	3687034	3	0	ID=Ciclev10019026m.g
S03_3858757	3	3858757	3	2.276	ID=Ciclev10019772m.g
S03_4052993	3	4052993	3	6.86	ID=Ciclev10018616m.g
S03_3956389	3	3956389	3	6.86	ID=Ciclev10018473m.g
S03_4124145	3	4124145	3	8.529	ID=Ciclev10019777m.g
S08_17853708	8	17853708	3	10.197	ID=Ciclev10027695m.g
S03_4195669	3	4195669	3	11.866	ID=Ciclev10019499m.g
S03_4155381	3	4155381	3	13.946	ID=Ciclev10024278m.g
S03_5374120	3	5374120	3	20.526	ID=Ciclev10019196m.g
S03_5397061	3	5397061	3	20.526	ID=Ciclev10018892m.g
S03_5509981	3	5509981	3	22.195	ID=Ciclev10019315m.g
S03_5874405	3	5874405	3	25.985	ID=Ciclev10022476m.g
S03_6226351	3	6226351	3	28.138	ID=Ciclev10019076m.g
S03_5432814	3	5432814	3	32.562	ID=Ciclev10018818m.g
S03_6942069	3	6942069	3	40.225	ID=Ciclev10019482m.g
S03_7576692	3	7576692	3	43.124	ID=Ciclev10022490m.g
S03_7792917	3	7792917	3	51.837	ID=Ciclev10019841m.g
S03_7951424	3	7951424	3	54.738	ID=Ciclev10018461m.g
S03_8268140	3	8268140	3	56.536	ID=Ciclev10022497m.g
S03_8862771	3	8862771	3	58.941	ID=Ciclev10021913m.g
S03_9019697	3	9019697	3	63.917	ID=Ciclev10018566m.g
S03_8642668	3	8642668	3	63.917	ID=Ciclev10019790m.g
S03_8843362	3	8843362	3	63.917	ID=Ciclev10019201m.g
S03_9881907	3	9881907	3	65.596	ID=Ciclev10023895m.g
S03_33679413	3	33679413	3	72.672	ID=Ciclev10020201m.g
S03_33749222	3	33749222	3	74.525	ID=Ciclev10018724m.g
S03_30868014	3	30868014	3	83.493	ID=Ciclev10021233m.g
S03_10898832	3	10898832	3	92.445	ID=Ciclev10018922m.g
S03_11412180	3	11412180	3	94.112	ID=Ciclev10018947m.g
S03_11509117	3	11509117	3	97.503	ID=Ciclev10018464m.g
S03_13226646	3	13226646	3	99.171	ID=Ciclev10018879m.g
S03_16436655	3	16436655	3	99.171	ID=Ciclev10018592m.g
S03_18826689	3	18826689	3	100.838	ID=Ciclev10021039m.g
S03_18960504	3	18960504	3	100.838	ID=Ciclev10020311m.g
S03_22081343	3	22081343	3	109.79	ID=Ciclev10024511m.g
S09_29013556	9	29013556	3	109.79	ID=Ciclev10004124m.g
S03_21766712	3	21766712	3	109.79	ID=Ciclev10024345m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S03_25319532	3	25319532	3	112.014	ID=Ciclev10018594m.g
S03_24899680	3	24899680	3	112.014	ID=Ciclev10020147m.g
S03_21814687	3	21814687	3	116.6	ID=Ciclev10024358m.g
S03_21921563	3	21921563	3	116.6	ID=Ciclev10018760m.g
S03_24756994	3	24756994	3	121.777	ID=Ciclev10018885m.g
S03_24967194	3	24967194	3	121.777	ID=Ciclev10024611m.g
S04_10582718	4	10582718	3	126.955	ID=Ciclev10033279m.g
S03_27185376	3	27185376	3	126.955	ID=Ciclev10018865m.g
S03_27253390	3	27253390	3	126.955	ID=Ciclev10023613m.g
S03_37127543	3	37127543	3	133.984	ID=Ciclev10024050m.g
S03_37154233	3	37154233	3	133.984	ID=Ciclev10024176m.g
S03_39037995	3	39037995	3	142.113	ID=Ciclev10023340m.g
S03_39402669	3	39402669	3	142.113	ID=Ciclev10024181m.g
S03_39622190	3	39622190	3	148.077	ID=Ciclev10021532m.g
S03_40110353	3	40110353	3	148.077	ID=Ciclev10021732m.g
S03_41697624	3	41697624	3	151.478	ID=Ciclev10024637m.g
S03_41581682	3	41581682	3	151.478	ID=Ciclev10021716m.g
S03_41316130	3	41316130	3	151.478	ID=Ciclev10018850m.g
S03_42920283	3	42920283	3	162.454	ID=Ciclev10021493m.g
S03_43420965	3	43420965	3	164.471	ID=Ciclev10021699m.g
S03_44125894	3	44125894	3	168.598	ID=Ciclev10019959m.g
S03_45148656	3	45148656	3	172.712	ID=Ciclev10018730m.g
S03_44995940	3	44995940	3	174.386	ID=Ciclev10018843m.g
S03_44548514	3	44548514	3	177.796	ID=Ciclev10019087m.g
S03_45712095	3	45712095	3	181.201	ID=Ciclev10018482m.g
S03_46693444	3	46693444	3	183.889	ID=Ciclev10019644m.g
S03_45992925	3	45992925	3	189.501	ID=Ciclev10023723m.g
S03_46443520	3	46443520	3	191.524	ID=Ciclev10018936m.g
S03_47596044	3	47596044	3	200.516	ID=Ciclev10018449m.g
S03_47901346	3	47901346	3	200.516	ID=Ciclev10018638m.g
S03_47585078	3	47585078	3	202.193	ID=Ciclev10020210m.g
S03_47388743	3	47388743	3	207.395	ID=Ciclev10022564m.g
S03_47708693	3	47708693	3	210.792	ID=Ciclev10020168m.g
S03_50005808	3	50005808	3	225.643	ID=Ciclev10022917m.g
S03_49887003	3	49887003	3	225.643	ID=Ciclev10024598m.g
