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Effects of Fruit on Floral Gene Expression and Floral Intensity in Alternate Bearing *Citrus reticulata* Blanco

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I dedicate this dissertation to my beloved parents, CJ Tang and Joyce Han, and my amazing brother, Wei Tang.

ABSTRACT OF THE DISSERTATION

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by

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Dr. Carol J. Lovatt, Chairperson

In September, buds of 'Washington' navel orange trees (*Citrus sinensis*) grown under warm, well-irrigated conditions for five months expressed *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *APETALA2* (*AP2*); *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) were not expressed and no inflorescences developed. Subjecting the trees to low-temperature or water-deficit treatment had no effect on *FT*, *SOC1* and *LFY* expression, but increased *AP1* and *AP2* expression with concomitant activation of *SEP1*, *PI* and *AG* and significant flowering. Gibberellic acid (GA3) applied to buds of low-temperature- or water-deficit-treated trees did not affect *FT*, *SOC1* or *LFY* expression, but dramatically reduced *AP1* and *AP2* transcription, repressing *SEP1*, *PI* and *AG* and flowering. Similarly, buds of field-grown low-yield, off-crop 'Pixie' and 'Nules Clementine' mandarin trees (*C. reticulata*) collected in October expressed *FT*, *SOC1*, *LFY*, *AP1* and *AP2*, with *SEP1*, *PI* and *AG* expression delayed until March, one month

before the intense return bloom. For high-yield, on-crop trees, which failed to flower, bud *FT* transcripts were not detected in October through March, *LFY*, *AP1*, *AP2* and *SEP1* expression were lower than off-crop trees by March, and *PI* and *AG* transcripts never exceeded the limit of detection. Removing the on crop from 'Pixie' mandarin trees in November increased *FT* expression above detectable levels, *AP1* to the level of off-crop trees, and activated *PI* and *AG*, resulting in a small number of inflorescence (21% of off-crop trees). To mitigate the negative effects of the on crop on return bloom and yield, fruit would need to be removed before October. Results of this research demonstrated: (*i*) citrus buds initiate floral development prior to exposure to low temperature in fall-winter, consistent with transition from vegetative to floral development in summer, but leaving open the possibility all buds on adult citrus trees are induced to flower; (*ii*) sustained *FT* expression is required for increased *AP1* and *AP2* expression to levels sufficient for floral determinacy, activation of downstream floral organ identity genes and flowering; and (*iii*) GA3 and the on crop of citrus fruit inhibit flowering by preventing bud determinacy, not floral induction.

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Chapter 1

General introduction

Alternate bearing (AB) is the tendency of a fruit tree to produce a large number of fruit in one year (on-crop tree, on-crop year), followed by a light yield or even no fruit the next year (off-crop tree, off-crop year). The low number of fruit produced in the year following the on crop is due to the low number of inflorescences in spring, rather than poor fruit set or excessive abscission of the setting crop (Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969; Monselise and Goldschmidt, 1982; Moss, 1973). This phenomenon, also known as biennial or uneven bearing, is a common problem with negative economic consequences to growers of woody perennial fruit and nut crops, including citrus (*Citrus* spp.) (Monselise and Glodschmidt, 1982). In citrus, on-crop trees produce a large number of fruit that are small in size with little commercial value, whereas off-crop trees produce a very small number of large fruit with the majority having undesirable traits, such as coarse rinds and granulated juice vesicles (Hield and Hilgeman, 1969; Moss et al., 1974). Due to differences in microclimates, the number of fruit per tree (crop load) commonly varies among blocks within an orchard and among trees within a single block (Monselise and Glodschmidt, 1982), which complicates orchard management. Moreover, the alternating on and off yields negatively impact packinghouse operations, marketing and the sustainability of the commodity-based industry.

Typically, AB is initiated by climatic conditions that reduce yield, such as spring frost, which damages floral buds, or high summer temperatures that result in excessive fruit drop. Such events synchronize the fruiting behavior of the trees in an orchard or a climatic region (Monselise and Goldschmidt, 1982). In some cases, AB is initiated by optimal conditions during floral development, flowering and fruit set, such that natural fruit thinning fails to occur. This results in an on crop, which will be followed the next year by an off crop (Monselise and Glodschmidt, 1982). After initially being triggered by climatic conditions, AB behavior is entrained by changes in internal factors brought about by the crop load on a tree that ultimately impact the number of inflorescences at return bloom the following spring (Monselise and Goldschmidt, 1982). The earlier in the on-crop year fruit is removed, the greater the increase in floral intensity the following spring (Garcia-Luis et al., 1986; Martinez-Fuentes et al., 2010; Verreynne and Lovatt, 2009), providing further evidence that fruit of the on crop are the cause of reduced return flowering in AB citrus.

Internal tree factors perpetuating AB

Historically, in the study of AB, the carbohydrate status of the tree has been the first factor suggested to regulate flowering. Crop load has been demonstrated to be negatively correlated with leaf starch concentrations during winter and spring and with the number of inflorescences at spring bloom in 'Valencia' orange (*C. sinensis* L. Osbeck) (Hilgeman et al., 1967; Jones et al., 1970, 1974), 'Wilking' mandarin (*C. reticulata* Blanco) (Goldschmidt and Golomb, 1982; Goldschmidt et al., 1985; Monselise et al., 1981) and

'Moncada' mandarin (*C. clementina* × *C. unshui* × *C. nobilis* Lour.) (Martínez-Alcántara et al., 2015). In contrast, results of other studies demonstrated no consistent relationship between tree carbohydrate status and floral intensity for 'Valencia' orange (Dovis et al., 2014; Martinez-Fuentes et al., 2010), 'Shamouti' orange (*C. sinensis* L. Osbeck) (Goldschmidt et al., 1985), Satsuma mandarin (*C. unshiu*) (Garcia-Luis et al., 1995; Yahata et al., 1995) and 'Pixie' mandarin (*C. reticulata* Blanco) (Verrynne, 2005), suggesting that tree carbohydrate status is not the predominant factor controlling flowering. In 'Pixie' mandarin, a strongly AB cultivar, removal of all fruit from on-crop trees in July had no effect on shoot or root carbohydrate concentrations but stimulated summer vegetative shoot growth and increased return bloom, providing evidence that summer vegetative shoot growth and its substantial contribution to return bloom are independent of carbohydrate availability (Verreynne, 2005; Verreynne and Lovatt, 2009). Removal of summer vegetative shoots from off-crop trees resulted in reduced inflorescence number during return bloom without any change in the concentrations of shoot and root carbohydrates (starch and glucose) in August or January. Similar results were also reported for 'Valencia' orange, for which removal of fruit in May did not have an effect on leaf carbohydrate concentrations from May through July, but resulted in an increase in bud break of summer vegetative shoots and floral intensity the following spring (Dovis et al., 2014).

A role for plant hormones in modulating flowering in AB citrus has been proposed. The results of Verreynne (2005) provided evidence that crop load caused changes in hormone ratios that inhibited bud break at two stages in the phenology of on-crop 'Pixie'

mandarin trees and thereby reduced floral intensity at return bloom. First, compared to off-crop trees, on-crop trees had greater concentrations of the auxin indole-3-aetic acid (IAA) and lower concentrations of the cytokinin isopentenyladenine (2iP) in the apical buds of vegetative shoots and produced less summer vegetative shoot growth than off-crop trees. Vegetative shoots that develop during the summer have the potential to increase the total number of inflorescences that develop the following spring by 40%. The role of the on crop in these changes was confirmed by removing all fruit from on-crop trees in July. This reduced bud IAA concentrations and increased 2iP concentrations to that of off-crop trees by August and restored summer vegetative shoot development and floral intensity the following spring to that of off-crop trees. Similarly, in 'Murcott' mandarin (*C. reticulata*), buds of on-crop trees had greater IAA levels in August and a lower number of inflorescences the following April compared to those of off-crop trees (Shalom et al., 2014). Removal of the on crop in August resulted in reduced IAA concentrations to a level equal to off-crop trees by September, with an increase in spring floral intensity. Second, abscisic acid (ABA), as well as IAA, accumulated in the buds of on-crop 'Pixie' mandarin trees starting in January, resulting in higher ratio of both ABA and IAA to 2iP than in buds of off-crop trees, corresponding to the lower percent spring bud break of on-crop trees compared to off-crop trees (Arbona and Lovatt, unpublished results; Verreynne, 2005; Verreynne and Lovatt, 2009). Removal of the on crop in December or January reduced bud concentrations of both ABA and IAA and increased bud 2iP concentrations, percent spring bud break, and floral intensity compared to on-crop trees with no fruit removed. Fruit removal this late in the season did not restore

flowering to the level off-crop trees due to the reduced development of summer vegetative shoots and the loss of their contribution to spring bloom (Verreynne and Lovatt, 2009).

Compared to seedless citrus cultivars, seeded cultivars tend to have more severe AB behavior (Monselise and Goldschmidt, 1982). Given that seeds are a source of hormones, including gibberellins (GA), it has been proposed that endogenous GA produced by seeds, or the placenta of seedless fruit, inhibits flowering in citrus following a heavy on crop (Iglesias et al., 2007; Monselise and Goldschmidt, 1982; Plummer et al., 1989; Talon et al., 1990). Bearing shoots of Satsuma mandarin, on which only a few inflorescences developed the next spring, had greater leaf GA concentrations in October than nonbearing shoots that produced a greater number of inflorescences (Koshita et al., 1999), consistent with fruit being the source of GA and with a negative effect of GA on citrus flowering. Whereas GA_3 application promotes flowering in many annual and biennial plants, including *Arabidopsis thaliana* (Blazquez et al., 1998, 2000; Moon et al., 2003; Wilson et al., 1992), the inhibitory effect of exogenous GA_3 on citrus flowering is well documented (Garcia-Luis et al., 1986; Goldberg-Moeller et al., 2013; Lord and Eckard, 1987; Muñoz-Fambuena et al., 2012a). In 'Washington' navel orange (*C. sinensis* L. Osbeck), exogenous GA_3 inhibits flowering by continuing vegetative development of the shoot apical meristem (SAM) when applied before the bud becomes determined (irreversibly committed to floral development), which is coincident with sepal formation (Lord and Eckard, 1987). Once the citrus bud is determined, GA_3 no longer has a negative effect on flowering. In 'Moncada' mandarin, a single spray of GA_3 (40 mg L^{-1})

in December resulted in a reduction in floral intensity and an increase in vegetative shoot number the following spring compared to untreated control trees (Muñoz-Fambuena et al., 2012a). In contrast, 'Moncada' mandarin trees treated with a single spray of paclobutrazol (PBZ) (2000 mg L^{-1}), a synthetic inhibitor of GA biosynthesis, in December produced a greater number of inflorescences than untreated control trees and GA_3-treated trees, with an accompanying decrease in vegetative shoot number (Muñoz-Fambuena et al., 2012a). The opposite effects of PBZ on citrus flowering to those of GA_3 were also reported in 'Salustiana' and 'Navelina' sweet orange (*C. sinensis* L. Osbeck), and 'Hernandina' Clementine mandarin (*C. clementina* Hort. ex. Tanaka) (Martinez-Fuentes et al., 2013), suggesting that an endogenous form of GA, the synthesis of which is inhibited by PBZ, has a negative effect on citrus flowering analogous to exogenously applied GA_3.

In 2012, differences in bud expression of several genes regulating floral development, which paralleled the differences in floral intensity the following spring, were reported for on- and off-crop 'Moncada' mandarin trees (Muñoz-Fambuena et al., 2012b). The results of this study provided the first evidence that reduced transcription of genes in the citrus flowering pathway was contributing to the perpetuation of alternating on and off return blooms and yields. Three genes have now been documented to be downregulated in buds of on-crop 'Moncada' and 'Murcott' mandarin trees, *FLOWERING LOUCS T* (*FT*), *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Muñoz-Fambuena et al., 2012b; Shalom et al., 2012). The role of these genes and others in the flowering process is discussed with

reference to the model plant *A. thaliana* and in context of what is currently known about the regulation of floral development in *Citrus* spp.

Floral development in *Arabidopsis thaliana*

Knowledge of sexual reproduction in the Anthophyta has been greatly advanced by the continued development and application of new genomic and transcriptomic tools to *A. thaliana.* In *A. thaliana*, there are two distinct developmental phases, juvenile and adult. After germination, the seedling plant, which is in the juvenile phase of development, is restricted to vegetative growth and asexual reproduction. In *A. thaliana*, four classic floral inductive pathways have been identified that initiate the transition from the juvenile to the adult phase, resulting in the SAM being competent. The pathways include vernalization (low temperature), autonomous (developmental), photoperiod (long day), and GA. After reaching the adult vegetative phase, the plant is competent to respond to signals that promote floral induction (transition from vegetative to floral development) and initiate sexual reproduction (Huijser and Schmid, 2011; Poethig, 1990, 2010). An overview of the genetic regulation of flowering via the four pathways in *A. thaliana* is described below and illustrated in Figure 1.1. This information serves as the background for interpreting current published information on floral development in *Citrus* spp. reviewed further below and for the analysis of new data on citrus floral gene expression presented in the research chapters that follow this introduction.

FLOWERING LOCUS C (*FLC*), a central floral repressor, targets the floral timing genes *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in the

SAM. Regulation of *FLC* involves epigenetic histone modifications brought about in response to low temperature (via the vernalization pathway) or by endogenous developmental events independent of environmental cues (via the autonomous pathway), which lead to the repression of *FLC* and upregulation of *FT* and *SOC1* (Bastow et al., 2004; He et al., 2003; Kwak et al., 2017; Searle et al., 2006; Sung and Amasino, 2004). Flowering in *A. thaliana* is also regulated by photoperiod. Long days (short nights) upregulate *CONSTANS* (*CO*), a floral promoter gene that activates *FT* in leaves and *SOC1* in the SAM (Amasino, 2010; Corbesier et al., 2007; Hepworth et al., 2002; Pajoro et al., 2014; Samach et al., 2000). The FT protein synthesized in leaves is translocated in the phloem to the SAM and subsequently upregulates the downstream target gene *SOC1* in *A. thaliana.* Current literature identifies the FT protein as the long-sought mobile flowering signal "florigen" (Corbesier et al., 2007; Pajoro et al., 2014). Short-day (long-night) conditions also upregulate flowering in *A. thaliana* via genes also common to the endogenous GA pathway, *SOC1* and *LFY* (Blazquez et al., 1998; Moon et al., 2003; Pajoro et al., 2014; Wilson et al., 1992). Due to their role in flowering time control, *FT*, *SOC1* and *LFY* were classified as floral timing genes. These genes collectively compose a checkpoint where the individual flowering pathways converge and thus, they also have been termed floral integrator genes and floral promoter genes. Following the integration of floral signals, these genes upregulate the genes that establish and maintain floral meristem identity, including *LFY*, which is also categorized as a floral meristem identity gene, and *AP1* (Lee and Lee, 2010; Pajoro et al., 2014; Ratcliffe et al., 1999; Weigel et al., 1992).

In the shoot apex, *SOC1* directly targets *LFY*, whereas *FT* activates both *LFY* and *AP1* expression (Abe et al., 2005; Lee and Lee 2010; Lee et al., 2008; Pajoro et al., 2014; Samach et al., 2000). In *A thaliana*, *LFY* and *AP1* also reciprocally upregulate each other (Adrian et al., 2009; Liljegren et al., 1999; Pajoro et al., 2014). In contrast, *TERMINAL FLOWER1* (*TFL1*) maintains indeterminacy of the SAM by suppressing the activity of *LFY* and *AP1* independently (Adrian et al., 2009; Ratcliffe et al, 1998, 1999; Shannon and Meeks-Wagner, 1993). Conversely, *TFL1* activity is repressed by *LFY* or *AP1*, leading to a determinate floral meristem (Adrian et al., 2009; Liljegren et al., 1999; Ratcliffe et al, 1999).

Once the SAM is committed to a flowering fate, the floral meristem undergoes differentiation, resulting in a series of sequential steps for floral organ development (Krizek and Fletcher, 2005). Formation of the four whorls of floral organs that collectively compose a perfect flower is explained by the classic ABCE model constructed on the basis of homeotic mutants in *A. thaliana* and *Antirrhinum majus*, in which *AP1* and *APETALA2* (*AP2*) are identified as class A genes, *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) are, respectively, in classes B and C, and four functionally redundant *SEPALLATA* (*SEP*) genes, *SEP1*, *SEP2*, *SEP3*, and *SEP4*, are class E genes (Bowman et al., 1991; Causier et al., 2010; Coen and Meyerowitz, 1991; Ditta et al., 2004; Krizek and Fletcher, 2005; Pelaz et al., 2000). In addition, *SEP4* also has a role in floral meristem identity in *A. thaliana* (Ditta et al., 2004). The ABCE model proposes that normal development of sepals in the first whorl requires the activity of the class A genes; the combined activity of the class A and B genes is required for petal formation in the second

whorl; the activity of the class B and C genes is collectively required for formation of stamens in the third whorl; development of carpels in the fourth whorl solely relies on class C gene activity; and class E gene expression is required for proper development of the floral organs in conjunction with the class A, B and C genes (Bowman et al., 1991; Causier et al., 2010; Coen and Meyerowitz, 1991; Ditta et al., 2004; Krizek and Fletcher, 2005; Pelaz et al., 2000).

Floral development in *Citrus* spp.

Citrus spp. are evergreen woody perennials, which undergo phase transition from the juvenile to the adult stage, with buds in the adult stage reported to undergo an annual transition from vegetative to floral development (Bergonzi and Albani, 2011; McDaniel et al., 1992). Due to the length of the juvenile stage (5-10 years), commercial citrus orchards are established with trees that were produced by grafting buds collected from adult (competent) scion cultivar trees to seedling (juvenile) rootstocks. After a relatively short immature adult vegetative growth stage (2-5 years), the tree scions are capable of flowering. For mature adult citrus trees, flowering can occur autonomously in response to endogenous developmental events without environmental influences or in response to low temperature or water-deficit stress, but, unlike *A. thaliana*, flowering in *Citrus* spp. is insensitive to day length (i.e., day neutral) (Garcia-Luis et al., 1992; Lovatt et al., 1988; Moss, 1969, 1976; Nishikawa et al., 2007; Southwick and Davenport, 1986). It is generally accepted that low temperature triggers the annual transition of the SAM from vegetative to reproductive development in late-fall and/or winter (mid-November through

late December) in the subtropics of the Northern Hemisphere, albeit the timing can vary depending on cultivar and climate (Krajewski and Rabe, 1995; Lord and Eckard, 1985, 1987; Nishikawa et al., 2009). Periods of water deficit augment the shorter periods of low temperature in the semi-tropics and tropics.

Citrus sinensis, like many other *Citrus* spp., produces a cymose inflorescence, which develops from the apex to the base. Once the terminal SAM is determined, the lateral buds also become floral. Lord and Eckard (1985, 1987) documented the histological changes in floral buds of 'Washington' navel orange (*C. sinensis*) over the period from fall to spring bloom in southern California. Fall resting (dormant, inactive) buds comprised three bracts, six or seven leaves and the dormant SAM. The first indication of the initiation of the terminal flower of the inflorescence was the loosening of the scales surrounding the bud (microscopic bud break) in mid-November to late December; new primorida were not visible by scanning electron microscopy at this point. Initiation of the first sepal of the terminal flower (macroscopic bud break) occurred during mid-January. Once one or more sepals were formed, the bud was determined (insensitive to GA_3 treatment, i.e., irreversibly committed to floral development) and the floral organs proceeded to develop in each whorl, respectively, with anthesis taking place in March to April. Once the terminal SAM formed sepals, the lateral buds also formed sepals and then flowers. This description of flower development was adapted to the phenology of Pixie and Nules Clementine mandarin, two cultivars of *C. reticulata* used in the research presented herein in addition to the 'Washington' navel orange (Figure 1.2).

Not all buds of mature adult citrus trees become floral. A population of buds continues the vegetative growth of the tree, producing vegetative shoots in spring, in parallel with the floral shoot flush, or during summer and fall. Tan and Swain (2006) proposed two possible mechanisms for the presence of vegetative meristems in adult citrus trees. In the first, meristems are competent, having transitioned from juvenile to adult, but subsequently fail to undergo the annual transition from vegetative to reproductive development and remain vegetative SAMs, whereas other SAMs successfully transition to floral development and subsequently produce flowers. In the second possibility, all buds of adult citrus trees are not only competent but also have been induced to flower as the result of phase transition. In both scenarios, the population of vegetative buds persists because prevailing local environmental or physiological conditions (shoot age, bud endogenous GA concentration, warm temperature, insufficient exposure to low temperature, lack of water-deficit stress, or presence of fruit) prevent the buds from becoming determined. In the second scenario, vegetative buds would be analogous to buds that later produce flowers, at least during the early stages of development. Thus, both floral and vegetative buds would express floral promoter genes and therefore would be indistinguishable at the level of floral gene transcription during early development. In contrast, in the first scenario, floral promoter genes would be expressed only in citrus buds that have undergone annual phase transition with the potential to subsequently flower, but not in buds that continue vegetative development; in other words, the two types of buds would be distinguishable at the level of gene transcription during early development. To date, the genes essential for citrus floral bud

determinacy have not been identified and the transcription of floral organ identity genes in citrus has not been investigated. Thus, resolution of the two possibilities proposed by Tan and Swain (2006) awaits a complete analysis of floral timing, floral meristem identity and floral organ identity gene transcription in populations of citrus buds that go on to flower and those that do not.

Of the key genes regulating floral development in *A. thaliana* (Figure 1.1), the following genes have been isolated from *Citrus* spp., characterized and demonstrated to be functional equivalents of their *A. thaliana* counterparts: *TFL1*, *FT*, *SOC1*, *LFY* and *AP1*. The role of each of these genes in upregulating floral development in *Citrus* spp. and in mediating the response of the SAM to conditions that promote or inhibit flowering are depicted in Figure 1.3. Overexpression of *C. sinensis TFL1* (*CsTFL1*) in *A. thaliana* resulted in delayed flowering and an increase in the number of rosette leaves, a juvenile trait of *A. thaliana* (Pillitteri et al., 2004b), suggesting a correlation of *CsTFL1* with juvenility maintenance. Moreover, buds of juvenile 'Washington' navel orange and Satsuma mandarin plants, had high levels of endogenous *TFL1* transcripts and failed to flower in response to low temperature, whereas buds of adult trees were characterized by very low *TFL1* expression and flowered in response to the low temperature treatment (Nishikawa et al., 2007; Pillitteri et al., 2004b). For adult Satsuma mandarin trees under field conditions, transcripts of *TFL1* were detected in buds in June, but the level decreased rapidly in July and remained extremely low through April when the trees flowered profusely (Nishikawa et al., 2007).

Expression of *C. unshiu FT* (*CiFT*) and *C. sinensis SOC1-like* genes (*CsSL1* and *CsSL2*), *LFY* (*CsLFY*) and *AP1* (*CsAP1*) also complemented the phenotypes of their respective *A. thaliana* mutants, confirming the functional equivalence of the citrus genes with their *A. thaliana* homologs (Kobayashi et al., 1999; Tan and Swain, 2007; Pillitteri et al., 2004a). Overexpression of *C. unshiu FT* in trifoliate orange seedlings (*Poncirus trifoliata*) resulted in early flowering by shortening the juvenile period (Endo et al., 2005), indicating that *FT* activity alone was sufficient for flowering in the juvenile background (Figure 1.3). Two other *FT* homologs, *CiFT2* and *CiFT3*, were characterized in *C. unshiu* based on cloning analysis (Nishikawa et al., 2007). Later, the full genome sequence of *C. clementina* (Phytozone database, http://www.phytozome.org/) suggested that *CiFT* and *CiFT2* were encoded by the same gene (Ciclev10013731m), which was renamed *CiFT1*, and that *CiFT3* was encoded by a different *FT* (Ciclev10012905m), renamed *CiFT2* (Samach, 2012). However, a third FT-encoding gene (Ciclev10012629m), not previously reported by Nishikawa et al. (2007), was identified in the genome data set and named *CiFT3* (Samach, 2012). For field-grown Satsuma mandarin, 'Murcott' mandarin, and 'Orri' mandarin (*C. reticulata* Blanco × *C. temple* Hort. ex Y. Tanaka) trees that bloomed in April, bud transcript levels of *FT* (currently named *CiFT2*) were very low starting in May through September but increased in November (five months before bloom). Maximum *FT* expression was in January (three months before bloom) (Goldberg-Moeller, et al., 2013; Nishikawa, 2007; Shalom et al., 2012, 2014), coinciding with the commonly regarded floral induction period triggered by low temperature. The seasonal changes in *FT* expression in leaves of Satsuma mandarin and 'Moncada' mandarin exhibited the

same patterns as buds (Muñoz-Fambuena et al., 2011; Nishikawa et al., 2007). In *A. thaliana*, the mobile signal produced by *FT* in leaves is translocated in the phloem to the SAM, where it upregulates *SOC1* and the downstream homeotic genes for floral development (Corbesier et al., 2007; Pajoro et al., 2014). An analogous pathway in citrus has not been demonstrated. In early research, citrus buds in the absence of leaves were proven to be capable of flowering (Garcia-Luis et al., 1992) and defoliation did not affect floral intensity (Davenport, 1990; Wilkie et al., 2008), suggesting that *FT* activity generated locally in buds is sufficient to promote flowering in citrus.

Bud expression of the floral timing gene *SOC1* was observed in 'Orri' mandarin, 'Moncada' mandarin and 'Murcott' mandarin as early as May (11 months before bloom). However, reports of the pattern of *SOC1* transcript accumulation over time were contradictory and no clear role for *SOC1* in citrus floral development has emerged (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012a, b; Shalom et al., 2012).

