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PhD in AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCE

CYCLE XXXII

Citrus reproductive biology: physiological and genetic aspects of sterility, seedlessness and fruiting

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Summary

Abstract

Citrus exhibit a complex genetic and reproductive biology as high heterozygosity, long juvenile period, parthenocarpy, apomixis and self-incompatibility, that hampers the use of traditional strategies to study and improve citrus traits. Flowering is regulated by different interacting factors, including both environmental and endogenous factors that affect gametes fertility, fruit set and the duration of the juvenile period. This dissertation project investigates on environmental and genetic factors regulating flowering by histological analysis, transcriptomic and biotechnological strategies providing novel insight helpful for planning citrus improvement programs. Histological and in vitro analyses showed that hot temperature stress during male gametophyte development reduced pollen performance by morphological alteration on tapetum and reduction in starch accumulation in clementine mandarin. The citrus FLOWERING LOCUS T (CiFT) gene is the main component of the flower induction together with exposition to cold temperature. Here by the analysis of the expression levels of three CiFT homologs, CiFT1, CiFT2 and CiFT3, it was observed that rootstocks influence flower induction and flowering intensity in the following spring in sweet orange. Finally, CiFT overexpression was induced in citrange to reduce the juvenile period and enable characterization of a gene potentially involved in seedlessness, selected by genome comparison from the seedless mutant Tango against the Clementine mandarin reference genome, through silencing by RNA interference (RNAi). Transgenic plants started to flower five months after the transformation experiments. Despite further analysis is required for pollen, ovary and fruit development,

this study proves the usefulness of biotechnological strategy for functional genomics studies in citrus.

Sommario

Gli agrumi presentano una complessa genetica e biologia riproduttiva (elevata eterozigosi, lunga fase giovanile, partenocarpia, apomissia, auto-incompatibilità) che ostacolano lo studio e il miglioramento dei caratteri di interesse attraverso le tradizionali strategie di miglioramento genetico. La fioritura è regolata da diversi fattori, sia ambientali che endogeni, che influenzano la fertilità dei gameti, la produzione dei frutti e la durata della fase giovanile. Questo progetto di tesi indaga sui fattori ambientali e genetici che regolano la fioritura attraverso analisi istologiche, di trascrittomica e tecniche biotecnologiche allo scopo di fornire informazioni utili nella pianificazione di programmi di miglioramento in agrumi. Analisi istologiche e in vitro hanno mostrato che l'esposizione ad alte temperature durante lo sviluppo del gametofito maschile in Clementine riduce la funzionalità del polline attraverso alterazioni morfologiche del tapetum e la riduzione nel contenuto di amido. Il FLOWERING LOCUS T (CiFT) è il principale componente che controlla l'induzione antogena insieme all'esposizione alle basse temperature. Qui dall'analisi dei livelli di espressione di tre omologhi, CiFT1, CiFT2 and CiFT3, è stato osservato come i portainnesti influenzano l'induzione antogena e l'intensità della fioritura in arancio dolce. Infine, la sovra espressione del gene CiFT è stata indotta in citrange per ridurre il periodo giovanile e favorire la caratterizzazione di un gene candidato coinvolto nell'apirenia, selezionato dal confronto di dati di sequenziamento del mandarino-simile apireno Tango e il genoma di riferimento di Clementine attraverso silenziamento da RNA ad interferenza (RNAi). Le piante

transgeniche hanno mostrato una precoce fioritura dopo cinque mesi dall'esperimento dimostrando l'utilità della strategia biotecnologica negli studi di genomica funzionale in agrumi.

CHAPTER I

General introduction

General introduction

The genus *Citrus* belongs to the order *Geraniales*, family *Rutaceae*, subfamily *Aurantioideae*, tribe *Citreae*. The main cultivated species are sweet oranges (*Citrus sinensis* (L.) Osb), mandarins (*C. clementina* Hort. ex Tan. and *C. unshiu* (Mak.) Marc., mainly), grapefruits (*C. paradisi* Macf.), pummelos (*C. grandis* (L.) Osb. or *C. maxima*), and lemons (*C. limon* L. Burm. f.). Genomic, phylogenetic and biogeographic analyses showed that citrus diversified during the late Miocene through a radiation from the southeast foothills of the Himalayas, in a region that includes the eastern area of Assam, northern Myanmar and western Yunnan that coincided with a pronounced transition from wet monsoon conditions to a drier climate (Wu et al., 2018). Biochemical and genomic studies suggest that many citrus species and/or cultivars derived from the natural interspecific hybridization between four ancestors: pummelo (*C. grandis* Osbeck), common mandarin (*C. reticulata* Blanco), citron (*C. medica* Linn.) and a Papeda cv. wild citrus (*C. micrantha* Wester) (Cuenca et al., 2018).

Citrus (*Citrus* spp.) include the most widely and economically important fruit tree crops grown throughout tropical and subtropical region of the world, with more than of 146 million tons produced in 2017 (FAO, 2017). The main cultivated citrus species include sweet oranges with about 50% of the world production, followed by mandarins (22.8%), lemons and limes (11.7%), and pummelos and grapefruits (6.2%). The importance of citrus fruits to the world's economy is demonstrated by its wide distribution and large-scale production. According to FAO, in 2017, the worldwide cultivation of citrus utilized more than 9 million

hectares and yielded more than 17 tons/ha, with a gross production value of 56.14 billion US dollars. The top ten citrus-producing countries are China with 26,5 % of the world production, followed by Brazil (13,5 %), India (7,8 %), Mexico (5,6 %), USA (4,8 %), Spain (4,3 %), Turkey (3,3 %), Egypt (3,0 %), Nigeria (2,8 %) and Iran (2,3 %). Italy ranks 12th in world production by 1,8 % of the world production (FAO, 2017). More of two-thirds of the total citrus production (84,1%) are destined for fresh consumption, while the remaining 15,9 % for juice production. Recently, the citrus consumption worldwide has increased principally thanks to the improvements made in quality traits to respond to the demand of "high-quality fresh citrus" (Cuenca et al., 2018).

Citrus exhibit very complex genetics and reproductive biology, with a wide range of reproductive systems among the different varieties of citrus species that include sexual reproduction, parthenocarpy, apomixis, self-incompatibility and mixtures of these systems. In addition, the high level of heterozygosity and the long juvenile period (5-10 years) make the citrus improvement through conventional strategies a very difficult and time-consuming process.

Flowering is the transition from vegetative to reproductive growth and represents one of the most critical phases during the plant life cycle it since ensure reproductive success and fruits set. The timing of flowering is regulated by a complex coordination of environmental and endogenous signals: photoperiod, vernalization, ambient temperature, florigen and autonomous flowering pathways (Amasino, 2010). In general, under subtropical conditions, citrus bloom during spring after a period of bud quiescence and the exposure to

short days and the low temperatures of winter (Tan and Swain, 2007). The citrus *FLOWERING LOCUS T* (*CiFT*) gene, encoding the long distance flowering signal florigen, has been recognized as the main component of the flowering signal cascade associated with flowering induction in citrus (Nishikawa et al., 2007, 2009; Muñoz-Fambuena et al., 2011). In general, fruit set depends on the success of pollination and fertilization since the presence of fertilized ovules normally triggers fruit development. Lack of fertilization results in arrest of the gynoecium development and subsequent flower abscission. For example, in the seeded 'Pineapple' sweet orange, lack of fertilization leads to abscission of the ovary (Ben-Cheikh et al., 1997). Anyhow, in citrus there are several varieties with the capability to produce parthenocarpic fruits. Parthenocarpy is the development of fruit without fertilization that gives rise to seedless fruit (Vardi et al., 2008). Seedlessness is a desirable economic trait for both the fresh and processed citrus markets, since it avoids seed removal process for consumption and processing increasing fruit quality. The male/female sterility and self-incompatibility are genetically inherited traits that allow production of seedless fruit when combined with parthenocarpy (Vardi et al., 2008; Aleza et al., 2010; Qin et al., 2015; Goto et al., 2016). Several kinds of sterility have been described in citrus so far. Satsuma mandarin (*Citrus unshiu* Marcovitch) shows cytoplasmic male sterility that results in abortion of anthers during initial stages of pollen development and although some ovules are functional most of them abort (Iwamasa, 1966; Yamamoto et al., 1997). Navel orange [*Citrus sinensis* (L.) Osbeck] produces no viable pollen because of the degeneration of

pollen mother cells, but it also shows ovule abortion because of degeneration before meiosis (Iwamasa, 1966). In 'Wilking' mandarin seedlessness is due to a recessive gene that results in pollen and ovule abortion (Vardi et al., 2008). In addition, environmental factors, such as temperature stresses, chromosomal aberrations, chemical treatments and genetic factors controlling the meiosis process can induce seedlessness (Vardi et al., 2008). For example, temperature stresses during gametophyte development could affect gamete fertility. The male gametophyte development seems to be the most vulnerable phase of the reproductive process because of its high sensitivity to temperature stresses resulting in reduced fertility or sterility (Zinn et al., 2010). Several studies carried out on herbaceous and woody species showed that the reduction in pollen viability induced by temperature stress is linked to anomalies occurring during the meiotic cell division such as erratic chromosome behaviour, cytokinesis, cytomixis or a lack of nutrients due to alteration in the functionality of nutritive tapetal cells surrounding microspores (Cavalcante et al., 2000; Pécrix et al., 2011; Parish et al., 2012; De Storme and Geelen, 2014; Omidi et al., 2014; Sage et al., 2015). Investigate on effects of temperature stress in biological processes represents an important issue considering the current climate change context characterized by the rise in the average global temperature, often accompanied by extreme fluctuations.

Self-incompatibility (SI) is a genetically controlled mechanism that prevents self-fertilization (De Nettancourt, 2001). Clementine mandarin (*Citrus clementina* Hort. ex Tan.) is self-incompatible and although pollen is capable of germination on the stigma, the ovary develops and results in

seedless fruit if cross-pollination does not occur (Distefano et al., 2009). However, parthenocarpic varieties can set seedy fruit under cross-pollination conditions. Different horticultural techniques have been reported to reduce cross-pollination: growing citrus orchard far away from any citrus intercompatible pollen source (Chao et al., 2005); covering trees with insect-proof nets (Gambetta et al., 2013); enhancing ovule abortion or reducing pollen tube growth by the application of gibberellic acid (GA_3) or copper sulfate ($CuSO_4$) at bloom (Mesejo et al., 2006, 2008).

Breeding strategies were implemented to produce seedless varieties such as the production of triploid varieties and mutagenesis induced by gamma irradiation (Spiegel-Roy and Vardi, 1992; Vardi et al., 2008; Aleza et al., 2012, 2016; Goldenberg et al., 2018). Triploidy is achieved by cross-hybridization of diploid and tetraploid varieties that result from unreduced gametes (Aleza et al., 2016). The resulting triploid varieties are sterile due to the formation of abnormal gametes or embryo-sac abortions (Aleza et al., 2012). In the gamma irradiation mutagenesis, the young buds are exposed to different doses of radiation, which create a wide range of random mutations often resulting in pollen or ovule abortion (Vardi et al., 2008).

Among varieties obtained by gamma irradiation mutagenesis there is ‘Tango’ mandarin, a mutant of W. Murcott (http://www.citrusvariety.ucr.edu/citrus/tango.html) and ‘Orri’ mandarin, a mutant of ‘Orah’ (Spiegel-Roy and Vardi, 1992). Anyway, the mentioned methods require large growing areas and are time expensive due to the long juvenile period typical of citrus species (5-15 years). Biotechnological and transgenic approaches have been exploited to overcome

problems of the conventional breeding. Li and colleagues (2002) successfully expressed the *barnase* suicide gene in embryogenic calluses of Ponkan mandarin enabling the production of male-sterile mandarins. Also, the release of sweet orange (*Citrus sinensis*) (Xu et al., 2012) and clementine genomes (Wu et al., 2014) represents a useful resource for understanding and improving many important citrus traits. The use of haploid material (Aleza et al., 2009) allowed to obtain a high quality clementine genome compared to those of sweet-orange making it the primary reference genome for all citrus and related genera. By transcriptome studies and high-throughput sequencing technologies several genes were identified as potential targets related to seedlessness in citrus (Garcia-Lor et al., 2012; Zheng et al., 2012; Fang et al., 2016; Zhang et al., 2017). The reduction of juvenile period by using biotechnological strategies is a promising tool for accelerating research and breeding in crop species that show delayed flowering. Early flowering and fruiting were induced in citrus plants by ectopic expression of flowering-time genes (Peña et al., 2001; Endo et al., 2005; Pons et al., 2014). Despite transgenic approaches cannot be used for commercial utilization because legal and ethical issues, are useful for functional genomic studies of candidate genes regulating quality traits. New breeding techniques such as CRISPR/Cas system seem to be the most promising strategy to accelerate breeding programs, which could overcome the legal issues inherent transgenic approach.

Dissertation overview

This dissertation project provides novel insight on physiological and genetic aspects regulating flowering in citrus that help to understand such a complex mechanism and could be exploited in breeding programs. The effects on flowering of environmental and agronomic factors were also studied and contributed to a more exhaustive comprehension of the first stages of fruitification process in citrus.

Results will be presented in the following chapters.

Chapter II describes morphological and physiological effects of cold and hot temperature stress during male gametophyte development in clementine mandarin by histological and *in vitro* analysis. This chapter provides information about phenomena influencing pollen vitality as well as male sterility in mandarin in relation to climate change context. The manuscript has been published in Annals of Applied Biology (2019) 175:1–13. DOI: 10.1111/aab.12508.

Chapter III investigates about the influence of rootstocks on flower induction in sweet orange by the regulation of the expression pattern of the citrus homologs of flowering-time gene *FLOWERING LOCUS T* (*CiFT*), *CiFT1*, *CiFT2* and *CiFT3*, in the leaves of 'Tarocco Scirè' respect 'Swingle' citrumelo and 'C35' citrange. The results provided new information about flowering regulation that could help in the choice of better rootstock in breeding programs.

Chapter IV assesses a biotechnological strategy based on early flowering phenotype induction in citrange by overexpression of *CiFT* for functional characterization of a

gene coding an RNA binding protein (RBP), potentially involved in seedlessness through silencing by RNA interference (RNAi). The results demonstrated the usefulness of the strategy in citrange to accelerate functional genomic studies in citrus. This work has been performed at the Laboratory of Citrus biotechnology of the Instituto de Biología Molecular y Celular de Plantas (IBMCP)-(UPV-CSIC), Valencia, Spain, under the supervision of Prof. Leandro Peña.

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CHAPTER II

Temperature stress interferes with male reproductive system development in clementine (*Citrus clementina* Hort. ex. Tan.)