S03_49846466	3	49846466	3	228.504	ID=Ciclev10024592m.g
S03_50768675	3	50768675	3	231.297	ID=Ciclev10018805m.g
S03_50636493	3	50636493	3	231.297	ID=Ciclev10022525m.g
S04_59320	4	59320	4	0	ID=Ciclev10033472m.g
S04_198356	4	198356	4	7.873	ID=Ciclev10030787m.g
S04_316443	4	316443	4	9.671	ID=Ciclev10030803m.g
S04_381014	4	381014	4	12.391	ID=Ciclev10033808m.g
S04_584532	4	584532	4	15.098	ID=Ciclev10031682m.g
S04_675715	4	675715	4	15.098	ID=Ciclev10030588m.g
S04_634976	4	634976	4	18.74	ID=Ciclev10030516m.g
S04_642286	4	642286	4	18.74	ID=Ciclev10031443m.g
S04_1139101	4	1139101	4	24.301	ID=Ciclev10031576m.g
S04_859028	4	859028	4	24.301	ID=Ciclev10032032m.g
S04_868982	4	868982	4	27.073	ID=Ciclev10033509m.g
S04_964598	4	964598	4	27.073	ID=Ciclev10031175m.g
S04_1260479	4	1260479	4	32.918	ID=Ciclev10031811m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S04_1773768	4	1773768	4	37.214	ID=Ciclev10030811m.g
S04_1793199	4	1793199	4	39.003	ID=Ciclev10032052m.g
S04_1794883	4	1794883	4	40.79	ID=Ciclev10033159m.g
S04_2710371	4	2710371	4	42.576	ID=Ciclev10030893m.g
S04_2408652	4	2408652	4	46.218	ID=Ciclev10033331m.g
S04_2264393	4	2264393	4	48.007	ID=Ciclev10030625m.g
S04_1829118	4	1829118	4	48.007	ID=Ciclev10032836m.g
S04_2243999	4	2243999	4	48.007	ID=Ciclev10030981m.g
S04_2717289	4	2717289	4	49.795	ID=Ciclev10030928m.g
S04_2863683	4	2863683	4	53.436	ID=Ciclev10032714m.g
S04_2927871	4	2927871	4	53.436	ID=Ciclev10032889m.g
S04_3209633	4	3209633	4	53.436	ID=Ciclev10030779m.g
S04_3244651	4	3244651	4	53.436	ID=Ciclev10033309m.g
S04_3612718	4	3612718	4	55.222	ID=Ciclev10031306m.g
S04_3790493	4	3790493	4	55.222	ID=Ciclev10031113m.g
S04_3902616	4	3902616	4	55.282	ID=Ciclev10033259m.g
S04_6089418	4	6089418	4	57.01	ID=Ciclev10033298m.g
S04_6370037	4	6370037	4	60.653	ID=Ciclev10031933m.g
S04_6811998	4	6811998	4	60.653	ID=Ciclev10031516m.g
S04_7027659	4	7027659	4	60.653	ID=Ciclev10030988m.g
S04_7334362	4	7334362	4	64.292	ID=Ciclev10033678m.g
S04_7197216	4	7197216	4	65.381	ID=Ciclev10031606m.g
S04_9170612	4	9170612	4	66.064	ID=Ciclev10033801m.g
S04_7382463	4	7382463	4	66.064	ID=Ciclev10031452m.g
S04_14071520	4	14071520	4	73.625	ID=Ciclev10030628m.g
S04_14511886	4	14511886	4	73.625	ID=Ciclev10030757m.g
S04_14423246	4	14423246	4	73.625	ID=Ciclev10031185m.g
S03_666739	3	666739	4	73.625	ID=Ciclev10018519m.g
S04_12323587	4	12323587	4	73.625	ID=Ciclev10032646m.g
S04_14038355	4	14038355	4	73.625	ID=Ciclev10030627m.g
S04_14165683	4	14165683	4	73.625	ID=Ciclev10030813m.g
S04_14046567	4	14046567	4	73.625	ID=Ciclev10033491m.g
S02_4036518	2	4036518	4	73.625	ID=Ciclev10015763m.g
S04_14762069	4	14762069	4	73.625	ID=Ciclev10031361m.g
S04_15046664	4	15046664	4	75.412	ID=Ciclev10031028m.g
S04_15172607	4	15172607	4	75.412	ID=Ciclev10033243m.g
S04_15203655	4	15203655	4	77.198	ID=Ciclev10031115m.g
S04_14438532	4	14438532	4	77.198	ID=Ciclev10030606m.g
S04_14328796	4	14328796	4	77.198	ID=Ciclev10033814m.g
S04_14677666	4	14677666	4	77.198	ID=Ciclev10032791m.g
S04_12356216	4	12356216	4	77.198	ID=Ciclev10032864m.g
S04_14461796	4	14461796	4	77.198	ID=Ciclev10030921m.g
S04_12922417	4	12922417	4	77.198	ID=Ciclev10033645m.g
S04_15049272	4	15049272	4	77.198	ID=Ciclev10031739m.g
S04_15280179	4	15280179	4	77.198	ID=Ciclev10031282m.g
S04_13905015	4	13905015	4	77.198	ID=Ciclev10030650m.g
S04_16085725	4	16085725	4	77.198	ID=Ciclev10033869m.g
S04_19092743	4	19092743	4	82.759	ID=Ciclev10032493m.g
S04_17795060	4	17795060	4	82.759	ID=Ciclev10033437m.g
S04_19049128	4	19049128	4	82.759	ID=Ciclev10031476m.g
S04_16339650	4	16339650	4	82.759	ID=Ciclev10030603m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S04_16519720	4	16519720	4	82.759	ID=Ciclev10031135m.g
S04_18659012	4	18659012	4	82.759	ID=Ciclev10033525m.g
S04_17800370	4	17800370	4	82.759	ID=Ciclev10032005m.g
S04_19420069	4	19420069	4	91.216	ID=Ciclev10031397m.g
S04_20045963	4	20045963	4	100.942	ID=Ciclev10033580m.g
S04_20906282	4	20906282	4	103.831	ID=Ciclev10030493m.g