Transcripts of *LFY* and *AP1* were detected early in the season (as early as May, approximately 11 months before bloom) in buds of Satsuma mandarin and 'Murcott' mandarin trees, similar to *FT*. However, in contrast to *FT*, peak expression of *LFY* and *AP1* occurred two to three months before bloom (Muñoz-Fambuena et al., 2012b; Nishikawa et al., 2007, 2009; Shalom et al., 2012), suggesting the activity of both genes is required in flower initiation (Figure 1.3), which is consistent with the role of their counterparts in *A. thaliana* as floral meristem identity genes (Lee and Lee, 2010; Pajoro et al., 2014; Ratcliffe et al., 1999; Weigel et al., 1992). Flowering in citrus is promoted by low temperature (Chica and Albrigo, 2013a, b; Lovatt et al., 1988; Nishikawa et al., 2007;

Pillitteri et al., 2004b; Southwick and Davenport, 1986) and water deficit (Barbera et al., 1981, 1985; Chica and Albrigo, 2013a; Garcia-Luis et al., 1986; Lovatt et al., 1988; Southwick and Davenport, 1986) but inhibited by GA_3 when it is applied before buds are determined (Goldberg-Moeller et al., 2013; Lord and Eckard, 1987; Muñoz-Fambuena et al., 2012a). Thus, low temperature, water deficit and GA_3 treatments have been used as tools to manipulate floral intensity for the study of floral development at the molecular level. Whereas the responses of *FT*, *SOC1*, *LFY* and *AP1* expression to low temperature has been documented in a number of publications (Chica and Albrigo, 2013a, b; Nishikawa et al., 2007; Pillitteri et al., 2004b), knowledge of the effects of water deficit on floral gene expression is limited to the results of a single study (Chica and Albrigo, 2013a). Use of low temperature and water deficit treatments to increase floral intensity resulted in an increase in leaf *FT* expression in Satsuma mandarin and 'Washington' navel orange (Chica and Albrigo, 2013a; Nishikawa et al., 2007). For 'Washington' navel orange, the increase in bud *SOC1* transcript accumulation occurred during the cold period for low-temperature-treated trees but was not observed in the water-deficit-treated trees until they were fully re-hydrated. For 'Washington' navel orange trees under either low temperature or water deficit, bud *LFY* and *AP1* expression increased only after the end of the treatment period, i.e. after transfer to the warm condition or re-hydration of the dehydrated trees. In these studies, expression of *SOC1*, *LFY* and *AP1* was not analyzed in buds of trees maintained under conditions that did not promote flowering for comparison. Currently, it remains unclear whether low-temperature and water-deficit treatments promote citrus flowering by upregulating floral induction or whether buds are already

induced and the treatments cause determinacy. Applying GA_3 resulted in a reduction in *FT* expression in leaves of 'Salustiana' orange and buds of 'Orri' mandarin with a decrease in floral intensity but no change in *SOC1* expression (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012a), suggesting the inhibitory effect of GA_3 on flowering might be mediated through *FT* expression. In contrast, the effects of GA_3 on the expression of *LFY* and *AP1* reported in the literature were contradictory (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012a), leaving the question of whether *LFY* and *AP1* are also targets of GA_3 and factors in the decrease in flowering unresolved.

At the present time, no expression data have been published for the floral organ identity genes downstream of *AP1* in citrus, with the exception of the seasonal changes in expression of the *SEP* homologs in *C. unshui*, *CuSEP1* and *CuSEP3*, which shortly before bloom were correlated with annual flowering in Satsuma mandarin (Nishikawa et al., 2009). With the use of floral-promoting low-temperature and water-deficit treatments as tools to increase floral intensity relative to the non-floral-promoting warm, well-irrigated condition, expression of key floral genes necessary for citrus flowering can be identified. An evaluation of the patterns of transcript accumulation for *AP2*, *PI* and *AG* would enhance our understanding of the timing of key events in floral development, i.e., floral induction, bud determinacy and floral organogenesis. Lord and Eckard (1987) provided evidence that GA_3 inhibited flowering by preventing bud determinacy, but had no inhibitory effect on flowering when bud determinacy preceded GA_3 application. Thus, GA_3 applications initiated at different times during bud development might shed light on which genes are involved in regulating this key developmental event.

Current knowledge of the effect of the on crop of fruit in alternate bearing mandarin trees is limited to bud expression of *FT, LFY* and *AP1*, which was inhibited, and *SOC1*, which was not affected (Muñoz-Fambuena et al., 2012b; Shalom et al., 2012). Whether bud transcript levels of *AP2*, *PI*, *AG* and *SEP* are sensitive to crop load is unknown and compromises the development of strategies to mitigate alternate bearing.

The overall goal of the research conducted as part of this dissertation was to broaden our basic knowledge of citrus floral development in general, and to gain insight into the inhibitory effect of the on crop of fruit on bud floral gene transcription in relationship to reduced flowering in alternate bearing citrus cultivars in particular. To fulfill this goal, there were two main objectives. The first was to determine the expression patterns of the genes regulating flowering time, floral meristem identity and floral organ identity, including *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG*, in buds of mature 'Washington' navel orange trees grown under floral-promoting low-temperature and water-deficit conditions and warm, well-irrigated conditions that do not result in flowering to identify the key genes regulating citrus floral development. As part of the research to meet this objective, GA_3 was applied at different times to the foliage of low-temperature- and water-deficit-treated trees to discover when bud determinacy occurred and to identify the genes essential to this event. The second objective was to quantify the effects of fruit number per tree (crop load) on transcription of the above eight genes during the six months before bloom in buds of field-grown off- and on-crop Pixie and Nules Clementine mandarin trees, late- and early-maturing cultivars, respectively. As part of this objective, all fruit were removed from on-crop trees monthly, starting four months

before bloom, to identify how early the on crop of fruit exerted its inhibitory effect on floral development at the level of gene transcription, when buds became determined in the two different cultivars growing in climatically different areas, and for how long buds on on-crop trees remained viable floral buds. This basic information is essential for the development of future strategies to mitigate alternate bearing and maintain the economic viability of the commercial citrus industry.

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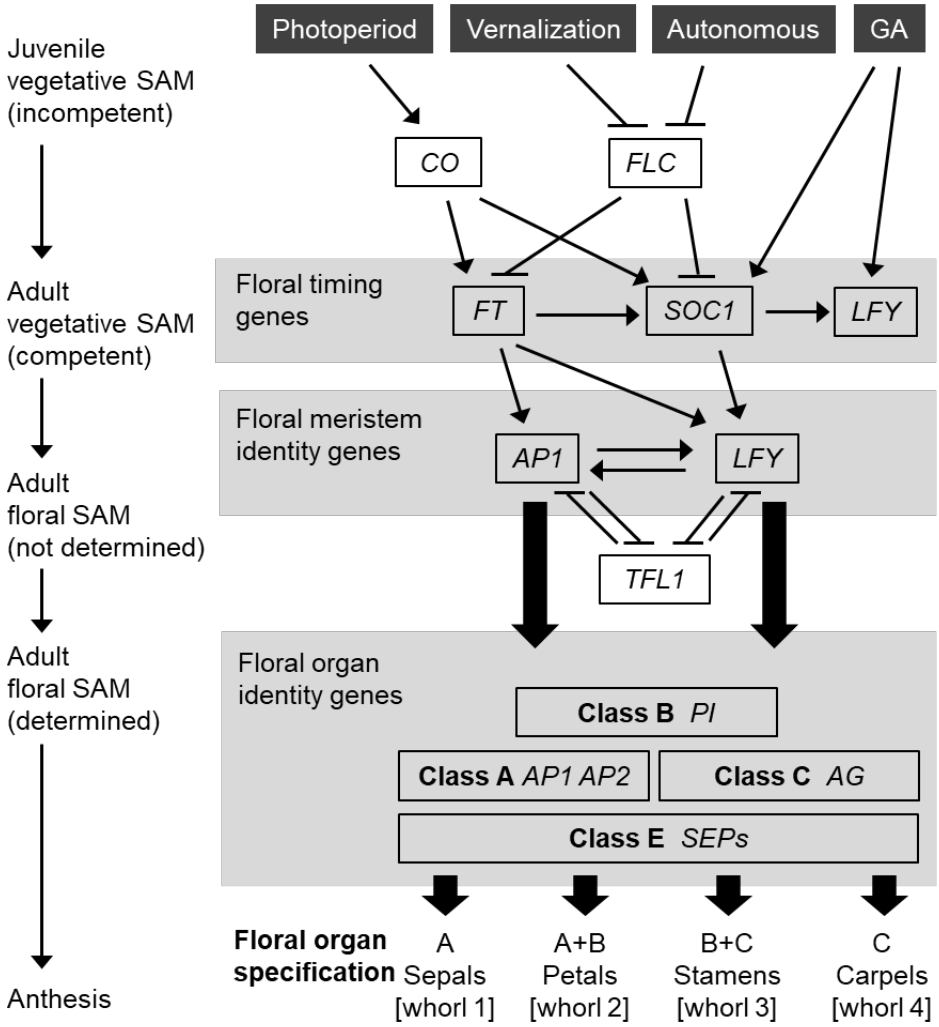

Figure 1.1 Simplified floral development pathways in *Arabidopsis thaliana* with associated changes in the shoot apical meristem (SAM) and gene expression of *CONSTANS* (*CO*); *FLOWERING LOCUS C* (*FLC*); *FLOWERING LOCUS T* (*FT*); *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*); *LEAFY* (*LFY*); *TERMINAL FLOWER1* (*TFL1*); *APETALA1* (*AP1*); *APETALA2* (*AP2*); *PISTILLATA* (*PI*); *AGAMOUS* (*AG*); and *SEPALLATA* (*SEP*).

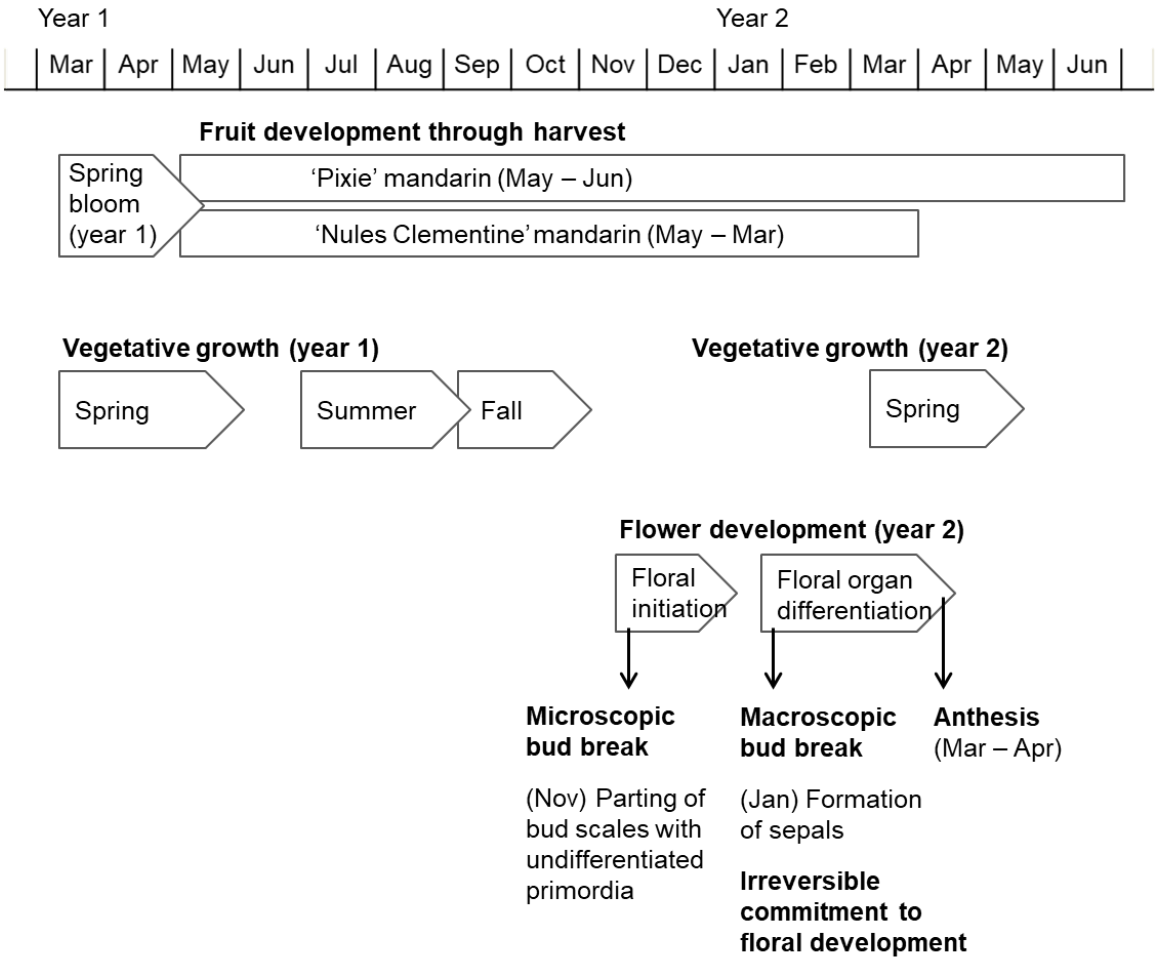

Figure 1.2 Phenology of 'Pixie' and 'Nules Clementine' mandarin (*Citrus reticulata* Blanco) trees in California based on field observations. Flower development based on the current literature (Lord and Eckard, 1985, 1987).

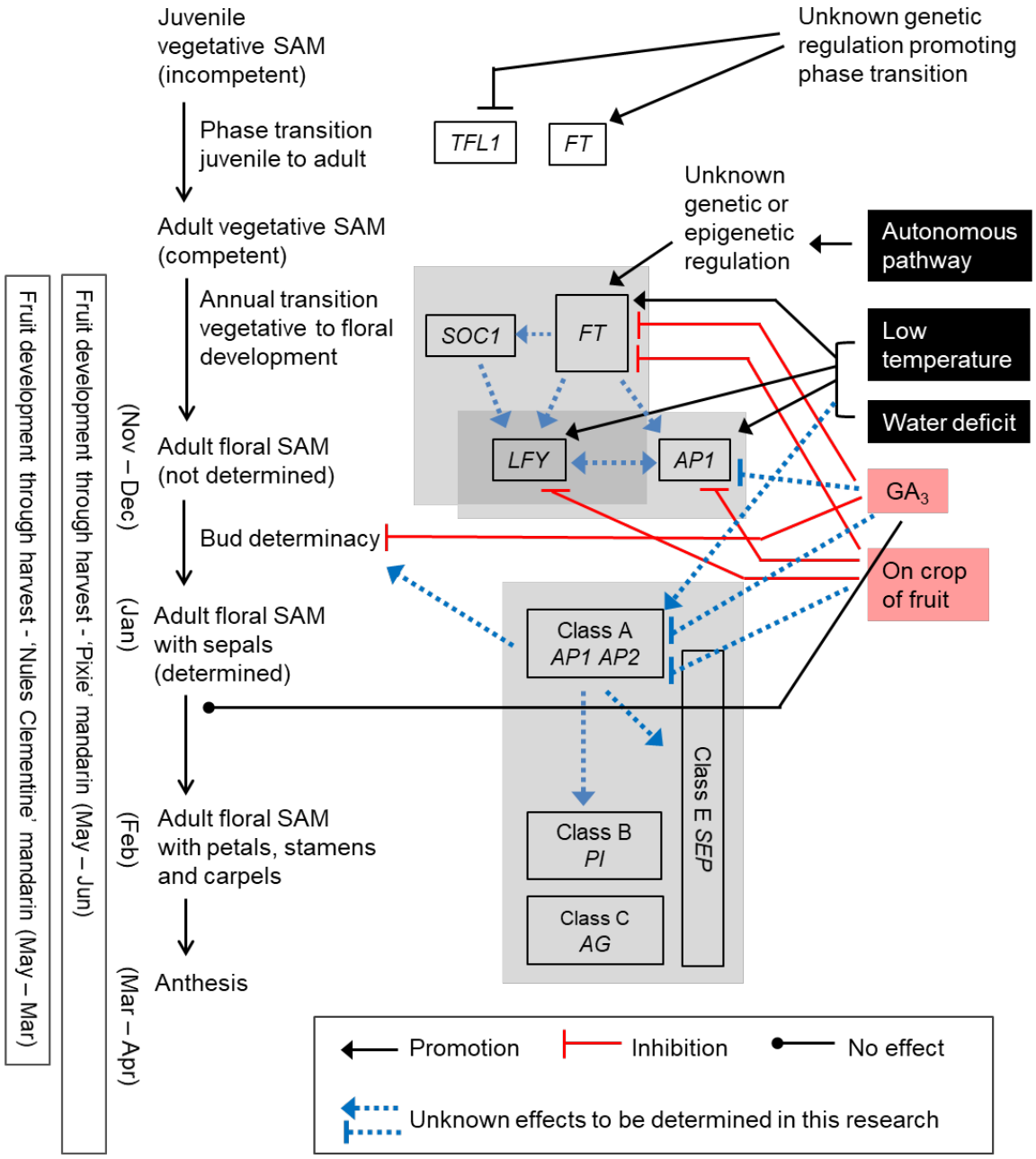

Figure 1.3 A model for floral regulation in buds of *Citrus* spp. based on the current literature.

Chapter 2

Relationship between floral intensity and floral gene expression in 'Washington' navel orange (*Citrus sinensis* L. Osbeck) in response to of low temperature and gibberellic acid

Abstract

Low temperature (LT) and gibberellic acid (GA_3) effects on 'Washington' navel orange (WNO) (*Citrus sinensis* L. Osbeck) floral gene expression and floral intensity were determined. Trees exposed to 8 weeks of LT (15/10 ˚C, day/night) and transferred to the warm condition (WC) (24/19 ˚C, day/night) for 3 weeks produced more inflorescences in week 11 than trees receiving 11 weeks of WC or 4 weeks of LT ($P < 0.001$). Buds from 8-week LT-treated trees had greater expression of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in week 2, *APETALA1* (*AP1*) and *APETALA2* (*AP2*) during weeks 8 through 10, and *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) after transfer to the WC than the 11-week WC-treated trees ($P < 0.05$). Foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 of LT treatment did not affect *FT*, *SOC1* or *LEAFY* (*LFY*) expression, but significantly reduced transcripts of *AP1* by week 8, *AP2*, *SEP1*, *PI* and *AG* after transfer to the WC, and inflorescence number to values equal to 11-week WC-treated trees. When GA_3 application was delayed to weeks 4 through 8 of LT treatment, *AP1*, *AP2*, *SEP1*, *PI* and *AG* expression and inflorescence number were intermediate to 11-week WC-treated and 8-week LT-treated trees. The results suggest inhibition of citrus floral development

by GA_3 is mediated through *AP1* and *AP2*, which also control bud determinacy and the downstream floral organ identity genes. The possibility that GA_3 reduces expression of each floral organ identity gene independently cannot be excluded.

Introduction

Flowering in citrus (*Citrus* spp.) is promoted by low temperature (LT) (García-Luís et al., 1992; Lovatt et al., 1988; Moss, 1969; Nishikawa et al., 2007; Southwick and Davenport, 1986). Two weeks of floral-promoting LT treatment stimulated 'Tahiti' lime (*C. latifolia* Tan.) trees to flower at a low level (Southwick and Davenport, 1986), but 4 weeks of LT were required to significantly increase flowering of 'Washington' navel orange (WNO) (*C. sinensis* L. Osbeck) (Lovatt et al., 1988; Moss, 1969) and Satsuma mandarin (*C. unshiu* Marc.) (Nishikawa et al., 2007). Floral intensity increased with the duration of the LT treatment, with maximum flowering occurring after 8 weeks of LT for 'Tahiti' lime (Southwick and Davenport, 1986) and WNO (Lovatt et al., 1988; Moss, 1976) and 10 weeks for Satsuma mandarin (Nishikawa et al., 2007). For WNO, maximum inflorescence number was achieved when trees were exposed to 15 ˚C during the day and 10 ˚C at night compared to warmer day or night temperatures (Moss, 1969). Using this LT treatment, Moss (1969) demonstrated that photoperiod had no effect on floral intensity of WNO.

In Satsuma mandarin, low temperature treatments known to increase floral intensity increased the expression of *FLOWERING LOCUS T* (*FT*) in buds and leaves and expression of *LEAFY* (*LFY*) in buds prior to morphological flower development

(Nishikawa et al., 2007). In the model plant *Arabidopsis thaliana, FT*, *LFY* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) control flowering time by integrating signals from different floral pathways and subsequently upregulating the genes that establish and maintain floral meristem identity, including *LFY*, which is also classified as a floral meristem identity gene, and *APETALA1* (*AP1*) (Horvath 2009; Lee and Lee, 2010; Michaels, 2009; Moon et al., 2005; Parcy, 2005; Ratcliffe et al., 1999). The homologs of these genes in citrus have been demonstrated to be functionally equivalent to their counterparts in *A. thaliana* (Endo et al., 2005; Pillitteri et al., 2004a, b; Tan and Swain, 2007). Similar to the results in Satsuma mandarin, LT treatments promoting flowering in WNO increased the expression of *FT* in leaves and *SOC1*, *LFY* and *AP1* in buds (Chica and Albrigo, 2013a, b; Pillitteri et al., 2004a, b). The expression of *FT* and *SOC1* increased during the LT period; *LFY* and *AP1* expression occurred only after the trees were transferred to the warm condition (WC). For WNO, *SOC1*, *LFY* and *AP1* expression has been analyzed only in buds of trees that flowered in response to LT, their expression in buds of trees that did not flower under WC remains unknown (Chica and Albrigo, 2013a, b; Pillitteri et al., 2004a, b).

Gibberellic acid (GA_3) inhibits flowering in citrus by continuing vegetative development of the shoot apical meristem (SAM) when applied before the SAM is determined (irreversibly committed to floral development) (Lord and Eckard, 1987). Once the citrus bud is determined, coincident with sepal formation, exogenously applied GA_3 no longer has an inhibitory effect on flowering. The effect of GA_3 on flowering might be mediated by *FT*, since its expression in leaves of 'Salustiana' sweet orange (*C.*

sinensis) was reduced 8, 32 and 50 days after a single spray of GA_3 (40 mg L^{-1}), which also reduced inflorescence number, and was increased 8, 32, 50 and 80 days after one application of paclobutrazol (2,000 mg L^{-1}), a GA biosynthesis inhibitor, which restored flowering (Muñoz-Fambuena et al., 2012). A single application of GA_3 (40 mg L^{-1}) or four applications of GA_3 (150 mg L^{-1}), which both reduced flowering, did not change the expression of *SOC1* in leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012) or buds of 'Orri' mandarin (*C. reticulata* Blanco x *C. temple* Hort. ex Y. Tanaka) (Goldberg-Moeller et al., 2013), respectively. In addition, expression of *LFY* and *AP1* in leaves of 'Salustiana' sweet orange was not affected by a single application of GA_3 (40 mg L^{-1}) in December that reduced flowering in spring (Muñoz-Fambuena et al., 2012). In contrast, four applications of GA_3 (150 mg L^{-1}) made every 2 weeks starting in mid-November increased *LFY* expression but reduced *AP1* expression in the buds of 'Orri' mandarin trees in December and January and reduced flowering (Goldberg-Moeller et al., 2013). Further research is needed to clarify the possible roles of *LFY* and *AP1* in GA_3-inhibition of flowering in citrus given the differences in cultivars, tissues analyzed, times and rates of GA_3 applied, and environmental conditions in the two experiments.

Currently, there are striking omissions in the evaluation of the floral development pathway in citrus. Notably, with the exception of a *SEPALLATA1* (*SEP1*) gene in Satsuma mandarin (Nishikawa et al., 2009), no expression data have been published for the floral organ identity genes downstream from *AP1*. It is of relevance to this research that the activity of both class A organ identity genes *AP1* and *APETALA2* (*AP2*) is necessary for sepal formation in *A. thaliana* (Bowman et al., 1991; Coen and Meyerowitz,

1991; Krizek and Fletcher, 2005) and that sepal formation was identified as the developmental marker that coincided with the irreversible commitment to floral development in WNO (Lord and Eckard, 1987). Comparative analysis of the expression of these genes under LT and WC and in response to GA_3 treatment would enhance our understanding of the floral-promoting effect of LT and the floral-inhibitory effect of GA_3 and broaden our overall knowledge of floral development in citrus.

In this regard, the first objective of the research presented herein was to compare the effects of a floral-promoting LT treatment (15/10 ˚C, day/night for 2, 4 and 8 weeks followed by 24/19 ˚C, day/night for 9, 7 and 3 weeks, respectively) with those of an 11-week WC treatment (24/19 ˚C, day/night), which did not result in flowering, on the expression sequence of eight classic genes regulating floral timing, floral meristem identity, and floral organ identity in the buds of WNO in relation to differences in floral intensity. The second objective was to quantify the effects of weekly foliar applications of GA_3 in weeks 2 through 8 compared to GA_3 applications delayed to weeks 4 through 8 of the LT treatment on the expression of each of the eight floral genes and inflorescence number in order to identify the genes associated with floral inhibition by GA_3 in citrus. To the author's knowledge, this is the first report of changes in the expression of citrus *AP2*, *SEP1*, *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) during both successful flower formation in response to LT treatment and inhibition of floral development by GA_3.

Materials and Methods

Plant material and treatment conditions

Five-year-old mature WNO scions on 'Carrizo' citrange rootstock (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) grown in 56-liter pots containing steam-sterilized University of California soil mix I (Baker, 1957) were used in this research. All fruit were removed from the trees before the initiation of the experiment in September. The research used a complete randomized design with four WNO trees (replications) per treatment and six treatments. In treatments 1 through 3, trees were exposed to LT (16-hr day [500 μmol m^{-2} s^{-1}] at 15 ˚C/8-hr night at 10˚C) (Percival PGW growth chamber; 2.3 x 1.5 x 2.0 m; Percival, Boone, IA) for 2, 4 and 8 weeks, respectively, and then transferred to the WC (16-hr day [500 μmol m^{-2} s^{-1}] at 24˚C/8-hr night at 19˚C) for the remainder of the experiment culminating with bloom in week 11. In treatments 4 and 5, two sets of LT-treated trees were sprayed weekly with 50 mg L^{-1} GA_3 (ProGibb 40%, Valent BioScience Corporation, Libertyville, IL), containing 0.01% Silwet L77 surfactant (Helena Chemical Company, Collierville, TN), in weeks 2 through 8 and weeks 4 through 8, respectively, of the LT treatment. In treatment 6, trees were maintained in the WC for 11 weeks. All trees used in this research had been maintained under WC conditions for the five months prior to the start of the experiment. With the exception of temperature, all trees were treated the same, including irrigation time and amount, fertilization, and relative humidity (~ 80%).

Sample collection and gene expression analysis

The apical five buds from 15 nonbearing shoots per tree were collected at weeks 2, 4, 6, 8, 9, and 10 from each of the four trees (four replications) in treatments three through six, with the exception that sample collection for the two sets of GA_3-treated trees was delayed until 2 weeks after the first GA_3 application, respectively. Collected buds were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab. Bud samples were quickly frozen in liquid nitrogen and stored at -80 ˚C until analyzed. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequences of *A. thaliana* homologs *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). Citrus *FT*, *SOC1*, *LFY* and *AP1* genes analyzed in this research were *CiFT2* (Nishikawa et al., 2007), *CsSOC-like2* (*CsSL2*) (Tan and Swain, 2007), *CsLFY* and *CsAP1* (Pillitteri et al., 2004a), *PtAP2* (Song et al., 2010), *CuSEP1* (Nishikawa et al., 2009), respectively; each gene was

selected based on its demonstrated functional equivalence in its respective *A. thaliana* mutant. In addition, expression of *CiFT2* was related to floral intensity in response to low temperature in *C. unshiu* (Nishikawa et al., 2007); *CsSL2* expression was also related to flowering in field-grown *C. reticulata* (Shalom et al., 2012). The sequences of *PI* and *AG* chosen in this research share high identity with *A. thaliana PI* and *AG*, respectively; the predicted protein sequences for the putative *PI* and *AG* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com/primerquest/Home/Index) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study as well as the BLAST results of PCR product sequence versus target sequence of each gene of interest are listed in Table 2.1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-µL reaction volume containing 1.2 µL cDNA (about 40 ng of input RNA), 0.6 µL gene-specific forward and reverse primer mix (10nM), 7.5 µL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 µL PCR-grade water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was performed at the end of each qPCR run to confirm that nonspecific products were not

formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with WNO flowers collected from orchard trees at spring bloom as the control (expression level of 1) and *β-ACTIN* (*ACT*) as the primary reference gene (endogenous control), and reported herein. The selection of *ACT* as the primary reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Results based on a second reference gene, *ELONGATION FACTOR 1-ALPHA* (*EF1-α*) (Nishikawa et al., 2009), are presented as supplemental results (Table S2.1). Treatment effects on the expression of all target genes were similar using *ACT* or *EF1-α* as the reference gene. The expression pattern of each floral gene with *ACT* as the endogenous control was strongly correlated with its expression pattern when *EF1-α* served as the reference gene (r = 0.71 to 0.99, $P < 0.001$ for all genes), confirming the consistency and reliability of the results. Gene expression data for each treatment and sample date were the mean of four biological replicates; each biological replicate was the mean of three qPCR technical replicates.