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Abstract

Male gametophyte development is a critical phase of the plant life cycle due to its high sensitivity to environmental stresses. The rise in the average global temperature, often accompanied by extreme fluctuations, has an important impact on biological processes. Among those, male gametophytes are particularly sensitive to temperature stress during flower bud development and anthesis. Male gametophyte development has been extensively studied in several plant species, but little information is available about the effects of temperature stress on male gametophyte development in the genus *Citrus*. We evaluated the effects of cold and hot temperatures during microsporogenesis and microgametogenesis on one of the most economically valuable citrus species, the "Comune" clementine (*Citrus clementina* Hort. ex. Tan.). The effect of constant temperature on the androecium was evaluated by a time course histological analysis performed on the anthers and by monitoring in vitro pollen germination. The results revealed how suboptimal hot and cold temperatures induce drastic alterations on the morphology of the tapetal cells, microspores and mature pollen grains. Shifting from the optimal temperature affected the timing of starch depletion in the anther walls, such as epidermis, endothecium and middle layer, influencing the pollen germination rate and pollen tube growth. To the best of our knowledge, this is the first study attempting to assess how temperature stress affects male reproductive development in citrus. A better understanding of the mechanisms underlining male sterility will provide novel insights to elucidate the physiology of this agronomical important quality trait.

1. Introduction

Earth's climate is experiencing the so-called global warming resulting in an increase in the average temperature and a higher frequency of extreme events related to both precipitation and temperature. The effects of temperature fluctuations outside the range of species tolerance during the flowering phase result in erratic final seed and fruit sets (Hedhly, 2011). Male gametophyte development is reported to be the most vulnerable phase of the reproductive process because of its high sensitivity to temperature stresses, leading to reduced fertility or sterility (Zinn et al., 2010). In flowering plants, pollen development occurs from the pollen mother cells to mature pollen grains within the anthers through a series of specific events, including microsporogenesis and microgametogenesis that involve the anther wall tissues. Tapetum in particular, is the innermost anther layer that provides the developing microspores with nutrients, enzymes and pollen wall components, while the surrounding layers are middle layer, endothecium and epidermis provide structural and support functions to promote dehiscence (Goldberg et al., 1993). Several studies carried out on the reproduction phases of different herbaceous species shed light on the stress-induced anomalies occurring during different stages of the meiotic cell division. These include erratic chromosome behavior, abnormal spindle orientation and cytokinesis, cytomixis resulting in an unbalanced ploidy level and reduced pollen viability (Cavalcante et al., 2000; Pécrix et al., 2011; De Storme and Geelen, 2014; Omidi et al., 2014; Sage et al., 2015). Temperature stress affects the functionality of the tapetum (Goldberg et al., 1993). It seems that a reduction in pollen production and viability is due to a lack of nutrients in

the tapetal cells that leads to their premature disappearance under heat stress or to ectopic persistence under cold stress while hypertrophy occurs in both extreme conditions (Parish et al., 2012; De Storme and Geelen, 2014). Carbohydrate accumulation in anthers serves as energy source either for developing pollen grains and for pollen maturation, and represents the major determinant for pollen viability and germination capability (Pacini, 2010; De Storme and Geelen, 2014). In optimal conditions, the starch and sugar contents in anther walls and developing pollen increase during the later stages of male gametogenesis (De Storme and Geelen, 2014), while under temperature stress conditions, the metabolic processes underlining starch and sugar metabolism are compromised (Shaked et al., 2004). The inhibition of the starch and soluble sugar turnover results in the disruption of the tapetum and pollen mother cells with negative consequences on the availability of pollen (Jain et al., 2007, 2010; Irenaeus and Mitra, 2014). In herbaceous plants, male sterility under temperature stress was correlated with a reduced accumulation of sugars in the anther walls and developing pollen (De Storme and Geelen, 2014), in rice in particular starch accumulation is used as an indicator of pollen viability (Gunawardena et al., 2003). The negative effect of temperature stress on male reproductive structure development is well documented in herbaceous plants, but little is known about the effect on fruit tree crops (Hedhly, 2011). In citrus fruit, the failure of the sexual reproductive process (e.g., male sterility) in parthenocarpic cultivars results in seedless fruits (Yamamoto et al., 1995). In contrast to other fruit crops, male sterility can be considered a benefit to avoid the presence of seeds, which is one of the most

important quality traits for fresh citrus fruit consumption. In particular, among mandarin and mandarin like cultivars, self-incompatible clementines have great economic importance and, even though clementines produce fertile pollen, they bear seedless parthenocarpic fruit. However, due to the spread of commercial cultivars with sexually intercompatible pollen, a conspicuous increase in the presence of seeds in seedless genotypes has been observed (Distefano et al., 2009). Understanding the effects of temperature stress on reproductive organs may be important to clarify the role of different temperatures in the reproduction process. It was previously reported that the genotype–temperature interaction during the progamic phase of the development of three ancestral citrus species affected pollen performance (Distefano et al., 2012). Additionally, exposure to different temperatures during flower bud development alters the expression of the self-incompatibility reaction affecting pollen tube growth along the pistil. However, little is known about the effects of temperature stresses during male gametophyte development in citrus (Yang and Nakagawa, 1970; Distefano et al., 2018). In the present study, we evaluated the morpho-physiological effects of heat/cold stress on the male gametophyte development of "Comune" clementine mandarin. Three different temperature regimes (15, 25, 30°C) were tested. These regimes simulate the thermal range, from cold to hot, recorded in the Mediterranean area during the citrus flowering season (Distefano et al., 2018). Histological analyses were performed on the anthers of the flower buds from the appearance of the first petal until anthesis. Histological analysis was used to evaluate the effects of temperature stress

during microsporogenesis and microgametogenesis. Pollen performance was monitored by an *in vitro* test to evaluate the effects of temperature on pollen germination and pollen tube growth.

2. Material and methods

2.1 Plant material

The study was conducted on the self-incompatible cultivar "Comune" clementine (*Citrus clementina* Hort. ex. Tan.). This cultivar is a known reference for self-incompatibility tests (Distefano et al., 2009). Four-year-old plants were grown in pots and subjected to three different constant temperatures (15, 25 or 30°C; three plants per treatment) in growth chambers from early flower bud appearance until full anthesis under 16/8 hr, light/dark cycle. To evaluate anther development under the three temperature regimes, flower buds were collected at five phenological stages: Stage A, when the first petal appeared and the buds had a longitudinal length of 2 mm (pollen mother cell stage); Stage B, during elongation when the buds were 4 mm long (tetrad stage); Stage C, when the flower buds were 6 mm long at the young microspore stage (from the early microspore stage to microspore vacuolation stage); Stage D, when the flower buds were 10 mm long at the mature microspore stage (from polarised microspore stage to early mature pollen grain stage) and Stage E, at flower anthesis with immature anthers (Fig. 1). Prior to phenotyping, plant flower buds showing a flowering stage subsequent to Stage A were removed.

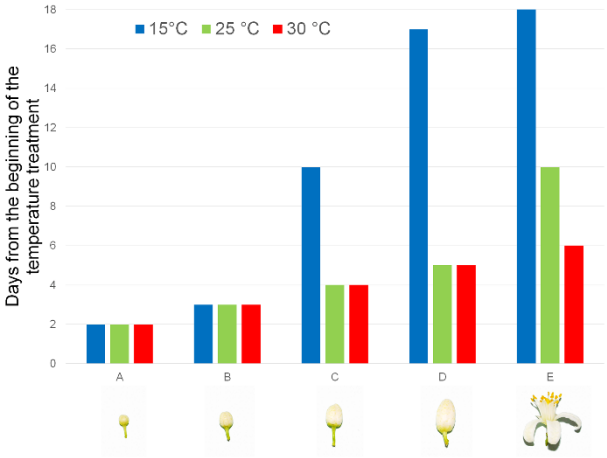


Figure 1. Sampling days from the beginning of the temperature treatments at 15, 25 and 30°C for the "Comune" clementine flower buds collected at five phenological stages (A–E). (A) At the appearance of the first petal with a longitudinal length of 2 mm ("pollen mother cell stage"). (B) During elongation with flower buds with a longitudinal length of 4 mm ("tetrad stage"). (C) Flower buds 6 mm long during the young microspore stage (from the "early microspore stage" to "microspore vacuolation stage"). (D) Near full growth with buds with a longitudinal length of approximately 10 mm during the mature microspore stage (from the "polarised microspore stage" to "early mature pollen grain stage"). (E) Flowers at anthesis with immature anthers.

2.2 Microscopic observations

Ten flower buds per stage (A–E) and temperature regime (15, 25, 30°C) were fixed in 2.5% glutaraldehyde in 0.03 M phosphate buffer (pH 7.0), dehydrated through a graded ethanol series (70 and 95%), embedded in JB-4 resin (Polysciences Co. Ltd, Eppelheim, Germany) and finally sectioned transversely into 5-µm-thick sections using a Jung

Multicut Leica microtome (Leica, Wetzlar, Germany). The sections were stained with 0.02% toluidine blue O (O'Brien et al., 1964) and periodic acid-Schiff's reagent (PAS) (Jensen, 1962) for general histological observations and insoluble carbohydrate analyses, respectively; 0.07% calcofluor (Hughes and McCully, 1975) for cellulose; 0.01% auramine (Heslop-Harrison and Shivanna, 1977) for cutin and a solution of 2% iodine and 5% potassium iodide (IKI) (Johansen, 1940) for starch. To observe the nuclei and meiotic activity during pollen development, double staining with 40,6-diamidino-2-phenylindole (DAPI) and acridine orange (AO) was performed according to the procedure described by Dudley et al., (1987) by mounting the slides in water. The sections were observed under Leica DM 2500 microscope (Leica Wetzlar, Germany) by bright-field microscopy for the sections stained with PAS, toluidine blue O and IKI and by fluorescence microscopy for the sections stained with calcofluor, auramine and DAPI/AO as described by Distefano et al., (2011). Images were acquired with a Leica DFC 320 (Leica) digital camera attached to the microscope. ImageJ software (https://imagej.nih.gov) was used for the quantitative analysis of the starch grains that accumulated in the anther tissues during microgametogenesis via an analysis of 50 images per anther.

2.3 In vitro pollen germination

To evaluate the effect of the tested temperature regimes (15, 25 or 30°C) on pollen behavior, fresh pollen was obtained from 20 flowers per treatment via extraction in a sterile water-based solution and immediately used for the *in vitro* germination test with an incubation at 25°C. The pollen was

transferred to Petri dishes on solidified germination medium consisting of 100 g/L sucrose, 0.1 g/L H_3BO_3, 0.3 g/L $Ca(NO_3)_2$, 0.1 g/L KNO_3 and 10 g/L agar. The culture medium was adjusted to pH 5.4 ± 0.1 as described by Mesejo et al., (2006). Pollen germination was arrested after 6, 12, 24 and 36 hr by immediately freezing the Petri dishes at −20°C. This procedure was adopted for its efficacy in preserving the pollen morphology for the following microscopic observation (Hedhly et al., 2005). A pollen grain was scored as germinated when the pollen tube length exceeded the diameter of the grain. For each treatment, the germination percentage and pollen tube length were recorded in three Petri dishes by counting three complete fields per dish (six replicates) and by counting at least 100 pollen grains per field.

2.4 Statistical analysis

The data were analysed using STATISTICA 6.0 software (Statsoft Inc., Tulsa, OK). The germination percentage and pollen tube length were first submitted to an analysis of variance with least significant differences (LSDs) ($p < 0.01$) and then to Levene's test for homogeneity of variance (Brown and Forsythe, 1974). To compare the production of starch grains among treatments, a one-way analysis of variance was applied, statistical differences between the three tested temperatures were assessed using the LSD test for $\alpha = 0.05$.

3. Results

3.1 Flower bud development

To evaluate the effects of the temperature regimes (15, 25, 30°C) during anthers development, we collected flower buds at five phenological stages from the appearance of the first petal until anthesis (Fig. 1). The occurrence of phenological stage occurred at different time points according to the treatment. The low temperature (15°C) reduced the speed of the growth of the flower buds, which reached phenological Stage C 6 days later than the flower buds grown at 25 and 30°C. The increase in temperature led to faster flower bud growth and earlier anthesis. The flower buds reached anthesis after 18, 10 and 6 days from the beginning of the temperature treatment at 15, 25 and 30°C, respectively.

3.2 Anther tissue analysis and microspores development during microsporogenesis

During the first stage of microsporogenesis, at the "pollen mother cell stage," no morphological differences between the three temperature regimes (15, 25, 30°C) were observed (Fig. 2a–f). In each anther lobe, the four layers were distinguishable showing from outside to inside: epidermis, endothecium, middle layer and tapetum surrounding a spherical loculus. In particular, tapetum consisted of a single layer of expanded multinucleated cells (Fig. 2d–f) with dense cytosols and without cell walls (Supporting information Fig. 1a–c). Under 15°C, all anthers tissues and pollen mother cells showed a decreased DAPI staining intensity compared to the other two thermal conditions (Fig. 2d–f). The chromosomes became evident during prophase of meiosis within the pollen

mother cells (Fig. 2d–f). Within the loculus, meiocytes appeared to be expanded and attached to each other without any intercellular space and with a thin layer of cellulose in the wall reducing locular fluid (Supporting information Fig. 1a–c). At the "tetrad stage," after meiosis, tetrads of young microspores became visible (Fig. 3a–f) and were enclosed by a dense callose wall (Supporting information Fig. 1d–f). No differences were observed among the three treatments for the tetrads, middle layer and tapetum (Fig. 2d–f and 3d–f; Supporting information Fig. 1d–f). During microsporogenesis, insoluble carbohydrates appeared to accumulate in the cells of the epidermis, endothecium and middle layer, while no accumulation was detected in the tapetum, pollen mother cells and tetrads due to the absence of carbohydrate grains. The PAS staining highlighted no differences in the insoluble carbohydrate content of the anther tissues among the three growing temperatures (Fig. 2a–c and 3a–c).

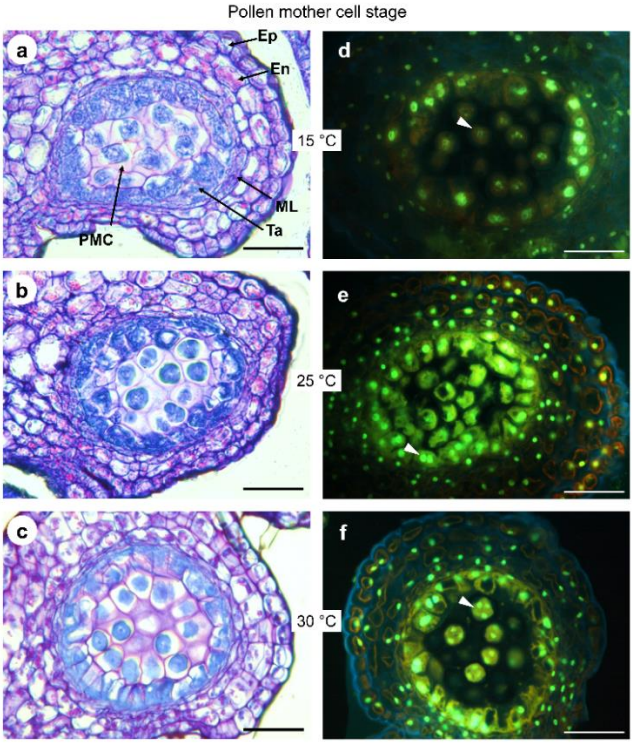


Figure 2. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e) and 30°C (c, f) during the "pollen mother cell stage" (a–f). (a) Each anther lobe consisted of an epidermis (Ep), an endothecium (En), a middle layer (ML), and a tapetum (Ta) surrounding the loculus where the pollen mother cell (PMC) (a–f) are located. The tapetal cells are large and multinucleated, and the PMC are surrounded by chromosomes during prophase I of meiosis (arrowheads). (a–c) PAS and toluidine blue O staining. (d–f) DAPI and AO staining. Scale bars: (a–f) = 50 μm.