S04_20723608	4	20723608	4	103.831	ID=Ciclev10033781m.g
S04_21629642	4	21629642	4	105.618	ID=Ciclev10031257m.g
S04_21362418	4	21362418	4	105.618	ID=Ciclev10031347m.g
S04_21181265	4	21181265	4	107.405	ID=Ciclev10033913m.g
S04_21955586	4	21955586	4	109.196	ID=Ciclev10033411m.g
S04_22086968	4	22086968	4	110.985	ID=Ciclev10033123m.g
S04_22416855	4	22416855	4	110.987	ID=Ciclev10032926m.g
S04_22612244	4	22612244	4	114.629	ID=Ciclev10033458m.g
S04_22347717	4	22347717	4	116.419	ID=Ciclev10030794m.g
S04_21992170	4	21992170	4	118.514	ID=Ciclev10032380m.g
S04_23375997	4	23375997	4	130.023	ID=Ciclev10033337m.g
S04_23203515	4	23203515	4	130.023	ID=Ciclev10030551m.g
S04_23209008	4	23209008	4	130.023	ID=Ciclev10030507m.g
S04_23246578	4	23246578	4	130.023	ID=Ciclev10030976m.g
S04_23268179	4	23268179	4	130.023	ID=Ciclev10030999m.g
S04_24067545	4	24067545	4	139.668	ID=Ciclev10030675m.g
S04_25050901	4	25050901	4	139.668	ID=Ciclev10031896m.g
S04_24949892	4	24949892	4	139.668	ID=Ciclev10033817m.g
S04_25548240	4	25548240	4	143.306	ID=Ciclev10033633m.g
S04_25467223	4	25467223	4	143.306	ID=Ciclev10030635m.g
S04_25359868	4	25359868	4	143.306	ID=Ciclev10031826m.g
S04_25232341	4	25232341	4	145.094	ID=Ciclev10033853m.g
S04_25593241	4	25593241	4	146.881	ID=Ciclev10033595m.g
S04_25119807	4	25119807	4	146.881	ID=Ciclev10031170m.g
S04_24780978	4	24780978	4	149.228	ID=Ciclev10030586m.g
S04_24688301	4	24688301	4	149.228	ID=Ciclev10031389m.g
S04_24742800	4	24742800	4	154.597	ID=Ciclev10032994m.g
S05_478920	5	478920	5	0	ID=Ciclev10000657m.g
S05_263132	5	263132	5	5.268	ID=Ciclev10002464m.g
S05_2046191	5	2046191	5	5.268	ID=Ciclev10000216m.g
S05_113701	5	113701	5	5.268	ID=Ciclev10000600m.g
S05_598359	5	598359	5	6.288	ID=Ciclev10000109m.g
S05_287702	5	287702	5	6.922	ID=Ciclev10001802m.g
S05_834208	5	834208	5	6.922	ID=Ciclev10000869m.g
S05_2476340	5	2476340	5	10.315	ID=Ciclev10000427m.g
S05_2202098	5	2202098	5	10.315	ID=Ciclev10000456m.g
S05_3490768	5	3490768	5	11.984	ID=Ciclev10003345m.g
S05_3514227	5	3514227	5	11.984	ID=Ciclev10003972m.g
S05_5838417	5	5838417	5	20.937	ID=Ciclev10003600m.g
S05_6483047	5	6483047	5	22.605	ID=Ciclev10000702m.g
S05_8360132	5	8360132	5	25.998	ID=Ciclev10002201m.g
S05_8643904	5	8643904	5	25.998	ID=Ciclev10000024m.g
S05_20933381	5	20933381	5	33.028	ID=Ciclev10002295m.g
S05_19034218	5	19034218	5	33.028	ID=Ciclev10003129m.g
S05_20820688	5	20820688	5	33.028	ID=Ciclev10002378m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S05_26146784	5	26146784	5	44.007	ID=Ciclev10003207m.g
S05_27376540	5	27376540	5	45.689	ID=Ciclev10003137m.g
S05_25967138	5	25967138	5	45.695	ID=Ciclev10000735m.g
S05_25843225	5	25843225	5	45.695	ID=Ciclev10000944m.g
S05_25877697	5	25877697	5	45.695	ID=Ciclev10001344m.g
S05_29129984	5	29129984	5	49.09	ID=Ciclev10000893m.g
S05_29693342	5	29693342	5	52.485	ID=Ciclev10000348m.g
S05_29850237	5	29850237	5	52.485	ID=Ciclev10003382m.g
S05_30528148	5	30528148	5	55.881	ID=Ciclev10002937m.g
S05_30839319	5	30839319	5	59.661	ID=Ciclev10001604m.g
S05_31634928	5	31634928	5	74.191	ID=Ciclev10000179m.g
S05_32774195	5	32774195	5	76.058	ID=Ciclev10000202m.g
S05_33220895	5	33220895	5	76.058	ID=Ciclev10000982m.g
S05_32549750	5	32549750	5	76.058	ID=Ciclev10001229m.g
S05_33394480	5	33394480	5	79.45	ID=Ciclev10002007m.g
S05_33705664	5	33705664	5	79.45	ID=Ciclev10003191m.g
S05_33985308	5	33985308	5	81.117	ID=Ciclev10003420m.g
S05_34179449	5	34179449	5	81.117	ID=Ciclev10003854m.g
S05_35521985	5	35521985	5	82.784	ID=Ciclev10001318m.g
S05_35118372	5	35118372	5	82.784	ID=Ciclev10000073m.g
S05_35660846	5	35660846	5	84.609	ID=Ciclev10002205m.g
S05_35897640	5	35897640	5	86.643	ID=Ciclev10003510m.g
S05_37048667	5	37048667	5	103.464	ID=Ciclev10000313m.g
S05_37123343	5	37123343	5	103.464	ID=Ciclev10001037m.g
S05_37091522	5	37091522	5	103.464	ID=Ciclev10000652m.g
S05_37315469	5	37315469	5	103.464	ID=Ciclev10003517m.g
S05_37639069	5	37639069	5	108.642	ID=Ciclev10000736m.g