Treatment effects on bud development

Maximum bloom occurred in trees exposed to 8 weeks of LT at week 11. At this time, the fate of the apical five buds on each of the 15 randomly selected nonbearing shoots per tree was determined as the number of leafless (one to many flowers with no leaves), leafy (one to many flowers with one to many leaves), and total inflorescences (sum of leafless plus leafy inflorescences), vegetative shoots, and inactive (dormant) buds for trees in all

treatments. Results for the five apical buds on the 15 shoots per tree were averaged for the four individual trees (replications) per treatment and reported as the average value per tree.

Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots and inactive buds per tree and the relative expression levels of genes, using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$. Pearson's correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $P \leq 0.05$) between the duration of the low temperature treatment and the developmental fate of buds and between gene expression level and inflorescence number, respectively. Significant correlations were subjected to regression analyses, using the least squares method for the generalized linear model.

Results

Effects of low temperature and GA_3 on flowering

WNO trees maintained in the WC for 11 weeks produced an average of only 0.8 total inflorescence per tree (based in all cases on 5 buds/15 shoots/4 trees/treatment) (Table 2.2). Thus, the majority of the buds collected and analyzed in this research were not committed to floral development at the initiation of the experiment. Two weeks of LT

treatment resulted in a non-significant increase to 2.3 total inflorescences per tree, whereas 4 weeks of LT resulted in 17.0 total inflorescences per tree, which was significantly greater than trees receiving 0 or 2 weeks of LT treatment ($P < 0.0001$). Trees exposed to 8 weeks of LT produced significantly more inflorescences (63 inflorescences/tree) than trees in all other treatments ($P < 0.0001$). There was a significant positive correlation between the duration of the LT period and the number of inflorescences produced per tree ($r = 0.95$, $P < 0.0001$) (Figure 2.1). The duration of the LT period explained 91% of the variation in inflorescence number. The number of leafy inflorescences was also significantly (positively) correlated with the duration of the LT treatment ($r = 0.82$, $P < 0.001$). Moreover, leafy inflorescences dominated bloom, comprising 66%, 89% and 74% of total inflorescences produced by trees exposed to 0, 2 and 4 weeks of LT, respectively (Table 2.2). In contrast, leafless inflorescences were only produced in significant number by trees receiving 8 weeks of LT. Thus, the number of leafless inflorescences was not as strongly correlated with the duration of the LT treatment ($r = 0.75$, $P < 0.001$) as leafy inflorescences ($r = 0.82$, $P < 0.001$). The total number of inflorescences produced by trees receiving 8 weeks of LT treatment was reduced 96% when trees were treated with six foliar applications of GA_3 in weeks 2 through 8, resulting in only 2.3 inflorescences per tree, a number equal to that of trees maintained in the WC for 11 weeks ($P < 0.0001$) (Table 2.2). When the GA_3 treatment was restricted to four applications in weeks 4 through 8, floral intensity was 22.5 inflorescences per tree, a value intermediate to trees exposed to 8 weeks of LT without and with six weekly applications of GA_3. The floral response to the four applications of

GA_3 was equal to that of trees exposed to 4 weeks of LT without GA_3 treatment but significantly greater than trees maintained in the WC for 11 weeks ($P < 0.0001$). Taken together, the results suggest that 4 weeks of LT treatment are sufficient for a significant proportion (23-30%) of the buds to become committed to floral development.

The length of the LT treatment had no effect on the number of vegetative shoots produced per tree (Table 2.2). Trees exposed to 8 weeks of LT or 11 weeks of WC produced an equivalent number of vegetative shoots. In contrast, application of GA_3 during the LT treatment significantly increased the number of vegetative shoots produced per tree ($P < 0.0001$) (Table 2.2). Six applications of GA_3 starting in week 2 of the LT treatment resulted in the greatest number of vegetative shoots (24) per tree ($P < 0.0001$). When GA_3 application was delayed to weeks 4 through 8, vegetative shoot number per tree was reduced by 50% ($P < 0.0001$), with a concomitant increase in inflorescence number. These results are consistent with a proportion of the bud population being committed to floral development by week 4 of LT treatment.

For trees receiving 11 weeks of WC or only 2 weeks of LT, the majority (73.8 and 70.0, respectively) of the 75 buds observed per tree remained inactive (dormant) (Table 2.2). There was a progressive and significant decrease in the number of inactive buds per tree related to the increase in the number of weeks at LT, e.g., 55.3 and 11.0 buds remained inactive per tree after 4 and 8 weeks of LT treatment, respectively ($P < 0.0001$) (Table 2.2). The number of inactive buds was significantly (negatively) correlated with the duration of the LT treatment ($r = -0.96$, $P < 0.001$) (Figure 2.1), with the duration of the LT treatment explaining 93% of the variation in the number of inactive buds per tree.

The negative relationship between LT and the number of inactive buds was largely due to the positive effect of LT on inflorescence development. The number of inactive buds was significantly (negatively) correlated with the total number of inflorescences per tree across all treatments, including GA_3 (r = -0.99, $P < 0.0001$), but not with vegetative shoot number (r = -0.20, P = 0.464).

Effects of warm and low temperatures and GA_3 on the expression of citrus floral timing genes

Transcripts of *FT* were detected in buds of trees maintained in the WC for 11 weeks on all sampling dates, except week 8 (Table 2.3). Expression of *FT* fluctuated significantly across the six sample dates ($P < 0.05$). For buds of LT-treated trees, *FT* expression was significantly greater only at week 2 of the LT treatment compared to trees in the WC treatment ($P < 0.05$). Four or six weekly applications of GA_3 to LT-treated trees had no significant effect on *FT* transcript levels on any sampling data (Table 2.3). Similarly, *SOC1* expression occurred in buds of trees in the WC on each of the six sample dates. Buds of LT-treated trees also had significantly greater *SOC1* expression by week 2 compared to the WC-treated trees ($P < 0.05$) (Table 2.3). Bud *SOC1* expression, although elevated, was not significantly greater during weeks 4 through 9 for LT-treated trees than trees in the 11-week WC treatment. Four or six weekly applications of GA_3 to LT-treated trees did not significantly affect *SOC1* expression (Table 2.3). The expression of *LFY* was also detected in buds of trees in the WC for 11 weeks on all sampling dates except at week 8, with *LFY* expression significantly greater by week 10 of the 11-week

WC treatment than all previous weeks ($P < 0.05$) (Table 2.3). For buds of LT-treated trees, *LFY* expression reached a maximum after transfer to the WC in week 9 ($P < 0.05$), but was never significantly greater than *LFY* expression in buds of trees in the 11-week WC treatment. Neither GA_3 treatment had a significant effect on *LFY* expression (Table 2.3). The values reported for *LFY* expression in Table 2.3 are high because *LFY* expression was very low in the WNO flower, which served as the control (expression level of 1). This was not the case for *FT* or *SOC1*. To assess the validity of the results reported for the treatment effects on *LFY* expression, the data were also analyzed using buds collected from trees at the start of the experiment (time zero). The expression levels for *LFY* were reduced, but no substantive differences in the data resulted. It should be noted that *FT*, *SOC1* and *LFY* transcripts were present in WNO buds at the initiation of the experiment in September, despite the trees being maintained under the WC for the previous five months. Total inflorescence number was not significantly related to the expression pattern of any floral timing gene across all treatments on any sampling date.

Effects of warm and low temperatures and GA_3 on the expression of citrus genes having class A activity

For trees in the 11-week WC treatment, bud *AP1* expression decreased over time, resulting in significantly greater expression during weeks 2 through 6 than weeks 8 through 10 ($P < 0.001$) (Table 2.4). In contrast, *AP1* expression did not change in the buds of LT-treated trees from week 2 through 10. As a result, transcript levels of *AP1* in buds of LT-treated trees were 7-, 5-, 3-fold greater than those of WC-treated trees in

weeks 8 ($P < 0.001$), 9 ($P < 0.01$) and 10 ($P < 0.05$), respectively. Six foliar applications of GA_3 during weeks 2 through 8 of the LT treatment significantly reduced bud *AP1* expression by 54%, 57% and 46% during weeks 8 ($P < 0.001$), 9 ($P < 0.01$) and 10 ($P < 0.05$), respectively, relative to LT-treated trees not receiving GA_3 (Table 2.4). Reducing the GA_3 treatment to four applications in weeks 4 through 8 resulted in *AP1* transcript levels that were not significantly different from those of trees receiving six GA_3 applications (Table 2.4). For weeks 8 and 10, both GA_3 treatments reduced *AP1* expression to a value significantly less than that of the 8-week LT-treated trees not receiving a GA_3 treatment, but still significantly greater than that of 11-week WC-treated trees. Bud *AP2* expression was more than 3- to 10-fold lower than *AP1* expression over the six sample dates for trees receiving 11 weeks of WC, despite a significant increase in *AP2* expression after week 4 of the WC treatment ($P < 0.01$) (Table 2.4). Bud *AP2* expression in 8-week LT-treated trees significantly increased from week 2 through 10 ($P < 0.0001$) to levels greater than that of the WC-treated trees on all sampling dates except weeks 2 and 6 ($P < 0.05$). For the 8-week LT-treated trees, maximum *AP2* expression occurred in week 10, after transfer of the trees to the WC, and was significantly greater than all other treatments ($P < 0.001$). Six foliar applications of GA_3 during weeks 2 through 8 of the LT treatment reduced *AP2* expression in weeks 4 and 6 ($P \leq 0.05$) and again in week 10 ($P < 0.001$) compared to LT-treated trees not receiving GA_3 (Table 2.4). Similarly, four applications of GA_3 in weeks 4 through 8 of the LT treatment reduced *AP2* expression in weeks 6 and 8 ($P \leq 0.05$) and again in week 10 ($P < 0.001$) relative to *AP2* expression in buds of LT-treated trees not treated with GA_3. Interestingly, for trees

receiving four applications of GA_3 in weeks 4 through 8, bud expression of both *AP1* and *AP2* significantly increased after transfer of the trees from LT to the WC ($P < 0.05$) (Table 2.4). Inflorescence number was strongly correlated across all treatments with the expression patterns of *AP1* at weeks 8 ($r = 0.91$, $P < 0.001$), 9 ($r = 0.73$, $P = 0.001$) and 10 ($r = 0.84$, $P < 0.001$) and *AP2* at weeks 8 ($r = 0.64$, $P = 0.015$), 9 ($r = 0.66$, $P = 0.008$) and 10 ($r = 0.94$, $P < 0.001$).

Effects of warm and low temperatures and GA_3 on the expression of citrus floral organ identity genes downstream from AP2

Transcripts of *SEP1* were detected at very low levels in weeks 2 and 4 and not detected thereafter in the buds of trees receiving 11 weeks of WC (Table 2.5). For buds of trees exposed to 8 weeks of LT, *SEP1* was not expressed at significant levels until week 8, with *SEP1* expression increasing significantly after transfer of the trees from LT to the WC ($P < 0.01$). This significant increase in *SEP1* expression after transfer of LT-treated trees to the WC failed to occur in buds of LT-treated trees receiving six weekly GA_3 applications during weeks 2 through 8 (Table 2.5). In contrast, for buds of LT-treated trees receiving only four GA_3 applications during weeks 4 through 8, *SEP1* expression occurred during the 2 weeks after transfer to the WC. Transcripts of *PI* were never detected in buds of trees maintained in the WC for 11 weeks, except for a very low detectable transcript level in week 8. For 8-week LT-treated trees, *PI* transcripts were only expressed at significant levels in buds after the trees were transferred from the LT to the WC, with the value 2-fold greater in week 10 than week 9 (Table 2.5). As observed

for *SEP1*, six applications of GA_3 from week 2 through 8 of the LT treatment blocked the increase in *PI* expression that occurred after LT-treated trees were transferred to the WC, whereas four applications of GA_3 in weeks 4 through 8 of the LT treatment resulted in *PI* expression after the trees were transferred from the LT to the WC. Thus, by week 10, *PI* expression was greater than that of trees in 11-week WC treatment, but significantly lower than 8-week LT-treated trees ($P < 0.01$). Transcripts of *AG* were below the limit of detection in weeks 2 through 8 and its expression occurred at very low levels during weeks 9 and 10 in buds of trees in the 11-week WC treatment (Table 2.5). For buds of trees exposed to 8 weeks of LT, *AG* was expressed at low levels in weeks 4 and 8 of the LT treatment. Expression of *AG* increased after the trees were transferred from the LT to the WC ($P < 0.0001$) to a maximum value at week 10 that was significantly greater than that of trees in all other treatments ($P < 0.001$). Expression of *AG* in buds of trees receiving 8 weeks of LT with six weekly applications of GA_3 during weeks 2 through 8 was significantly reduced by week 10 to a level less than trees receiving 8 weeks of LT and equal to trees receiving 11 weeks of the WC ($P < 0.01$) (Table 2.5). In contrast, four GA_3 applications in weeks 4 through 8 of the LT treatment reduced *AG* expression by week 10 to a value significantly less than that of 8-week LT-treated trees not treated with GA_3, but significantly greater than that of 11-week WC-treated trees ($P < 0.001$). Inflorescence number was strongly correlated across all treatments with the expression patterns of *SEP1* at weeks 9 ($r = 0.94$, $P < 0.001$) and 10 ($r = 0.85$, $P < 0.001$), *PI* at weeks 9 ($r = 0.77$, $P < 0.001$) and 10 ($r = 0.98$, $P < 0.001$) and *AG* at weeks 9 ($r = 0.63$, $P = 0.012$) and 10 ($r = 0.90$, $P < 0.001$).

Discussion

The research reported herein was the first to compare the relative expression of floral timing genes, floral meristem identity genes, and floral organ identity genes in the buds of citrus trees grown for a prolonged period (~eight months) under warm temperature conditions (24 ˚C day/19 ˚C night). In light of the fact that these trees did not flower (< 1 inflorescence/75 buds/tree), it is of significant interest that buds of WNO trees expressed *FT*, *SOC1*, *LFY*, *AP1* and *AP2* (at a low level) at the start of the experiment (September) and throughout the 11 weeks of WC treatment. In *A. thaliana*, expression of *LFY* and *AP1* is one of the first indications that the SAM has been induced to flower (Melzer et al., 1999). Based on the results of Chico and Albrigo (2013b) demonstrating that a drop in night temperature below 20 ˚C increased *FT* expression in leaves, it is possible that prolonged exposure to average night temperatures of 19 ˚C was sufficient to support the initial stages of floral development but not to achieve flowering. For buds of WC-treated and LT-treated trees, *FT*, *SOC1* and *LFY* expression patterns were variable over time. With the exception of *FT* and *SOC1* expression in week 2, no significant differences in expression were observed between the two treatments during the 11 weeks. Importantly, WC-treated trees never expressed *SEP1* or *PI* at significant levels, and *AG* expression was present only at a very low level during weeks 9 and 10. It should also be noted that for the 11-week WC-treated trees, bud *AP1* transcript levels significantly decreased over time ($P < 0.001$) and *AP2* transcript levels remained very low over the 11 weeks. In contrast, the 8-week LT-treated trees maintained bud *AP1* transcript levels at a constant high level and bud *AP2* transcript levels significantly increased over time starting at week

6 of the LT treatment ($P < 0.0001$). As a result, *AP1* and *AP2* expression were significantly greater in buds of 8-week LT-treated trees than WC-treated trees for weeks 8, 9 and 10 prior to flowering in week 11. Expression of *SEP1*, *PI* and *AG* at significant levels was only observed in buds from 8-week LT-treated trees and only after transfer of the trees to the WC (weeks 9 and 10) just prior to flowering.

In this research, as previously reported, floral intensity increased with the duration of LT treatment (Lovatt et al., 1988; Moss 1976; Nishikawa et al., 2007; Southwick and Davenport, 1986), from a low of 0.8 inflorescence per 75 buds per tree with no LT treatment (11 weeks of WC) to a high of 63 inflorescences per 75 buds per tree for 8-week LT-treated WNO trees, with 4 weeks of LT treatment sufficient to promote moderate flowering (17 inflorescences/75 buds/tree) in WNO (Lovatt et al., 1988; Moss, 1976). By comparing the floral intensity of trees exposed to only 2 and 4 weeks of LT treatment with that of trees receiving weekly foliar applications of GA_3 starting in weeks 2 and 4 of the 8-week LT treatment, respectively, the results of the current research provided evidence that GA_3 blocked floral development in buds that were not determined (irreversibly committed to floral development) at the time of application as proposed by Lord and Eckard (1987). For example, for trees receiving 2 weeks of LT treatment, only 3% of the bud population completed floral development (2.25 inflorescences/75 buds/tree). Initiating weekly GA_3 applications in week 2 of the 8-week LT treatment blocked floral development in all buds except the 3% that were determined by week 2 of the LT treatment, resulting in 2.25 inflorescences per 75 buds per tree. Similarly, 4 weeks of LT treatment were sufficient for ~25% of the bud population to be determined (17

inflorescences/75 buds/tree). When weekly applications of GA_3 were delayed to week 4 of the LT treatment, the trees produced 22.5 inflorescences per 75 buds per tree. The capacity of some buds, but not others, to flower after only 2 or 4 weeks of LT suggests that shoot age might be a factor in the response of citrus buds to conditions that promote flower formation.

In light of the above effects of GA_3 on WNO floral intensity, the results of this research clearly indicated that expression of *FT*, *SOC1* and *LFY* was not sufficient for bud determinacy. Both GA_3 treatments significantly reduced inflorescence number (96% and 60% when applied in weeks 2-8 and 4-8 of the LT treatment, respectively), but neither GA_3 treatment had an effect on *FT*, *SOC1* or *LFY* expression, demonstrating that the negative effect of GA_3 on citrus flowering was not mediated through expression of *FT*, *SOC1* or *LFY* in buds of WNO trees. The results are in sharp contrast to the analysis of *FT* in leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012), but are consistent with the results of studies analyzing *SOC1* and *LFY* expression in buds of 'Orri' mandarin (Goldberg-Moeller et al., 2013) and leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012).

Importantly, the results demonstrated that *AP1* and *AP2* were the earliest genes in the floral development pathway for which bud transcript levels were responsive to both temperature and GA_3 treatments in a manner strongly related to inflorescence number. First, there were significant differences in the expression patterns of both *AP1* and *AP2* in buds of 11-week WC-treated trees compared to those from 8-week LT-treated trees during weeks 8, 9 and 10 that correlated with floral intensity. Second, weekly foliar

applications of GA_3 in weeks 2 through 8 of the LT treatment reduced the expression of *AP1* in weeks 9 and 10 and *AP2* in week 10 to levels equaling those of 11-week WC-treated trees with a concomitant decrease in floral intensity (from 63 to 2.25 inflorescences/tree for 8-week LT-treated trees without and with GA_3 treatment, respectively). Third, delaying the weekly GA_3 applications to weeks 4 through 8 of the LT treatment resulted in intermediate flowering (22.5 inflorescences/tree) with bud *AP1* and *AP2* expression intermediate to that of the 8-week LT-treated trees and 11-week WC-treated trees in weeks 8 and 9 and weeks 8 and 10, respectively. Inflorescence number was most strongly correlated across all treatments with the expression patterns of *AP1* at week 8 ($r = 0.91$, $P < 0.001$) and *AP2* at week 10 ($r = 0.94$, $P < 0.001$). Goldberg-Moeller et al. (2013) previously reported that a GA_3 treatment that reduced flowering in 'Orri' mandarin also reduced bud *AP1* expression, with no negative effect on *LFY* expression. Taken together, the results provide compelling evidence that expression of *AP1* and *AP2* to the levels achieved by 8 weeks of LT treatment resulted in bud determinacy in WNO. Removal of the LT stimulus upon transfer of the trees to WC at the end of week 8 did not interfere with the continued accumulation of *AP1* and *AP2* transcripts or the increased expression of the downstream floral organ identity genes, resulting in flowering. The low transcript levels of *AP1* and *AP2* observed in buds of 11-week WC treated trees were apparently insufficient to activate *SEP1* or *PI* expression or to increase *AG* expression and thus, flowering did not occur.

Consistent with *AP1* and *AP2* regulating bud determinacy, the floral organ identity genes downstream from *AP2* were only expressed in buds of 8-week LT-treated WNO

trees and only at significant levels after transfer to the WC (weeks 9 and 10). Moreover, weekly GA_3 applications in weeks 2 through 8 of the LT treatment, which reduced the levels *AP1* and *AP2* expression to those of WC-treated trees in weeks 9 and 10 and week 10, respectively, repressed *SEP1* and *PI* transcription to the limit of detection or below it, and reduced *AG* expression to that of the 11-week WC-treated trees. Delaying the weekly GA_3 applications to week 4 reduced bud expression of *SEP1*, *PI* and *AG* after transfer of the trees to the WC to levels intermediate to those of the 8-week LT-treated and 11-week WC-treated trees, which paralleled the changes in *AP1* and *AP2* expression, with an equivalent effect on floral intensity. Across all treatments, inflorescence number was strongly correlated with the expression of *SEP1*, *PI* and *AG* in weeks 9 and 10 ($r \geq 0.90$, $P < 0.001$ for the three genes). Taken together, the parallel relationships among bud expression of *AP1* and *AP2* and expression of the downstream floral organ identity genes *SEP1*, *PI* and *AG*, and the resulting inflorescence number strongly suggest that inhibition of citrus floral development by GA_3 is mediated through *AP1* and *AP2,* providing further evidence that the two genes control the developmental fate of WNO buds. This interpretation is supported by the fact that the two GA_3 treatments reduced inflorescence number with a reciprocal increase in vegetative shoot number and no significant effect on the number of inactive (dormant) buds (i.e., the GA_3 treatments did not increase bud break). Two points are of interest here. First, according to the ABCE model for floral organ specification, expression of both class A genes, *AP1* and *AP2*, is required in *A. thaliana* for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). Second, sepal formation was the developmental marker coincident

with irreversible commitment to floral development in WNO identified by Lord and Eckard (1987). Based on the ABCE model, *SEP1* gene activity might additionally be required for sepal formation (Ditta et al., 2004; Krizek and Fletcher, 2005; Pelaz et al., 2000). The *A. thaliana SEP1* homolog in Satsuma mandarin was expressed 4 weeks before flowering in field-grown trees (Nishikawa et al., 2009) and, in this study, was first expressed in week 8 of the LT-treatment, 3 weeks before flowering in WNO. The results are consistent with the role of *SEP1* in the early stages of flower formation in citrus.

The results of this research do not preclude the possibility that GA_3 regulates the expression of each floral organ identity gene independently. Further, research utilizing the treatments presented herein but delaying single or weekly GA_3 applications to weeks 6 or 7 through 8 and after transfer of the 8-week LT-treated trees to the WC is required to confirm that bud expression of *AP1* alone or together with *AP2* is required for the citrus bud to be determined and to test for possible independent control of *SEP1*, *PI* or *AG* by GA_3. The results suggest that *AP1*, which responded to both GA_3 treatments initially in week 8, and *AP2*, which responded to GA_3 two weeks after each application, are independently regulated by GA_3. The results also leave open the possibility of post-transcriptional regulation of the floral genes in response to GA_3 and/or LT. In support of this possibility, it was recently reported (Shalom et al., 2015) that citrus buds expressed a member of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL*), an *A. thaliana* gene family of transcription factors related to floral transition, flower and fruit development, and GA signaling, with members that are post-transcriptionally regulated by microRNAs (miRNAs) (Yu et al., 2012). Shalom et al. (2015) demonstrated that

removal of fruit, a potential source of GA, from 'Murcott' mandarin (*C. reticulata*) trees increased bud expression of a *SPL* gene, miRNA172 activity, and flowering. Additional research is necessary to clarify the regulatory mechanisms associated with *SPL* transcription factors in citrus, especially in relation to miRNAs, GA signaling, and flowering.

The results of the research reported here demonstrated for the first time that temperature conditions that did not support flowering were sufficient to initiate floral development in WNO, i.e., *FT*, *SOC1*, *LFY*, *AP1* and *AP2* were expressed in buds of WNO trees during 11 weeks of WC that did not result in flowering. The results confirmed that the SAM of buds that produced vegetative or floral shoots or remained inactive were indistinguishable at the level of floral gene expression during the early stages of development, consistent with the possibility that all buds of adult citrus trees are competent and have been induced to flower as a result of the transition from juvenile to adult (Tan and Swain, 2006). An alternative possibility, given that *FT*, *SOC1*, *LFY*, *AP1* and *AP2* were already expressed in WNO buds collected in September from trees maintained under WC for five months, is that transition from vegetative to reproductive SAM occurs annually, but much earlier and through a different pathway than classic low temperature induction in fall (Nishikawa et al., 2009), possibly during the summer at the end of the summer vegetative shoot flush. This proposal is consistent with age-dependent (autonomous) flowering in citrus (Shalom et al., 2015; Zhang and Hu, 2013) and with citrus being developmentally similar to other evergreen perennial tree crops, such as avocado (*Persea americana* Mill.) (Salazar-García et al., 1998) and olive (*Olea europaea*

L.) (Cuevas et al., 1999), known to transition from vegetative to reproductive development in summer (July). In olive, floral and vegetative buds can only be distinguished by an increase in floral bud size in November; no differences in the SAM were observed until the initiation of the sepals at bud break in February. This is analogous to WNO, for which floral and vegetative resting buds remained indistinguishable until November when resting buds of floral shoots underwent microscopic bud break compared to December for the vegetative shoot resting buds, with the SAM of each bud remaining indistinguishable until sepals formed in January (Lord and Eckard, 1985).