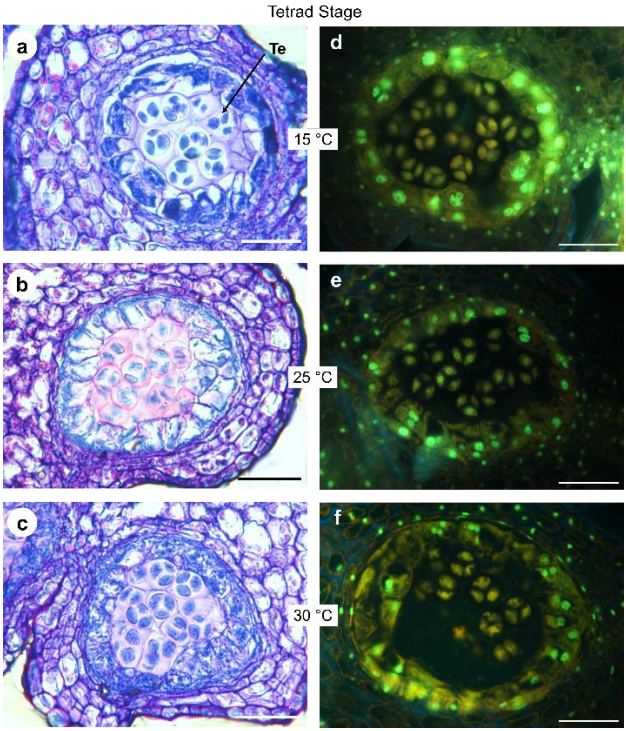


Figure 3. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e), and 30°C (c, f) during the "tetrad stage" (a–f). After meiosis, tetrads (Te) of young microspores became visible PAS and toluidine blue O staining. (d–f) DAPI and AO staining. Scale bars: (a–f) = 50 μm.

3.3 Anther tissue analysis and pollen development during microgametogenesis

At the “early microspore stage,” the young microspores were released by the tetrads. They appeared to be small with a central nucleus (Fig. 4a–c and 5a–c). Under cold/heat stress, the cytoplasm of the microspores appeared less dense (Fig. 4a, c) compared to that of the microspores developed at 25°C (Fig. 4b), although at 15°C it showed the presence of a protoplast but with a strong plasmolysis (Fig. 4a). At this stage, the tapetal cells of the flower buds grown at 15°C (Fig. 4a) appeared compact and full of secretions compared to those of the flower buds grown at 25°C (Fig. 4b) and especially at 30°C (Fig. 4c), which were less dense. During the “microspore vacuolation stage,” the microspores were enlarged by vacuolation that pushed the nucleus to a peripheral position (Fig. 4d–f and 5d–f). Compared to the tapetal cells from the flower buds grown at 25 and 30°C (Fig. 4b–c), at this stage, a significant delay in tapetum development was evident in the flower buds grown at 15°C, in which the cells of the tapetum appeared full of secretions, and this appearance was similar to what was observed during the “early microspore stage” (Fig. 4a). At the “polarised microspore stage,” the tapeta of flowers grown at 15°C (Fig. 6a and 7a) and 30°C (Fig. 6c and 7c) were composed of irregular layers of cells that were highly vacuolated, while the tapeta of the anthers grown at 25°C (Fig. 6b and 7b) were composed of regular layers of cells with dense cytosols, suggesting an early PCD (programmed cell death) of the tapetal cells under temperature stress. At the “early mature pollen grain stage” tapetum layer disappeared in all

temperatures tested (Fig. 6d–f and 7d–f**)** indicating that PCD occurred in the previous "polarised microspore stage" (Fig. 6a–c and 7a–c). Anyhow, the presence of residues of the tapetal cells were still evident under temperature stress (Fig. 7d–f) compared to control temperature (Fig. 7e). Nuclei in the tapetal cells were not visible, while inside the microspores, the nuclei were shown to be in a peripheral position (Fig. 7a–c). Starting from the "polarised microspore cells" stage, the microspores of anthers grown at 15°C (Fig. 6a and 7a) had an irregular contour. At 30°C the pollen grains showed an even more irregular contour than that observed at 15°C (Fig. 6b and 7b), showing a roundish shape. In the "early mature pollen grain stage," residues of the tapetal cells were still evident at 15 and 30°C (Fig. 6d–f and 7d–f). An unambiguous change in starch grain accumulation under the three different temperatures was evident during the "microspore vacuolation stage" (Fig. 8a–c). At this stage, compared to the flower buds grown at 25°C (Fig. 8b), an increased accumulation of starch grains was observed in the anther walls (epidermis, endothecium and middle layer) grown at 15°C (Fig. 8a), while a reduced accumulation of starch was observed in the anther walls of the flower buds grown at 30°C (Fig. 8c). The statistical analysis showed a significant difference in starch grain accumulation among the three temperature treatments (Fig. 9). In the "early mature pollen grain stage", the starch content was drastically reduced in the pollen grains developed at 30°C compared to that of the pollen grains developed at 15 and 25°C (Fig. 8d–f); a higher content of soluble sugars (glucose, fructose and sucrose) derived from starch hydrolysis appeared at 25°C in comparison to at 15°C (Fig. 6d–e). The formation of lignified bands with a radial

orientation, due to lignin deposition in the epidermis and in some cells of the middle layer of the anthers, was evident in the three treatments during the “early mature pollen grain stage” (Supporting information Fig. 2a–c). During the same stage, the pollen grains from the samples grown at 30°C were irregular in shape compared to those developed at 15 and 25°C (Supporting information Fig. 2a–c).

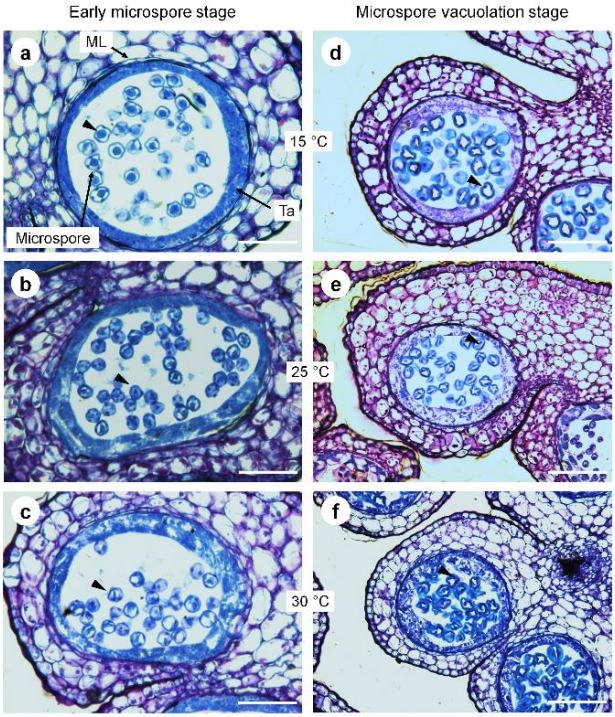


Figure 4. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e), and 30°C (c, f) during the "early microspore stage" (a–c) and "microspore vacuolation stage" (d–f). Different tissues are reported in a, endothecium (n), middle layer (ML) and tapetum (Ta) surrounding the loculus. (a–c), small young microspores after tetrads release show nuclei at the central position. The tapetal cells of the flower buds grown at 15°C (a) appeared compact and full of secretions compared to those of the flower buds grown at 25°C (b) and 30°C (c). Under cold (a) and hot (c) temperatures, the microspores (arrowheads) appeared less dense. (d–f) microspore during vacuolation: the young microspores become enlarged and push the nuclei to a peripheral position (arrowheads). A delay in Ta development was evident in the flower buds grown at 15°C

(d), in which the cells of the Ta appeared still full of secretions compared to (e) and (f). (a–f) PAS and toluidine blue O staining. Scale bars: (a–c) = 50 μm; (d– f) = 100 μm.

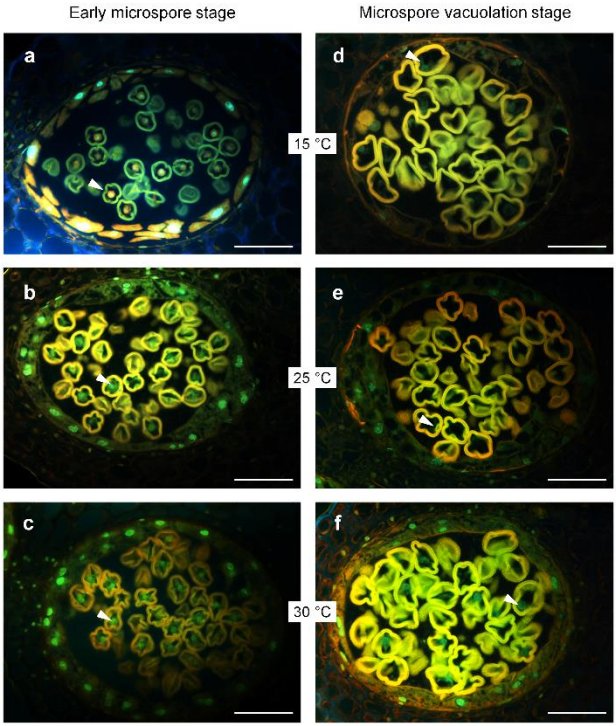


Figure 5. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e), and 30°C (c, f). (a–c), "early microspore stage," the young microspores showed central nuclei (arrowheads). (d–f), "microspore vacuolation stage," microspore nuclei are pushed by vacuolation to a peripheral position (arrowheads). (a–f) DAPI and AO staining. Scale bars: (a–f) = 50 μm.

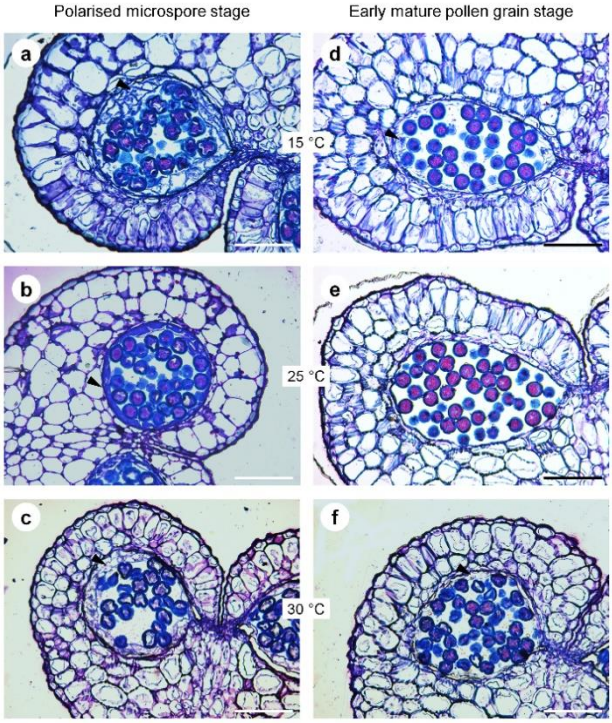


Figure 6. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e), and 30°C (c, f). (a–c), "polarised microspore stage". The tapeta of the flower buds (arrowhead) grown at 15°C (a) and 30°C (c) are composed of irregular and vacuolated cells while the tapetum of the flower buds grown at 25°C (b) is composed of a regular layer of cells full of secretion. In addition, the microspores of anthers grown at 15°C (a) and 30°C (c) have an irregular contour (especially at 30°C) compared to those grown at 25°C (b). (d–f), "early mature pollen grain stage". Soluble carbohydrates (pink color) are evident in the pollen grain at 25°C (e). Residues of the tapetal cells (arrowhead) were still evident at 15°C (d) and 30°C (f) compared to 25°C (e). (a–f) PAS and toluidine blue O staining. Scale bars: (a–f) = 100 μm.

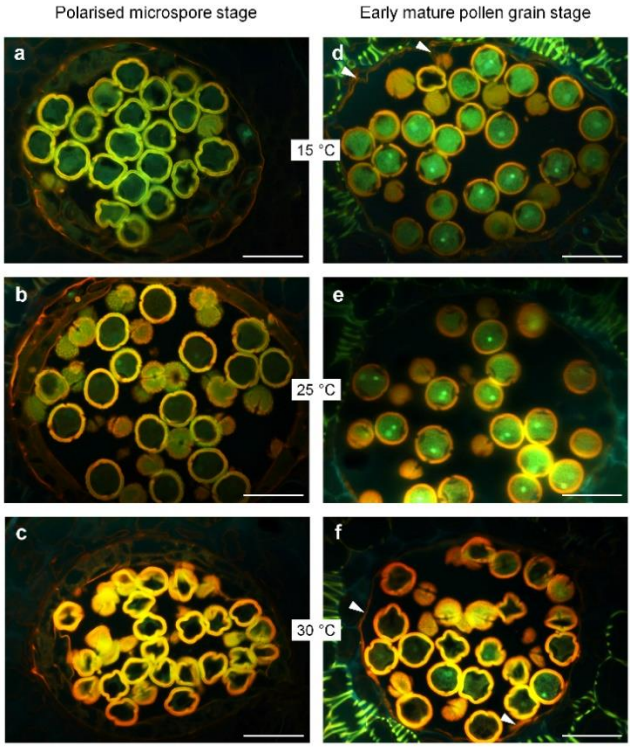


Figure 7. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e) and 30°C (c, f). (a–c), "polarised microspore stage", the nuclei are pushed to a peripheral position (arrowhead). The tapetal cells of the anthers grown at 15°C (a) and 30°C (c) undergo rapid degeneration. (d–f), "early mature pollen grain stage". Residues of the tapetal cells (arrowhead) were still evident at 15°C (d) and 30°C (f) compared to 25°C (e). (f) Flower buds grown at 30°C show pollen grains with irregular shapes. (a–f) DAPI and AO staining. Scale bars: (a–f) = 50 μm.