S05_37570889	5	37570889	5	110.316	ID=Ciclev10000010m.g
S05_37821300	5	37821300	5	115.608	ID=Ciclev10000005m.g
S05_38659929	5	38659929	5	118.148	ID=Ciclev10000593m.g
S05_38347207	5	38347207	5	118.148	ID=Ciclev10001443m.g
S05_38150240	5	38150240	5	118.148	ID=Ciclev10001753m.g
S05_38469518	5	38469518	5	124.122	ID=Ciclev10002106m.g
S05_39301459	5	39301459	5	127.517	ID=Ciclev10002068m.g
S05_39929037	5	39929037	5	131.926	ID=Ciclev10001636m.g
S05_39858985	5	39858985	5	131.926	ID=Ciclev10000651m.g
S05_39694014	5	39694014	5	135.193	ID=Ciclev10000804m.g
S05_41139108	5	41139108	5	145.38	ID=Ciclev10000483m.g
S05_40876126	5	40876126	5	145.38	ID=Ciclev10000910m.g
S06_6172051	6	6172051	6	0	ID=Ciclev10013854m.g
S06_12995157	6	12995157	6	5.57	ID=Ciclev10012001m.g
S06_11412517	6	11412517	6	8.226	ID=Ciclev10011495m.g
S06_10236328	6	10236328	6	10.801	ID=Ciclev10011194m.g
S06_6817114	6	6817114	6	10.801	ID=Ciclev10011454m.g
S06_12237196	6	12237196	6	10.801	ID=Ciclev10012157m.g
S06_1854693	6	1854693	6	10.801	ID=Ciclev10011527m.g
S06_11103054	6	11103054	6	10.801	ID=Ciclev10012548m.g
S06_7809349	6	7809349	6	11.656	ID=Ciclev10012906m.g
S06_6183348	6	6183348	6	12.511	ID=Ciclev10012634m.g
S06_10036597	6	10036597	6	12.511	ID=Ciclev10010923m.g
S06_7874714	6	7874714	6	13.363	ID=Ciclev10011634m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S06_12704873	6	12704873	6	14.222	ID=Ciclev10011951m.g
S06_434944	6	434944	6	15.08	ID=Ciclev10013842m.g
S06_9297331	6	9297331	6	15.933	ID=Ciclev10013851m.g
S06_11462087	6	11462087	6	15.933	ID=Ciclev10011862m.g
S06_13425421	6	13425421	6	15.933	ID=Ciclev10012277m.g
S06_497155	6	497155	6	19.443	ID=Ciclev10011912m.g
S06_7125989	6	7125989	6	19.443	ID=Ciclev10012732m.g
S06_11704723	6	11704723	6	21.17	ID=Ciclev10012554m.g
S06_1942988	6	1942988	6	22.893	ID=Ciclev10010916m.g
S06_7803973	6	7803973	6	22.893	ID=Ciclev10012397m.g
S06_547770	6	547770	6	22.893	ID=Ciclev10010929m.g
S06_11597766	6	11597766	6	22.893	ID=Ciclev10010897m.g
S06_10488380	6	10488380	6	22.893	ID=Ciclev10011997m.g
S06_9545096	6	9545096	6	22.893	ID=Ciclev10013718m.g
S06_8284587	6	8284587	6	22.893	ID=Ciclev10011737m.g
S06_12673030	6	12673030	6	24.619	ID=Ciclev10011445m.g
S06_67124	6	67124	6	26.344	ID=Ciclev10013905m.g
S06_11142919	6	11142919	6	26.344	ID=Ciclev10011496m.g
S06_10520920	6	10520920	6	26.344	ID=Ciclev10011672m.g
S08_16675906	8	16675906	6	26.344	ID=Ciclev10028674m.g
S08_16548478	8	16548478	6	26.344	ID=Ciclev10028530m.g
S06_1109843	6	1109843	6	27.199	ID=Ciclev10011295m.g
S06_12552313	6	12552313	6	28.055	ID=Ciclev10011715m.g
S06_2028031	6	2028031	6	28.055	ID=Ciclev10013638m.g
S06_6334460	6	6334460	6	28.055	ID=Ciclev10011420m.g
S06_5773811	6	5773811	6	28.055	ID=Ciclev10013516m.g
S06_9686010	6	9686010	6	30.226	ID=Ciclev10011376m.g
S06_14402785	6	14402785	6	34.68	ID=Ciclev10012000m.g
S06_14679690	6	14679690	6	36.843	ID=Ciclev10012493m.g
S06_17510041	6	17510041	6	52.127	ID=Ciclev10013499m.g
S06_17835964	6	17835964	6	54.323	ID=Ciclev10011331m.g
S06_17837547	6	17837547	6	54.323	ID=Ciclev10012066m.g
S06_18050816	6	18050816	6	60.699	ID=Ciclev10011085m.g
S06_18370599	6	18370599	6	67.069	ID=Ciclev10011572m.g
S06_18727456	6	18727456	6	68.809	ID=Ciclev10011385m.g
S06_19160290	6	19160290	6	70.544	ID=Ciclev10013737m.g
S06_19411339	6	19411339	6	72.284	ID=Ciclev10011545m.g
S06_19722643	6	19722643	6	75.581	ID=Ciclev10013876m.g
S06_19621621	6	19621621	6	78.889	ID=Ciclev10011945m.g
S06_20132087	6	20132087	6	82.179	ID=Ciclev10012455m.g
S06_19712134	6	19712134	6	82.179	ID=Ciclev10013007m.g
S06_20271006	6	20271006	6	82.18	ID=Ciclev10012797m.g
S06_20545773	6	20545773	6	86.53	ID=Ciclev10011609m.g
S06_20655993	6	20655993	6	88.654	ID=Ciclev10012247m.g
S06_20822896	6	20822896	6	88.654	ID=Ciclev10011627m.g
S06_21165558	6	21165558	6	94.994	ID=Ciclev10013017m.g
S06_21768932	6	21768932	6	103.175	ID=Ciclev10010989m.g
S06_21496705	6	21496705	6	106.399	ID=Ciclev10013010m.g
S06_25193819	6	25193819	6	115.663	ID=Ciclev10010991m.g