For WNO, the earliest detectable differences between buds that produced inflorescences and those that did not were the significantly greater transcript levels of *AP1* and *AP2* observed for floral buds by week 8 of the LT-treated trees and after transfer of the trees to the WC. The results provided evidence suggesting that the greater expression of *AP1* and *AP2*, which are necessary for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005), conferred determinacy and upregulated the expression of the downstream floral organ identity genes *SEP1*, *PI* and *AG* in the buds of the 8-week LT-treated trees, resulting in maximum flowering. Significant expression of *SEP1*, *PI* and *AG* only occurred after the 8-week LT-treated trees were transferred to the WC, suggesting a possible mechanism to synchronize flowering with the warmer temperatures of spring. For WNO, sepal formation was previously identified as the developmental marker indicating irreversible commitment to floral development and delineating when GA_3 application no longer inhibited floral

development (Lord and Eckard, 1987). This result, taken together with the results of the current research demonstrating that *AP1* and *AP2* expression were reduced by GA_3 applied during early floral development, suggests that normal expression of *AP1* and *AP2* would negate the inhibitory effect of GA_3 on flowering in citrus, a possibility that remains to be tested. Taken together, the results suggest that all adult citrus buds are competent and have been induced to flower or alternatively, that annual transition from vegetative to floral development occurs earlier than fall, but in either case, prevailing local environmental or physiological conditions (shoot age, bud endogenous GA concentration, warm temperature, insufficient exposure to low temperature, lack of water-deficit stress, or presence of fruit) prevent adequate expression of *AP1* and *AP2* for the SAM to become determined and to activate the downstream floral organ identity genes necessary for flower development. The results documented that LT promoted flower formation but also served as an inhibitor of floral organ development subsequent to sepal formation to prevent flower production under adverse climate conditions.

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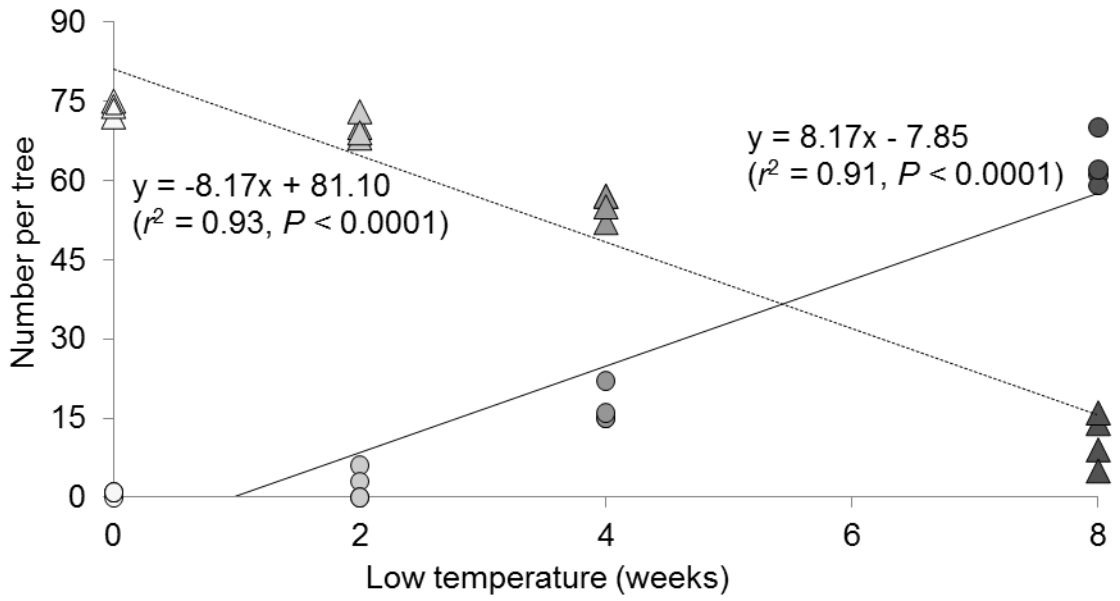

Figure 2.1 Effect of low temperature (LT) (15/10 ˚C, day/night) on the number of inflorescences (– ● –) and inactive buds (-- ▲ --) of 'Washington' navel orange trees exposed to 2, 4, 6 and 8 weeks of LT and transferred to the warm condition (WC) (24/19 ˚C, day/night) for 9, 7, 5 and 3 weeks, respectively; trees receiving no LT treatment remained in the WC for 11 weeks. Data are the means of five apical buds per 15 shoots per tree averaged across four trees per treatment.

Table 2.1 Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence BLAST against target gene sequence	
				E-value	Identity
FT	AB027456.1 (*C. unshiu*)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
SOC1	EU032532.1 (*C. sinensis*)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
LFY	AY338976.1 (*C. sinensis*)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
AP1	AY338974.1 (*C. sinensis*)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
AP2	EU883665.1 (*C. trifoliata*)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEP1	AB329715.1 (*C. unshiu*)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PI	XM_ 006472790.1 (*C. sinensis*)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AG	HM246523.1 (*C. sinensis*)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov).

Table 2.2 Developmental fate of buds of 'Washington' navel orange trees exposed to 2, 4 and 8 weeks of low temperature (LT) (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[z].

LT	WC	GA_3	Inflorescences (no./tree)			Vegetative shoots (no./tree)	Inactive buds (no./tree)
			Total	Leafless	Leafy		
0 wk	11 wks	No GA_3	0.8 c	0.3 b	0.5 c	0.5 c	73.8 a
2 wks	9 wks	No GA_3	2.3 c	0.3 b	2.0 c	2.8 c	70.0 a
4 wks	7 wks	No GA_3	17.0 b	4.5 b	12.5 bc	2.8 c	55.3 b
8 wks	3 wks	No GA_3	63.0 a	31.5 a	31.5 a	1.0 c	11.0 d
8 wks	3 wks	Wk 2 to 8	2.3 c	0.3 b	2.0 c	24.0 a	48.8 bc
8 wks	3 wks	Wk 4 to 8	22.5 b	3.8 b	18.8 ab	11.8 b	40.8 c
P-value			****	****	****	****	****

[z] Data are the means for four trees (5 apical buds/15 shoots/tree) per treatment. Means followed by different letters within a vertical column are significantly different according to Fisher's least significant difference (LSD) test in which ** refers to a significant effect at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

Table 2.3 Expression of *FT*, *SOC1* and *LFY* in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[y].

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
FT expression									
0 wks	11 wks	No GA_3	0.2 b^{B}	0.8 a^{B}	7.8 a^{A}	ND[z]	4.3 a^{AB}	5.6 a^{A}	*
8 wks	3 wks	No GA_3	1.5 a^{A}	9.2 a^{A}	4.6 a^{A}	9.2 a^{A}	19.0 a^{A}	2.7 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		3.0 a^{A}	1.4 a^{A}	0.9 a^{A}	18.9 a^{A}	1.7 a^{A}	ns
8 wks	3 wks	Wk 4 to 8			1.1 a^{B}	14.4 a^{A}	3.6 a^{B}	3.5 a^{B}	**
P-value			*	ns	ns	ns	ns	ns	
SOC1 expression									
0 wks	11 wks	No GA_3	3.4 b^{C}	3.0 a^{C}	15.9 a^{A}	6.6 a^{BC}	8.4 a^{BC}	11.1 a^{AB}	**
8 wks	3 wks	No GA_3	5.0 a^{C}	20.8 a^{AB}	22.6 a^{A}	16.0 a^{ABC}	23.0 a^{A}	10.5 a^{BC}	*
8 wks	3 wks	Wk 2 to 8		13.4 a^{A}	10.8 a^{A}	14.3 a^{A}	18.9 a^{A}	7.9 a^{A}	ns
8 wks	3 wks	Wk 4 to 8			14.8 a^{A}	11.9 a^{A}	13.7 a^{A}	7.4 a^{A}	ns
P-value			*	ns	ns	ns	ns	ns	
LFY expression									
0 wks	11 wks	No GA_3	6.0 a^{B}	8.0 a^{B}	475.7 a^{B}	ND	537.0 a^{B}	1662.9 a^{A}	*
8 wks	3 wks	No GA_3	4.6 a^{B}	425.2 a^{B}	120.4 a^{B}	681.0 a^{B}	1859.9 a^{A}	364.3 a^{B}	*
8 wks	3 wks	Wk 2 to 8		376.1 a^{A}	104.1 a^{A}	264.1 a^{A}	3951.3 a^{A}	313.2 a^{A}	ns
8 wks	3 wks	Wk 4 to 8			ND	1511.7 a^{A}	514.9 a^{A}	580.9 a^{A}	ns
P-value			ns	ns	ns	ns	ns	ns	

[y] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$; ns, not significant.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 2.4 Expression of *AP1* and *AP2* in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[z].

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
AP1 expression									
0 wks	11 wks	No GA_3	7.3 a^{A}	7.7 a^{A}	7.8 a^{A}	2.2 c^{B}	3.7 c^{B}	4.3 b^{B}	***
8 wks	3 wks	No GA_3	14.9 a^{A}	11.6 a^{A}	13.9 a^{A}	16.0 a^{A}	21.3 a^{A}	13.4 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		8.0 a^{A}	5.9 a^{A}	7.4 b^{A}	9.1 bc^{A}	7.2 b^{A}	ns
8 wks	3 wks	Wk 4 to 8			7.0 a^{B}	10.2 b^{B}	16.5 ab^{A}	7.3 b^{B}	*
P-value			ns	ns	ns	***	**	*	
AP2 expression									
0 wks	11 wks	No GA_3	0.2 a^{CD}	0.2 b^{D}	0.6 ab^{A}	0.4 c^{BC}	0.4 b^{BC}	0.4 c^{AB}	**
8 wks	3 wks	No GA_3	0.2 a^{E}	1.4 a^{C}	0.9 a^{D}	1.5 a^{C}	2.6 a^{B}	4.1 a^{A}	****
8 wks	3 wks	Wk 2 to 8		0.8 ab^{BC}	0.5 b^{C}	1.3 ab^{AB}	1.9 a^{A}	0.7 c^{C}	**
8 wks	3 wks	Wk 4 to 8			0.5 b^{B}	1.1 b^{B}	2.4 a^{A}	2.6 b^{A}	*
P-value			ns	*	*	***	*	***	

[z] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P \leq 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

Table 2.5 Expression of *SEP1*, *PI* and *AG* in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[x].

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
SEP1 expression									
0 wks	11 wks	No GA_3	< 0.05[y]	< 0.05	ND[z]	ND	ND	ND	--
8 wks	3 wks	No GA_3	< 0.05	ND	ND	0.1 a^C	1.9 a^B	5.0 a^A	**
8 wks	3 wks	Wk 2 to 8		ND	ND	0.1 a	ND	ND	--
8 wks	3 wks	Wk 4 to 8			ND	ND	1.0 a^A	3.5 a^A	ns
P-value			--	--	--	ns	ns	ns	
PI expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	ND	--
8 wks	3 wks	No GA_3	ND	ND	< 0.05	ND	1.6 a^A	3.2 a^A	ns
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	ND	< 0.05	--
8 wks	3 wks	Wk 4 to 8			< 0.05	< 0.05	0.8 a^A	1.2 b^A	ns
P-value			--	--	--	--	ns	**	
AG expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	0.1 a^A	0.1 c^A	ns
8 wks	3 wks	No GA_3	ND	0.1 a^B	ND	0.1 a^B	2.1 a^B	10.8 a^A	***
8 wks	3 wks	Wk 2 to 8		0.1 a^A	ND	D	1.1 a^A	0.1 c^A	ns
8 wks	3 wks	Wk 4 to 8			ND	0.1 a^B	1.7 a^B	5.5 b^A	*
P-value			ns	ns	ns	ns	ns	***	

[x] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the four biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Supplemental Results to Chapter 2

Table S2.1 Expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* relative to *EF1-α* expression in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[x].

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
FT expression									
0 wks	11 wks	No GA_3	0.1 b^{A}	0.4 a^{A}	2.3 a^{A}	ND[y]	1.2 a^{A}	1.3 a^{A}	ns
8 wks	3 wks	No GA_3	0.7 a^{A}	2.3 a^{A}	1.8 a^{A}	2.5 a^{A}	3.9 a^{A}	1.0 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		1.5 a^{A}	0.7 a^{A}	0.3 a^{A}	2.3 a^{A}	0.4 a^{A}	ns
8 wks	3 wks	Wk 4 to 8			0.4 a^{B}	4.0 a^{A}	1.6 a^{B}	0.9 a^{B}	*
P-value			*	ns	ns	ns	ns	ns	
SOC1 expression									
0 wks	11 wks	No GA_3	1.4 a^{B}	1.6 a^{B}	6.0 a^{A}	2.3 a^{B}	2.5 a^{B}	2.5 b^{B}	*
8 wks	3 wks	No GA_3	2.4 a^{A}	6.4 a^{A}	9.7 a^{A}	3.8 a^{A}	6.5 a^{A}	5.0 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		9.3 a^{A}	4.7 a^{B}	2.9 a^{B}	3.0 a^{B}	2.7 b^{B}	*
8 wks	3 wks	Wk 4 to 8			4.9 a^{A}	2.7 a^{B}	5.1 a^{A}	2.0 b^{B}	*
P-value			ns	ns	ns	ns	ns	*	
LFY expression									
0 wks	11 wks	No GA_3	2.6 a^{B}	8.1 a^{B}	71.0 a^{B}	ND	198.7 a^{B}	784.0 a^{A}	*
8 wks	3 wks	No GA_3	2.1 a^{C}	30.4 a^{BC}	88.9 a^{BC}	255.4 a^{B}	527.6 a^{A}	127.7 b^{BC}	**
8 wks	3 wks	Wk 2 to 8		56.1 a^{A}	78.1 a^{A}	99.0 a^{A}	613.7 a^{A}	91.3 b^{A}	ns
8 wks	3 wks	Wk 4 to 8			ND	456.4 a^{A}	366.2 a^{A}	167.5 b^{A}	ns
P-value			ns	ns	ns	ns	ns	*	
AP1 expression									
0 wks	11 wks	No GA_3	3.1 b^{B}	5.0 a^{A}	2.0 a^{BC}	0.6 c^{C}	1.0 b^{C}	0.9 a^{C}	***
8 wks	3 wks	No GA_3	8.8 a^{A}	3.7 a^{A}	3.8 a^{A}	3.2 a^{A}	5.5 a^{A}	5.3 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		5.0 a^{A}	2.4 a^{B}	1.6 bc^{B}	1.6 b^{B}	1.9 a^{B}	*
8 wks	3 wks	Wk 4 to 8			2.6 a^{B}	2.0 ab^{B}	5.2 a^{A}	1.9 a^{B}	**
P-value			*	ns	ns	*	*	ns	

Table S2.1 continued.

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
AP2 expression									
0 wks	11 wks	No GA_3	0.1 a^A	0.1 a^A	0.2 a^A	0.2 a^A	0.1 a^A	0.1 a^A	ns
8 wks	3 wks	No GA_3	0.1 a^A	0.4 a^A	0.3 a^A	0.3 a^A	0.7 a^A	2.0 a^A	ns
8 wks	3 wks	Wk 2 to 8		0.4 a^A	0.2 a^A	0.4 a^A	0.2 a^A	0.2 a^A	ns
8 wks	3 wks	Wk 4 to 8			0.3 a^B	0.3 a^B	0.8 a^A	0.7 a^A	*
P-value			ns	ns	ns	ns	ns	ns	
SEP1 expression									
0 wks	11 wks	No GA_3	ND	< 0.05	ND	ND	ND	ND	--
8 wks	3 wks	No GA_3	< 0.05[z]	ND	ND	< 0.05	0.4 a^B	1.3 a^A	****
8 wks	3 wks	Wk 2 to 8		ND	ND	< 0.05	ND	ND	--
8 wks	3 wks	Wk 4 to 8			ND	ND	0.3 a^B	1.0 a^A	**
P-value			--	--	--	--	ns	ns	
PI expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	ND	--
8 wks	3 wks	No GA_3	ND	ND	ND	ND	0.7 a^A	0.7 a^A	*
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	ND	< 0.05	--
8 wks	3 wks	Wk 4 to 8			< 0.05	< 0.05	0.3 a^A	0.4 a^A	*
P-value			--	--	--	--	ns	ns	
AG expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	< 0.05	--
8 wks	3 wks	No GA_3	ND	0.1 a^B	ND	< 0.05	0.7 a^B	2.9 a^A	****
8 wks	3 wks	Wk 2 to 8		< 0.05	ND	< 0.05	0.1 a^A	0.1 b^B	*
8 wks	3 wks	Wk 4 to 8			ND	0.1 ^{B}	0.5 a^B	1.6 a^A	*
P-value			--	--	--	--	ns	*	

[x] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] ND, not detected, the expression level in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

[z] < 0.05, detected, the mean expression level of the four biological replications was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

Chapter 3

Relationship between floral intensity and floral gene expression in 'Washington' navel orange (*Citrus sinensis* L. Osbeck) in response to water deficit and gibberellic acid

Abstract

Effects of water deficit (WD) and gibberellic acid (GA_3) on 'Washington' navel orange (WNO) (*Citrus sinensis* L. Osbeck) floral gene expression and floral intensity were quantified. Trees subjected to 8 weeks of WD (average midday stem water potential [SWP] -2.86 MPa) followed by 3 weeks of re-irrigation (SWP recovered to > -1.00 MPa) produced more inflorescences in week 11 than trees well irrigated (WI) (SWP > -1.00 MPa) for 11 weeks ($P < 0.001$). For 8-week WD-treated trees, bud expression of *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*), *APETALA2* (*AP2*), *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) increased after re-irrigation, but only expression of *AP1*, *AP2*, *SEP1*, *PI* and *AG* was greater than that of 11-week WI trees. Foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 of WD treatment did not affect *FT*, *SOC1* or *LFY* expression, but reduced *AP1* and *AP2* expression, repressed *SEP1*, *PI* and *AG* expression in weeks 9 and 10 despite re-irrigation, and reduced inflorescence number equal to that of 11-week WI trees. Thus, under WD, GA_3 inhibited citrus floral development at *AP1* and *AP2*, which likely regulated downstream floral organ identity gene activity. The same results were obtained using low temperature (LT) and GA_3 to promote and inhibit WNO

flowering, respectively (Chapter 2). Together, the results suggest WD and LT regulate citrus floral development through overlapping pathways, whereby *AP1* and *AP2* expression are necessary for bud determinacy and downstream activation of *SEP1*, *PI* and *AG*.

Introduction

It is well known that flowering in citrus (*Citrus* spp.) is stimulated by water deficit (WD) (Barbera et al., 1981, 1985; Chica and Albrigo, 2013; Lovatt et al., 1988; Southwick and Davenport, 1986). The ancient technique of *forzatura di limone* was developed in Sicily to provide summer (*verdelli*) lemons (*Citrus limon* L. Burm. f.) (Barbera et al., 1981, 1985). The technique "forces" trees to flower by imposing an 8-week period of water-deficit stress during the hot summer months, the trees flower within 4 weeks of re-irrigation, and the crop is harvested the following summer. For 'Frost Lisbon' lemon trees subjected to modified *forzatura* treatments, floral intensity increased with the severity or duration of the water-deficit period (Lovatt et al., 1988). Two weeks of moderate WD treatment (stem water potential [SWP] -2.25 MPa) triggered 'Tahiti' lime (*C. latifolia* Tan.) trees to produce a small number of flowers (Southwick and Davenport, 1986). However, to dramatically increase floral intensity in 'Tahiti' lime and 'Washington' navel orange (WNO) (*C. sinensis* L. Osbeck) at least 5 weeks of moderate WD (SWP -2.25 and -2.00 MPa, respectively) were required (Chica and Albrigo, 2013; Southwick and Davenport, 1986). For 'Tahiti' lime trees, maintaining the treatment duration at 5 weeks, but increasing the severity of the WD (SWP -3.50 MPa) resulted in a

greater number of inflorescences than obtained with moderate WD (Southwick and Davenport, 1986). Knowledge of the effects of WD on floral development at the level of gene transcription is limited to a single study, in which 6 weeks of moderate WD (SWP -2.00 MPa) resulted in significant flowering in WNO (Chica and Albrigo, 2013). Leaf expression of *FLOWERING LOCUS T* (*FT*) increased during WD treatment but bud expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *LEAFY* (*LFY*) and *APETALA1* (*AP1*) increased only after the trees were re-irrigated. Effects of WD on the expression of the genes downstream from *AP1* that function in floral organ specification have not been reported.

Flowering in citrus is inhibited by GA_3, which continues the vegetative development of the shoot apical meristem (SAM) when it is applied before the SAM is determined (irreversibly committed to floral development) (Lord and Eckard, 1987). Interestingly, the inhibitory effect of GA_3 has only been documented under conditions, in which low temperature (LT) is the stimulus for flowering. For example, a single spray of GA_3 (40 mg L^{-1}) in early December and four applications of GA_3 (150 mg L^{-1}) made every two weeks starting in mid-November, both of which decreased floral intensity under LT, resulted in reduced *FT* expression in leaves of 'Salustiana' sweet orange (*C. sinensis*) and buds of 'Orri' mandarin (*C. reticulata* Blanco x *C. temple* Hort. ex Y. Tanaka), respectively (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012), suggesting the possible role of *FT* in mediating the inhibitory effect of GA_3 on citrus flowering. In contrast, results of recent research (Chapter 2) demonstrated that six weekly applications of GA_3 (50 mg L^{-1}) to WNO trees during weeks 2 through 8 of an 8-week LT treatment

had no significant effect on bud expression of *FT*, *SOC1* or *LFY*, but reduced bud expression of *AP1* and *APETALA2* (*AP2*), totally repressed *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) expression, and reduced inflorescence number to that of control trees under warm temperature well-irrigated conditions. The results suggested that inhibition of floral development by GA_3 was mediated through *AP1* and *AP2*, which regulated the subsequent activity of the downstream floral organ identity genes. Activity of *AP1* and *AP2* is necessary for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005), which was identified as the developmental marker indicating irreversible commitment to floral development (bud determinacy) in WNO (Lord and Eckard, 1987). Whether WD and GA_3 promote and inhibit flowering in citrus, respectively, through developmental events analogous to those observed under LT has not previously been tested.

Thus, the overall goal of the research reported herein was to determine whether WD and LT promote flowering through overlapping floral developmental pathways, specifically that bud determinacy and the inhibitory effect of GA_3 on citrus floral intensity are mediated through *AP1* and *AP2* under WD as they are under LT conditions. The first objective of this research was to compare the effects of a floral-promoting WD treatment (SWP $\leq$ -2.40 MPa for 8 weeks followed by 3 weeks of re-irrigation and recovery to SWP > -1.00 MPa) with those of an 11-week well-irrigated (WI) treatment (SWP > -1.00 MPa), which did not result in flowering, on the expression sequence of eight classic genes regulating flowering time, floral meristem identity, and floral organ identity in the buds of WNO in relation to differences in floral intensity. The second

objective was to evaluate the effects of six weekly foliar applications of GA_3 in weeks 2 through 8 of WD treatment on the expression of each of the eight floral genes and inflorescence number in order to identify the genes associated with floral inhibition by GA_3 in citrus. The final objective was to compare the effects of WD on the expression patterns of the eight floral genes and resulting inflorescence number presented herein with those observed under LT (Chapter 2), including the effects of the same GA_3 treatment under WD versus LT conditions. To the author's knowledge, this is the first research to report the expression patterns of citrus *AP2*, *SEP1*, *PI* and *AG* in relation to floral intensity under WD stress. This study is also the first to use WD as a tool to increase floral intensity in order to quantify the effects of GA_3 on the expression of genes in the floral developmental pathway in citrus. In addition, SWP of WNO trees in all treatments was determined throughout the course of the experiment, including the subsequent re-irrigation period, to thoroughly document the relationships among WD and floral gene expression and floral intensity.

Materials and Methods

Plant material and treatment conditions

Five-year-old mature WNO scions on 'Carrizo' citrange rootstock (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) grown in 56-liter pots containing steam-sterilized University of California soil mix I (Baker, 1957) were used in this research. All fruit were removed from the trees before the initiation of the experiment in September. The research used a complete randomized design with four WNO trees (replications) per

treatment and three treatments. Midday SWP reported herein was measured according to McCutchan and Shackel (1992). For all trees in all treatments, the evening prior to the day of SWP measurement, three leaves per tree were randomly selected and each leaf was covered with a plastic zip lock bag and then wrapped with aluminum foil to prevent exposure to light. Between 8:00 and 10:00 am the following day, each leaf was detached and SWP was determined, using a pressure chamber (PMS Instrument Company, Albany, OR). This process was repeated every 2 to 5 days for the duration of the experiment. For the WI treatment, SWP of the trees was maintained around -1.00 MPa for the 11 weeks of the experiment by daily irrigation (Figure 3.1). For the WD treatments, trees were maintained at an SWP less than or equal to -2.40 MPa by deficit irrigation for 8 weeks (day 0 to day 54) (Southwick and Davenport, 1986). Note that day 0 refers to the first day that WD-treated trees reached a SWP less than -2.4 MPa. On day 55, the WD trees were re-irrigated and by day 60, tree SWP had recovered to a non-stress level (> -1.00 MPa). For the WD-treated trees, WD was interrupted briefly on days 14 and 15 (Figure 3.1). A set of trees subjected to 8 weeks of WD treatment were sprayed weekly with 50 mg L^{-1} GA_3 (ProGibb 40%, Valent BioScience Corporation, Libertyville, IL), containing 0.01% Silwet L77 surfactant (Helena Chemical Company, Collierville, TN), in weeks 2 through 8. Trees used in this research were grown in a glasshouse. With the exception of irrigation regime, all trees were treated the same, including temperature (24/19 ˚C, day/night), day length (16-hr day [500 μmol m^{-2} s^{-1}]/8-hr night), fertilization, and relative humidity (~ 80%). All trees used in this research were grown as described above under

WI warm conditions (WC) (24/19 ˚C, day/night) for five months prior to the start of the experiment.

Sample collection and gene expression analysis

The apical five buds from 15 nonbearing shoots per tree were collected at weeks 2, 4, 6, 8, 9, and 10 from each of the four trees (four replications) in all treatments, with the exception that sample collection for the GA_3-treated trees was delayed until 2 weeks after the first GA_3 application. Collected buds were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab. Bud samples were quickly frozen in liquid nitrogen and stored at -80 ˚C until analyzed. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequences of *Arabidopsis thaliana* homologs *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). Citrus *FT*, *SOC1*, *LFY* and *AP1* genes analyzed in this

research were *CiFT2* (Nishikawa et al., 2007), *CsSOC-like2* (*CsSL2*) (Tan and Swain, 2007), *CsLFY* and *CsAP1* (Pillitteri et al., 2004), *PtAP2* (Song et al., 2010), *CuSEP1* (Nishikawa et al., 2009), respectively; each gene was selected based on its demonstrated functional equivalence in its respective *A. thaliana* mutant. In addition, expression of *CiFT2* was related to floral intensity in response to low temperature in *C. unshiu* (Nishikawa et al., 2007); *CsSL2* expression was also related to flowering in field-grown *C. reticulata* (Shalom et al., 2012). The sequences of *PI* and *AG* chosen in this research share high identity with *A. thaliana PI* and *AG*, respectively; the predicted protein sequences for the putative *PI* and *AG* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com/primerquest/Home/Index) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study as well as the BLAST results of PCR product sequence versus target sequence of each gene of interest are listed in Table 3.1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-µL reaction volume containing 1.2 µL cDNA (about 40 ng of input RNA), 0.6 µL gene-specific forward and reverse primer mix (10nM), 7.5 µL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 µL PCR-grade

water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was performed at the end of each qPCR run to confirm that nonspecific products were not formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with WNO flowers collected from orchard trees at spring bloom as the control (expression level of 1) and *β-ACTIN* (*ACT*) as the primary reference gene (endogenous control), and reported herein. The selection of *ACT* as the primary reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Results based on a second reference gene, *ELONGATION FACTOR 1-ALPHA* (*EF1-α*) (Nishikawa et al., 2009), are presented as supplemental results (Table S3.1). Treatment effects on the expression of all target genes were similar using *ACT* or *EF1-α* as the reference gene. The expression pattern of each floral gene with *ACT* as the endogenous control was strongly correlated with its expression pattern when *EF1-α* served as the reference gene (r = 0.73 to 0.99, $P < 0.001$ for all genes), confirming the consistency and reliability of the results. Gene expression data for each treatment and sample date were the mean of four biological replicates; each biological replicate was the mean of three qPCR technical replicates.