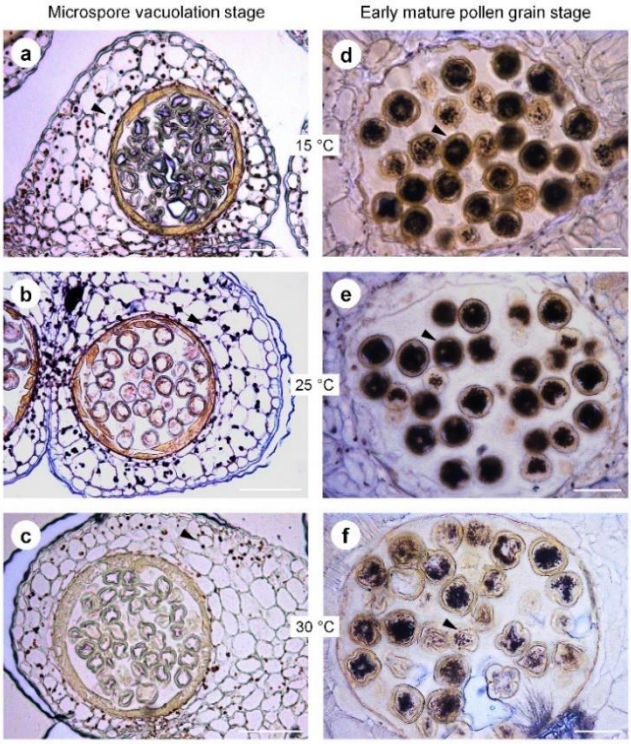


Figure 8. Anther cross-sections of "Comune" clementine grown at 15°C (a, d), 25°C (b, e) and 30°C (c, f) during the "microspore vacuolation stage" (a–c) and "early mature pollen grain stage" (d-f). (a–c), starch (arrowheads) accumulates in the epidermis, endothecium and middle layer. (a), at 15°C, an increased accumulation of starch grains was observed in the anther walls compared to in those of the flower buds grown at 25°C (b) and 30°C (c). (d–f), starch (arrowheads) accumulates in the pollen grains. (d), at 15°C, the starch amount within the pollen grains is similar to those of the flower buds grown at 25°C. At 30°C (f), the pollen grains showed fewer starch granules compared to those

observed in (d) and (e). (a–f) IKI staining. Scale bars: (a– c) = 100 μm; (d–f) = 50 μm.

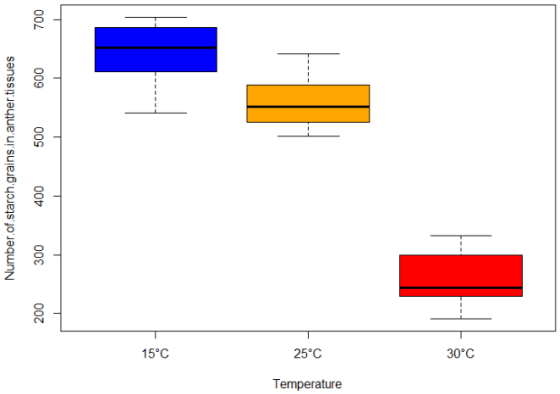


Figure 9. Effects of the temperature treatment (15, 25 and 30°C) on starch grain accumulation in the anther walls (epidermis, endothecium and middle layer) during the "microspore vacuolation stage." Analysis of variance was calculated for the three temperatures tested (degrees of freedom = 87, F-value = 674.6, p-value $<2^{-16}$). The starch grain accumulation was statistically different in all temperatures tested: least significant difference = 21.8 ($\alpha = 0.05$, mean square of residuals = 1,801) while pairwise means of treatments were equal to 298.7 (30–25°C), 84.3 (25–15°C) and 383 (30–15°C).

3.4 In vitro pollen germination and tube length

To evaluate pollen germination and tube elongation in response to temperatures, pollen that was collected from the plants growing at the three different temperatures (15, 25 and 30°C) was germinated *in vitro* at 25°C and analysed after 6, 12, 24 and 36 hr. The pollen germination rate was significantly affected by the treatments (Fig. 10a). In particular, the germination percentage was the highest at 25°C starting at the first time point, while at 15 and 30°C, it decreased remarkably. After 12 and 24 hr of incubation, the length of the pollen tubes from the flowers grown at 25°C was significantly longer than those from the flowers grown at 15 and particularly at 30°C (Fig. 10b). However, at 36 hr of incubation, the length of the pollen tubes reached a plateau, showing differences among treatments (Fig. 10b).

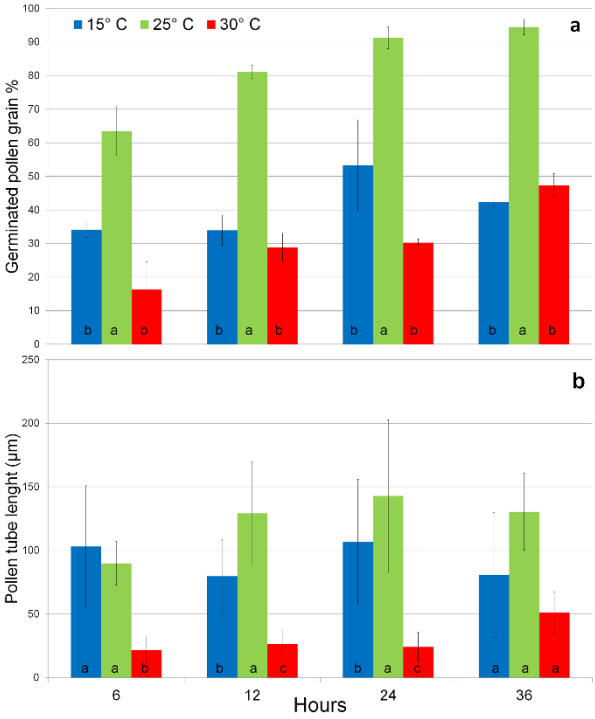


Figure 10. Effect of flower bud growth and incubation temperatures on *in vitro* pollen germination in clementine. (a) Percentage of germinated pollen grains collected from flower buds grown at 15, 25 or 30°C and incubated for 6, 12, 24, 36 hr at 25°C. (b) Pollen tube length measured in flower buds grown at 15, 25 or 30°C and incubated for 6, 12, 24, 36 hr at 25°C.

4. Discussion

4.1 Temperature stress effects on flower bud development

Temperature stress induces different alterations to plant reproductive system physiology and phenology, including changes in flowering initiation (De Storme and Geelen, 2014). In citrus, temperature stress has been demonstrated to be effective in affecting pollen germination, self-incompatibility reactions and early fruit development (Distefano et al., 2018). In the current study, focused on clementine, one of the most important citrus species, we monitored the effect of three temperatures in the pollen development process. We observed that low temperature (15°C) resulted in slower growth of the flower buds starting at the young microspore stage (Stage C in Fig. 1), which is in accordance with our previous results (Distefano et al., 2018). The flower exposure to higher temperatures resulted in earlier anthesis and flower maturation without any evidence of flower functionality alterations. Nevertheless, anther dehiscence occurred in the flowers grown at all the temperature regimes once the flowers were open.

4.2 Temperature stress effects on anther tissue and pollen development during microgametogenesis

Male gametophyte development is a particularly sensitive phase of the reproductive process during which temperature stress induces defects in the structure and function of the male gametophytes, resulting in an overall reduction of the fertility (Zinn et al., 2010). During the first stages of microsporogenesis, no visible alterations were detected under any of the imposed treatments. In a number of cases,

temperature stress has been identified as one of the main causes linked with meiotic abnormalities leading to ploidy alterations and pollen sterility (Cavalcante et al., 2000; Sage et al., 2015). A putative relationship between the occurrence of cytomixis (i.e., chromatin migration between meiocytes) and other meiotic abnormalities in pollen mother cells, pollen viability and reduced fertility have been observed in *Citrus* species (Fatta Del Bosco et al., 2009). Nevertheless, in this study, no evidence of altered meiotic behaviour due to temperature stress was revealed. The tapetum is the secretory anther tissue that surrounds the microspores and supports them with nutrients (Goldberg et al., 1993). During normal microsporogenesis, the tapetum undergoes a period of intense metabolic activity because it receives nutritive substances from the vascular bundles. The nutritive substances are then collected and released by exocytosis or through the membrane into the locular fluid, providing either resources to promote microspore development (Pacini et al., 1985; Scott et al., 2004) and enzymes for the release of microspores from tetrads (Goldberg et al., 1993). In many species, the cell walls in tapetal cells disappear to facilitate therelease of substances into the locular fluid (Pacini, 1997). Here, we observed that the cell walls of tapetal cells were not detected, this might imply that tapetal cell leakage started during the "pollen mother cell stage," indicating a strong relationship between pollen development and tapetum nutritive activity. Boyer and McLaughlin (2007) observed that after the release of the young microspores from tetrads, the sensitivity of the male organs to stress increases dramatically. In our study, after tetrads release, the early microspores under heat stress showed reduced quantity of cytoplasm compared to 25°C, in

addition the staining at 15°C indicated the presence of protoplast with cytosol (albeit showing strong plasmolysis) (Fig. 4a–c). Similar results were observed in a sterile mandarin mutant in which the abnormal development of the microspores led to pollen abortion (Hu et al., 2007). Temperature stress has been proven to induce defects in the nutritive function of tapetal cells (Parish et al., 2012; De Storme and Geelen, 2014), and this evidence is in accordance with the reduced density of the cytoplasm in the microspores under cold and heat stress observed in this work. In our study, the temperature stress effects became more evident from the "early microspore stage" until the "early mature pollen grain stage," during which aberrations in the timing of tapetal cell development and degeneration were observed. Under normal conditions, during the final stage of microgametogenesis, the tapetum undergoes PCD to promote pollen maturation and fertility (Sanders et al., 1999; Parish and Li, 2010). It has been previously reported that heat and cold stress hamper the nutritive function of tapetal cells resulting in their premature disappearance and excessive persistence, respectively (De Storme and Geelen, 2014; Müller and Rieu, 2016). However, changes in temperatures did not significantly affect the dynamic of the tapetal cell disruption during "polarised microspore stage." In *Actinidia*, male sterility was associated with the prolonged secretory activity of the anther wall tissues caused by a delay in PCD (Falasca et al., 2013). In our study, a drastic alteration in the tapetum, leading to a premature PCD, was observed under both hot and cold temperature conditions; this alteration was also coupled with the presence of tapetal cell residuals and altered microspore morphology at the "early mature pollen grain stage." Large

reductions in the number of starch grains and polysaccharide content in the anther wall layers and pollen grains, respectively, were detected for the anthers grown under 30°C compared with those grown under 25°C and mainly under 15°C. This result suggests that the effect of temperature stress on carbohydrate dynamics could be different for high and low temperatures. During their development, microspores have a strong requirement for carbohydrates to sustain pollen production. It was proposed that the carbohydrate-related defects under high temperature stress could be linked to increased respiration to sustain metabolic activity in developing pollen or to a decreased hexose supply by the tapetum (Pressman et al., 2006; Jain et al., 2007; Müller and Rieu, 2016). In contrast, low temperature exposure during the young microspore stage induced the downregulation of cell wall invertase expression in the tapetum, leading to a disruption in hexose production and causing starch accumulation in the pollen grains and pollen sterility (Oliver et al., 2005). Therefore, the altered tapetal cell development and the different carbohydrate dynamics could be part of a more complex system activated in response to stress conditions (Sage et al., 2015), which also involves hormones, reactive oxygen species and transcriptional factors (De Storme and Geelen, 2014). In agreement with results reported for other species, the reduction in the transient accumulation of starch and sugars in maturing pollen grains seems to be affected by high temperatures, causing a reduction in pollen viability similar to the effect that has already been observed in "Ougan" mandarin (Hu et al., 2007). During the last stage of microgametogenesis, the pollen grains were irregularly shaped under heat stress conditions.

From the “tetrad stage” to PCD, the tapetum provides the cell wall components, such as sporopollenin, for the construction of the pollen outer exine layer, while the inner intine layer is formed by cytoplasm (Shivanna and Johri, 1986; Pacini, 2010; Ariizumi and Toriyama, 2011). Defects in providing cell wall components could explain the irregular shapes of the pollen grains observed at the early mature pollen grain stage. It has been proposed that the deposition of lignin in the cell walls of the endothecium has a mechanical function to strengthen the residual anther wall during dehiscence (Sanders et al., 2005). In our study, we observed the presence of lignified bands not only in the endothecium but also in some cells of the middle layer. Similar results were observed in the middle layer of kiwifruit anthers in a study by Falasca et al. (2013), who suggested that this deposition of lignin could be involved in the transition of the function of the middle layer from transport and secretion to a mechanical function.

4.3 In vitro pollen germination and tube length

Anther tissue development and carbohydrate metabolism play a central role in ensuring pollen viability and germination (Pacini, 1996). Increases in temperature negatively affect pollen viability, pollen germinative ability and the pollen tube growth rate (Hedhly et al., 2009). The reduction in pollen performance is linked to abnormalities during microsporogenesis and, in particular, to the interruption of nutrient transportation required to ensure the correct development of pollen grains (Parish et al., 2012; De Storme and Geelen, 2014). In fact, initial pollen tube growth is autotrophic and metabolises the reserves stored in the

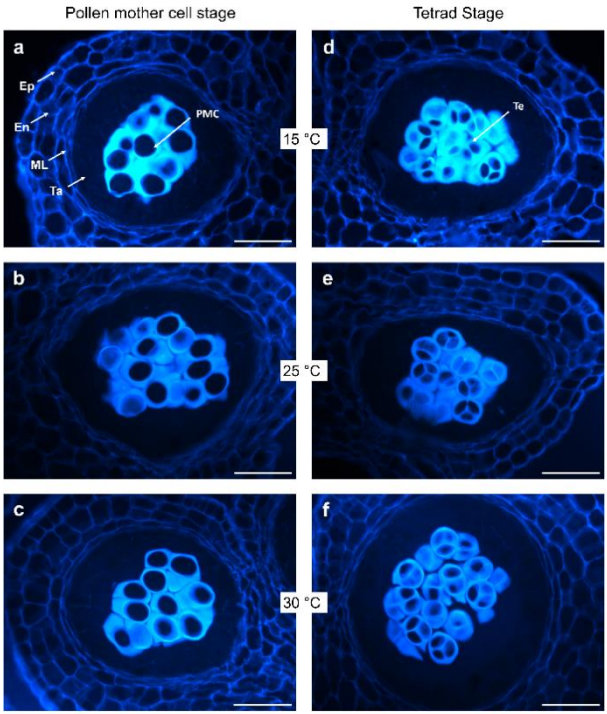
pollen grain, especially starch and sucrose (Shivanna, 2003; Obermeyer et al., 2013). The results of our study demonstrate that both high and low temperatures affect *in vitro* pollen germination. This result is likely due to the reduced availability of soluble carbohydrates in the case of pollen grains developed at 15°C, whereas a reduction in pollen viability was mainly observed at 30°C. These results agree with previous studies in which the pollen germination rate under different *in vitro* incubation temperatures increased from 15 to 25°C, which is the optimum temperature for pollen germination of citrus *in vitro*, and sharply decreased when the temperature reached 30°C (Distefano et al., 2012, 2018). During the first 24 hr after incubation, a clear increase in the pollen tube growth rate from 15 to 25°C and an evident decrease at 30°C were observed. After 36 hr, the difference in pollen tube elongation among the temperature regimes was no longer evident, perhaps due to the depletion of autotrophic reserves.