S06_23780859	6	23780859	6	115.663	ID=Ciclev10013726m.g
S06_24679060	6	24679060	6	117.39	ID=Ciclev10013770m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S06_23921861	6	23921861	6	119.115	ID=Ciclev10010885m.g
S06_24322387	6	24322387	6	119.115	ID=Ciclev10010936m.g
S06_25254271	6	25254271	6	120.84	ID=Ciclev10010958m.g
S06_24147535	6	24147535	6	123.454	ID=Ciclev10010995m.g
S06_24767234	6	24767234	6	126.065	ID=Ciclev10011709m.g
S06_23939498	6	23939498	6	126.066	ID=Ciclev10012892m.g
S06_24440235	6	24440235	6	126.066	ID=Ciclev10011199m.g
S06_24188615	6	24188615	6	126.066	ID=Ciclev10013597m.g
S06_23041131	6	23041131	6	126.921	ID=Ciclev10013755m.g
S06_22804828	6	22804828	6	127.775	ID=Ciclev10010910m.g
S03_23487944	3	23487944	6	127.775	ID=Ciclev10024661m.g
S06_22485541	6	22485541	6	127.775	ID=Ciclev10013828m.g
S06_23619085	6	23619085	6	127.775	ID=Ciclev10013175m.g
S06_21927823	6	21927823	6	127.775	ID=Ciclev10013654m.g
S06_23001602	6	23001602	6	127.775	ID=Ciclev10011179m.g
S06_23068547	6	23068547	6	133.235	ID=Ciclev10011430m.g
S06_22525889	6	22525889	6	133.235	ID=Ciclev10011546m.g
S06_23147646	6	23147646	6	133.235	ID=Ciclev10012575m.g
S07_31008	7	31008	7	0	ID=Ciclev10025351m.g
S07_540443	7	540443	7	0	ID=Ciclev10024840m.g
S07_869201	7	869201	7	3.391	ID=Ciclev10025759m.g
S07_926250	7	926250	7	3.391	ID=Ciclev10027108m.g
S07_877476	7	877476	7	3.391	ID=Ciclev10026691m.g
S07_1310473	7	1310473	7	5.06	ID=Ciclev10026549m.g
S07_1363046	7	1363046	7	9.247	ID=Ciclev10027574m.g
S07_1678038	7	1678038	7	11.292	ID=Ciclev10025725m.g
S07_1942759	7	1942759	7	14.398	ID=Ciclev10025926m.g
S07_2032631	7	2032631	7	16.706	ID=Ciclev10026226m.g
S07_2133318	7	2133318	7	19.009	ID=Ciclev10025093m.g
S07_2074830	7	2074830	7	19.009	ID=Ciclev10025149m.g
S07_2278993	7	2278993	7	23.755	ID=Ciclev10025963m.g
S07_2228812	7	2228812	7	23.755	ID=Ciclev10025048m.g
S07_2608149	7	2608149	7	30.796	ID=Ciclev10026303m.g
S07_3149760	7	3149760	7	32.472	ID=Ciclev10024681m.g
S07_3137839	7	3137839	7	32.472	ID=Ciclev10026429m.g
S07_4114176	7	4114176	7	34.146	ID=Ciclev10024730m.g
S07_4243356	7	4243356	7	35.82	ID=Ciclev10027120m.g
S07_4337210	7	4337210	7	35.82	ID=Ciclev10026641m.g
S07_4584302	7	4584302	7	35.82	ID=Ciclev10025260m.g
S07_4822906	7	4822906	7	39.822	ID=Ciclev10024875m.g
S07_4589575	7	4589575	7	39.822	ID=Ciclev10025588m.g
S07_5361896	7	5361896	7	47.459	ID=Ciclev10025455m.g
S07_5774466	7	5774466	7	51.475	ID=Ciclev10025026m.g
S07_5803212	7	5803212	7	51.475	ID=Ciclev10027599m.g
S07_5997050	7	5997050	7	54.866	ID=Ciclev10025152m.g
S07_5847409	7	5847409	7	54.866	ID=Ciclev10024761m.g
S07_5985765	7	5985765	7	54.866	ID=Ciclev10024878m.g
S07_5964026	7	5964026	7	54.866	ID=Ciclev10025161m.g
S07_6824995	7	6824995	7	59.463	ID=Ciclev10024696m.g
S07_6818695	7	6818695	7	59.463	ID=Ciclev10025353m.g
S07_6871314	7	6871314	7	61.695	ID=Ciclev10025998m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S07_8395126	7	8395126	7	65.094	ID=Ciclev10024743m.g
S07_7642167	7	7642167	7	65.094	ID=Ciclev10025604m.g
S07_8909809	7	8909809	7	73.684	ID=Ciclev10027173m.g
S07_9956446	7	9956446	7	75.68	ID=Ciclev10024839m.g
S07_9170423	7	9170423	7	75.68	ID=Ciclev10024988m.g
S07_10010136	7	10010136	7	75.68	ID=Ciclev10025498m.g
S07_9538028	7	9538028	7	75.68	ID=Ciclev10025245m.g
S07_10751343	7	10751343	7	80.859	ID=Ciclev10026835m.g
S07_10807662	7	10807662	7	80.859	ID=Ciclev10025851m.g
S07_13368456	7	13368456	7	88.002	ID=Ciclev10025693m.g
S07_14137140	7	14137140	7	91.358	ID=Ciclev10024841m.g
S07_12999657	7	12999657	7	91.358	ID=Ciclev10024946m.g
S07_13833581	7	13833581	7	95.966	ID=Ciclev10027021m.g
S07_14284267	7	14284267	7	98.201	ID=Ciclev10026151m.g
S07_14643946	7	14643946	7	99.874	ID=Ciclev10025681m.g
S07_14385687	7	14385687	7	99.874	ID=Ciclev10024814m.g
S07_15091727	7	15091727	7	105.056	ID=Ciclev10026430m.g
S04_5802709	4	5802709	7	105.056	ID=Ciclev10030470m.g
S07_15100404	7	15100404	7	106.933	ID=Ciclev10026162m.g