Treatment effects on bud development

Maximum bloom occurred at week 11 for trees exposed to 8 weeks of WD and re-irrigated for 3 weeks. At this time, the fate of the apical five buds on each of the 15

randomly selected nonbearing shoots per tree was determined as the number of leafless (one to many flowers with no leaves), leafy (one to many flowers with one to many leaves), and total inflorescences (sum of leafless plus leafy inflorescences), vegetative shoots, and inactive (dormant) buds per tree for trees in all treatments. Results for the five apical buds on the 15 shoots per tree were averaged for the four individual trees (replications) per treatment and reported as the average value per tree.

Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots and inactive buds per tree, and the relative expression levels of genes using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$. Pearson's correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $P \leq 0.05$) between average SWP and the developmental fate of buds and between gene expression level and inflorescence number, respectively. Significant correlations were subjected to regression analyses, using the least squares method for the generalized linear model.

Results

Effects of water deficit and GA_3 on flowering

For WNO trees grown under WI conditions for 11 weeks, SWP averaged -0.70 MPa (Table 3.2) and was never less than -1.50 MPa (Figure 3.1), indicating that the trees were never subjected to WD stress. For trees in WD treatment, SWP decreased to -2.70 MPa after irrigation had been withheld for 5 days and was subsequently maintained at less than or equal to -2.40 MPa for the majority of the 54-day treatment period (Figure 3.1). Averaged across the 54 days of WD, the SWP for the two sets of WD-treated trees was not significantly different; compare -2.86 ± 0.65 for WD trees not treated with GA_3 to -2.70 ± 0.62 for WD trees treated with GA_3, indicating that the trees in the two treatments were subjected to an equivalent severity of WD and that differences in floral intensity and gene expression were solely due to the effect of the GA_3 treatment. Importantly, both sets of trees subjected to WD had SWPs significantly lower than that of the WI trees throughout the 54-day treatment period ($P < 0.001$) (Table 3.2).

Trees of WNO grown under WI conditions for 11 weeks produced only 0.8 total inflorescence per tree (based in all cases on 5 buds/15 shoots/tree) (Table 3.2), indicating that the majority of the buds collected and analyzed in this research was not committed to floral development at the initiation of the experiment. In contrast, trees subjected to 8 weeks of WD produced 51 inflorescences per tree, which was significantly greater than that of WI trees ($P < 0.001$). Average SWP during the first 8 weeks of the experiment was significantly negatively correlated with the number of inflorescences produced per tree ($r = -0.97$, $P < 0.001$), with average SWP explaining 93% of the variation in

inflorescence number (Figure 3.2). Leafless and leafy inflorescences comprised 57% and 43%, respectively, of the total inflorescences produced by WD-treated trees. The number of leafless inflorescences produced by WD-treated trees was greater than that of WI trees (Table 3.2), but not significantly greater due to the variation in the number of leafless inflorescences produce by the four trees (replications) in the WD treatment. The number of leafy inflorescences was significantly increased by the 8-week WD treatment compared to the WI trees ($P < 0.01$) and was significantly correlated with average SWP ($r = -0.80$, $P = 0.018$). The WD treatment had no effect on the number of vegetative shoots produced per tree (Table 3.2).

Weekly foliar applications of GA_3 during weeks 2 through 8 of the WD treatment significantly reduced the total number of inflorescences produced by 95% compared to 8-week WD trees not treated with GA_3, resulting in only 0.3 inflorescence per tree, a number equal to that of 11-week WI trees ($P < 0.001$) (Table 3.2). In contrast, six foliar applications of GA_3 to WD-treated trees increased vegetative shoot number (> 2.5-fold) to 23.3 per tree compared to WD-treated trees not treated with GA_3 ($P < 0.01$). For the 11-week WI trees, the majority (73.8) of the 75 buds observed per tree remained inactive (dormant) (Table 3.2). Eight weeks of WD significantly reduced the number of inactive buds to 15.3 per tree compared to 11-week WI trees ($P < 0.001$). The number of inactive buds was significantly and positively correlated with the average SWP during the 8 weeks of the treatments ($r = 0.99$, $P < 0.001$) (Figure 3.2). Average SWP explained 98% of the variation in inactive bud number. The relationship between SWP and the number of inactive buds was largely due to the positive effect of WD on inflorescence

development. The number of inactive buds was significantly negatively correlated with the total number of inflorescence per tree across all treatments, including GA_3 (r = -0.96, $P < 0.001$), but not with vegetative shoot number (r = -0.42, P = 0.299).

Effects of water deficit and GA_3 on the expression of citrus floral timing genes

Transcripts of *FT* were detected in buds of 11-week WI-treated trees on each of the six sampling dates, except week 8 (Table 3.3). Bud expression of *FT* was very low during weeks 2 through 4, but increased 10-fold by week 6 and remained elevated during weeks 9 and 10 ($P < 0.01$). For WD-treated trees, the significant increase in bud *FT* expression was delayed until weeks 9 and 10 when the trees were re-irrigated ($P < 0.0001$). The expression of *FT* was not significantly greater in the buds of 8-week WD-treated trees than that of 11-week WI-treated trees on any sampling date. For trees in both the 11-week WI treatment and 8-week WD treatment, expression of *SOC1* was detected in buds on all sampling dates and fluctuated significantly over time in both treatments ($P < 0.01$ and $P < 0.0001$, respectively) (Table 3.3). Expression of *SOC1* in buds of WI-treated trees increased significantly by week 6 (5-fold), decreased more than 50% by week 8, and returned to the level of expression observed in week 6 during weeks 9 and 10 ($P < 0.01$). Bud *SOC1* expression in WD-treated trees also increased significantly by week 6 (more than 3-fold) and decreased 45% by week 8, but a transcript level equal to that of week 6 was only observed after 2 weeks of re-irrigation in week 10 ($P < 0.001$). Thus, bud *SOC1* expression was significantly greater in WD-treated trees than WI trees only in week 8 ($P < 0.05$). Transcripts of *LFY* were also detected in buds of trees WI for 11 weeks on all

sampling dates except at week 8, with *LFY* expression significantly greater by week 10 of the 11-week WI treatment than in all previous weeks ($P < 0.05$) (Table 3.3). In buds of WD-treated trees, *LFY* expression remained relatively low from weeks 2 through 8, but increased to a maximum value during re-irrigation in weeks 9 and 10 ($P < 0.0001$). The expression of *LFY* in buds of WD-treated trees was only greater than that of WI trees at week 8, when the level of *LFY* transcripts was below the limit of detection in buds of WI trees. The values reported for *LFY* expression in Table 3.3 are high because *LFY* expression was very low in the WNO flower, which served as the control (expression level of 1). This was not the case for *FT* or *SOC1*. To assess the validity of the results reported for the treatment effects on *LFY* expression, the data were also analyzed using buds collected from trees at the start of the experiment (time zero) as the control (expression level of 1). The expression levels for *LFY* were reduced, but no substantive differences in the data resulted. Although it is interesting that in week 8 of the 11-week WI treatment that bud transcript levels for both *FT* and *LFY* dropped below the limit of detection and *SOC1* activity declined by more than 50%, the developmental significance of the timing of this change in expression is unclear. It should be noted that *FT*, *SOC1* and *LFY* transcripts were present in WNO buds at the initiation of the experiment in September, despite the trees being maintained for the prior five months under WI and WC (24/19 ˚C, day/night) that did not result in flowering.

For trees receiving six applications of GA_3 starting in week 2 of the 8-week WD treatment, bud expression of *FT* ($P < 0.05$), *SOC1* ($P < 0.05$) and *LFY* ($P < 0.01$) was significantly greater in the GA_3-treated trees by week 4 than the WD and WI control trees

not receiving GA_3 (Table 3.3). For weeks 6 through 10, there were no significant differences in the expression of *FT*, *SOC1* or *LFY* among the three treatments, with the exception that the expression of all three genes was present in buds of GA_3-treated trees but not in buds of WI trees at week 8 (Table 3.3). Thus, GA_3 had no inhibitory effect on *FT*, *SOC1* or *LFY* expression in buds of WNO trees in the 8-week WD treatment. In addition, total inflorescence number was not significantly related to the expression pattern of any floral timing gene across all treatments on any sampling date.

Effects of water deficit and GA_3 on the expression of citrus genes with class A activity

For buds of 11-week WI trees, *AP1* expression decreased over time, leading to significantly greater expression during weeks 2 through 6 than weeks 8 through 10 ($P < 0.001$) (Table 3.4). In contrast, for buds of WD-treated trees, *AP1* expression did not change significantly during weeks 2 through 8 but there was a linear, non-significant increase in *AP1* expression after re-irrigation in weeks 9 and 10. As a result, transcript levels of *AP1* in buds of WD-treated trees were 3- and 4-fold greater than those of WI trees in weeks 9 ($P < 0.01$) and 10 ($P < 0.01$), respectively. In buds of trees WI for 11 weeks, *AP2* expression was more than 10-fold lower than *AP1* expression and remained low for the duration of the experiment (Table 3.4). For WD-treated trees, bud *AP2* expression did not change significantly from weeks 2 through 9, but increased 2.7-fold after re-irrigation to a maximum value at week 10 ($P < 0.001$). At weeks 9 and 10, buds of WD-treated trees exhibited greater expression of *AP2* than those of WI trees ($P < 0.05$).

Six weekly applications of GA_3 to WD-treated trees in weeks 2 through 8 significantly reduced bud *AP1* expression 40% and 63% during weeks 9 ($P < 0.01$) and 10 ($P < 0.01$), respectively, relative to WD-treated trees not receiving GA_3. As a result, bud *AP1* transcript levels were equal to those of WI trees by week 10 (Table 3.4). Similarly, six applications of GA_3 in weeks 2 through 8 of the WD treatment reduced bud *AP2* expression 58% by week 10 compared to WD-treated trees not treated with GA_3 and equal to that of trees WI for 11 weeks ($P < 0.05$) (Table 3.4). Inflorescence number was significantly correlated across all treatments with the expression patterns of *AP1* at weeks 9 ($r = 0.89$, $P < 0.001$) and 10 ($r = 0.91$, $P < 0.001$) and *AP2* at weeks 9 ($r = 0.63$, $P = 0.028$) and 10 ($r = 0.89$, $P < 0.001$).

Effects of water deficit and GA_3 on the expression of citrus floral organ identity genes downstream from AP2

Transcripts of *SEP1* were detected at very low levels in weeks 2 and 4 and not detected on any sampling date thereafter in buds of trees maintained under the WI condition for 11 weeks (Table 3.5). In buds of WD-treated trees, *SEP1* was only expressed at significant levels after SWP returned to a non-stress level during the 2-week re-irrigation period that followed the 8-week WD treatment, with *SEP1* expression level 5-fold greater in week 10 than week 9. Similarly, *PI* was never expressed at significant levels in WI trees for the duration of the experiment (Table 3.5). For WD-treated trees, *PI* expression also occurred only in buds after re-irrigation, with a greater value at week 10. Trace levels of *AG* expression were detected during weeks 9 and 10 in the buds of trees

in the 11-week WI treatment (Table 3.5). In buds of WD-treated trees, *AG* transcripts were not detected during the 8 weeks of WD treatment, but after re-irrigation, *AG* was expressed at significant levels, with the value at week 10 9-fold greater than that at week 9.

Buds of WNO trees in the 8-week WD treatment did not express *SEP1*, *PI* or *AG* until the trees were re-irrigated, reaching maximum expression in week 10 (Table 3.5). When GA_3 was applied during weeks 2 through 8 of the WD treatment, the increases in *SEP1* and *PI* expression that occurred after re-irrigation did not occur, with only very low levels of *AG* expression at weeks 9 and 10 (Table 3.5). The resulting transcript levels for *SEP1* and *PI* were at the limit of detection or below it in buds of 8-week WD-treated trees receiving GA_3. Whereas *AG* expression was still detectable in buds of 8-week WD-treated trees receiving GA_3, the transcript levels at weeks 9 and 10 were 9- and 79-fold lower than those of the WD-treated trees not receiving GA_3, respectively, and equal to those of the 11-week WI trees. Inflorescence number was strongly correlated across all treatments with the expression patterns of *SEP1* at weeks 9 ($r = 0.83$, $P = 0.003$) and 10 ($r = 0.88$, $P < 0.001$), *PI* at week 10 ($r = 0.90$, $P < 0.001$) and *AG* at week 10 ($r = 0.91$, $P < 0.001$).

Discussion

Whereas the commercial use of WD to promote flowering in citrus, especially lemons and limes, has a long history characterized by numerous experiments designed to maximize the effect of WD treatment on floral intensity (Barbera et al., 1981, 1985;

Lovatt et al., 1988; Maranto and Hake, 1985; Southwick and Davenport, 1986), knowledge of the role of WD in promoting floral development at the level of gene transcription previously has been limited to one investigation until the current study. In the research of Chica and Albrigo (2013), WNO trees flowered (2 inflorescences/shoot) in response to 6 weeks of moderate WD (SWP -2.00 MPa), whereas WI trees flowered poorly (0.2 inflorescence/shoot). During the period of WD, *FT* transcripts accumulated in leaves, but decreased immediately after re-irrigation to levels equaling the low expression of WI trees. For buds of WD-treated trees, expression of *SOC1*, *LFY* and *AP1* remained at a constant low level (equal to WI trees) during the WD period, but increased shortly after re-irrigation to levels greater than those of WI trees for approximately 10 days. The results of Chica and Albrigo (2013) suggested that flowering in citrus in response to WD might be mediated through leaf *FT*, which subsequently upregulated *SOC1*, *LFY* and *AP1* in buds.

Results of the current research are consistent with the results reported by Chica and Albrigo (2013). The research presented herein demonstrated that 8 weeks of WD, with an average SWP of -2.86 MPa, significantly increased total inflorescence number in WNO trees (51 inflorescences/75 buds/tree) compared to 11-week WI trees (SWP > -1.0 MPa; 0.8 inflorescence/75 buds/tree). In addition, thorough quantification of SWP for all trees for the duration of the experiment made it possible to demonstrate that inflorescence number for an individual tree was strongly correlated with its average SWP during the first 8 weeks of the experiment ($r = -0.97$, $P < 0.001$). A major contribution of the current research was the analysis of transcript levels of additional genes downstream from *AP1*,

including *AP2*, *SEP1*, *PI* and *AG*, in buds of both WI and WD-treated trees. In this regard, it is noteworthy that the expression of all floral genes analyzed in buds of 8-week WD-treated trees increased, but only after full irrigation had been restored for 1 to 2 weeks, with full bloom 1 week later (week 11). For trees subjected to 8 weeks of WD, it took 6 days from the restoration of full irrigation for SWP to recover to greater than -1.0 MPa. Transcript levels of *FT* in buds did not change during the 8 weeks of WD but increased significantly 1 week after irrigation was restored and remained significantly greater during weeks 9 and 10 of the re-irrigation period than during the 8-week WD period ($P < 0.0001$). Bud expression of *SOC1* fluctuated during the 8 weeks of WD but was significantly greater after 2 weeks of re-irrigation in week 10 ($P < 0.001$). Bud *LFY* expression remained low throughout the WD treatment but was significantly greater in both weeks 9 and 10 of the re-irrigation period ($P < 0.0001$). For 8-week WD trees, re-irrigation resulted in a linear increase in bud *AP1* expression through week 10 (non-significant), but *AP2* transcript level was significantly greater by week 10 than the previous weeks ($P < 0.001$). Expression of the downstream floral organ identity genes *SEP1*, *PI* and *AG*, which were never detected during the 8-week WD period, were expressed at significant levels in week 10, approximately one week after SWP had recovered to greater than -1.0 MPa following the 8-week WD treatment.

Despite the significant increases in bud expression of *FT*, *SOC1* and *LFY* after re-irrigation of the 8-week WD-treated trees reported above, bud *FT*, *SOC1* and *LFY* transcript levels were never greater than those in buds of 11-week WI trees, with the exception of week 8 of the experiment when, inexplicably, the expression of *SOC1*

decreased and *FT* and *LFY* dropped below the limit of detection in buds of WI trees. This result did not change even when the data for all three genes were analyzed using buds collected from trees at the start of the experiment (time zero) as the control (expression level of 1) instead of WNO flowers. Thus, in the current study, re-irrigation of WD-treated trees resulted in significantly greater bud *AP1* expression (more than 4-fold in weeks 9 and 10) compared to that of WI trees ($P < 0.05$) and also significantly greater bud *AP2* expression (7.5-fold by week 10) compared to that of WI trees ($P < 0.01$). Strikingly, 11-week WI trees never expressed *SEP1* or *PI* at signficant levels and expressed *AG* only at very low levels, but 8 weeks of WD treatment followed by full irrigation dramatically increased *SEP1*, *PI* and *AG* expression above detectable levels by week 9, with transcripts continuing to accumulate to maximum levels in week 10, at which time *AG* expression was 79-fold greater than that of WI trees. These data suggest that the increase in *AP1* and *AP2* transcript levels, i.e., to values significantly greater than those of WI trees, played a significant role in successful flowering. According to the ABCE model for floral organ specification, activity of both the class A genes, *AP1* and *AP2*, is required for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). Of relevance to this research, sepal formation was identified as the developmental marker coincident with bud determinacy in WNO (Lord and Eckard, 1987). Thus, increased bud expression of *AP1* and *AP2*, which occurred during re-irrigation of WD-treated trees, was likely the developmental event required for upregulation of the downstream floral organ identity genes *SEP1, PI* and *AG* and

successful flower formation. For WI WNO trees, bud *AP1* and *AP2* transcript levels were likely too low to upregulate the floral organ identity genes so flower development failed.

The above interpretation is supported by the effects of the GA_3 treatment on floral intensity and floral gene expression. Use of a GA_3 treatment to inhibit floral development under WD conditions in order to determine which genes mediated the effect of GA_3 and which genes must be expressed for intense flowering to occur is the second important contribution of the current research. Six weekly GA_3 applications in weeks 2 through 8 of the 8-week WD period significantly reduced the number of inflorescences per tree below that of 8-week WD-treated trees not receiving GA_3 and equal to the low inflorescence number of WI trees ($P < 0.001$). However, GA_3 treatment actually increased bud expression of *FT* ($P < 0.05$), *SOC1* ($P < 0.05$) and *LFY* ($P < 0.01$) above that of both the WI and WD-treated trees not treated with GA_3 during week 4 and did not significantly reduce transcription of any of the three genes at any time below that of the 8-week WD-treated trees not receiving GA_3. In contrast, the GA_3 treatment prevented the increase in bud expression of *AP1*, *AP2*, *SEP1*, *PI,* and *AG* that occurred after WD-treated trees were re-irrigated, resulting in transcript levels that were lower than those of WD-treated trees not receiving GA_3 and equal to those of WI trees. Specifically, when compared to 8-week WD-treated trees not treated with GA_3, expression of *AP1* was reduced by 40% in week 9 and 65% in week 10 ($P < 0.01$), *AP2* by 57% in week 10 ($P < 0.05$), *SEP1* and *PI* expression were reduced to the limit of detection or below it in weeks 9 and 10, and *AG* expression was reduced 79-fold to the very low level of the WI trees by week 10. These results provide strong evidence that GA_3 inhibits citrus flowering by downregulating *AP1*

and *AP2* transcription and concomitantly downstream *SEP1*, *PI* and *AG* activities in buds of WD-treated trees. It remains unresolved whether the inhibitory effect of GA_3 on *SEP1*, *PI* and *AG* expression is mediated indirectly through *AP1* and/or *AP2* or is a direct effect of GA_3 on each gene independently. Across all treatments, inflorescence number was not significantly related to the expression of *FT*, *SOC1* or *LFY*, but was strongly correlated with the expression of *AP1* at weeks 9 ($r = 0.89$, $P < 0.001$) and 10 ($r = 0.91$, $P < 0.001$), *AP2* at week 10 ($r = 0.89$, $P < 0.001$) and *SEP1*, *PI* and *AG* in week 10 ($r \geq 0.88$, $P < 0.01$ for the three genes).

The results reported herein and those of Chica and Albrigo (2013) and Chapter 2 are consistent in providing evidence that citrus buds that produced inflorescences and those that produced vegetative shoots or remained inactive (dormant) were indistinguishable at the level of gene transcription during the early stages of development. In all cases, under conditions that resulted in inflorescence development (WD and LT), those that resulted in inactive (dormant) buds (WI and WC) and those that increased vegetative shoot number (WD and LT with six weekly applications of GA_3 during weeks 2 through 8 of treatment, respectively), buds of WNO trees expressed *FT*, *SOC1*, *LFY*, *AP1* and *AP2* at a low level. Tan and Swain (2006) proposed that this scenario indicated the possibility that all buds of adult citrus trees were competent and were induced to flower as a result of the transition from juvenile to adult, but prevailing local environmental or endogenous conditions (shoot age, bud endogenous GA concentration, warm temperature, insufficient exposure to low temperature, lack of water-deficit stress, or presence of fruit) prevented some buds from becoming determined and producing inflorescences. In this scenario, the potential

role for a leaf-produced *FT* signal in response to WD is unclear (Chica and Albrigo, 2013), but upregulation of *FT* would be required for transition from juvenile to adult (Endo et al., 2005). Since *FT*, *SOC1*, *LFY*, *AP1* and *AP2* were expressed in WNO buds collected in September after five months of WI and WC, the alternative scenario that transition from vegetative to floral development might occur in adult citrus buds annually but much earlier than previously proposed, i.e., in the summer, as proposed in Chapter 2, is also proposed herein. In this scenario, a leaf-produced *FT* floral signal that was age-dependent, or possibly in response to increasing day length, might play a critical role in citrus flowering. At present, both scenarios and the role of the putative leaf-produced *FT* floral signal in citrus await resolution. However, the results reported herein and those of Chapter 2 provide three lines of evidence that bud *AP1* and *AP2* expression determine whether flowering will occur or not. First, maximum flowering occurred only after the 8-week WD-treated trees were re-irrigated and after 8-week LT-treated trees were transferred to the WC compared to trees maintained in WI and WC for the duration of the experiment. In both cases, once the stress conditions were eliminated, bud expression of *AP1* and *AP2* increased to levels significantly greater than those of the WI and WC control trees. Second, bud *AP1* and *AP2* expression was downregulated when floral intensity was dramatically reduced in WNO trees receiving foliar-applied GA_3 during weeks 2 through 8 of the 8-week WD and 8-week LT treatment periods compared to trees in the WD and LT treatments not treated with GA_3, which exhibited significantly greater bud *AP1* and *AP2* transcript levels and maximum flowering. Moreover, the GA_3 treatment reduced bud *AP1* and *AP2* expression to the low level of the WI and WC

control trees with a concomitant reduction in inflorescence number. Third, regulation of citrus flowering at *AP1* and *AP2* is consistent with the role of class A activity genes in sepal development (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005) and the relationship between sepal formation and bud determinacy in WNO (Lord and Eckard, 1987). In all cases, the expression of the floral organ identity genes downstream from *AP2* paralleled the expression of *AP1* and *AP2* and when their activity was repressed, flowering did not occur. It is possible that bud determinacy also requires expression of at least one or possibly all four *SEP* genes (Ditta et al., 2004; Pelaz et al., 2000).

A potential key role for *AP1* in the regulation of flowering in citrus in response to WD and LT is commensurate with the unique features of the promoter region of WNO *AP1*. *In silico* analysis conducted to compare transcriptional regulation of *LFY*, *AP1*, and *TERMINAL FLOWER* (*TFL*) by WD, LT, and abscisic acid (ABA) in *C. sinensis* (WNO), *A. thaliana* and *Populus trichocarpa* revealed a striking enrichment of response elements (RE) upregulated by WD present only in the WNO *AP1* promoter (not *LFY* or *TFL*) and not in the promoters of *A. thaliana AP1* (*AtAP1*) and *P. trichocarpa AP1-1 (PtAP1-1)* (Becerra et al., 2016). Notably, a tandem array of three RE, each containing a *LFY* binding site (CCANTG) (Benlloch et al., 2011), a dehydration-responsive element (DRE, RCCGACA) (Dubouzet et al., 2003), and a coupling element 3 site (CE3, GCGTGTC) (Shen et al., 1996), was found within a 100-bp region approximately 500 bp upstream from the translation start codon of the WNO *AP1* promoter. The CE3 site is associated with an ABA-dependent signaling pathway (Shen et al., 1996) and DRE with an ABA-

independent pathway (Liu et al., 1998; Yamaguchi-Shinozaki and Shinozaki, 2005). The reverse-strand CE3 sequence was found in *AtAP1*, but not in *PtAP1-1*. The DRE site was not present in the promoter region of either *AtAP1* or *PtAP1-1*. The *AP1* promoter of all three species have *LFY* binding sites. The unique 100-bp regulon of the WNO *AP1* promoter is consistent with WD-promoted flowering in *C. sinensis* mediated at *AP1* by ABA-dependent and ABA-independent signaling pathways. The WNO *AP1* and *AtAP1*, but not *PtAP1-1,* promoters also contain multiple copies of the core motif for the C-repeat binding factor (CBFHV) response element (RYCGAC) (Gu and Cheng, 2014), which is a low-temperature and ABA-independent RE (Xue, 2002), consistent with LT-promoted flowering.

The possibility proposed by Tan and Swain (2006) that all buds of adult citrus trees are competent and have been induced to flower as the consequence of the transition from juvenile to adult and indistinguishable through early development at the level of gene transcription is supported by evidence first presented in Chapter 2 and by additional documentation present herein. However, the results of these two studies are also consistent with the alternative possibility proposed in Chapter 2 that adult citrus buds transition from vegetative to floral development annually in the summer, much earlier than has been investigated to date. Regardless of which proves to be correct, the results of Chapter 2 taken together with those reported here provide clear evidence that bud determinacy is regulated by *AP1* and *AP2*. This provides citrus buds with a unique failsafe system, whereby transcription of *AP1* and *AP2* increases in a manner paralleling the duration of the LT or WD stress period, but *only* after the stress has been alleviated.