5. Conclusion

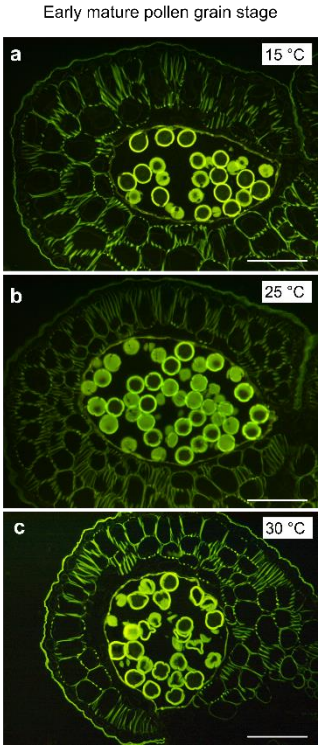
Temperature stresses have been reported to affect gamete development, performance and selection of many temperate, sub-tropical and tropical fruit tree species, and these effects are also reported to play roles in evolution, determining the ability of a genotype to adapt to different environmental conditions (Irenaeus and Mitra, 2014; Distefano et al., 2018). However, literature on the possible mechanisms leading to pollen impairment due to temperature stress in specific fruit crops is poor. In this study, we evaluated the effects of different temperature regimes on the whole male gametophyte development process of clementine and

demonstrated how temperature stresses during the flowering stage affect male gametophyte development. The main stress effects were evident especially during the microgametogenesis stage rather than during the microsporogenesis stage, resulting in a drastic reduction in pollen performance. Important alterations in tapetum layer morphology and function were observed, including reduced cytoplasm content in the microspores, abnormal timing of development and degeneration in the tapetal cells, a reduction in the starch content in the anther walls during microspore development and in mature pollen, and different contents of soluble sugar among pollen grains with abnormal shapes. However, the reasons for the tapetum alteration are unknown and could be due to several processes that are more or less evident, such as starch and soluble sugar metabolism, the timing of tapetal cell PCD, premature callose degradation, the inhibition of vacuolar invertase, advanced DNA fragmentation and a dysfunctional endoplasmic reticulum (Parish et al., 2012; De Storme and Geelen, 2014). Overall, the results of our study shed light on the principal modifications occurring in anther tissues during pollen development in response to both nonoptimal hot and cold thermal conditions. Histological analysis enabled the identification of physiological mechanisms that are likely involved in determining pollen performance under stress conditions in citrus. This information could be useful in understanding the processes related to pollen sterility in citrus.

Appendix



Supporting information Figure 1 Anther cross sections of 'Comune' clementine grown at 15 (a, d), 25 (b, e), and 30 °C (c, f). (a-f), a cell wall is present in the cells of the epidermis (Ep), endothecium (En) and middle layer (ML), while it is absent in tapetal cells. (a-c), during the "pollen mother cell stage", a thin layer of cellulose is present in the wall of pollen mother cells (PMC). (d-f), the tetrads (Te) of the young microspores are enclosed by a dense cellulose wall. (a-f), Calcofluor staining. Scale bars: (a-f) = 50 µm.



Supporting information Figure 2 Anther cross sections of 'Comune' clementine grown at 15 (a), 25 (b), and 30 °C (c) during the "early mature pollen grain stage". (a-c), lignin deposition in the endothecium and in some cells of the middle layer. (c), at 30 °C, the pollen grains have irregular shapes compared to those at 15 and 25 °C. (a-c) Auramine staining. Scale bars: (a-c) = 100 µm.

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CHAPTER III

Deciphering the rootstock influence on floral induction in sweet orange by analysis of citrus *FLOWERING LOCUS T* expression patterns

Abstract

In *Citrus*, flower induction represents the transition from vegetative to reproductive growth. The regulation of the flower induction in subtropical areas is mainly triggered by exposure to low temperature that activate the signaling cascade leading to an increased expression of the citrus *FLOWERING LOCUS T* homologs (*CiFT*s). In this study the relationship between rootstock and flower induction under Mediterranean field conditon was investigated by monitoring the expression levels of *CiFT*s, *CiFT1*, *CiFT2* and *CiFT3* genes, in leaves of Tarocco Scirè sweet orange plants grafted into two different rootstocks, 'C35' citrange and 'Swingle' citrumelo. All three *CiFT*s showed a seasonal expression with a peak during the inductive period in January triggered by cold temperature in winter. Compared to 'Swingle' citrumelo, 'C35' citrange showed the highest expression levels for *CiFT*s and a positive correlation with the number of flowers recorded in the following spring. These results suggested that rootstocks affect flower induction in sweet orange by the regulation of expression of *CiFT*s. By contrast, increase of expression levels for CiFTs was observed in summer when flowering was over, suggesting that other factors, environmental or endogenous, could be involved in regulation of their expression.

1. Introduction

Flowering transition promotes the transition from vegetative to reproductive growth and it is regulated by a complex interaction of environmental factors (photoperiod, vernalization, temperature etc.) and endogenous genetic

regulation systems (Amasino, 2010). Citrus plants do not show a true dormancy, this is certainly influenced by its nature as tropical-subtropical evergreen species (Spiegel-Roy and Goldschmidt, 1996). Several works indicated that floral induction occurs during fall and the chilling winter temperatures independently from the photoperiod (Spiegel-Roy and Goldschmidt, 1996; Nishikawa, 2013). Low temperatures (15-20 °C) are recognized as the main factor involved in the induction of flowering in citrus (Lenz, 1969; Moss, 1976; Inoue, 1989; Garcia-Luis et al., 1992; Valiente and Albrigo, 2004; Nishikawa et al., 2007; Wilkie et al., 2008; Nishikawa, 2013) while water stress condition seems to be the major flower-inducing signal in tropical areas (Cassin et al., 1969; Chica and Albrigo, 2013). Although the environmental stimuli described above seem to play a key role in determining the natural control of citrus flowering, there are many evidences suggesting that the flowering response to inductive conditions could also be influenced by developmental factors such as: fruit load, altered nutritional and carbohydrate metabolism together with increased levels of gibberellin (Khan et al., 2014). In citrus, the flowering inhibition is strictly related to juvenility and alternate bearing with important consequences for cultivation and also for breeding programs. The juvenility period is caused by a suppression of flowering in young plants, this phenomenon lasts several years (usually more than 5) after germination or grafting (Spiegel-Roy and Goldschmidt, 1996). The alternate bearing mainly results from suppression of flowering by heavy fruit production and late harvest in one year, determining a reduction of the flower number (thus the fruits on the tree) in the following year (Monselise and Halevy,

1964). While several studies are available on the effect of the above mentioned developmental factors on flower induction, few reports focused on the role of other factors such as the choice of the rootstock while, at the best of our knowledge, no studies focused on the rootstock effect on induction of flowering in citrus. In perennial fruit tree crops, such as apple, mango and avocado the effect of rootstocks in the flowering process was demonstrated (Foster et al., 2014; Sharma et al., 2019). Similar studies were conducted in sweet cherry and pear in which it was observed that rootstock affects scion precocity and abundance of flowering as well as the propensity of flowers to set fruit (Webster, 2010). Several studies around the world are addressing the selection and evaluation of rootstocks that are tolerant to *Citrus tristeza virus* (CTV) and can be efficiently adopted in different citrus production countries. Therefore, rootstock choice should consider, among other aspects, productive and qualitative features in relation to different environmental conditions. Continella and colleagues (2018) highlighted the effect of several rootstocks in determining different vegetative, productive and qualitative aspects of 'Tarocco Scirè' pigmented sweet orange.

In recent years, several studies have been carried out to elucidate the mechanisms of florigen signaling in plants studying the complex network of genetic pathway involved in the regulation of flowering (Khan et al., 2014). In *Arabidopsis* several flowering-related genes have been characterized (Komeda, 2004; Albani and Coupland, 2010; Pin and Ove, 2012) and appeared to be conserved in citrus (Dornelas et al., 2007). Among these genes, the citrus *FLOWERING LOCUS T* homologs (*CiFT*s), showed a close

correlation with floral induction (Velázquez et al., 2016). In Satsuma mandarin, three *CiFT*s, namely *CiFT*1, *CiFT*2 and *CiFT*3, involved in flowering induction were characterized using a cloning approach (Nishikawa et al., 2007) and homology analysis revealed that *CiFT*1 and *CiFT*2 are likely alleles of the same gene (Samach, 2013). The role of these genes in modulating flowering time was also investigated in other *Citrus* crops (mandarins, sweet orange, pummelo) through transcriptomic approaches (Muñoz-Fambuena et al., 2011; Shalom et al., 2012; Chica and Albrigo, 2013; Endo et al., 2017; Pajon et al., 2017; Agustí et al., 2019). In experiments on transgenic plants, ectopic expression of *CiFT*s resulted in early flowering in *A. thaliana* as well as in *Citrus* species such as trifoliate orange (*Poncirus trifoliata* L. Raf) and sweet orange (Kobayashi et al., 1999; Endo et al., 2005; Nishikawa et al., 2010; Pons et al., 2014). Studies on the endogenous expression of the *CiFT*s showed that their expression levels was regulated by the floral inductive signals while environmental factors such as cold temperature and water stress have been associated with a seasonal increase in the expression of the *CiFT*s (Nishikawa et al., 2007, 2009, 2011; Muñoz-Fambuena et al., 2011; Chica and Albrigo, 2013; Pajon et al., 2017). Recently, increased expression of *CiFT*s were observed in response to endogenous accumulation of abscisic acid (ABA) in the shoots of Satsuma mandarin trees during the floral induction period under low temperatures (Endo et al., 2017). The inductive signals are perceived in leaves triggering the *FT* gene expression, then the FT gene product, florigen, moves to the apices to influence meristem identity inducing the flowering response (Abe et al., 2005; Huang et al., 2005; Wigge et al., 2005;

Corbesier et al., 2007). Furthermore, several works reported the active role of fruit bearing in modulating changes in the *CiFT*s expression of mandarin and mandarin-like genotypes. Ectopic somministration of gibberellin (GA) in mandarin reduced *CiFT*s expression in buds and leaves (Muñoz-Fambuena et al., 2012; Goldberg-Moeller et al., 2013) while somministration of a Giberellic Acid biosynthesis inhibitor in sweet orange resulted in an upregulation of *CiFT*s and in an increased flower number per plant (Muñoz-Fambuena et al., 2012). On the whole, it is well known as hormonal signaling is strictly related to rootstock–scion communication (Aloni et al., 2010). Despite numerous studies have been conducted on environmental and endogenous factors affecting flowering, the role of rootstock in flower induction in *Citrus* was not sufficiently investigated.

In the present study, the expression patterns of the citrus flowering-related homologs, *CiFT1*, *CiFT2* and *CiFT3*, were evaluated in leaves of sweet orange grafted on 'C35' citrange and 'Swingle' citrumelo to investigate (i) the differential expression of the CiFTs in the two rootstocks, (ii) the flower bud induction period in relation to the temperature regimes, (iii) the flowering intensity in spring.

2. Materials and Methods

2.1 Plant material

The study was performed on 7-year-old trees of the cv 'Tarocco Scirè' [*Citrus sinensis* (L.) Osb.] grafted onto two rootstocks: 'C35' citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] and 'Swingle' citrumelo [*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.]. Orchard is

located in the experimental field of Scordia (Catania, Italy) and subjected to standard cultural practices.

2.2 Sampling

Four 'Tarocco Scirè' trees for each rootstock were selected for their uniformity in size and vigour. Seven fully developed mature leaves per tree were collected monthly from September 2017 to July 2018. The leaves were pooled in one group, quickly frozen in liquid nitrogen and stored at −80 °C until RNA extractions. The flowering intensity was evaluated in spring (April 2018) as follow: three branches per tree, similar in size, with some 300 nodes, were chosen from around the tree at about 2 m above the soil level. The number of flowers per sprout was counted. Temperature data registerd during the sampling period were provided by Servizio Informativo Agrometeorologico Siciliano (SIAS) (http://www.sias.regione.sicilia.it/).

2.3 Total RNA extraction and cDNA syntesis

Total RNA was extracted from frozen leaves using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) and treated with with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA) according to the manufacturer's instructions. For each rootstock three RNA extractions were performed (three biological replicates). RNA concentration and purity were assessed using the a spectrophotometer (NanoDrop-2000, Thermo Scientific, USA) while RNA integrity was verified by agarose gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, USA) according to the procedure indicated by the manufacturer.

2.4 Real Time-PCR

The Real Time-PCR (RT-PCR) assays were performed on the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) in 20 µL total reaction volume containing 1x PCR buffer II, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of forward and reverse primer (Eurofins Genomics), 1.5 µM SYTO9 (Life Technologies, UK), 50 ng of cDNA and 1U of MyTaq DNA polymerase (Bioline, UK). Thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95 °C for 5 s, 58 °C for 20 s and 72 °C for 2 min. The results were based on the average of two replicate reactions for each gene. The quantification was normalized against citrus Elongation Factor 1-alpha (EF-1α, accession AY498567) used as housekeeping genes. The threshold cycle (C_T) values were used to calculate the expression level of each target gene relative to elongation factor 1-alpha transcript using the Rotor-Gene Q software (v2.1.0). Sequences of primers used to analyze the three *CiFT* and the internal control EF-1α are shown in Table 1.

Target	Forward primer (5'–3')	Reverse primer (5'–3')	Reference
EF-1α	ATTGACAAGCGTGTGATTGGC	TCCACAAGGCAATATCAATGGTA	(Distefano et al., 2008)
CiFT1	CTACCAACAAAATTTCATCACTTGAATAG	GATCTCTCTCCCTGCTAGACATATCA	(Nishikawa et al., 2007)
CiFT2	CTACCAACAAAATTTCATCACTTGAATAG	GGGTCTCTCTCCCTGCTAGACA	(Nishikawa et al., 2007)
CiFT3	CAACAAAATTTCATCACTTGAATAGTC	AAACACTCAACAACACTTAGCACAAA	(Nishikawa et al., 2007)

Table 1. Oligonucleotide sequences used for real-time PCR assays.

2.5 Statistical analysis

The statistical analysis was performed with R (R Core Team, 2017). The Welch Two Sample *t*-test is used to determine statistical differences between samples. *P*-values < 0.05 were considered significant.

3. Results and Discussion

Seasonal changes were observed in the mRNA level of *CiFT*s throughout the experimental period (Fig. 1). The expression levels for all *CiFT* transcripts began to increase in autumn (*CiFT2* and *CiFT3* in particular showed a significant difference in expression in October) in coincidence with the decrease in field temperature. All three *CiFT*s showed a peak in January, after which the mRNA levels decreased rapidly. Then, mRNA level of *CiFTs* increased slightly in spring with a peak in July (Fig. 1). The yearly changes in *CiFTs* expression were correlated with the seasonal periodicity: the increased *CiFTs* expression in January for example was in agreement with previous reports indicating that the flower bud induction occurs between autumn and winter during floral induction (Sherman and Beckman, 2003; Nishikawa, 2013). Previous reports highlighted the tight connection between temperature and flower-gene expression. An overexpression of *CiFT* genes during the transition from winter to spring was already observed in Satsuma (*Citrus unshiu* (Macf.) Marc.) suggesting a strong association between *CiFT* expression level and floral induction triggered by low temperature of winter (Nishikawa et al., 2007, 2009) or by the exposure to higher temperatures (15°C) for longer periods (1.5 months) (Inoue, 1989; Nishikawa et al., 2007).