S07_15212820	7	15212820	7	108.817	ID=Ciclev10027095m.g
S05_17808301	5	17808301	7	120.668	ID=Ciclev10003286m.g
S05_18160081	5	18160081	7	120.668	ID=Ciclev10000740m.g
S07_18436704	7	18436704	7	126.576	ID=Ciclev10027629m.g
S07_20114847	7	20114847	7	132.042	ID=Ciclev10025730m.g
S07_20104451	7	20104451	7	132.042	ID=Ciclev10024684m.g
S07_20305471	7	20305471	7	133.929	ID=Ciclev10026247m.g
S07_20540492	7	20540492	7	133.929	ID=Ciclev10027510m.g
S08_1493068	8	1493068	8	0	ID=Ciclev10028513m.g
S08_1359809	8	1359809	8	0	ID=Ciclev10028967m.g
S08_1649129	8	1649129	8	0	ID=Ciclev10027661m.g
S08_1422214	8	1422214	8	0	ID=Ciclev10028083m.g
S08_826272	8	826272	8	5.46	ID=Ciclev10029887m.g
S08_999173	8	999173	8	5.46	ID=Ciclev10028931m.g
S08_689245	8	689245	8	7.215	ID=Ciclev10028093m.g
S08_864884	8	864884	8	7.215	ID=Ciclev10029529m.g
S08_503462	8	503462	8	10.787	ID=Ciclev10029088m.g
S08_109287	8	109287	8	10.787	ID=Ciclev10028022m.g
S08_112996	8	112996	8	10.787	ID=Ciclev10028471m.g
S08_47411	8	47411	8	10.787	ID=Ciclev10028825m.g
S08_286783	8	286783	8	10.787	ID=Ciclev10028003m.g
S08_294978	8	294978	8	10.787	ID=Ciclev10028938m.g
S08_146252	8	146252	8	10.787	ID=Ciclev10028691m.g
S08_1041921	8	1041921	8	14.36	ID=Ciclev10027670m.g
S08_1006651	8	1006651	8	14.36	ID=Ciclev10027936m.g
S08_832105	8	832105	8	14.36	ID=Ciclev10030163m.g
S08_1165513	8	1165513	8	16.115	ID=Ciclev10028863m.g
S08_927557	8	927557	8	16.115	ID=Ciclev10028228m.g
S08_661020	8	661020	8	16.115	ID=Ciclev10028731m.g
S08_1204715	8	1204715	8	16.115	ID=Ciclev10027976m.g
S08_1095478	8	1095478	8	16.115	ID=Ciclev10027859m.g
S08_791814	8	791814	8	16.115	ID=Ciclev10028457m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S08_1214214	8	1214214	8	19.688	ID=Ciclev10029600m.g
S08_1241936	8	1241936	8	19.688	ID=Ciclev10029765m.g
S08_1640724	8	1640724	8	21.442	ID=Ciclev10027822m.g
S08_1605876	8	1605876	8	21.442	ID=Ciclev10028366m.g
S08_1618894	8	1618894	8	21.442	ID=Ciclev10030142m.g
S08_1433038	8	1433038	8	21.442	ID=Ciclev10028868m.g
S08_1411532	8	1411532	8	21.442	ID=Ciclev10027748m.g
S08_1716603	8	1716603	8	23.197	ID=Ciclev10028052m.g
S08_1814724	8	1814724	8	23.197	ID=Ciclev10029877m.g
S08_1796166	8	1796166	8	23.197	ID=Ciclev10030194m.g
S08_2429542	8	2429542	8	26.771	ID=Ciclev10028186m.g
S08_2529040	8	2529040	8	27.705	ID=Ciclev10029973m.g
S08_2107212	8	2107212	8	28.512	ID=Ciclev10028015m.g
S08_2289097	8	2289097	8	28.512	ID=Ciclev10028343m.g
S08_2172917	8	2172917	8	28.512	ID=Ciclev10029274m.g
S08_2263871	8	2263871	8	28.512	ID=Ciclev10028649m.g
S08_2188117	8	2188117	8	28.512	ID=Ciclev10029597m.g
S08_2720245	8	2720245	8	30.267	ID=Ciclev10028040m.g
S08_2850376	8	2850376	8	30.267	ID=Ciclev10027734m.g
S08_2844093	8	2844093	8	30.267	ID=Ciclev10027715m.g
S08_2635803	8	2635803	8	30.267	ID=Ciclev10027853m.g
S08_2854197	8	2854197	8	30.267	ID=Ciclev10030127m.g
S08_2788922	8	2788922	8	30.267	ID=Ciclev10029184m.g
S08_2818887	8	2818887	8	30.267	ID=Ciclev10029198m.g
S08_3026111	8	3026111	8	32.615	ID=Ciclev10028435m.g
S08_3000113	8	3000113	8	37.459	ID=Ciclev10027863m.g
S08_2971896	8	2971896	8	37.459	ID=Ciclev10027850m.g
S08_3494245	8	3494245	8	39.217	ID=Ciclev10028454m.g
S08_4026112	8	4026112	8	40.974	ID=Ciclev10028201m.g
S08_5326450	8	5326450	8	46.436	ID=Ciclev10028904m.g
S08_3701588	8	3701588	8	46.436	ID=Ciclev10027659m.g
S08_3998172	8	3998172	8	46.436	ID=Ciclev10029698m.g
S08_4892310	8	4892310	8	46.436	ID=Ciclev10028655m.g
S08_4417545	8	4417545	8	46.436	ID=Ciclev10029642m.g
S08_5343601	8	5343601	8	46.436	ID=Ciclev10029896m.g
S08_4871092	8	4871092	8	46.436	ID=Ciclev10029936m.g
S08_3130127	8	3130127	8	46.436	ID=Ciclev10029362m.g
S08_4297257	8	4297257	8	46.436	ID=Ciclev10028264m.g
S08_5052181	8	5052181	8	46.436	ID=Ciclev10030203m.g
S08_4889493	8	4889493	8	46.436	ID=Ciclev10027789m.g
S08_4461663	8	4461663	8	46.436	ID=Ciclev10029365m.g
S08_4553146	8	4553146	8	46.436	ID=Ciclev10029521m.g
S08_5461426	8	5461426	8	46.436	ID=Ciclev10028890m.g