Then in the absence of stress, downstream floral organ identity gene activity is upregulated leading to flower development under conditions of adequate water and warm temperature.

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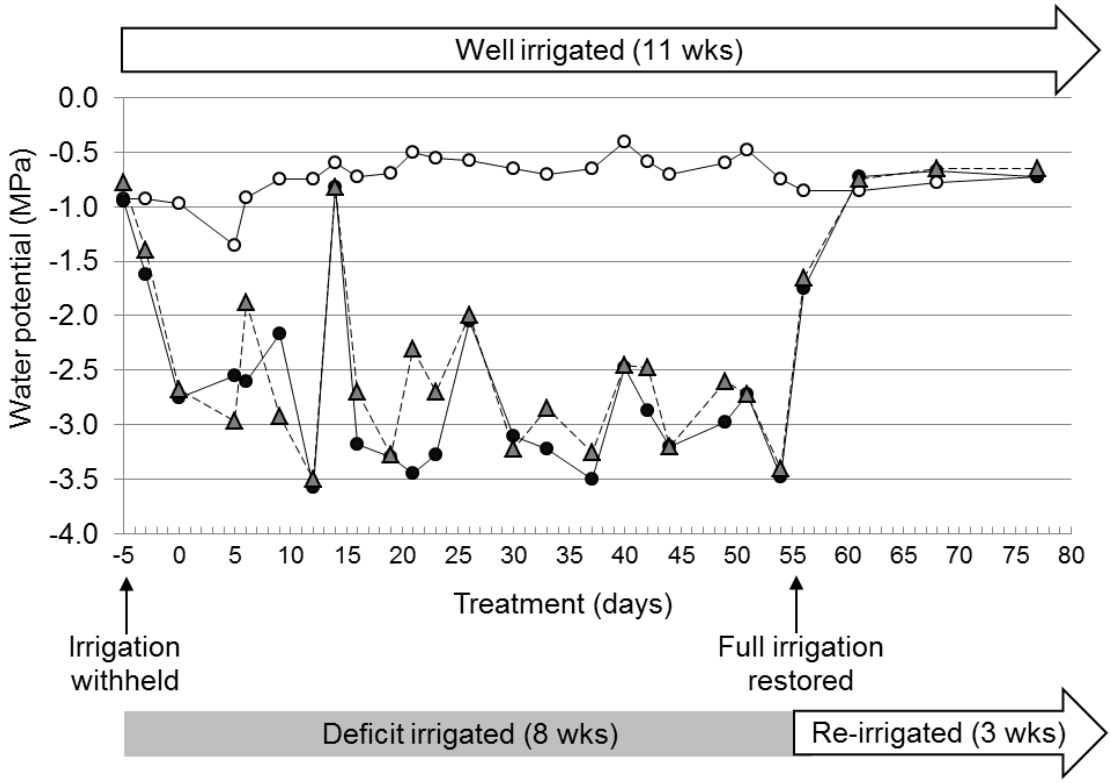

Figure 3.1 Midday stem water potentials (SWP) for 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation (– ● –), trees subjected to 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) from week 2 to 8 (-- ▲ --), and trees maintained well-irrigated (SWP > -1.00 MPa) for 11 weeks (– ○ –).

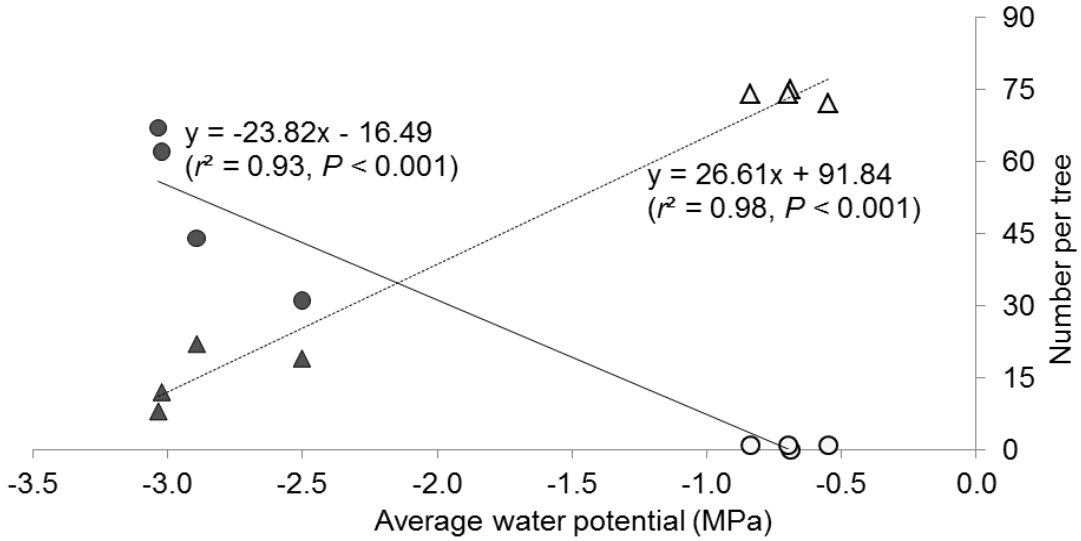

Figure 3.2 Effect of water deficit (WD) (midday stem water potential ≤ -2.40 MPa) on the number of inflorescences (– ● –) and inactive buds (-- ▲ --) of 'Washington' navel orange trees subjected to 8 weeks of WD followed by 3 weeks of re-irrigation and trees maintained well-irrigated (SWP > -1.00 MPa) for 11 weeks. Data are the means of five apical buds per 15 shoots per tree averaged across four trees per treatment.

Table 3.1 Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence BLAST against target gene sequence	
				E-value	Identity
FT	AB027456.1 (*C. unshiu*)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
SOC1	EU032532.1 (*C. sinensis*)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
LFY	AY338976.1 (*C. sinensis*)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
AP1	AY338974.1 (*C. sinensis*)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
AP2	EU883665.1 (*C. trifoliata*)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEP1	AB329715.1 (*C. unshiu*)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PI	XM_ 006472790.1 (*C. sinensis*)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AG	HM246523.1 (*C. sinensis*)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov).

Table 3.2 Average midday stem water potential (SWP) during the first 8 weeks of treatment (from day 0 to day 54) and the developmental fate of buds of 'Washington' navel orange trees maintained under well-irrigated (WI) (SWP > -1.00 MPa) conditions for 11 weeks and trees subjected to 8 weeks of water deficit (WD) and trees exposed to 8 weeks of WD and receiving foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8[z].

WD	WI	GA_3	Average SWP (MPa)	Inflorescences (no./tree)			Vegetative shoots (no./tree)	Inactive buds (no./tree)
				Total	Leafless	Leafy		
0 wks	11 wks	No GA_3	-0.70 a	0.8 b	0.3 a	0.5 b	0.5 b	73.8 a
8 wks	3 wks	No GA_3	-2.86 b	51.0 a	22.0 a	29.0 a	8.8 b	15.3 c
8 wks	3 wks	Wk 2 to 8	-2.70 b	0.3 b	0.3 a	0.0 b	23.3 a	51.5 b
P-value			****	***	ns	**	**	****

[z] Data are the means for four trees (5 apical buds/15 shoots/tree) per treatment. Means followed by different letters within a vertical column are significantly different according to Fisher's least significant difference (LSD) test in which ** refers to a significant effect at $P < 0.01$, *** at $P < 0.001$, **** $P < 0.0001$; ns, not significant.

Table 3.3 Expression of *FT*, *SOC1* and *LFY* in buds of 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (midday stem water potential ≤ -2.40 MPa), 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8, or well-irrigated for 11 weeks (WI) (SWP > -1.00 MPa)[y].

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
FT expression									
0 wks	11 wks	No GA_3	0.2 a^{B}	0.8 b^{B}	7.8 a^{A}	ND[z]	4.3 a^{AB}	5.6 a^{A}	*
8 wks	3 wks	No GA_3	0.2 a^{B}	0.4 b^{B}	1.6 a^{B}	1.3 a^{B}	6.5 a^{A}	6.2 a^{A}	****
8 wks	3 wks	Wk 2 to 8		1.8 a^{A}	0.9 a^{A}	0.9 a^{A}	4.7 a^{A}	1.8 a^{A}	ns
P-value			ns	*	ns	ns	ns	ns	
SOC1 expression									
0 wks	11 wks	No GA_3	3.4 a^{C}	3.0 b^{C}	15.9 a^{A}	6.6 b^{BC}	8.4 a^{AB}	11.1 a^{AB}	**
8 wks	3 wks	No GA_3	6.0 a^{B}	4.6 b^{B}	22.0 a^{A}	12.2 a^{B}	11.2 a^{B}	24.2 a^{A}	***
8 wks	3 wks	Wk 2 to 8		12.4 a^{B}	10.1 a^{B}	11.4 a^{B}	10.5 a^{B}	26.4 a^{A}	**
P-value			ns	*	ns	*	ns	ns	
LFY expression									
0 wks	11 wks	No GA_3	6.0 a^{B}	8.0 b^{B}	476 a^{B}	ND_	537 a^{B}	1663 a^{A}	*
8 wks	3 wks	No GA_3	5.0 a^{B}	2.1 b^{B}	368 a^{B}	209.4 a^{B}	1747 a^{A}	1447 a^{A}	****
8 wks	3 wks	Wk 2 to 8		362.7 a^{B}	82 a^{B}	185.9 a^{B}	1434 a^{A}	273 a^{B}	*
P-value			ns	**	ns	ns	ns	ns	

[y] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 3.4 Expression of *AP1* and *AP2* in buds of 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (midday stem water potential ≤ -2.40 MPa), 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8, or well-irrgated for 11 weeks (WI) (SWP > -1.00 MPa)[z].

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
AP1 expression									
0 wks	11 wks	No GA_3	7.3 a^{A}	7.7 a^{A}	7.8 a^{A}	2.2 a^{B}	3.7 c^{B}	4.3 b^{B}	***
8 wks	3 wks	No GA_3	5.6 a^{A}	8.5 a^{A}	11.7 a^{A}	9.7 a^{A}	12.7 a^{A}	17.6 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		10.0 a^{A}	9.6 a^{A}	5.9 a^{A}	7.6 b^{A}	6.5 b^{A}	ns
P-value			ns	ns	ns	ns	**	**	
AP2 expression									
0 wks	11 wks	No GA_3	0.2 b^{CD}	0.2 a^{D}	0.6 b^{A}	0.4 b^{BC}	0.4 b^{BC}	0.4 b^{AB}	**
8 wks	3 wks	No GA_3	0.5 a^{B}	0.5 a^{B}	1.0 a^{B}	0.5 b^{B}	1.1 a^{B}	3.0 a^{A}	***
8 wks	3 wks	Wk 2 to 8		0.4 a^{A}	0.7 b^{A}	0.8 a^{A}	0.7 ab^{A}	1.3 b^{A}	ns
P-value			*	ns	*	**	*	*	

[z] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$; ns, not significant.

Table 3.5 Expression of *SEP1*, *PI* and *AG* in buds of 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (midday stem water potential ≤ -2.40 MPa), 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8, or well-irrigated for 11 weeks (WI) (SWP > -1.00 MPa)[x].

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
SEP1 expression									
0 wks	11 wks	No GA_3	< 0.05[y]	< 0.05	ND[z]	ND	ND	ND	--
8 wks	3 wks	No GA_3	< 0.05	< 0.05	ND	ND	0.6 [A]	2.9 [A]	ns
8 wks	3 wks	Wk 2 to 8		ND	< 0.05	0.1	ND	ND	--
P-value			--	--	--	--	--	--	
PI expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	ND	--
8 wks	3 wks	No GA_3	ND	ND	ND	ND	0.4 [A]	1.9 [A]	ns
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	ND	< 0.05	--
P-value			--	--	--	--	--	--	
AG expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	0.1 a[A]	0.1 a[A]	ns
8 wks	3 wks	No GA_3	ND	ND	ND	ND	0.9 a[A]	7.9 a[A]	ns
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	0.1 a[A]	0.1 a[A]	ns
P-value			--	--	--	--	ns	ns	

[z] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the four biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Supplemental Results to Chapter 3

Table S3.1 Expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP*, *PI* and *AG* relative to *EF1-α* expression in buds of 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (midday stem water potential ≤ -2.40 MPa), 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8, or well-irrigated for 11 weeks (WI) (SWP > -1.00 MPa)[x].

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
FT expression									
0 wks	11 wks	No GA_3	0.1 a^{A}	0.4 a^{A}	2.3 a^{A}	ND[y]	1.2 a^{A}	1.3 a^{A}	ns
8 wks	3 wks	No GA_3	0.1 a^{C}	0.2 a^{C}	0.8 a^{BC}	0.4 a^{C}	1.7 a^{A}	1.4 a^{AB}	**
8 wks	3 wks	Wk 2 to 8		0.5 a^{B}	0.1 a^{B}	0.3 a^{B}	1.4 a^{A}	0.5 a^{B}	*
P-value			ns	ns	ns	ns	ns	ns	
SOC1 expression									
0 wks	11 wks	No GA_3	1.4 b^{B}	1.6 c^{B}	6.0 a^{A}	2.3 a^{B}	2.5 a^{B}	2.5 b^{B}	*
8 wks	3 wks	No GA_3	2.7 a^{A}	2.4 b^{A}	5.3 a^{A}	3.8 a^{A}	2.9 a^{A}	6.6 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		3.7 a^{B}	3.0 a^{BC}	3.3 a^{B}	2.0 a^{C}	6.6 a^{A}	****
P-value			*	**	ns	ns	ns	**	
LFY expression									
0 wks	11 wks	No GA_3	2.6 a^{B}	8.1 a^{B}	71.0 a^{B}	ND	198.7 a^{B}	783.9 a^{A}	*
8 wks	3 wks	No GA_3	2.3 b^{B}	5.2 a^{B}	65.7 a^{B}	97.7 a^{B}	496.4 a^{A}	502.1 a^{A}	****
8 wks	3 wks	Wk 2 to 8		16.1 a^{B}	65.6 a^{B}	65.6 a^{B}	368.4 a^{A}	113.5 a^{B}	**
P-value			**	ns	ns	**	ns	ns	
AP1 expression									
0 wks	11 wks	No GA_3	3.1 a^{B}	5.0 a^{A}	2.0 a^{BC}	0.6 a^{C}	0.9 c^{C}	0.9 a^{C}	***
8 wks	3 wks	No GA_3	3.1 a^{A}	5.4 a^{A}	3.1 a^{A}	3.3 a^{A}	3.6 a^{A}	4.7 a^{A}	ns
8 wks	3 wks	Wk 2 to 8		3.3 a^{A}	2.7 a^{A}	1.8 a^{A}	1.6 b^{A}	3.1 a^{A}	ns
P-value			ns	ns	ns	ns	****	ns	

Table S3.1 continued.

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
AP2 expression									
0 wks	11 wks	No GA_3	0.1 b^A	0.1 b^A	0.2 a^A	0.2 a^A	0.1 a^A	0.1 c^A	ns
8 wks	3 wks	No GA_3	0.3 a^B	0.2 a^B	0.3 a^B	0.2 a^B	0.3 a^B	0.8 a^A	***
8 wks	3 wks	Wk 2 to 8		0.1 b^C	0.1 a^C	0.2 a^B	0.1 a^C	0.3 b^A	***
P-value			*	*	ns	ns	ns	**	
SEP1 expression									
0 wks	11 wks	No GA_3	ND	< 0.05[z]	ND	ND	ND	ND	--
8 wks	3 wks	No GA_3	< 0.05	ND	ND	ND	0.2 a^A	0.5 a^A	ns
8 wks	3 wks	Wk 2 to 8		ND	< 0.05	< 0.05	ND	ND	--
P-value			--	--	--	--	--	--	
PI expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	ND	--
8 wks	3 wks	No GA_3	ND	ND	ND	ND	< 0.05	0.4 a^A	**
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	ND	< 0.05	--
P-value			--	--	--	--	--	--	
AG expression									
0 wks	11 wks	No GA_3	ND	ND	ND	ND	< 0.05	< 0.05	--
8 wks	3 wks	No GA_3	ND	ND	ND	ND	0.3 a^B	1.7 a^A	**
8 wks	3 wks	Wk 2 to 8		ND	ND	ND	< 0.05	< 0.05	--
P-value			--	--	--	--	--	--	

[x] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

[z] < 0.05, detected, the mean relative expression level of the four biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

Chapter 4

Alternate bearing in *Citrus reticulata* Blanco — Effect of fruit on floral gene expression and floral intensity of 'Pixie' and 'Nules Clementine' mandarin

Abstract

For alternate bearing (AB) mandarin trees (*Citrus reticulata* Blanco), high on-crop yields alternate almost annually with low off-crop yields. Effects of crop load on floral gene expression and inflorescence number the following spring were compared for Pixie mandarin, a late-maturing cultivar harvested after bloom in June, and Nules Clementine mandarin, an early-maturing cultivar harvested before bloom in March. For both cultivars, trees producing an off crop flowered profusely the following April. In October, six months before bloom, transcripts were detected in buds of off-crop 'Pixie' and 'Nules Clementine' mandarin trees for *FLOWERING LOCUS T* (*FT*) (December for 'Nules Clementine' mandarin), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *APETALA2* (*AP2*), but the downstream floral organ identity genes *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) were not expressed until one month before bloom. On-crop 'Pixie' and 'Nules Clementine' mandarin trees did not produce any inflorescences in April. For buds of on-crop trees, *FT* transcripts were not detected from October through March, *SOC1* transcript levels were equal to those of off-crop trees in February and March, and *LFY* transcripts, which were equal to those of off-crop trees in October through January, were not detected in March. Transcript levels of *AP1* and *AP2* were also equal to those of off-

crop trees in October through January but significantly lower in February and/or March. Transcripts of *SEP1*, *PI* and *AG* were never detected at significant levels in buds of on-crop trees. Harvesting the on crop from 'Pixie' mandarin trees in February and 'Nules Clementine' mandarin trees in January did not increase floral gene expression or floral intensity relative to on-crop trees harvested in June and March, respectively. Taken together, the timing of transcript accumulation for *FT*, *SOC1*, *LFY*, *AP1* and *AP2* in buds of off-crop trees of both cultivars indicates that floral development is initiated before October, whereas transcript accumulation for *SEP1*, *PI* and *AG* suggests that floral organogenesis occurs one month before bloom. Removing the on crop one to two months before flower development, which failed to restore flowering, but increased vegetative shoot growth, provides evidence that repression of bud *FT* from October through December is sufficient to prevent the upregulation of *LFY*, *AP1* and *AP2* and the subsequent expression of *SEP1*, *PI* and *AG*. Taken together, the results indicate that repression of *FT* from October through December in buds of on-crop mandarin trees does not inhibit inflorescence development by preventing floral induction, but rather by preventing floral determinacy. The results further suggest that the on crop of mandarin fruit would need to be removed before October to fully restore floral intensity, but how early in the season is unresolved.

Introduction

Alternate bearing (AB), repeating cycles of a light off-crop year followed by a heavy on-crop year, is a widespread phenomenon in both evergreen and deciduous woody

perennial fruit and nut crops (Wheaton, 1992). In *Citrus* spp., especially mandarin cultivars (*C. reticulata* Blanco), AB is a significant economic problem. On-crop trees produce a large number of small fruit with reduced commercial value. Whereas off-crop trees produce large fruit, there are too few to provide adequate grower income and the majority are frequently too large and have undesirable fruit quality (rough peels, granulated juice vesicles) (El-Zeftawi, 1973; Moss et al., 1974). In addition, alternating light and heavy crops compromise orchard management, packinghouse operation and marketing (Hield and Hilgeman, 1969; Moss et al., 1974). Once initiated, the cycles of on and off citrus yields are perpetuated through the effect of crop load (fruit number) on floral intensity at return bloom the following spring. Thus, the light off crop is the result of the low number of inflorescences produced during spring bloom following the heavy on crop, not poor fruit set (Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969; Monselise and Goldschmidt, 1982). The role of fruit number in determining the intensity of the return bloom has been confirmed in numerous experiments demonstrating that early fruit removal (June through December) increased inflorescence number to varying degrees the following spring compared to on-crop trees with no fruit removed (Hield and Hilgeman, 1969; Garcia-Luis et al., 1986; Muñoz-Fambuena et al., 2011; Shalom et al., 2014; Verreynne and Lovatt, 2009). Moreover, floral intensity of the spring bloom following early fruit removal increased with the greater proportion of the on crop removed (Muñoz-Fambuena et al., 2011).

The effects of crop load and fruit removal on the expression of several key genes regulating citrus floral development have been demonstrated to parallel their effects on

inflorescence number at spring bloom. In both 'Moncada' mandarin (Clementine 'Oroval' [*C. clementina* Hort *ex* Tanaka] x 'Kara' mandarin [*C. unshiu* Marc. x *C. nobilis* Lou.]) and 'Murcott' mandarin (*C. reticulata* Blanco), the on crop of fruit reduced bud expression of genes regulating floral timing and floral meristem identity, including *FLOWERING LOCUS T* (*FT*) (as early as November) and *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (as early as January), with a concomitant decrease in inflorescence number at spring bloom (~April) compared to off-crop trees (Muñoz-Fambuena et al., 2012; Shalom et al., 2012). For both cultivars, the on- or off-crop status of the trees had no effect on bud expression of the floral timing gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), despite dramatic differences in floral intensity at spring bloom (Muñoz-Fambuena et al., 2012; Shalom et al., 2012). For on-crop 'Murcott' mandarin trees, early fruit removal (August) significantly increased bud expression of *FT* and *LFY* within one week relative to their expression in buds of on-crop trees with no fruit removed (Shalom et al., 2014).

The effects of crop load and fruit removal on the expression of the floral organ identity genes downstream from *AP1* have not been investigated in *Citrus* spp. Furthermore, the basic pattern of transcript accumulation for *APETALA2* (*AP2*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*), which are respectively classified as class A, B and C floral organ identity genes (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005), relative to the timing of *FT*, *SOC1*, *LFY* and *AP1* expression and full bloom has not been published for commercially bearing citrus trees under field conditions. The expression patterns of *SEPALLATA1* (*SEP1*) and *SEPALLATA3* (*SEP3)*,

the *Arabidopsis thaliana* homologs with class E activity (Ditta et al., 2004; Krizek and Fletcher, 2005; Pelaz et al., 2000), in Satsuma mandarin (*C. unshiu* Marc.), an evergreen woody perennial, and trifoliate orange (*Poncirus trifoliata* [L.] Raf), a deciduous citrus relative, correlated with seasonal flowering in the two species (Nishikawa et al., 2009). For both *SEP* genes, maximum transcript accumulation occurred the month before bloom, providing evidence that *SEP1* and *SEP3* were more involved in flower development than floral induction (Nishikawa et al., 2009).

Similar analyses of the timing of *AP2*, *PI* and *AG* expression and, additionally, the effects of crop load on the expression of each gene, would increase our knowledge of the role these genes play with regard to floral induction, bud determinacy (irreversible commitment to floral development), and flower development in *Citrus* spp. From a practical point of view, this information would be useful for properly timing cultural practices, e.g., GA_3 applications and pruning. It might also provide insight into the exacerbation of AB caused by the commercial practice of holding the on crop of fruit on the tree past the standard harvest period to increase fruit size at a late harvest (El-Otmani et al., 2004, Hilgeman et al., 1967) and provide guidance as to how early the on crop must be harvested to reverse its negative effect on floral gene expression and return bloom.

With the goal of expanding our knowledge of floral development in *Citrus* spp., the first objective of this research was to analyze the pattern of transcript accumulation of eight genes, which included floral timing genes, floral meristem identity genes and floral organ identity genes, during the six months before bloom for two cultivars of *C.*

reticulata. Pixie mandarin is a late-maturing cultivar harvested in June (two months after bloom) and Nules Clementine mandarin is an early-maturing cultivar harvested in March (one month before bloom). The two cultivars were in commercial orchards located in a coastal and inland growing area, respectively, in order to evaluate the potential effect of microclimate on gene activity. To increase our understanding of AB at the level of floral gene transcription, the second objective was to quantify the effects of the on crop of fruit during the six months before bloom on transcript accumulation of the four genes previously studied (*FT*, *SOC1*, *LFY* and *AP1*) relative to that of genes downstream from *AP1* (*AP2*, *SEP1*, *PI* and *AG*) that have not been investigated. The capacity of an early harvest to restore floral gene expression and inflorescence number the following spring was determined by harvesting on-crop 'Pixie' and 'Nules Clementine' mandarin trees in February and January, two and three months before bloom, respectively, The results of this research provided new information regarding the genetic control and timing of citrus floral induction, floral determinacy and flower organogenesis and the effects of the on crop on floral development.

Materials and Methods

Plant materials

Fifteen-year old 'Pixie' mandarin (*C. reticulata* Blanco) trees on 'Troyer' citrange (*C. sinensis* L. Osbeck x *P. trifoliata* L. Raf.) rootstock in a commercial orchard in a coastal valley in Ojai, California (34˚27'N, 119˚15'W; elevation 298 m above sea level [asl]) and 10-year old 'Nules' Clementine mandarin (*C. reticulata* Blanco) grafted on 'Carrizo'

citrange (*C. sinensis* L. Osbeck x *P. trifoliata* L. Raf.) rootstock in a commercial inland orchard in Fillmore, California (34˚22'N, 118˚54W; elevation 136 m asl) were used in this research. Monthly average maximum and minimum air temperatures for the two years of the research were downloaded from the California Irrigation Management Information System (CIMIS) website (California Department of Water Resources, 2009) for the closest station to each orchard. In each orchard, there were five blocks (replications), each containing one tree setting an off crop and two nearby trees setting an on crop subjected to early and late harvest, respectively. Both orchards reached full bloom in April. For each block of 'Pixie' mandarin, the off-crop tree was harvested in March, one on-crop tree was harvested in February, two months before bloom, and the second on-crop tree was harvested in June (the typical harvest time), two months after bloom. For each block of 'Nules Clementine' mandarin trees, one on-crop tree was harvested in January, three months before bloom, and the off-crop tree and the second on-crop tree were harvested in March (the typical harvest time), one month before bloom. For each cultivar, the three treatments were each replicated five times.