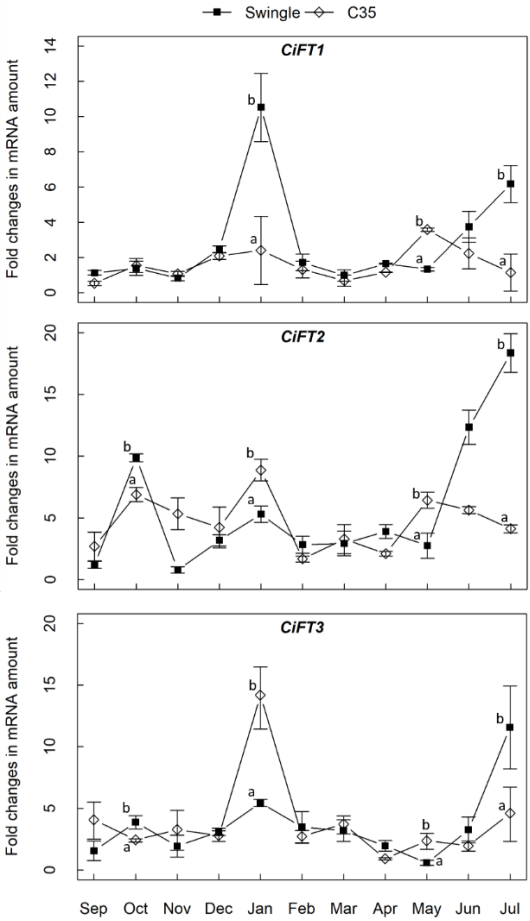

Figure 1. Mean expression and standard deviation of *CiFT1*, *CiFT2* and *CiFT3* in leaves of 'Tarocco Scirè' on 'C35' citrange and 'Swingle' citrumelo rootstocks throughout 11 months. Different letters for the same sampling date indicate significant difference between rootstocks (*P*-value < 0.05).

Temperature data registered during the experimental period (Fig. 2) showed a marked decrease in autumn, temperatures reached their lowest value (5 °C) in December 2017, just before the increase in *CiFT*s expression levels and confirming a direct effect of low temperature on the flower induction and expression levels of *CiFT*s. On the other hand, the increase in expression levels of *CiFT*s observed when flowering is over and the temperature increased (May and July), could be linked to other environmental factors than temperature. Similar results were observed by Pajon and colleagues (2017) in plant of pummelo (*Citrus grandis* Osbeck) and 'Pineapple' sweet orange suggesting that the increase in leaves expression of *CiFT* genes soon after flowering is probably associated to changes in light conditions or other internal signals regulation. According to previous studies, all three *CiFT* genes were demonstrated to be involved in flower induction (Nishikawa et al., 2007, 2009, 2012; Shalom et al., 2012; Endo et al., 2017; Pajon et al., 2017). In the deduced amino acid sequences, *CiFT1* and *CiFT2* show more than 95% homology, sequence identity decreased to 80% when *CiFT3* is compared either to *CiFT1* or *CiFT2* (Nishikawa, 2013). According to the Clementine mandarin (*Citrus reticulata* Blanco) reference genome (https://phytozome.jgi.doe.gov), *CiFT1* and *CiFT2* appear to be two alleles of the same gene while *CiFT*3 is encoded by a different genetic region (Samach, 2013). However, by recent studies it seems that expression of *CiFT2* is highly correlated to floral induction compared to the other two *CiFT* genes (Muñoz-Fambuena et al., 2011; Agustí et al., 2019).

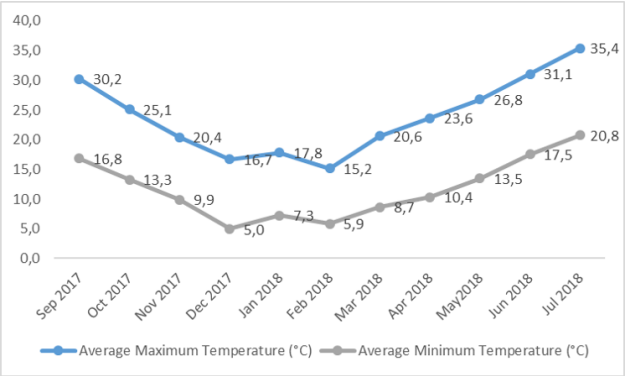

Figure 2. Average maximum and minimum temperature (°C) registered during the sampling period (source SIAS).

Significant differences were found in mRNA transcripts between 'C35' citrange and 'Swingle' citrumelo revealing an effect of rootstocks on the regulation of expression of *CiFT*s in leaves of sweet orange. 'C35' citrange showed the highest levels of *CiFT2* and *CiFT3* transcripts during the inductive period in January, while 'Swingle' citrumelo showed the highest *CiFT1* expression level (Fig. 1). A significant difference in gene expression between the two rootstocks is also observed in July, all three genes were significantly more expressed in 'Tarocco Scirè' leaves on 'Swingle' then on 'C35'. The same pattern was also observed in October in which both *CiFT*2 and *CiFT*3 resulted significantly more expressed in 'Swingle' compared to 'C35'. It is reported that *CiFT* mRNA levels during floral induction in citrus trees appeared to be closely related with the number of flowers in the following spring (Nishikawa et al., 2012, 2017). Here, flowering intensity was estimated in spring (April 2018) by count number of flowers per 300 nodes and revealed

differences among rootstocks (Fig. 3). ‘Tarocco Scirè’ trees on ‘C35’ citrange showed the highest number of flowers compared to ‘Swingle’ citrumelo although the difference was slight above the significant threshold (*p*-value = 0.056).

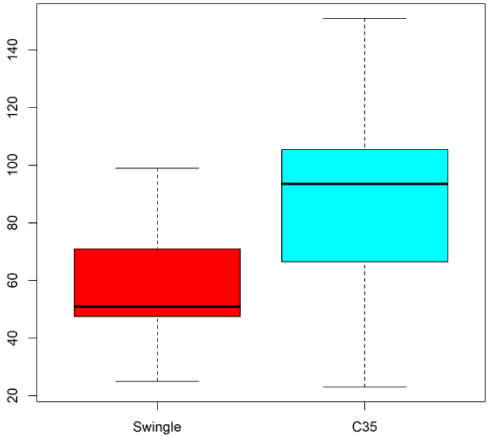

Figure 3. Number of flowers per 300 nodes recorded on ‘Tarocco Scirè’ on ‘C35’ citrange and ‘Swingle’ citrumelo rootstoks in spring 2018. Each value is the mean of four trees per rootstock.

These results support the hypothesis that rootstocks affected floral induction in sweet orange even though further studies are needed to confirm our preliminary results. The increased expression for all *CiFT*s during spring and summer is not registered in Satsuma in which floral induction and overexpression of *CiFTs* occurred only during early winter (Nishikawa et al., 2009). The increase in *CiFT*s expression during warmer months could be due to the genetic contribution in both rootstocks of trifoliate orange origin, the

latter is characterized by the occurrence of floral induction and flower bud development during early summer. In citrus flower induction appear to be strongly affected by exposure to low temperature and regulation of expression of *CiFTs*. In this study, the effect of rootstock on flower induction was investigated by the analysis of the *CiFT*s expression in leaves of cv. 'Tarocco Scirè' sweet orange grafted onto 'C35' citrange or 'Swingle' citrumelo during 11 month in relation to temperature and flower intensity.

4. Conclusion

The aim of the work was the evaluation of the expression pattern of the flowering-related genes, *CiFT1*, *CiFT2* and *CiFT3*, in plant of sweet orange grafted onto two citrus rootstocks: 'C35' citrange and 'Swingle' citrumelo. Results showed that rootstocks do affect floral induction in sweet orange by overexpression of *CiFT*s in January during the induction period. In this month, 'C35' citrange in particular showed the highest expression levels for *CiFT2* and *CiFT3* resulting in an increased flowering intensity in the following spring. The overexpression of these two genes is of particular interest since *CiFT3* is expressed in the leaves while *CiFT2* is the most widely used gene in present studies. The overall results get insights in the regulation of flower induction in citrus that could guide the choice of rootstock combining fruit crop yield and quality. This aspect is of particular importance due to the widespread presence of CTV in the Mediterranean basin that imposes the adoption of alternative rootstocks from sour orange (*Citrus aurantium* L.).

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CHAPTER IV

Towards the characterization of a candidate gene potentially involved in sterility by induction of early flowering into Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.).

Abstract

Citrus species are among the most important fruit tree crops grown in the world. Their long juvenile period, joined to their complex genetic and reproductive characteristics, severely hampers the use of traditional strategies to improve citrus traits. Genetic engineering is the fastest way to develop better cultivars, even more if using flowering-time genes, which allow early flowering and fruiting, reducing the time required for genetic improvement programs and genetic studies. In this study, early flowering phenotype was induced in Troyer citrange by overexpression of the citrus ortholog of the floral integrator *FLOWERING LOCUS T* gene (*CiFT*). Concurrently, a gene coding an RNA binding protein (RBP), potentially related to sterility, was silenced by RNA interference (RNAi). Transgenic plants showed an early flowering phenotype starting to flower five months after the transformation experiments. Silencing of RBP resulted in normal flowers without anomalies on anthers indicating that may not affect male fertility. Despite further observations are necessary for functional characterization of RBP by analysis of pollen, ovary and fruit development, this study showed that the reduction of juvenile period in citrange by overexpression of *CiFT* is a useful strategy to accelerate functional genomic studies in citrus.

1. Introduction

Citrus is one of the most economically important fruit crops worldwide, with more than of 146 million tons produced in 2017 (FAO, 2017). The improvement of citrus traits by traditional breeding is a very time-consuming process since

because their long juvenile period most citrus types require 5-15 years to begin flowering and fruiting (Peña et al., 2008). Besides, complex genetics and reproductive biology of citrus, including high heterozygosity, cross and self-incompatibility and facultative apomixes, hampers even more traditional breeding. Genetic engineering and methods of transformation and regeneration have been developed to accelerate the selection in citrus breeding programs and genetic studies (Cuenca et al., 2018). Flowering-time genes from *Arabidopsis*, such as *LEAFY* or *APETALA1*, involved in regulating the transition from the vegetative to reproductive phase, have been used to induce early flowering and fruiting in juvenile citrus plants (Peña et al., 2001). Similarly, the overexpression of the *FT* homolog (*CiFT*) from satsuma mandarin (*Citrus unshiu* Marc.) (Hisada et al., 1997) was shown to induce an early flowering phenotype in *Arabidopsis* (Kobayashi et al., 1999) and in trifoliate orange (Endo et al., 2005). These reductions of the juvenile period by ectopic expression of flowering-time genes could be a helpful tool for functional genomics studies in citrus (Peña et al., 2008). In fact, *CiFT* overexpression was induced in transgenic plants of Pineapple sweet orange to obtain fruits with increased content in β-carotene in an extremely short period of time (Pons et al., 2014). More recently, it has been suggested that, from all the genes identified up to date, the main component of the flowering signal is the floral pathway integrator *FLOWERING LOCUS T* (*FT*) (Velázquez et al., 2016). These approaches can be used to tackle genetic and functional studies on citrus. Nowadays, the function of most citrus genes is envisaged from their transcription profiles and/or by homology of the encoded proteins with that of other species,

in which the activity has been already demonstrated. However, most times these comparisons are related to model species such as *Arabidopsis* (with no edible fruits) or tomato (a very different fruit). In some cases, ortholog genes do not show the same function. For example, *inner no outer* (*ino*) mutants, lacking the outer integument of the ovary, have been reported in both *Arabidopsis* (Villanueva et al., 1999) and a natural *Annona squamosa* seedless mutant (Lora et al., 2011). Although pollen tubes and embryo sacs are not observed in the ovules of *Arabidopsis ino*, both are partially present in *Annona ino* suggesting that the INO ortholog in *Annona* is not as tightly associated with overall ovule development and function, as it is in *Arabidopsis* (Lora et al., 2019). Genetic engineering allows to induce or repress the expression of individual or group of genes to irrevocably demonstrate their function and their influence in metabolic pathways. This work demonstrates the usefulness of inducing early flowering in citrus juvenile material to test a biotechnological approach before attempting it in adult material, much more laborious.

Tango is a mandarin selection developed from an irradiated bud of the diploid mandarin cultivar W. Murcott, also known as Nadorcott, produced under the UCR Breeding Program (http://www.citrusvariety.ucr). Tango mandarin distinguishes itself by producing a very low seeded fruit (<1 seed/fruit) under cross-pollination conditions, contrary to Nadorcott which sets up to 10-20 seeds/fruit in cross-pollinated conditions (Roose and Williams, 2007). The gamma irradiation mutagenesis often results in diminished fertility, because of the high frequency of pollen or ovule abortion (Vardi et al., 2008). Here by comparison of sequencing data from Tango and Nadorcott Single-

Nucleotide Polymorphisms (SNPs) were identified. Some of these SNPs were found affecting the homolog gene of *Ciclev10027731m* from clementina mandarin, which codes for an RNA-binding protein (RBP). RBPs play a key role in the regulation of gene expression by control of the post-transcriptional fate of mRNA (Iadevaia and Gerber, 2015), that seems to be involved in seed development in *Arabidopsis thaliana* (Kourmpetli et al., 2013) and cytoplasmic male sterility (CMS) in *Brassica rapa* and in cybrid pummelo (Zheng et al., 2014; Jeong et al., 2017). Male sterility (MS) in plants results in the inability to produce functional pollen. This in combination with female sterility and parthenocarpy allows to obtain seedless citrus fruits, a desirable trait for both fresh and processed fruit in the citrus industry (Yamamoto et al., 1995; Vardi et al., 2008). Considering the wide metabolic pathway in which RBPs could be involved and that mutations affecting RBP in Tango could be the main underlying genetic cause of its seedlessness, it would be useful to characterize their function in citrus using a biotechnological approach that reduce the juvenile period and concurrently repress expression of the RBPs. RNA interference (RNAi) is a strategy widely used for functional genomics studies by using double-stranded RNA (dsRNA) homologous to the target locus to trigger RNA silencing. This approach has been exploited in plants by genetic transformation with sense and antisense DNA target sequence separated by an intron (intron-hairpin constructs) (Smith et al., 2000). In this study early flowering phenotype was induced in transgenic plants of citrange by overexpression of the citrus *FLOWERING LOCUS T* (*CiFT*) in order to assess the usefulness of this strategy for functional characterization of a gene coding an

RBP potentially related to sterility through silencing by RNAi.

2. Materials and methods

2.1 Identification of the candidate gene for RNAi

Illumina sequencing data from Tango (confidential data) have been aligned against *Citrus clementina* v1.0 genome (https://phytozome.jgi.doe.gov). Genome comparison showed that homolog to *Ciclev10027731m* from Tango respect clementine mandarin presented three SNPs in nucleotide positions 1454 (T>C), 2247 (C>G) and 2662 (A>G) of the 8391-bp genomic sequence of the gene, of which the second one affect an exon (Fig. 1a). The gene sequence was translated into amino acid sequence, using the programme EMBOSS Transeq (www.ebi.ac.uk/emboss/transeq/, http://www.ebi.ac.uk/emboss/transeq/) and compared with the peptide sequence of *Ciclev10027731m* from clementine using BLASTp. Alignment showed that the SNPs in position 2247 (C>G) resulted in a change Cys > Ser in position 222 of the peptide sequence (Fig. 1b). *Ciclev10027731m* from clementine enables RNA binding function by RRM and Zinc-finger domains (https://www.uniprot.org). A BLASTp search was conducted against *Arabidopsis thaliana* genome in TAIR database (https://www.arabidopsis.org/) using *Ciclev10027731m* protein sequences as query sequences finding homology with AT5G60170.1 (identity 50.05%) that codes an RNA binding (RRM/RBD/RNP motifs) family protein. In order to verify if mutations affecting *Ciclev10027731m* are responsible for seedless phenotype in Tango RNAi strategy was used.