S08_5153846	8	5153846	8	46.436	ID=Ciclev10028494m.g
S08_5469542	8	5469542	8	46.436	ID=Ciclev10030368m.g
S08_5491094	8	5491094	8	46.436	ID=Ciclev10027908m.g
S08_4140439	8	4140439	8	46.436	ID=Ciclev10029477m.g
S08_4969549	8	4969549	8	46.436	ID=Ciclev10028120m.g
S08_5201102	8	5201102	8	46.436	ID=Ciclev10029966m.g
S08_4075187	8	4075187	8	46.436	ID=Ciclev10028081m.g
S08_4555396	8	4555396	8	47.306	ID=Ciclev10029826m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S08_2438501	8	2438501	8	48.176	ID=Ciclev10028922m.g
S08_4860794	8	4860794	8	48.176	ID=Ciclev10027804m.g
S08_5896067	8	5896067	8	51.75	ID=Ciclev10029285m.g
S08_5956341	8	5956341	8	53.506	ID=Ciclev10027795m.g
S08_6142645	8	6142645	8	53.506	ID=Ciclev10028670m.g
S08_6050573	8	6050573	8	53.506	ID=Ciclev10027768m.g
S08_6899421	8	6899421	8	53.506	ID=Ciclev10028796m.g
S08_6026790	8	6026790	8	53.506	ID=Ciclev10027952m.g
S08_7354821	8	7354821	8	57.079	ID=Ciclev10029039m.g
S08_7889541	8	7889541	8	57.079	ID=Ciclev10029225m.g
S08_7969048	8	7969048	8	59.28	ID=Ciclev10027800m.g
S09_12220521	9	12220521	8	62.401	ID=Ciclev10004129m.g
S09_14455630	9	14455630	8	62.401	ID=Ciclev10004125m.g
S08_14374136	8	14374136	8	65.974	ID=Ciclev10027821m.g
S09_13316193	9	13316193	8	65.974	ID=Ciclev10005704m.g
S08_12955479	8	12955479	8	66.844	ID=Ciclev10028315m.g
S09_13377446	9	13377446	8	67.714	ID=Ciclev10004979m.g
S03_34838063	3	34838063	8	67.714	ID=Ciclev10020693m.g
S08_15046539	8	15046539	8	67.714	ID=Ciclev10030175m.g
S08_17500054	8	17500054	8	67.714	ID=Ciclev10028402m.g
S09_14406157	9	14406157	8	67.714	ID=Ciclev10004140m.g
S08_13963276	8	13963276	8	68.582	ID=Ciclev10030354m.g
S08_8442079	8	8442079	8	69.453	ID=Ciclev10030161m.g
S03_35357083	3	35357083	8	69.453	ID=Ciclev10018972m.g
S09_13242376	9	13242376	8	70.324	ID=Ciclev10005525m.g
S08_12933198	8	12933198	8	71.192	ID=Ciclev10029860m.g
S08_9913551	8	9913551	8	71.192	ID=Ciclev10028239m.g
S08_17432779	8	17432779	8	72.062	ID=Ciclev10029749m.g
S08_10985632	8	10985632	8	72.932	ID=Ciclev10030243m.g
S08_15392183	8	15392183	8	72.932	ID=Ciclev10027956m.g
S09_13605848	9	13605848	8	72.94	ID=Ciclev10004203m.g
S03_34605853	3	34605853	8	75.59	ID=Ciclev10018643m.g
S08_9625033	8	9625033	8	78.239	ID=Ciclev10029974m.g
S09_11451292	9	11451292	8	78.247	ID=Ciclev10004119m.g
S09_12024152	9	12024152	8	78.247	ID=Ciclev10006553m.g
S09_13043853	9	13043853	8	78.247	ID=Ciclev10005064m.g
S08_10896979	8	10896979	8	78.247	ID=Ciclev10028905m.g
S09_12329281	9	12329281	8	78.247	ID=Ciclev10006572m.g
S08_18745520	8	18745520	8	80.002	ID=Ciclev10027754m.g
S08_18007669	8	18007669	8	80.002	ID=Ciclev10030283m.g
S08_17785184	8	17785184	8	80.002	ID=Ciclev10027700m.g
S08_13554869	8	13554869	8	83.582	ID=Ciclev10029098m.g
S03_7447097	3	7447097	8	85.343	ID=Ciclev10019899m.g
S08_10523167	8	10523167	8	85.343	ID=Ciclev10029296m.g
S08_14935534	8	14935534	8	85.343	ID=Ciclev10030258m.g
S08_7914908	8	7914908	8	87.1	ID=Ciclev10030126m.g
S08_17805504	8	17805504	8	90.675	ID=Ciclev10027942m.g
S08_18863606	8	18863606	8	90.675	ID=Ciclev10029347m.g
S08_17860999	8	17860999	8	90.675	ID=Ciclev10029179m.g
S08_19484009	8	19484009	8	93.334	ID=Ciclev10027862m.g
S08_19394032	8	19394032	8	93.334	ID=Ciclev10028229m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S08_19570883	8	19570883	8	95.998	ID=Ciclev10028361m.g
S08_19741034	8	19741034	8	97.763	ID=Ciclev10027891m.g
S08_19818219	8	19818219	8	97.763	ID=Ciclev10027719m.g
S08_20271875	8	20271875	8	101.349	ID=Ciclev10028244m.g
S08_20501245	8	20501245	8	101.349	ID=Ciclev10028164m.g
S08_20481785	8	20481785	8	101.349	ID=Ciclev10028932m.g
S08_21029321	8	21029321	8	101.349	ID=Ciclev10027713m.g
S08_21462224	8	21462224	8	103.11	ID=Ciclev10028099m.g
S08_21560988	8	21560988	8	104.871	ID=Ciclev10027892m.g
S08_21579912	8	21579912	8	104.871	ID=Ciclev10028085m.g
S08_21586066	8	21586066	8	104.871	ID=Ciclev10030284m.g
S08_21834125	8	21834125	8	104.871	ID=Ciclev10030090m.g
S08_22014423	8	22014423	8	106.633	ID=Ciclev10030432m.g