Sample collection and gene expression analysis

The apical five buds on nonbearing shoots (NBS) of off-crop trees and bearing shoots (BS) of on-crop trees for each harvest date were collected from 20 shoots of each type per five trees for each of the three treatments per cultivar and used for gene expression analysis. For both cultivars, bud collection was initiated in October and continued through March since full bloom was in April. For on-crop trees harvested early,

collection of buds on BS was initiated one month after harvest and ended in March. At the time of collection, buds were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab. Bud samples were quickly frozen in liquid nitrogen and stored at -80 ˚C until analysis. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequences of *Arabidopsis* homologs *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). Citrus *FT*, *SOC1*, *LFY* and *AP1* genes analyzed in this research were *CiFT2* (Nishikawa et al., 2007), *CsSOC-like2* (*CsSL2*) (Tan and Swain, 2007), *CsLFY* and *CsAP1* (Pillitteri et al., 2004), *PtAP2* (Song et al., 2010), and *CuSEP1* (Nishikawa et al., 2009), respectively; each gene was selected based on its demonstrated functional equivalence in its respective *A. thaliana* mutant. In addition, expression of *CiFT2* was related to floral intensity in response to low temperature in *C. unshiu* (Nishikawa et al., 2007) and crop load in *C. reticulata* (Shalom et al., 2012, 2014); *CsSL2* expression was also related to

the effect of crop load on flowering in *C. reticulata* (Shalom et al., 2012). The sequences of *PI* and *AG* chosen in this research share high identity with *A. thaliana PI* and *AG*, respectively; the predicted protein sequences for the putative *PI* and *AG* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com/primerquest/Home/Index) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study as well as the BLAST results of PCR product sequence versus target sequence of each gene of interest are listed in Table 4.1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-µL reaction system containing 1.2 µL cDNA (about 40 ng of input RNA), 0.6 µL gene-specific forward and reverse primer mix (10nM), 7.5 µL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 µL PCR-grade water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was run at the end of each qPCR run to confirm that nonspecific products were not formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with the flowers of ‘Pixie’ mandarin and ‘Nules Clementine’ mandarin

collected from off-crop trees during spring bloom as the control (expression level of 1), respectively, and *β-ACTIN* (*ACT*) as the primary reference gene (endogenous control), and reported herein. The selection of *ACT* as the primary reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Results based on a second reference gene, *ELONGATION FACTOR 1-ALPHA* (*EF1-α*) from *C. unshiu* (Nishikawa et al., 2009), are presented as supplemental results (Tables S4.1, S4.2). The expression pattern of each floral gene with *ACT* as the endogenous control was strongly correlated with its expression pattern when *EF1-α* served as the reference gene (r = 0.68 to 0.99, $P < 0.05$ for all genes), confirming the consistency and reliability of the results. Gene expression data for each sampling date were the mean of five biological replicates; each biological replicate was the mean of three qPCR technical replicates.

Effects of fruit on bud development

At full bloom in April, the developmental fate of the apical five buds on each of 20 shoots randomly selected before bloom on NBS and BS of the five trees in each treatment, respectively, was determined. For 'Pixie' mandarin, the number of leafless (one to many flowers with no leaves), leafy (one to many flowers with one to many leaves), and total inflorescences (sum of leafless plus leafy inflorescences), vegetative shoots, and inactive (dormant) buds per 20 shoots per tree was counted. However, at bloom, 'Nules Clementine' mandarin trees were sequestered under netting to prevent pollen transfer by bees. Thus, floral intensity was estimated one month later in May based on the number of young fruit set per shoot, which underestimated inflorescence number and overestimated

vegetative shoot number. Results for the five apical buds on the 20 shoots of each type per tree were averaged for the five individual trees (replications) per treatment and reported as the average value per five apical buds per 20 shoots per five trees per treatment (100 buds/tree).

Yield data

Total yield (kg/tree) for each data tree was recorded in the field during each harvest. For off-crop trees, the number of fruit per tree was counted during harvest. For on-crop trees, a randomly selected sample of 200 fruit per tree, which represented about 5 to 8 % of the average total number of fruit per tree, was collected for each tree and the transverse diameter of each fruit was measured with an electronic caliper. Based on its diameter, each fruit was assigned to a fruit size category. The fruit weight of a specified number of fruit in each fruit size category was determined in order to estimate the total number of fruit per tree.

Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots and inactive buds per tree and the relative expression levels of genes, using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$. Pearson's correlation coefficients were calculated to

identify significant relationships ($r > 0.5$, $P \leq 0.05$) between crop load (kg/tree and number/tree) and floral intensity of the return bloom.

Results

Effects of fruit on floral intensity of the return bloom in 'Pixie' mandarin

There was a significant difference in yield between the off- and on-crop 'Pixie' mandarin trees used in this research. Off-crop trees harvested in March set a final crop of 19.8 kg per tree (185 fruit/tree), whereas on-crop trees harvested in June produced 12- and 18-fold more fruit by weight (235.9 kg/tree) and number per tree (3381 fruit/tree), respectively ($P < 0.0001$ for both) (Table 4.2). The light crop load resulted in off-crop trees producing an average of 96.4 inflorescences (based on 5 apical buds/20 shoots/tree) at return bloom the following year in April (Table 4.2). Bloom was dominated by leafless inflorescences, which constituted 91% of total inflorescences produced by off-crop trees. In contrast, the heavy yielding on-crop trees did not produce any inflorescences on either NBS or BS. Furthermore, no inflorescences developed for on-crop trees after normal spring bloom in April through June, indicating flowering was inhibited, rather than delayed, by the on crop of fruit. Early harvest of a second set of on-crop trees (178.0 kg/tree, 3633 fruit/tree) in February (two months before bloom) did not significantly increase flowering (Table 4.2). For the trees in the data set, floral intensity (inflorescence number) at return bloom was significantly (negatively) correlated with yield per tree as kg ($r = -0.95$, $P < 0.0001$) and fruit number ($r = -0.99$, $P < 0.0001$).

For on-crop trees, all 100 apical buds remained inactive during spring, producing neither inflorescences, nor vegetative shoots. Thus, the number of inactive buds of on-crop trees was significantly greater than off-crop trees ($P < 0.0001$). Harvesting on-crop trees in February resulted in 41.8 vegetative shoots per 100 buds per tree, a number significantly greater than that of both off-crop trees harvested in March and on-crop trees harvested in June ($P < 0.0001$). With the increase in vegetative shoot number, on-crop trees harvested in February had a significantly lower number of inactive buds (58.0/tree) compared to that of on-crop trees harvested in June (100 inactive buds/tree) ($P < 0.0001$). These results indicated that both floral and vegetative buds were inhibited from undergoing spring bud break due to the presence of on crop of fruit on the tree through June. Moreover, the results provide evidence that the presence of the on crop of fruit on the tree through February was sufficient to prevent the shoot apical meristem from being determined.

Effects of fruit on the expression of citrus floral timing genes in 'Pixie' mandarin

For buds of off-crop trees, it is of interest that *FT* was expressed as early as October, six months before bloom (Table 4.3). Whereas *FT* transcripts were detected at a low level in November, expression increased in December. Maximum *FT* expression was in January ($P < 0.05$), with bud *FT* expression significantly lower during February and March ($P < 0.05$), but equal to the level of expression in October. In contrast, transcripts of *FT* were not detected in buds of on-crop trees on any sampling date. Early harvest of on-crop trees in February did not result in a significant level of *FT* expression in March.

Transcripts of *SOC1* were consistently detected in buds of all trees in the data set from October through March (Table 4.3). For buds of off-crop trees, greater expression levels of *SOC1* occurred in October, December and January compared to November, February and March, with the lowest expression in March ($P < 0.0001$). For buds of on-crop trees, *SOC1* expression varied over time, being greater in October and January than November, December, February and March ($P < 0.01$), but significantly lower than *SOC1* expression in buds of off-crop trees only in October ($P < 0.01$), December ($P < 0.01$) and January ($P < 0.05$). Thus, no differences in *SOC1* expression were observed in any buds in February or March, including buds of on-crop trees harvested in February.

For buds of off-crop trees, *LFY* expression occurred at a constant low level from October through December, but dropped below the limit of detection in January. Maximum *LFY* expression occurred in February ($P < 0.05$), returning to a lower level in March, the month prior to bloom (Table 4.3). For buds of on-crop trees, *LFY* transcript accumulation followed the same pattern as off-crop trees, with no significant differences in transcript levels in the two bud types from October through January, when transcripts were not detected in either one. Whereas *LFY* expression was restored in buds of off-crop trees in February and March, *LFY* expression was low in February and not detected in March for buds of on-crop trees. As a result, by March, only buds of off-crop trees expressed *LFY*; even on-crop 'Pixie' mandarin trees harvested in February had no detectable levels of *LFY* expression in March.

Effects of fruit on the expression of citrus genes with class A activity in 'Pixie' mandarin

Buds of off-crop trees expressed *AP1* in October, but at a lower level in October through January than in February and March, two months and one month before bloom, respectively ($P < 0.0001$) (Table 4.4). Buds of on-crop trees also expressed *AP1* in October. For these buds, *AP1* expression was at a maximum in October ($P < 0.0001$) and significantly greater than in buds of off-crop trees ($P < 0.01$). Although expression of *AP1* in buds of on-crop trees decreased after October, it remained equal to that of off-crop trees through January but was significantly lower than in buds of off-crop trees in February ($P < 0.001$) and March ($P < 0.0001$). Early harvest of on-crop trees in February did not increase bud *AP1* transcript levels by March. Thus, one month before bloom, buds of off-crop trees had significantly greater *AP1* expression than buds of on-crop 'Pixie' mandarin trees, including those harvested in February ($P < 0.0001$).

Buds of both off- and on-crop trees expressed *AP2* at a low level in October (Table 4.4). Expression remained low and not significantly different between the two sample types from October through December. However, in January and February, *AP2* transcript levels were significantly lower in buds of off-crop trees than on-crop trees ($P < 0.05$ for both months). For buds of off-crop trees, the *AP2* expression level observed in February increased four-fold to its maximum value in March ($P < 0.001$), coincident with a 50% decrease in *AP2* expression to the lowest level observed in buds of on-crop trees ($P < 0.01$) (Table 4.4). This resulted in significantly greater *AP2* expression in buds of off-crop trees than buds of on-crop trees in March ($P < 0.05$), one month before bloom. Harvesting on-crop trees in February resulted in bud *AP2* transcript levels in March that

were significantly lower than those in buds of off-crop trees ($P < 0.05$) and equal to those of on-crop trees harvested in June.

Effects of fruit on the expression of citrus floral organ identity genes downstream from AP2 in 'Pixie' mandarin

Transcripts of *SEP1* in buds of off-crop trees were detected in October through December, not detected in January, and detected again in February, with maximum *SEP1* expression in March, one month before bloom (Table 4.5). In contrast, *SEP1* expression was stronger in buds of on-crop trees than off-crop trees in October through February, but the reverse was true the month before bloom. For buds of off-crop trees, transcripts of *PI* and *AG* were either below the limits of detection or at very low, detectable levels in October through February; both genes were expressed at a significant level in March, one month prior to anthesis (Table 4.5). For buds of on-crop trees, *PI* transcripts were never detected, but *AG* transcripts were detected in all months from October through March, with the exception of January. For buds of on-crop trees harvested in February, transcripts of *SEP1* and *AG*, but not *PI*, were detected one month after harvest (Table 4.5). However, in March, one month before bloom, only buds of off-crop trees expressed *SEP1*, *PI* and *AG* at significant levels (Table 4.5).

Consistent with the observation that no inflorescences developed in on-crop trees after the normal spring bloom in April, analysis of floral gene expression in April documented that transcripts of *FT* and *LFY* were still below the limits of detection. Expression of *SOC1* in buds of on-crop trees remained equal to that of off-crop trees,

whereas bud expression of *AP1* and *AP2* remained significantly lower than that of off-crop trees. In April, transcripts of *SEP1* were still detected, but *PI* and *AG* expression was below the limit of detection in buds of on-crop trees (unpublished results). These results rule out the possibility that the on crop of fruit delays floral development. Moreover, the failure of February harvest to restore floral gene expression in buds of on-crop trees indicates that the negative effect of the on crop of fruit on floral gene expression and floral development is irreversible in 'Pixie' mandarin by February.

Effects of fruit on floral intensity of the return bloom in 'Nules Clementine' mandarin

Off-crop 'Nules Clementine' mandarin trees (23.9 kg/tree; 203 fruit/tree), produced 43.4 inflorescences (based on the number of fruit set by 5 apical buds/20 shoots/tree) the following April (Table 4.6). In contrast, on-crop trees setting a heavier crop (86.5 kg/tree, 1262 fruit/tree) did not flower (< 1 inflorescence/100 buds/tree). The data confirm visual observations that off-crop trees flowered intensely and on-crop trees did not flower. Inflorescence number was significantly (negatively) correlated with total fruit weight (r = -0.93, $P < 0.0001$) and total fruit number per tree at harvest (r = -0.90, $P < 0.001$). Harvesting on-crop trees in January, two months earlier than the regular harvest time, did not increase inflorescence number but led to the production of vegetative shoots, resulting in 33.2 vegetative shoots per tree, a significantly greater number than that of both off-crop trees and on-crop trees harvested in March ($P < 0.001$) (Table 4.6).

Effects of fruit on the expression of citrus floral timing genes in 'Nules Clementine' mandarin

The pattern of *FT* expression in 'Nules Clementine' mandarin was different from that of 'Pixie' mandarin. Bud *FT* transcripts were not detected in off-crop 'Nules Clementine' mandarin trees in October. No samples were collected in November. Thus, *FT* expression in buds of off-crop trees was first observed in December, and also observed in January, but was below the limit of detection in February, with maximum transcript accumulation in March, one month before bloom (Table 4.7). Consistent with the results obtained with 'Pixie' mandarin trees, transcripts of *FT* were not detected in buds of on-crop 'Nules Clementine' mandarin trees on any sampling date. Early harvest of on-crop 'Nules Clementine' mandarin trees in January (three months before bloom) did not result in detectable levels of *FT* transcripts (Table 4.7).

Like 'Pixie' mandarin, buds of off-crop and on-crop 'Nules Clementine' mandarin trees also expressed *SOC1* in October, but overall expression was lower in 'Nules Clementine' than 'Pixie' mandarin through February. There were no significant differences in *SOC1* expression in buds from off- and on-crop trees from October through March, nor with *SOC1* expression in buds from on-crop trees harvested in January for the two months before bloom (Table 4.7).

For buds of off-crop 'Nules Clementine' mandarin trees, *LFY* was expressed in October at a level not significantly different than two months later in December, but was not detected in January or February, with maximum expression in March ($P < 0.01$) (Table 4.7). For comparison, expression of *LFY* in buds of off-crop 'Pixie' mandarin tree

was only below the limit of detection in January, with recovery and maximum expression in February (Table 4.3). Like off-crop trees, *LFY* was also expressed in buds of on-crop trees in October and December at a level equal to that of off-crop trees, and not detected in January and February. However, for buds of on-crop trees, *LFY* expression did not recover in March. Only buds of off-crop trees expressed *LFY* in March, with the exception of buds of on-crop trees harvested in January. For the early-harvested trees, *LFY* transcripts were not detected in February but were detected in March at a level lower than but not significantly different from that observed for buds of off-crop trees.

Effects of fruit on the expression of citrus genes with class A activity in 'Nules Clementine' mandarin

Consistent with the results in 'Pixie' mandarin, the expression of *AP1* was detected as early as October (six months before bloom) in buds of both off-crop and on-crop 'Nules Clementine' mandarin trees. For buds of off-crop 'Nules Clementine' mandarin trees, *AP1* expression was not significantly different from October through February, but increased significantly to its maximum level in March, one month before bloom ($P < 0.0001$) (Table 4.8). Thus, *AP1* expression was not significantly different between buds of off- and on-crop trees from October through February, but in March, one month before bloom, buds of off-crop trees had greater expression of *AP1* than buds of on-crop trees ($P < 0.001$). Although January harvest of on-crop 'Nules Clementine' mandarin trees significantly increased *LFY* expression by March, the early harvest had no effect on *AP1* expression in February or March. Consequently, one month before bloom, bud *AP1*

transcript levels were significantly greater in buds of off-crop trees than those of on-crop trees harvested in January or March ($P < 0.001$).

The pattern of *AP2* expression in 'Nules Clementine' mandarin was similar to that of 'Pixie' mandarin. Transcripts of *AP2* were detected in October in buds of off-crop and on-crop trees, with no difference in the level of *AP2* expression between the two sample types in October (Table 4.8). In addition, there were no significant differences in *AP2* expression levels in buds of off-crop trees compared to buds of on-crop trees in December or January. By February, expression of *AP2* was significantly greater for buds of on-crop trees than all other buds, including buds of on-crop trees harvested in January ($P < 0.05$). However, by March, similar to the results obtained with 'Pixie' mandarin, bud *AP2* expression was significantly greater in buds of off-crop 'Nules Clementine' mandarin trees compared to that of buds of on-crop trees harvested in January or March ($P < 0.01$).

Effects of fruit on the expression of citrus floral organ identity genes downstream from AP2 in 'Nules Clementine' mandarin

For buds of both off-crop and on-crop 'Nules Clementine' mandarin trees, transcripts of *SEP1* were detected in October, six months before bloom, through March, when *SEP1* transcripts accumulated in buds of off-crop trees, but not on-crop trees (Table 4.9). Expression of *PI* occurred in October in buds of both off- and on-crop trees, but thereafter *PI* transcripts were not detected in either bud type; in March, the month before bloom, *PI* transcripts accumulated to a maximum value in buds of off-crop 'Nules

Clementine' mandarin trees (Table 4.9). Transcripts of *AG* were only detected in buds of on-crop trees in October and not detected in any other bud samples until March, when *AG* transcripts accumulated to a maximum level in buds of off-crop trees (Table 4.9). Early harvest of on-crop 'Nules Clementine' mandarin trees in January resulted in detectable levels of *SEP1* and *AG*, but not *PI*, in February and March (Table 4.9). Thus, full expression of *SEP1*, *PI* and *AG* only occurred in buds of off-crop trees and only in March, the month before bloom, consistent with the results with 'Pixie' mandarin. The results also demonstrated that the on crop of 'Nules Clementine' mandarin fruit had an irreversible effect on floral gene expression and floral development as early as January, three months before bloom, indicating that fruit would need to be removed at least by December, and likely earlier, to have a positive effect on return bloom.

Relationship between air temperatures and the transcript accumulation pattern for citrus floral genes

Given the synchrony in the timing and pattern of floral gene expression for the two cultivars, a clear relationship with air temperatures could not be established. Moreover, it is clear from the results that floral initiation actually occurred prior to October, but exactly when is unclear. Maximum and minimum air temperatures for the 'Pixie' mandarin orchard were on average 2 to 5 ºC warmer and 5 to 8 ºC colder from July through June the following year than the 'Nules Clementine' orchard, respectively. Minimum temperatures in January and February were less than 1 ºC in the 'Pixie' mandarin orchard, but 8 ºC in the 'Nules Clementine mandarin orchard. However, in

March and April, both orchards had average maximum temperatures above 20 ºC but less than 25 ºC and average minimum temperatures above 5 ºC but less than 10ºC, which might have synchronized the upregulation of *LFY*, *AP1* and *AP2* and activation of *SEP1*, *PI* and *AG* in March and bloom in April of the off-crop 'Pixie' and 'Nules Clementine' mandarin trees in the two orchards.

Discussion

In this research, off-crop trees of two cultivars of *C. reticulata*, Pixie and Nules Clementine mandarin, flowered profusely the following spring in April. The pattern of floral gene expression in the buds of these trees documented the presence of transcripts of floral timing genes (*FT*, *SOC1* and *LFY*), floral meristem identity genes (*LFY* and *AP1*) and floral organ identity genes (*AP2, SEP1*) in October, six months before bloom. For 'Nules Clementine' mandarin trees, bud expression of *FT* was first detected in December; it was not detected in October and no buds were analyzed in November. However, it can be inferred that *FT* had been expressed earlier in buds of 'Nules Clementine' mandarin since in October all genes downstream from *FT* through *AP2* were expressed at significant levels and *SEP1* transcripts were detected. The possibility that *FT* transcripts cycle between detectable and undetectable levels is consistent with *FT* transcripts being below the limits of detection in buds of off-crop 'Nules Clementine' mandarin trees again in February. Similarly, fluctuations in bud transcript levels were observed for *LFY* in both cultivars. First detected in October in buds of off-crop trees, *LFY* expression persisted through December, but was not detected in January in 'Pixie' and January or February in

'Nules Clementine' mandarin. In both cases, *LFY* expression was restored to a significant level a month later in February ($P < 0.05$) and March ($P < 0.01$), respectively. Months in which *FT* or *LFY* expression were not detected were not related to reduced expression of any downstream gene in off-crop trees. However, restoration of *LFY* expression in buds of 'Pixie' mandarin in February was coincident with a marked increase in *AP1* expression in the same month through March ($P < 0.0001$). The later restoration of *LFY* expression in buds of 'Nules Clementine' mandarin in March was accompanied by a significant increase in *AP1* expression in the same month ($P < 0.0001$). In *A. thaliana*, *LFY* and *AP1* are capable of reciprocal activation (Pajoro et al, 2014), so it is equally possible that increased *AP1* expression restored *LFY* transcription. Both *LFY* and *AP1* are targets of *FT* and both upregulate the downstream floral organ identity genes in *A. thaliana* (Abe et al., 2005; Pajoro et al, 2014). For both cultivars, transcript accumulation for the downstream Class A floral organ identity gene *AP2* reached a maximum in March, one month before bloom, with a concomitant increase in the expression of the class B, C and E floral organ identity genes, *PI*, *AG* and *SEP1*, respectively, from not detected or detected in February to expressed in March.

'Pixie' and 'Nules Clementine' mandarin trees producing an on crop failed to develop any inflorescences the following April. The typical harvest dates in June and March, respectively, did not result in different effects on floral gene expression. For both cultivars, the on crop repressed bud expression of *FT* below the limits of detection from October through March. Despite this, bud *SOC1* transcript levels were only sometimes lower or never lower in on-crop than off-crop 'Pixie' and 'Nules Clementine' mandarin

trees, respectively, but importantly equal to those of off-crop trees of both cultivars in February and March. The results provided evidence that *SOC1* activity was neither directly regulated by the on crop of fruit, nor indirectly regulated by *FT* expression. In addition, for buds of on-crop trees of both cultivars, the expression pattern for *LFY* was not correlated with that of *FT,* nor *SOC1*, but it had the same pattern as buds of off-crop trees, respectively, with the noteworthy exception that *LFY* transcripts were not detected in buds of on-crop trees in March, the month before bloom. For buds of on-crop trees of both cultivars, in the month before bloom, expression of the genes downstream from *LFY* was either significantly reduced (*AP1* and *AP2*), or reduced to the limit of detection or below it (*SEP1, PI* and *AG*) compared to that of off-crop trees. Thus, results obtained with the two cultivars of *C. reticulata* confirmed the negative effects of the on crop on *FT* expression as early as six months before bloom and the subsequent downstream effects on *LFY* and *AP1* expression five months later in March, just one month before bloom. The results are similar to those previously reported for 'Moncada' and 'Murcott' mandarin demonstrating the reduced expression of *FT* in November and *LFY* and *AP1* in January (Muñoz-Fambuena et al., 2012; Shalom et al., 2012). In addition, the results of the present research documented for the first time that the negative effects of the on crop on *FT*, *LFY* and *AP1* expression resulted in reduced expression of the downstream Class A, B, C and E floral organ identity genes *AP2*, *PI*, *AG* and *SEP1*, respectively. Taken together, the results provided evidence that *LFY* or *AP1* or both are the targets of *FT* and that one or both are the activators of the downstream floral organ identity genes, *AP2*, *PI*,

AG and *SEP1*, in *C. reticulata,* consistent with the ABCE model of floral organ specification described for *A. thaliana* (Pajoro et al., 2014).

Early harvest of on-crop 'Pixie' mandarin trees in February and 'Nules Clementine' mandarin trees in January, two and three months before bloom, respectively, did not result in the production of any inflorescences, indicating that early effects of the on crop on floral development were not reversible at this point. February harvest of on-crop 'Pixie' mandarin trees increased *FT* expression to a detectable level, but *LFY* expression remained below the limit of detection. Bud transcript levels for the genes downstream from *LFY* remained the same for on-crop 'Pixie' mandarin trees harvested in February as trees harvested in June. For buds of on-crop 'Nules Clementine' mandarin trees harvested in January, *FT* transcripts were not detected, whereas *LFY* transcripts increased to a level lower but not significantly different from that of off-crop trees. In contrast to the increase in *LFY* expression, but consistent with *FT* not being expressed, *AP1* and *AP2* expression remained equal to or less than that of on-crop trees harvested in March, respectively, with no increase in *SEP1*, *PI* or *AG* expression. These results suggest the possibility that *AP1* alone might be the target of *FT* and that *AP1,* not *LFY*, regulates the activity of the downstream floral organ identity genes, *AP2*, *SEP1*, *PI*, and *AG*. It is clear from these data that bud expression of the floral timing gene *SOC1* in on-crop trees, which was equal to that of off-crop trees for both cultivars in February and March, was not able to upregulate *LFY* or *AP1* to promote flowering, indicating that *SOC1* activity could not "substitute" for the lack of *FT* activity in on-crop trees. Further, the results provided clear evidence that although *AP1* and *AP2* continued to be expressed in the buds of on-crop

trees, the level of expression was insufficient to activate the downstream floral organ identity genes required for flower formation. The results of the research presented herein provided strong evidence suggesting that, as in *A. thaliana*, persistent expression of *FT* is essential for the shoot apical meristem (SAM) to become committed to floral development (determined) (Müller-Xing et al., 2014). In *C. sinensis*, floral determinacy correlates with sepal formation (Lord and Eckard, 1987), the development of which is likely under the control of the two Class A genes, *AP1* and *AP2*, in citrus as it is in *A. thaliana* (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005).

Although February and January harvests of on-crop 'Pixie' and 'Nules Clementine' mandarin trees, respectively, did not result in inflorescence development, the early harvests significantly increased the number of vegetative shoots that developed the following April. This result confirmed the inhibitory effect of the on crop on spring bud break first reported by Verreynne and Lovatt (2009) and established that the buds of on-crop trees were not determined (i.e., committed to floral development). This result is also interesting because regardless of the different final fate of buds of off-crop trees, and buds of on-crop trees harvested in January or February; each expressed *SOC1*, *AP1* and *AP2,* exhibited *LFY* expression that cycled between levels that were significant and not detectable, and had detectable levels of *SEP1* during the six-month period from October through March. However, in contrast to buds of off-crop trees, which became committed to floral development, buds of the early harvested on-crop trees, which produced only vegetative shoots, never expressed *FT* during the six months before bloom, resulting in the failure to accumulate the substantial levels of *LFY, AP1* and *AP2* transcripts observed

in buds of off-crop trees by March that subsequently activate the downstream Class B, C and E genes. The results lend further support to the above proposal that persistent expression of *FT* is necessary to upregulate the genes required for floral determinacy of the SAM.