Figure 1. (a) Schematic representation of genomic sequence of *Ciclev10027731m* from *Citrus clementina*. Red bars indicate position of SNPs identificated in homolog from Tango; (b) aligning of the derived amino acid sequences from homolog of *Ciclev10027731m* from Tango (Sbjct) against peptide sequence of the same gene from clementine (Query) showing a change Cys > Ser in position 222 of the peptide sequence.

2.2 Vector construction

The vector pROKII-CsFT-A2i was generated using Goldenbraid cloning system v3.0 (https://gbcloning.upv.es/) and routinary enzyme digestion and ligation procedures. The details of the resulting construction are provided in the Supporting information. A schematic representation of the T-DNA region of the pROKII-CsFT-A2i vector is shown in Figure 2a. The vector includes the selectable marker gene neomycin phosphotransferase II (*nptII*) cassette under the control of *nos* (nopaline synthase) promoter and terminator sequences; an intron-hairpin (ihp) RNAi to silence the expression of the *Ciclev10027731m* gene (named A2) from clementine mandarin (*Citrus* × *clementina* cv. Clemenules) (GenBank: KI536978.1) and an CsFT overexpression cassette, both controlled by CaMV 35S promoter and *nos* terminator. After confirmation by plasmid restriction analysis and by sequencing, the pROKII-CsFT-A2i was transferred to *A. tumefaciens* strain EHA105 by thermal shock. The binary vector pROKII-CsFT, previously constructed (Pons et al., 2014) and containing *nptII* and CsFT overexpression cassettes (Fig. 2b), was used to transform control plants (CN) (Details provided in the Supporting information).

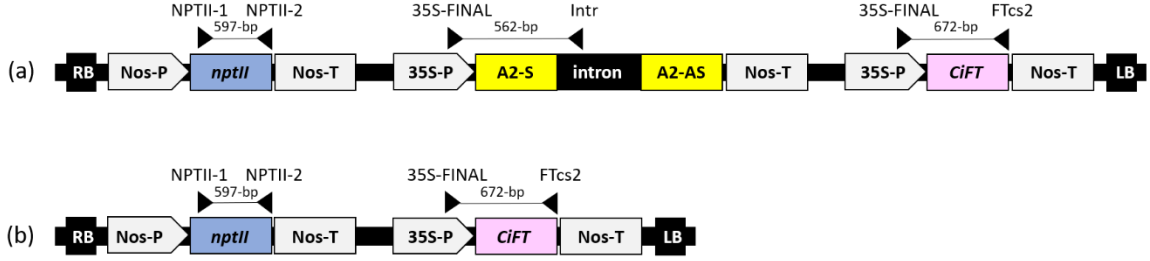


Figure 2. Schematic representation of the T-DNA region from plasmids used for plant transformation. (a) pROKII-CsFT-A2i plasmid containing both an intron-hairpin RNAi and an CsFT overexpression cassettes; (b) pROKII-CsFT containing FT overexpression cassette used to transform control plants. RB and LB, right and left T-DNA borders, respectively; Nos-P and Nos-T, nopaline synthase promoter and terminator sequences, respectively; *nptII*, the selectable marker gene neomycin phosphotransferase II conferring resistance to the antibiotic kanamycin; 35S-P, CaMV 35S promoter; *CiFT*, *FLOWERING LOCUS T* from sweet orange; A2-S and A2-AS, sense- and antisense-oriented sequences, respectively, designed to silence the expression of the *Ciclev10027731m* gene (A2), intron sequence from *S. lycopersicum*. Black arrows at the bottom of T-DNA indicate PCR primers used to amplify HRP-A2, *nptII* and *CiFT* genes and the respective size of PCR amplicons.

2.3 Plant transformation and regeneration

Five-week-old Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) *in vitro*-grown seedlings (Peña et al., 2008) were used as a source of epicotyl explants for transformation as described in Peña et al., (2004). Briefly, epicotyl explants (1 cm in length) were incubated for 5 min in bacterial suspension. After removing bacterial debris explants were maintained for 3 days in the semi dark at 25 °C on solid co-cultivation medium [4.4 g/L MS salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 100 mg/L *myo*-inositol, 1mg/L nicotinic acid, 1 mg/L pyridoxine hydrochloride, 0.2 mg/L thiamine hydrochloride, 2 mg/L indole-3-acetic acid (IAA), 1 mg/L 2-isopentenyl-adenine (2-ip), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 8 g/L agar, pH 5.7]. Then, the explants were transferred to solid selection medium [4.4 g/L MS salts, 3% (w/v) sucrose, 100 mg/L *myo*-inositol, 1mg/L nicotinic acid, 1 mg/L pyridoxine hydrochloride, 0.2 mg/L thiamine hydrochloride, 3 mg/L BAP, 10 g/L agar, pH 5.7] supplemented with 100 mg/L kanamycin for *nptII* selection and 250 mg/L of both cefotaxime and vancomycin to control bacterial growth. The plates were maintained in the dark at 25 °C for two weeks and then transferred to 16 h photoperiod at 25 °C. After four-five weeks regenerated shoots were shoot-tip grafted on Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) seedlings grown *in vitro* as described in Peña et al., (2008). After about four weeks, shoots leaves were screened by PCR to verify the integration of *nptII, CiFT* and HRPA2i for plants transformed with pROKII-CsFT-A2i vector (HRP-A2i) and *nptII* and *CiFT* for CN. After about four weeks the PCR-positive plantlets, HRP-A2i and CN, from the shoot-tip grafting, were grafted on

vigorous citrus rootstocks in greenhouse in April 2019. Transformation efficiency was evaluated as the number of transformed shoots regenerated from inoculated explants. For each transformation experiment, HRP-A2i and CN, 200 starting explants were inoculated.

2.4 PCR analysis

Genomic DNA was extracted from leaves using CTAB method as described by Mcgarvey & Kaper (1991). The PCR was performed using FIREPol® DNA Polymerase following the manufacturer's instructions. Reactions for *CiFT* and *nptII* amplification were carried out under following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 45 sec, followed by 72 °C for 10 min. Reactions for HRP amplification were carried out using conditions of 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 m, followed by 72 °C for 10 min. PCR products were detected by electrophoresis on 1% agarose gels. PCR reactions were performed using pairs of specific primers (Fig. 2) for the different expression cassettes: for *FT* amplifying the region encompassing the end of the 35S promoter and the entire *CiFT* transgene to avoid non-specific amplification of the endogenous *FT* gene; for *nptII* amplifying the neomycin phosphotransferase II selectable marker gene and the region encompassing the end of the 35S promoter; and for the HRP amplifying just the A2 sense and intron sequences to avoid intron-hairpin hairpin folding. Primer sequences used were: 35S-FINAL, 5'-ATCTCCACTGACGTAAGGGATGACG-3'; FTcs2, 5'-GGGATTGATCATCGTCTGA-3'; NPTII-1, 5'- GACGAGGCAGCGCGGCTAT-3'; NPTII-2, 5' AAGAAGGCGATAGAAGGCGA-3' (previously designed)

and Intr, 5’ GCGCCGTCTCGCTCAGGCTCTGTTCTCCAATCAAAT GGTTC-3’ (designed in this study).

3 Results and discussion

Genome comparison of Illumina sequencing data from the seedlessness mutant Tango against *Citrus clementina* v1.0 genome (https://phytozome.jgi.doe.gov) revelated the presence of three SNPs in the Tango *Ciclev10027731m* homolog (in nucleotide positions 1454 (T>C), 2247 (C>G) and 2662 (A>G) of the 8391-bp genomic sequence of the gene) resulting in a change Cys > Ser in position 222 of the peptide sequence (Fig. 1a, b). *Ciclev10027731m* from clementine encode an RBP containing both an RRM and Zinc-finger domains (https://www.uniprot.org) one of the most widely spread RBPs family that seems to be involved in seed development in *Arabidopsis thaliana* (Kourmpetli et al., 2013) and cytoplasmic male sterility (CMS) in *Brassica rapa* and in cybrid pummelo (Zheng et al., 2014; Jeong et al., 2017). In order to verify if mutations affecting *Ciclev10027731m* are responsible for seedless phenotype in Tango RNAi strategy was used.

In order to silence a gene coding an RBP, potentially related to sterility and concurrently reduce the juvenile period in citrange the vector pROKII-CsFT-A2i, containing both an intron-hairpin RNAi and an *CiFT* overexpression cassettes, was generated and used to transform Troyer citrange seedlings via *Agrobacterium tumefaciens* mediated T-DNA transfer. The binary vector pROKII-CsFT, previously generated (Pons et al., 2014) was used to transform control plants (CN). Regenerants buds resistant to kanamycin

selection were screened by PCR to verify the integration of *nptII*, *CiFT* and HRPA2i for HRP-A2i plants and *nptII* and *CiFT* for CN plants (Fig. 3). For CN experiment a frequency of transformed shoots of 33% and a transformation efficiency of 5% were obtained. This is low in comparison to that reported before for juvenile orange and Carrizo transformation (7.9 and 11 %, (Peña et al., 1995; Cervera et al., 1998)), but still enough to recover transgenic plants. For HRP-A2i experiment, 11 transgenic shoots were recovered from 24 regenerants analyzed, giving a frequency of transformed shoots of 46% and a total transformation efficiency of 5,5% (Table 1).

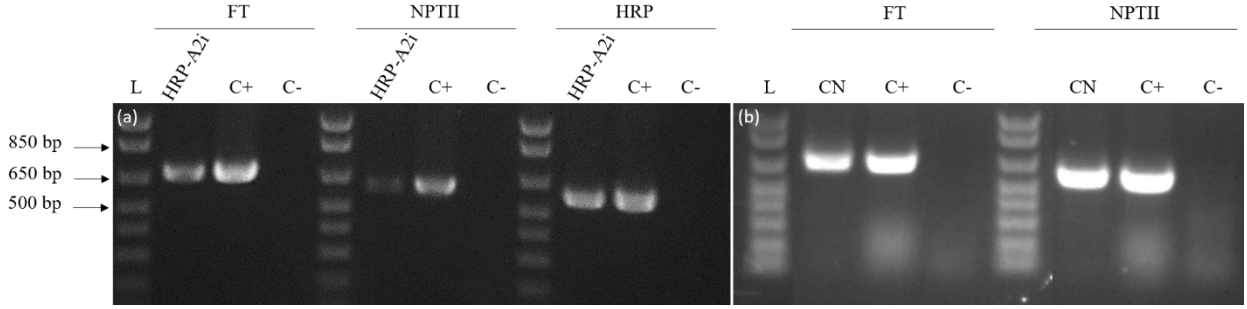


Figure 3. PCR screening of transgenic plants. Visualization on agarose gel of amplified PCR products of *CiFT* (672-bp), *nptII* (597-bp) and HRP (562-bp) from leaves of a representative transgenic plant HRP-A2i (a) and control plant (CN) (b). pROKII-CsFT-A2i and pROKII-CsFT plasmids extracted from *A. tumefaciens* strain EHA105 used for plant transformation of HRP-A2i and CN, respectively, were used as positive control (C+); non-transformed wild type plant (WT), was used as negative control (C-). 1 Kb Plus DNA Ladder (Invitrogen) (L).

Table 1 Transformation efficiency for HRPA2i and CN experiments.

Experiment	PCR positive selection	Shoots analysed by PCR	Positive shoots	Frequency of transformed shoots (%)[a]	Transformation efficiency (%)[b]
HRPA2i	*nptII, CiFT* or HRPA2i	24	11	46	5,5
	nptII+ *CiFT* + HRPA2i	24	5	21	2,5
CN	*nptII*+ *CiFT*	33	10	33	5

[a] positive shoots of total shoots analyzed

[b] positive shoots of total explants inoculated (200)

Just 5 out of the 11 transgenic shoots resulted PCR-positive for all three cassettes (*nptII*, *CiFT* and HRPA2i), reducing frequency of transformed shoots and transformation efficiency at 21% and 2,5%, respectively. The reduction found in transformation efficiencies for HRP-A2i experiment compared to CN is probably linked to the higher size of the T-DNA (7803-bp) plus the intron-hairpin construct folding that hamper its correct integration in the genome. The PCR positive plantlets, both HRP-A2i and CN, were grafted on vigorous citrus rootstocks in greenhouse in April 2019 and subjected to phenotypic observation. *CiFT* overexpression caused a clearly visible early flowering phenotype. Transgenic plants started to flower five months after the transformation experiment during *in vitro* regeneration and, when grafted on rootstocks in the greenhouse exhibited a terminal flower bud (Fig. 4a). Two months after the grafting in the greenhouse transgenic plants reached full flowering (Fig. 4b) and about one month later, in July 2019, they started fruiting showing two small fruits (Fig. 4c). The wild-type (WT) plants remained in the vegetative growth compared to the transgenic overexpressing-*FT* plants, both HRP-A2i and CN (Fig. 4d). Although Southern analysis are planned in a future, when enough leaf material is available for genomic DNA extraction, early flowering and fruiting already confirmed the effective integration and expression of the *CiFT* cassette in the HRP-A2i and CN transformants. The CN plants developed normally compared to WT, while the transgenic plants HRP-A2i showed smaller size than WT and CN plants, suggesting that RBP silencing could affect plant growth (Fig. 4d).

Figure 4. Induction of early flowering in transgenic Troyer citrange plants containing a citrus *FLOWERING LOCUS T* (*CsFT*) overexpression cassette. (a) a representative transgenic plantlet (HRP-A2i) carrying an intron-hairpin RNAi and an *CsFT* overexpression cassettes grafted on a vigorous citrus rootstocks in the greenhouse exhibits a precocious terminal flower bud five months after the transformation (April 2019); (b) HRP-A2i flowering two months after the grafting in the greenhouse (June 2019); (c), HRP-A2i starts to fruiting exhibiting small fruits eight months after the transformation (July 2019); (d), wild type plant (WT), control plant carrying the *CiFT* cassette (CN) and two representative transgenic plants HRP-A2i, showing a reduced size compared to WT and CN plants of the same age (July 2019).