S08_22179096	8	22179096	8	106.633	ID=Ciclev10030205m.g
S08_22380271	8	22380271	8	108.401	ID=Ciclev10027702m.g
S08_22481109	8	22481109	8	108.401	ID=Ciclev10027972m.g
S08_23491657	8	23491657	8	115.843	ID=Ciclev10028570m.g
S08_23922554	8	23922554	8	116.87	ID=Ciclev10028405m.g
S08_23951378	8	23951378	8	117.593	ID=Ciclev10027939m.g
S08_23698069	8	23698069	8	117.593	ID=Ciclev10029397m.g
S08_24256500	8	24256500	8	117.593	ID=Ciclev10028048m.g
S08_23766116	8	23766116	8	117.593	ID=Ciclev10030157m.g
S08_24260613	8	24260613	8	118.026	ID=Ciclev10030046m.g
S08_24263166	8	24263166	8	118.459	ID=Ciclev10029616m.g
S08_24236171	8	24236171	8	118.893	ID=Ciclev10028117m.g
S08_23791570	8	23791570	8	119.325	ID=Ciclev10030042m.g
S08_23773620	8	23773620	8	119.325	ID=Ciclev10030147m.g
S08_23947799	8	23947799	8	120.196	ID=Ciclev10029849m.g
S08_24121120	8	24121120	8	121.066	ID=Ciclev10027683m.g
S08_24153502	8	24153502	8	121.066	ID=Ciclev10028137m.g
S08_23856595	8	23856595	8	121.066	ID=Ciclev10029047m.g
S08_24012392	8	24012392	8	121.066	ID=Ciclev10029453m.g
S08_24319401	8	24319401	8	122.821	ID=Ciclev10029884m.g
S08_24316531	8	24316531	8	122.821	ID=Ciclev10028631m.g
S08_24304124	8	24304124	8	122.822	ID=Ciclev10030097m.g
S08_24346014	8	24346014	8	126.411	ID=Ciclev10028065m.g
S08_24462661	8	24462661	8	131.896	ID=Ciclev10029951m.g
S08_24701701	8	24701701	8	135.486	ID=Ciclev10030370m.g
S08_24705731	8	24705731	8	137.248	ID=Ciclev10027941m.g
S08_24964339	8	24964339	8	144.831	ID=Ciclev10029924m.g
S08_24992754	8	24992754	8	144.831	ID=Ciclev10028485m.g
S08_25035907	8	25035907	8	144.831	ID=Ciclev10028057m.g
S09_216316	9	216316	9	0	ID=Ciclev10005284m.g
S09_375490	9	375490	9	3.648	ID=Ciclev10004187m.g
S09_1301325	9	1301325	9	19.03	ID=Ciclev10005318m.g
S09_1642180	9	1642180	9	20.862	ID=Ciclev10004153m.g
S09_1823544	9	1823544	9	22.694	ID=Ciclev10007035m.g
S09_1896611	9	1896611	9	22.694	ID=Ciclev10006926m.g
S09_2346807	9	2346807	9	22.694	ID=Ciclev10005432m.g
S09_2340896	9	2340896	9	26.092	ID=Ciclev10007121m.g
S09_2284669	9	2284669	9	26.092	ID=Ciclev10004115m.g

Marker	Scaffold	Phisical position	LG	Genetic position	gene name (phytozome)
S09_2538649	9	2538649	9	26.092	ID=Ciclev10004552m.g
S09_3040164	9	3040164	9	32.4	ID=Ciclev10005035m.g
S09_3331684	9	3331684	9	36.508	ID=Ciclev10005615m.g
S09_3316690	9	3316690	9	36.508	ID=Ciclev10004440m.g
S09_4460987	9	4460987	9	41.695	ID=Ciclev10004951m.g
S09_4383972	9	4383972	9	41.695	ID=Ciclev10006419m.g
S09_3692012	9	3692012	9	45.093	ID=Ciclev10004753m.g
S09_3847675	9	3847675	9	45.093	ID=Ciclev10006599m.g
S09_4660017	9	4660017	9	46.76	ID=Ciclev10007173m.g
S09_4606133	9	4606133	9	46.76	ID=Ciclev10007078m.g
S09_4625005	9	4625005	9	46.76	ID=Ciclev10006968m.g
S09_4657185	9	4657185	9	46.76	ID=Ciclev10004701m.g
S09_5395222	9	5395222	9	50.151	ID=Ciclev10005820m.g
S09_5560231	9	5560231	9	50.151	ID=Ciclev10004803m.g
S09_4684722	9	4684722	9	50.151	ID=Ciclev10006941m.g
S09_4969998	9	4969998	9	50.151	ID=Ciclev10007055m.g
S09_5627681	9	5627681	9	50.151	ID=Ciclev10004847m.g
S09_9808962	9	9808962	9	53.544	ID=Ciclev10005408m.g
S09_22114569	9	22114569	9	55.213	ID=Ciclev10004493m.g
S09_15918070	9	15918070	9	55.213	ID=Ciclev10006793m.g
S09_10333149	9	10333149	9	55.213	ID=Ciclev10004351m.g
S09_22323899	9	22323899	9	55.213	ID=Ciclev10006934m.g
S09_14697132	9	14697132	9	55.213	ID=Ciclev10004255m.g
S09_28043147	9	28043147	9	60.39	ID=Ciclev10004838m.g
S09_27290558	9	27290558	9	60.39	ID=Ciclev10004476m.g
S09_28110760	9	28110760	9	62.056	ID=Ciclev10004870m.g
S09_28739861	9	28739861	9	69.085	ID=Ciclev10004762m.g
S09_29254875	9	29254875	9	76.541	ID=Ciclev10006929m.g
S09_29410378	9	29410378	9	81.358	ID=Ciclev10006134m.g
S09_29643157	9	29643157	9	83.062	ID=Ciclev10006482m.g
S09_29955707	9	29955707	9	88.347	ID=Ciclev10006155m.g
S09_30525399	9	30525399	9	95.388	ID=Ciclev10004921m.g
S09_30855335	9	30855335	9	103.381	ID=Ciclev10006575m.g
S09_31192430	9	31192430	9	103.381	ID=Ciclev10006542m.g