In eukaryotic, RBPs are involved in multiple aspects of mRNA metabolism playing a major role in reprogramming the transcriptome and controlling protein translation (Denis and Chen, 2003; Collart and Timmers, 2004). Although the presence of RBPs in plants has been reported, their functional role is less investigated than in animals, in which alterations on RBPs have been shown to affect alternative splicing and cause diseases (Lukong et al., 2008; Mayr and Bartel, 2009). In *Arabidopsis* some RBPs have been reported to be putatively involved in RNA processing, including translocation, modification, translation and degradation, influencing plant development and stimulus specific responses (Marondedze et al., 2016). HRP-A2i dwarf phenotype may be related with altered levels of phytohormones related to plant growth, such as gibberelins or brassinosteroids. Future metabolic and molecular analysis will unravel the basis of this phenotype. Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) is widely used as rootstock in commercial citrus production, and reduction of its growth could be profitable to be use as dwarfing-rootstock. A similar development has been previously addressed by gibberellin synthesis modification (Fagoaga et al., 2007). By the other hand, microarray and proteomic analysis showed that RBPs are involved in regulation of cytoplasmic male sterility in *Brassica rapa* (Jeong et al., 2017) and in cybrid pummelo (Zheng et al., 2014), respectively. Male sterility is widely used in citrus breeding programs because in combination with female sterility and parthenocarpy allows to obtain seedless fruits (Yamamoto et al., 1995; Vardi et al., 2008), which is a desirable trait for consumers. Genes associated with male sterility have been

investigated and exploited in efforts for seedlessness induction in citrus (Li et al., 2002; Tan et al., 2009; Qiu et al., 2012; Fang et al., 2016). In addiction sequencing technologies and the availability of reference sweet orange (*Citrus sinensis*) (Xu et al., 2012) and clementine mandarin (*C.* × *clementina* cv. Clemenules) genomes (Wu et al., 2014) represents a useful resource for understanding and improving many important citrus traits. Here a gene coding an RBP, *Ciclev10027731m,* was selected as candidate responsible for sterility because mutations were observed in its homolog in Tango by performing genome comparison with the clementine mandarin reference genome. The seedlessness phenotype in Tango is poorly understood. In the gamma irradiation mutagenesis, the exposition of young buds to radiation create a wide range of random mutations often resulting in diminished fertility, because of the high frequency of pollen or ovule abortion (Vardi et al., 2008). Chromosome misalignment was observed in anthers of Tango during the meiotic metaphase suggesting that this could be the main underlying genetic cause of its seedless phenotype (Crowley, 2011). Here, although more work is required to full characterization, visual observations indicate that transgenic plants developed normal flowers without anomalies on anthers indicating that silencing of RBP may not affect male fertility. Considering that under cross-pollination conditions Tango mandarin set a very low seeded fruits (<1 seed/fruit), contrary to Nadorcott (Roose and Williams, 2007) it could be hypothesized that seedlessness is linked to other anomalies as female sterility or seed abortion. Bioinformatics studies revealed that RPBs could regulate genes with roles in seed development in *Arabidopsis*

(Kourmpetli et al., 2013). Further analysis is necessary to characterize RPB loss of function phenotype by performing cross-pollination tests and ovary and fruit development analysis. Here, as an engineering approach was used to reduce the long citrus juvenile period, fruits with a repressed expression of RBP gene *Ciclev10027731m* will be obtained within a year. Further studies with this material will allow gain knowledge on RBPs involvement in sterility. Results will potentially lead to develop biotechnological strategies to obtain seedless fruits. Genetic modification to overcome the long juvenility period by which citrus require many years to begin to flower and set fruit has been described before for *Poncirus trifoliata* (Endo et al., 2005) and *Citrus sinensis* (Pons et al., (2014). In this work the same strategy was evaluated for Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) by overexpression of *CiFT*. Obtained transgenic citrange plants started to set flowers five months after the transformation experiment, demonstrating the validity of this approach also for this genotype. The reduction of the juvenile phase has important implications in citrus genetic improvement because help to accelerate citrus genetic improvement and to promote experimental studies within these species (Peña et al., 2001). Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) represent an excellent candidate for characterization of fruit quality traits in transgenic studies for its high vigor and transformation and regeneration efficiency (Peña et al., 2008).

In summary, this study shows that the reduction of juvenile period by *CiFT* overexpression is a useful strategy for developing transgenic precocious citrange plants and concurrently accelerate functional genomic studies in citrus.

In this work this strategy is been used to decipher the function of an *RBP* gene by silencing its expression via RNA interference. Silencing of RBP results in transgenic plants producing normal flowers without morphological alterations. Still further observations are necessary to characterize RPB loss of function phenotype by analysis of anthers, ovary and fruit development, performing cross pollination tests for at least two consecutive years. It has been found that this gene could plays a role on plant growth, but the exact mechanism has still to be unraveled by molecular and biochemical investigations. This strategy will allow to characterize more genes related to economically important traits that could be introduced into new citrus cultivars with improved fruit quality by using new breeding techniques.

Appendix

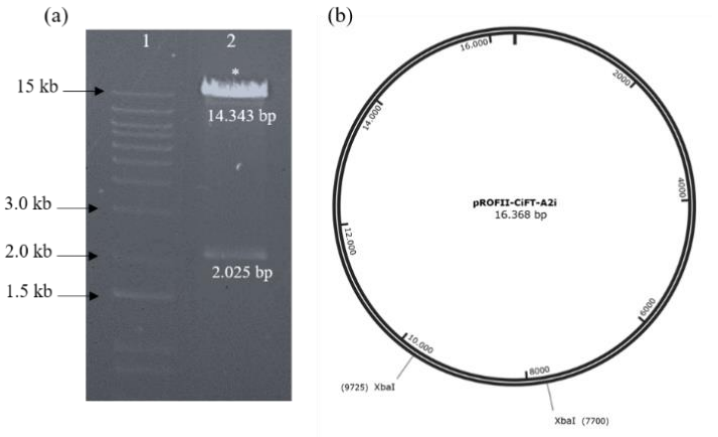
Supporting information

Vector construction

The pROKII-CsFT-A2i vector was constructed following instructions provided by Goldenbraid cloning system v3.0 (https://gbcloning.upv.es/), a modular DNA assembly technology based on type IIS restriction enzymes. The coding sequence of *Ciclev10027731m* (named A2) of clementine mandarin (*Citrus* × *clementina* cv. Clemenules), used for RNAi (A2i), was downloaded from Phytozome database v12.1 (https://phytozome.jgi.doe.gov). A 415 bp fragment, (nucleotide positions 1201-1615 of the 3123-bp complete coding sequence of A2), was chosen as target sequence for the hairpin-intron construction. The choose of the fragment was made verifying: the absence of restriction enzyme sites used by Goldenbraid system (*Bsa*I and *Bsm*BI) and sequence conservation only in plants performing Blast analysis (Altschul et al., 1990), to avoid that hairpin could induce RNA-interference in animal organisms.
The 415-bp fragment was PCR-amplified from a gBlocks Gene Fragments (IDT) using the specific primers 5'-GCGCCGTCTCGCTCGCCATCCAGCCTCTGTAAGTCTGAG-3' and 5'-GCGCCGTCTCGCTCAGGCTCTGTTCTCCAATCAAATGGTTC-3' for sense orientation and 5'-GCGCCGTCTCGCTCGTTCGCTGTTCTCCAATCAAATGGTTC-3' and 5'-GCGCCGTCTCGCTCAAAGCCCAGCCTCTGTAAGTCTGAG-3' for antisense orientation. PCR amplification was

performed by using the CloneAmp HiFi PCR Premix (Clontech, USA) following the manufacturer's instructions. PCR was analyzed by agarose 1% gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The gel-purified PCR parts were cloned in the pUPD2 entry vector with chloramphenicol resistance. Plasmids harboring sense and antisense clones were sequenced to confirm their identity and the lack of mistakes in the sequence. Sense and antisense fragments were assembled into the pDGB3-α2 vector with kanamycin resistance, separated by intron sequence from *S. lycopersicum* (Accession: GB01281) and under the control of the CaMV 35S promoter (Accession: GB0552) and the *nos* (nopaline synthase) terminator (Accession: GB0037). Assembly reactions were performed using *Bsa*I (for pDGB3-α2) and *Bsm*BI (for pUDP2) as restriction enzymes in 25 cycle digestion/ligation reactions (37°C for 2' and 16°C for 5'). The HRP cassette was excised from pDGB3-α2 by digestion with*Hind*III and ligated in the binary vector pROKII-CsFT (Pons et al., 2014) digested with the same restriction enzyme and de-phosphorilated, to obtain the final pROKII-FT-A2i plasmid (Fig. 1a). The T-DNA of the vector pROKII-CsFT includes the *FLOWERING LOCUS T* gene from sweet orange (*CiFT*), which sequence is identical to the *Citrus unshiu CiFT2* homolog (AB301934.1), in sense orientation, controlled by the CaMV 35S promoter and *nos* terminator, and the selectable marker gene neomycin phosphotransferase II (*nptII*) under the control of the *nos* promoter and terminator sequences. The binary vector pROKII-CsFT was used to transform control plants (Fig. 1b). The pROKII-CsFT-A2i construct was confirmed by plasmid

restriction analysis (*Xba*I, Supporting information Figure 1) and by sequencing. Restriction enzymes were purchased from New England Biolabs (USA). T4 DNA ligase was purchased from Promega. Plasmid extractions were made by using The E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). *Escherichia coli* XL1-Blue grown in LB medium under agitation (200 rpm) at 37°C was used for gene cloning. Medium was supplemented with chloramphenicol (25 μg/ml, pUPD2) or kanamycin (50 μg/ml, pDGB3 a2 and pROKII) to culture and recover and plasmids. Positive colonies were picked based on white/blue color selection after growing overnight on plates supplemented with IPTG (0.5mM) and Xgal (40 μg/ml).



Supporting information Figure 1. Plasmid restriction analysis. (a) agarose gel electrophoresis (1%) of restriction enzyme digestion of plasmid pROKII-CsFT-A2i digested with *XbaI* (lane 2) giving two fragments, 14,343 bp and 2,025 bp long, plus undigested plasmid (*). Lane 1: 1 Kb Plus DNA Ladder (Invitrogen); (b) map of pROKII-CsFT-A2i of 16,368 bp showing *XbaI* restriction sites.

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CHAPTER V

General conclusion

General conclusion

Flowering is the most important phase during the plant cycle life since ensures reproductive success and fruits set by the interaction of environmental and endogenous signals that regulate the transition from vegetative to reproductive growth. In this work, histological analysis, transcriptomic and biotechnological strategies were used in different *Citrus* species to investigate on environmental and genetic factors regulating flowering and affecting male sterility, flower induction and its time regulation, in order to study the genetic background involved in seedlessness.

Among environmental factors regulating flowering, temperature stress has a key role, especially considering the current climate change context characterized by the rise in the average global temperature, often accompanied by extreme fluctuations with an important impact on biological processes. Temperature stresses affect flowering, especially during gamete development determining their fertility (Zinn et al., 2010). Little information is available about the effects of temperature stress on male gametophyte development in citrus. Here histological and *in vitro* analysis were performed in flower in the self-incompatible "Comune" clementine mandarin (*Citrus clementina Hort*. ex. Tan.), one of the most economically valuable citrus species, during male gametophyte development under cold/hot temperature stresses. The effect of constant temperatures (15, 25 and 30 °C) was evaluated by a time course histological analysis performed on the anthers and by monitoring *in vitro* pollen germination. The results showed that suboptimal hot and cold temperatures induce drastic alterations, especially during the microgametogenesis stage affecting the morphology of the

tapetal cells, microspores and mature pollen grains. Also, the timing of starch depletion in the anther walls was affected, probably linked to the tapetum alteration, with a consequent reduction in starch accumulation in pollen grains, influencing the pollen germination rate and pollen tube growth. Despite the reasons for the tapetum alteration are unknown, information provided here could be useful in understanding the processes related to pollen sterility in citrus, a very desirable trait that results in seedless fruit when combined with parthenocarpy.

The citrus *FLOWERING LOCUS T* (*CiFT*) homologs are the main components regulating the flower induction timing by the transition from vegetative to reproductive development and flowering. Increase of the expression level of *CiFT*s was associated to flower induction in adult citrus trees by exposition to cold temperature and water stress under subtropical and tropical area respectively (Spiegel-Roy and Goldschmidt, 1996). Many evidences suggest that the flowering response to inductive conditions is also influenced by endogenous and developmental factors such as fruit load, nutritional state, gibberellin, acid abscisic (Khan et al., 2014; Agustí et al., 2019). However, less information is available about the role of rootstock on flower induction. Several studies are currently carried out in different citrus production areas in order to evaluate several *Citrus Tristeza Virus* (CTV) tolerant rootstocks that could be adopted (Continella et al., 2018). Here transcriptional analysis was performed to observe the influence of rootstock on flower induction and flower intensity, by the analysis of the expression levels of three *CiFT*s homologs, *CiFT1*, *CiFT2* and *CiFT3*, in leaves of sweet orange grafted onto ‘C35’ and ‘Swingle’ citrumelo.

The results showed that rootstocks influence flower induction in sweet orange by an increase of *CiFT*s expression levels in January during the induction period by the exposure to winter cold temperature with 'C35' citrange showing the highest expression levels for *CiFTs* and an increased flowering intensity in the following spring. These results provide useful information for rootstock breeding programs in order to couple satisfactory fruit crop yield and resistance to pathogens, since the presence of CTV in the Mediterranean area imposes the adoption of rootstocks different from sour orange.

Finally, the role of *CiFT*s on flowering time regulation was exploited for functional genomic studies of fruit quality traits. In experiments on transgenic plants, ectopic expression of *CiFT*s resulted in early flowering in *A. thaliana* as well as in Citrus species such as trifoliate orange (*Poncirus trifoliata* L. Raf) and sweet orange (Kobayashi et al., 1999; Endo et al., 2005; Nishikawa et al., 2010; Pons et al., 2014). Here, *CiFT* overexpression was induced in citrange to reduce the juvenile period and to enable the characterization of a gene potentially involved in seedlessness, selected by genome comparison between the seedless mutant Tango and the Clementine mandarin reference genome, through silencing by RNA interference (RNAi). The comparison of Illumina sequencing data from Tango and the availability of Clementine mandarin reference genomes allows us to identify a candidate gene, encoding an RNA binding protein (RBP) affected by mutation resulting in a change of peptide sequence that could be responsible for the seedless phenotype in Tango. *CiFT* overexpression allows transgenic plants to flower five months after the transformation experiments producing

normal flower without anomalies in anthers and with normal and plenty pollen indicating that silencing of RBP did not affect male fertility. Considering that seedlessness in Tango is less investigated, further analysis is required on ovary and fruit development to investigate on other anomalies that could explain its seedlessness, such as female sterility or seed abortion. Despite further analysis is required, this study proves the usefulness of biotechnological strategy for functional genomics studies in citrus. The reduction of juvenile period by using biotechnological strategy is a promising tool for accelerating research and breeding in perennial crop species with long juvenile phase. Transgenic approaches cannot be used for commercial utilization because of legal and ethical issues, but this approach is still useful for functional genomic studies of candidate genes regulating quality traits.

On the whole, the present thesis work provides new insights on both environmental and genetic factors regulating flowering behavior in *Citrus.* This research field is of particular relevance in light of the complex genetics and reproductive biology proper of *Citrus* (parthenocarpy, apomixis, self-incompatibility, high level of heterozygosity, long juvenile period). Some of these aspects, are also common to most of the tree crops, and make both the study of qualitative traits inheritance and the adoption of conventional breeding strategies a very difficult and time-consuming process. The outcome of this thesis could be exploited for the setup of novel citrus breeding programs for old and new challenges.

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