How early *FT* is first expressed in buds of 'Pixie' and 'Nules Clementine' mandarin and when the on crop first imposes a negative effect on the expression of *FT,* or other gene, in the citrus floral development pathway remains unknown, but the results presented herein strongly suggest these events occur before October, possibly as early as August (Shalom et al., 2014), and thus, more than six months before bloom. The results leave open the possibility that all buds on adult citrus trees are competent and have been induced to flower as a consequence of the transition from juvenile to adult (Tan and Swain, 2006). These data are also consistent with annual transition from vegetative to floral development earlier than October, possibly through a different pathway than classic low temperature induction in fall (Nishikawa et al., 2009). This proposal is consistent with age-dependent (autonomous) flowering in citrus (Shalom et al., 2015; Zhang and Hu, 2013) and with citrus being developmentally similar to other evergreen perennial tree crops, such as avocado (*Persea americana* Mill.) (Salazar-García et al., 1998) and olive (*Olea europaea* L.) (Cuevas et al., 1999), known to transition from vegetative to reproductive development in summer (July). Both possibilities are consistent with the essential role of *FT* in floral induction in *C. reticulata* demonstrated herein.

The negative effect of the on crop on *FT* expression as early as October in *C. reticulata* likely did not inhibit inflorescence development by inhibiting floral induction.

For buds of on-crop ’Pixie’ and ‘Nules Clementine’ mandarin trees, the presence of transcripts of *LFY*, *AP1*, *AP2* and *SEP1* in October supports the interpretation that floral induction had successfully occurred. Harvest of on-crop ‘Nules Clementine’ mandarin trees in January, three months before bloom, resulted in only vegetative shoot development the following spring, providing evidence that the reduced expression of *FT* to levels below the limit of detection beginning in October, but possibly earlier, and continuing through December, resulted in the failure of the SAM to become determined. Moreover, increased expression of *LFY, AP1*, and *AP2* observed in off-crop ‘Pixie’ and ‘Nules Clementine’ mandarin trees in March failed to occur in early harvested on-crop trees, providing additional evidence of the need for persistent expression of *FT* for the upregulation of *LFY* and/or *AP1,* and the essential role of one or more of the following genes *LFY*, *AP1* and *AP2* in floral determinacy in citrus.

Taken together, the results indicate that for both ‘Pixie’ and ‘Nules Clementine’ mandarin, the on crop would need to be harvested before October to fully restore flowering. How much earlier in the season is unknown. Harvest of the on crop in the several months before October, while not commercially viable, would be an excellent tool for manipulating floral gene expression not only to increase our understanding of alternate bearing, but also to resolve the intriguing question of floral induction in citrus.

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Table 4.1 Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence BLAST against target gene sequence	
				E-value	Identity
FT	AB027456.1 (*C. unshiu*)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
SOC1	EU032532.1 (*C. sinensis*)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
LFY	AY338976.1 (*C. sinensis*)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
AP1	AY338974.1 (*C. sinensis*)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
AP2	EU883665.1 (*C. trifoliata*)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEP1	AB329715.1 (*C. unshiu*)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PI	XM_ 006472790.1 (*C. sinensis*)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AG	HM246523.1 (*C. sinensis*)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov).

Table 4.2 Crop load and developmental fate of buds, as number of total, leafless and leafy inflorescences, vegetative shoots or inactive (dormant) buds, on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in February and June in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest time	Total yield[y] (kg/tree)	Total yield (no./tree)	Inflorescences[z] (no./tree)			Vegetative shoots (no./tree)	Inactive buds (no./tree)
					Total	Leafless	Leafy		
Off-crop	NBS	Mar	19.8 c	185 b	96.4 a	87.6 a	8.8 a	0.2 b	3.4 c
On-crop	BS	Jun	235.9 a	3381 a	0.0 b	0.0 b	0.0 b	0.0 b	100.0 a
On-crop	BS	Feb	178.0 b	3633 a	0.2 b	0.0 b	0.0 b	41.8 a	58.0 b
P-value			****	****	****	****	****	****	****

[x] Means followed by different letters within a vertical column are significantly different according to Fisher's least significant difference (LSD) test in which **** refers to a significant effect at $P < 0.0001$.

[y] For crop load, data are the means of five trees per treatment.

[z] For numbers of inflorescences (total, leafless, leafy), vegetative shoots and inactive buds, data are the average values per five apical buds per 20 shoots per five trees per treatment (100 buds/tree).

Table 4.3 Expression of *FT*, *SOC1* and *LFY* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in February and June in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest time	Relative expression						
			Oct	Nov	Dec	Jan	Feb	Mar	*P*-value
FT expression									
Off-crop	NBS	Mar	0.1 ^{B}	< 0.05[y]	0.4 ^{A}	0.5 ^{A}	0.2 ^{B}	0.1 ^{B}	*
On-crop	BS	Jun	ND[z]	ND	ND	ND	ND	ND	--
On-crop	BS	Feb						D	--
P-value			--	--	--	--	--	--	
SOC1 expression									
Off-crop	NBS	Mar	9.6 a^{A}	5.1 a^{B}	7.4 a^{A}	8.5 a^{A}	3.0 a^{BC}	1.8 a^{C}	****
On-crop	BS	Jun	6.6 b^{A}	4.0 a^{B}	4.0 b^{B}	5.7 b^{A}	3.3 a^{B}	3.1 a^{B}	**
On-crop	BS	Feb						3.4 a	--
P-value			**	ns	**	*	ns	ns	
LFY expression									
Off-crop	NBS	Mar	3.5 a^{B}	1.0 a^{B}	1.1 a^{B}	ND	9.0 a^{A}	2.1^{B}	*
On-crop	BS	Jun	2.2 a^{A}	0.9 a^{AB}	0.6 a^{AB}	ND	0.4 a^{B}	ND	ns
On-crop	BS	Feb						ND	--
P-value			ns	ns	ns	--	ns	--	

[x] Data are the means for five trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 4.4 Expression of *AP1* and *AP2* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in February and June in 'Pixie' mandarin[z].

Tree status	Shoot status	Harvest time	Relative expression						
			Oct	Nov	Dec	Jan	Feb	Mar	*P*-value
AP1 expression									
Off-crop	NBS	Mar	1.5 b^{C}	2.2 a^{B}	1.5 a^{C}	1.5 a^{C}	3.2 a^{A}	2.9 a^{A}	****
On-crop	BS	Jun	3.6 a^{A}	2.5 a^{B}	1.1 a^{CD}	0.8 a^{CD}	1.5 b^{C}	0.6 b^{D}	****
On-crop	BS	Feb						0.8 b	--
P-value			**	ns	ns	ns	***	****	
AP2 expression									
Off-crop	NBS	Mar	0.2 a^{B}	0.2 a^{B}	0.2 a^{B}	0.2 b^{B}	0.1 b^{C}	0.4 a^{A}	***
On-crop	BS	Jun	0.2 a^{AB}	0.2 a^{B}	0.2 a^{AB}	0.3 a^{A}	0.2 a^{B}	0.1 b^{C}	**
On-crop	BS	Feb						0.2 b	--
P-value			ns	ns	ns	*	**	*	

[z] Data are the means for five trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

Table 4.5 Expression of *SEP1*, *PI* and *AG* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in February and June in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest time	Relative expression						
			Oct	Nov	Dec	Jan	Feb	Mar	*P*-value
SEP1 expression									
Off-crop	NBS	Mar	< 0.05[y]	< 0.05	< 0.05	ND[z]	< 0.05	0.2	--
On-crop	BS	Jun	0.1 [A]	0.1 [A]	0.1 [A]	< 0.05	0.1 [A]	< 0.05	ns
On-crop	BS	Feb						< 0.05	--
P-value			--	--	--	--	--	--	
PI expression									
Off-crop	NBS	Mar	ND	ND	ND	ND	< 0.05	0.2	--
On-crop	BS	Jun	ND	ND	ND	ND	ND	ND	--
On-crop	BS	Feb						ND	--
P-value			--	--	--	--	--	--	
AG expression									
Off-crop	NBS	Mar	< 0.05	ND	< 0.05	ND	< 0.05	0.2	--
On-crop	BS	Jun	< 0.05	< 0.05	< 0.05	ND	< 0.05	< 0.05	--
On-crop	BS	Feb						ND	--
P-value			--	--	--	--	--	--	

[x] Data are the means for five trees (replications) per treatment. Means followed by the same upper-case letter within a horizontal row are not significantly different (ns) over time for the same treatment according to Fisher's least significant difference (LSD) test.
[y] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.
[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 4.6 Crop load and developmental fate of buds, as number of total inflorescences, vegetative shoots or inactive (dormant) buds, on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in January and March in 'Nules Clementine' mandarin[x].

Tree status	Shoot status	Harvest time	Total yield[y] (kg/tree)	Total yield (no./tree)	Total inflorescences[z] (no./tree)	Vegetative shoots (no./tree)	Inactive buds (no./tree)
Off-crop	NBS	Mar	23.9 b	203 b	43.4 a	9.4 b	47.2 c
On-crop	BS	Mar	85.5 a	1262 a	0.0 b	0.4 b	99.6 a
On-crop	BS	Jan	80.7 a	1335 a	0.4 b	33.2 a	66.4 b
P-value			**	**	****	***	****

[x] Means followed by different letters within a vertical column are significantly different according to Fisher's least significant difference (LSD) test in which ** refers to a significant effect at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$.
[y] For crop load, data are the means of five trees per treatment.
[z] For numbers of total inflorescences, vegetative shoots and inactive buds, data are the average values per five apical buds per 20 shoots per five trees per treatment (100 buds/tree).

Table 4.7 Expression of *FT*, *SOC1* and *LFY* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in January and March in 'Nules Clementine' mandarin[y].

Tree status	Shoot status	Harvest time	Relative expression					
			Oct	Dec	Jan	Feb	Mar	*P*-value
FT expression								
Off-crop	NBS	Mar	ND[z]	0.1 ^{A}	0.1 ^{A}	ND	0.3 ^{A}	ns
On-crop	BS	Mar	ND	ND	ND	ND	ND	--
On-crop	BS	Jan				ND	ND	--
P-value			--	--	--	--	--	
SOC1 expression								
Off-crop	NBS	Mar	2.2 a^{A}	1.2 a^{C}	1.0 a^{C}	0.9 a^{C}	1.7 a^{B}	****
On-crop	BS	Mar	1.6 a^{AB}	0.9 a^{B}	1.1 a^{B}	1.2 a^{B}	2.6 a^{A}	*
On-crop	BS	Jan				1.1 a^{A}	1.0 a^{A}	ns
P-value			ns	ns	ns	ns	ns	
LFY expression								
Off-crop	NBS	Mar	4.1 a^{B}	1.3 a^{B}	ND	ND	12.1 a^{A}	**
On-crop	BS	Mar	2.4 a^{A}	3.0 a^{A}	ND	ND	ND	ns
On-crop	BS	Jan				ND	2.5 a	--
P-value			ns	ns	--	--	ns	

[y] Data are the means for five trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, **** at $P < 0.0001$; ns, not significant.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 4.8 Expression of *AP1* and *AP2* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in January and March in 'Nules Clementine' mandarin[z].

Tree status	Shoot status	Harvest time	Relative expression					
			Oct	Dec	Jan	Feb	Mar	*P*-value
AP1 expression								
Off-crop	NBS	Mar	1.5 b^{B}	2.4 a^{B}	1.5 a^{B}	1.9 a^{B}	6.5 a^{A}	****
On-crop	BS	Mar	3.3 a^{A}	1.3 a^{C}	2.1 a^{BC}	2.5 a^{AB}	1.5 b^{C}	**
On-crop	BS	Jan				1.9 a^{A}	1.5 b^{A}	ns
P-value			**	ns	ns	ns	***	
AP2 expression								
Off-crop	NBS	Mar	0.5 a^{B}	0.3 a^{C}	0.4 a^{B}	0.2 b^{D}	0.8 a^{A}	****
On-crop	BS	Mar	0.4 a^{AB}	0.4 a^{B}	0.5 a^{A}	0.3 a^{B}	0.5 b^{A}	*
On-crop	BS	Jan				0.2 b^{A}	0.3 c^{A}	ns
P-value			ns	ns	ns	*	**	

[z] Data are the means for five trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

Table 4.9 Expression of *SEP1*, *PI* and *AG* in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in January and March in 'Nules Clementine' mandarin[x].

Tree status	Shoot status	Harvest time	Relative expression					
			Oct	Dec	Jan	Feb	Mar	*P*-value
SEP1 expression								
Off-crop	NBS	Mar	D[y]	D	D	D	0.2	--
On-crop	BS	Mar	0.1 [A]	0.1 [A]	D	D	D	ns
On-crop	BS	Jan				D	D	--
P-value			--	--	--	--	--	
PI expression								
Off-crop	NBS	Mar	0.1 a[B]	ND[z]	ND	ND	0.5 [A]	*
On-crop	BS	Mar	0.1 a	ND	ND	ND	ND	--
On-crop	BS	Jan				ND	ND	--
P-value			ns	--	--	--	--	
AG expression								
Off-crop	NBS	Mar	ND	ND	ND	ND	0.3	--
On-crop	BS	Mar	D	ND	ND	ND	ND	--
On-crop	BS	Jan				D	D	--
P-value			--	--	--	--	--	

[x] Data are the means for five trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$; ns, not significant.

[y] D, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Supplemental Results to Chapter 4

Table S4.1 Expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* relative to *EF1-α* expression in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in February and June in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest time	Relative expression						
			Oct	Nov	Dec	Jan	Feb	Mar	*P*-value
FT expression									
Off-crop	NBS	Mar	0.2 a^{BC}	0.1 a^{C}	1.2 a^{A}	0.7 a^{AB}	0.4 a^{BC}	0.5 a^{BC}	**
On-crop	BS	Jun	ND[y]	ND	ND	ND	ND	ND	--
On-crop	BS	Feb						< 0.05[z]	
P-value			--	--	--	--	--	--	
SOC1 expression									
Off-crop	NBS	Mar	24.0 a^{A}	10.0 a^{A}	18.5 a^{A}	23.5 a^{A}	8.0 a^{A}	10.9 a^{A}	ns
On-crop	BS	Jun	9.0 a^{BC}	5.3 a^{C}	8.9 b^{BC}	12.3 b^{BC}	7.7 a^{BC}	25.1 a^{A}	*
On-crop	BS	Feb						7.0 a	
P-value			ns	ns	*	**	ns	ns	
LFY expression									
Off-crop	NBS	Mar	9.8 a^{A}	1.8 a^{A}	2.6 a^{A}	ND	27.2 a^{A}	10.0 a^{A}	ns
On-crop	BS	Jun	2.5 a^{A}	1.4 a^{A}	1.4 a^{A}	ND	0.7 b^{A}	ND	ns
On-crop	BS	Feb						ND	
P-value			ns	ns	ns	--	*	--	
AP1 expression									
Off-crop	NBS	Mar	3.9 a^{B}	5.1 a^{B}	4.5 a^{B}	3.9 a^{B}	6.2 a^{B}	12.9 a^{A}	****
On-crop	BS	Jun	4.7 a^{A}	4.5 a^{A}	2.8 a^{A}	1.8 b^{A}	3.2 b^{A}	5.2 b^{A}	ns
On-crop	BS	Feb						1.7 b	
P-value			ns	ns	ns	**	**	**	
AP2 expression									
Off-crop	NBS	Mar	0.6 a^{B}	0.4 a^{B}	0.6 a^{B}	0.6 a^{B}	0.2 b^{B}	1.9 a^{A}	**
On-crop	BS	Jun	0.3 a^{B}	0.4 a^{B}	0.6 a^{B}	0.5 a^{B}	0.5 a^{B}	1.2 ab^{A}	**
On-crop	BS	Feb						0.4 b	
P-value			ns	ns	ns	ns	*	*	

Table S4.1 continued.

Tree status	Shoot status	Harvest time	Relative expression						
			Oct	Nov	Dec	Jan	Feb	Mar	*P*-value
SEP1 expression									
Off-crop	NBS	Mar	< 0.05	0.1 a^{B}	< 0.05	ND	< 0.05	1.0 a^{A}	*
On-crop	BS	Jun	0.1 a^{B}	0.1 a^{B}	0.2 a^{A}	0.1 b^{B}	0.1 a^{B}	0.3 b^{A}	**
On-crop	BS	Feb						0.1 b	
P-value			--	ns	--	--	--	***	
PI expression									
Off-crop	NBS	Mar	ND	ND	ND	ND	< 0.05	1.1 a^{A}	--
On-crop	BS	Jun	ND	ND	ND	ND	ND	ND	--
On-crop	BS	Feb						ND	
P-value			--	--	--	--	--	--	
AG expression									
Off-crop	NBS	Mar	< 0.05	ND	< 0.05	ND	< 0.05	0.8 a^{A}	--
On-crop	BS	Jun	< 0.05	< 0.05	< 0.05	ND	< 0.05	< 0.05	--
On-crop	BS	Feb						ND	
P-value			--	--	--	--	--	--	

[x] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

[z] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

Table S4.2 Expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* relative to *EF1-α* expression in buds on nonbearing shoots (NBS) of off-crop trees harvested in March and bearing shoots (BS) of on-crop trees harvested in Janurary and March in 'Nules Clementine' mandarin[x].

Tree status	Shoot status	Harvest time	Relative expression					
			Oct	Dec	Jan	Feb	Mar	*P*-value
FT expression								
Off-crop	NBS	Mar	ND[y]	< 0.05[z]	< 0.05	ND	0.3 a^{A}	--
On-crop	BS	Mar	ND	ND	ND	ND	ND	--
On-crop	BS	Jan				ND	ND	--
P-value			--	--	--	--	--	
SOC1 expression								
Off-crop	NBS	Mar	1.1 a^{B}	0.4 a^{C}	0.3 a^{C}	0.5 a^{C}	1.9 a^{A}	****
On-crop	BS	Mar	0.7 b^{A}	0.3 b^{A}	0.7 a^{A}	0.4 a^{A}	1.7 a^{A}	ns
On-crop	BS	Jan				0.9 a^{A}	0.5 a^{A}	ns
P-value			*	*	ns	ns	ns	
LFY expression								
Off-crop	NBS	Mar	2.2 a^{B}	0.4 ab^{B}	ND	ND	18.6 a^{A}	**
On-crop	BS	Mar	1.0 a^{A}	1.0 a^{A}	ND	ND	ND	ns
On-crop	BS	Jan				ND	1.4 a^{A}	--
P-value			ns	*	--	--	ns	
AP1 expression								
Off-crop	NBS	Mar	0.9 a^{B}	1.1 a^{B}	0.3 b^{B}	0.9 a^{B}	9.9 a^{A}	****
On-crop	BS	Mar	1.3 a^{A}	0.5 a^{A}	1.2 a^{A}	1.1 a^{A}	1.1 b^{A}	ns
On-crop	BS	Jan				1.6 a^{A}	1.0 b^{A}	ns
P-value			ns	ns	*	ns	***	
AP2 expression								
Off-crop	NBS	Mar	0.3 a^{B}	0.1 a^{B}	0.1 a^{B}	0.1 a^{B}	1.0 a^{A}	***
On-crop	BS	Mar	0.2 a^{B}	0.1 a^{B}	0.4 a^{B}	0.2 a^{B}	1.1 a^{A}	*
On-crop	BS	Jan				0.2 a^{A}	0.1 a^{A}	ns
P-value			ns	ns	ns	ns	ns	

Table S4.2 continued.

Tree status	Shoot status	Harvest time	Relative expression					
			Oct	Dec	Jan	Feb	Mar	*P*-value
SEP1 expression								
Off-crop	NBS	Mar	< 0.05	< 0.05	< 0.05	< 0.05	0.4 a[A]	--
On-crop	BS	Mar	< 0.05	< 0.05	< 0.05	< 0.05	0.1 b[A]	--
On-crop	BS	Jan				< 0.05	< 0.05	--
P-value			--	--	--	--	*	
PI expression								
Off-crop	NBS	Mar	0.1 a[B]	< 0.05	< 0.05	< 0.05	0.7 a[A]	*
On-crop	BS	Mar	0.1 a[A]	< 0.05	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Jan				< 0.05	< 0.05	--
P-value			ns	--	--	--	*	
AG expression								
Off-crop	NBS	Mar	ND	ND	ND	ND	0.4 a[A]	--
On-crop	BS	Mar	< 0.05	ND	ND	ND	ND	--
On-crop	BS	Jan				< 0.05	< 0.05	--
P-value			--	--	--	--	--	

[x] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[y] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

[z] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

Chapter 5

Effect of fruit removal on floral gene expression and floral intensity of on-crop 'Pixie' mandarin trees (*Citrus reticulata* Blanco)

Abstract

For alternate bearing (AB) citrus trees, repeating cycles of "off" (light yield) and "on" (heavy yield) crops are perpetuated by the inverse effect of fruit number on floral intensity the following spring. Off-crop 'Pixie' mandarin (*Citrus reticulata* Blanco) trees harvested in March flowered profusely on nonbearing shoots (NBS) at spring bloom (March), whereas on-crop trees harvested the same month did not produce any inflorescences on either NBS or bearing shoots (BS). The on crop reduced expression of *FLOWERING LOCUS T* (*FT*) to the limit of detection and below it for buds on NBS and BS, respectively, from December (three months before bloom) through February (one month before bloom), but increased the expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) on both shoot types to values greater than off-crop trees in February. *LEAFY* (*LFY*) transcripts were not detected in buds of off- or on-crop trees until one month before bloom and were 89% and 83% lower in buds on NBS and BS of on-crop trees than NBS of off-crop trees, respectively. From December to February, *APETALA1* (*AP1*) expression levels in buds of both NBS and BS of on-crop trees remained significantly lower than those of off-crop trees. In contrast, in December and January, *APETALA2* (*AP2*) expression in buds of both NBS and BS of on-crop trees was significantly greater than that of off-crop trees, but decreased to one-third the

expression of off-crop trees by February. For buds on BS of on-crop trees in February, *SEPALLATA1* (*SEP1*) was expressed at 30% of the level off-crop trees, *PISTILLATA* (*PI*) transcripts were not detected, and *AGAMOUS* (*AG*) transcripts were at the limit of detection. Fruit removal (FR) from BS of on-crop trees in November and December increased flowering to 21% and 9% of the number of inflorescences produced by off-crop trees, respectively; FR in January did not result in flowering. By March, bud *FT* expression increased in November and December FR trees, but never exceeded the limit of detection for January FR trees. For November and December FR trees, bud expression of *AP1* increased to levels equal to off-crop control trees in February through March with *PI* and *AG* expression at significant levels in March; whereas in buds of January FR trees, *AP1* expression remained low and transcripts of *PI* and *AG* were at the limit of detection. These results provide evidence that *FT* transcription above the limit of detection is a prerequisite for the upregulation of *AP1* expression to a level sufficient to activate the downstream floral organ identity genes, *PI* and *AG*. Progressively later FR treatments (Nov., Dec., and Jan.) became less effective in increasing floral intensity, but uniformly increased the number of vegetative shoots that developed in March, demonstrating that the buds of on-crop trees were not determined (irreversibly committed to floral development) by January and that spring bud break was inhibited by the presence of the on crop. The results suggest that to increase floral intensity to a level approaching that of off-crop trees, fruit would need to be removed before October but likely earlier. Whereas commercial harvest at this time is impractical in citrus production, earlier but less severe degrees of fruit thinning could prove valuable in mitigating AB.

Introduction

Flowering is the first developmental event in fruit production, with yield strongly correlated with the number of flowers produced at spring bloom for many *Citrus* spp. Conversely, crop load (number of fruit set per tree) is a major determinant of floral intensity the following spring (Monselise and Goldschmidt, 1982; Verreynne and Lovatt, 2009; Chapter 4). In citrus, the inverse relationship between crop load and return bloom perpetuates alternate bearing (AB). High yield in the on-crop year reduces return bloom the following year and results in an off crop. Low yield in the off-crop year leads to intense flowering the next spring, which sets an on crop. Alternate bearing compromises orchard management and causes revenue losses. The on crop is characterized by a large number of small fruit having reduced commercial value. The off crop is comprised of large fruit but too few to provide an adequate income to the grower and many larger fruit have undesirable fruit quality (El-Zeftawi, 1973; Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969; Monselise and Goldschmidt, 1982; Moss et al., 1974).

For 'Pixie' mandarin (*C. reticulata* Blanco) and 'Nules Clementine' mandarin (*C. reticulata* Blanco), buds of off-crop trees, which flowered profusely in April, expressed *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *APETALA2* (*AP2*) as early as October (six months before bloom) through March (one month before bloom), but only expressed *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) at significant levels in March (Chapter 4). The on crop of fruit for both cultivars initiated a sequence of events in the pattern of bud transcript accumulation relative to off-crop trees that included total

repression of *FT* starting in October or December, depending on the cultivar, reduced *LFY*, *AP1*, and *AP2* transcript levels by March and no expression of *SEP1*, *PI* and *AG* in buds of on-crop trees in March, resulting in total inhibition of flowering. Results of Chapter 4 provided strong evidence that persistent expression of *FT* was necessary for the upregulation of *AP1* and/or *LFY* and bud determinacy, the subsequent expression of the downstream floral organ identity genes with class A, B, C and E activity, *AP2*, *PI*, *AG* and *SEP1*, respectively, and flowering. This sequence of events is consistent with the role of *Arabidopsis thaliana FT* in flowering time control, *LFY* and *AP1* as the two major determinants of floral meristem identity, and subsequent specification of floral organs based on the ABCE model (Causier et al., 2010; Lee and Lee, 2010; Michaels, 2009; Moon et al., 2005; Pajoro et al., 2014; Ratcliffe et al., 1999; Weigel et al., 1992). For both 'Pixie' and 'Nules Clementine' mandarin, the inhibitory effect of the on crop of fruit on bud *FT* expression was not offset or reversed by the strong expression of *SOC1*, which was independent of crop load, despite the significant role of *SOC1* in *A. thaliana* floral development (Lee and Lee, 2010; Pajoro et al., 2014). Similarly, for both 'Moncada' mandarin [Clementine 'Oroval' (*C. clementina* Hort *ex* Tanaka) x 'Kara' mandarin (*C. unshiu* Marc. x *C. nobilis* Lou.)] and 'Murcott' mandarin (*C. reticulata* Blanco), bud expression of *FT*, *LFY* and *AP1* was reduced in on-crop trees characterized by decreased spring floral intensity compared to off-crop trees (Muñoz-Fambuena et al., 2012; Shalom et al., 2012). The pattern of bud *SOC1* expression in these two cultivars also was not related to crop load or floral intensity of the return bloom (Muñoz-Fambuena et al., 2012; Shalom et al., 2012).

Removal of all or part of the on crop has been used to study the effects of fruit number on floral intensity the following spring. However, changes in floral gene expression related to return bloom in response to fruit removal were documented only in a limited number of studies (Muñoz-Fambuena et al., 2011; Shalom et al., 2014; Chapter 4). Removing the on crop of fruit from 'Murcott' mandarin trees in late August (~eight months before bloom) increased bud *FT* and *LFY* transcript levels within one week. Elevated *FT* expression lasted for four weeks, with floral intensity fully recovered to the level off-crop trees at return bloom (Shalom et al., 2014). Although effective, harvest at this time is not practical for mitigating AB in commercial citrus production. However, harvesting the on crop from 'Nules Clementine' mandarin trees in January, which was three months before bloom and the normal harvest period, or from 'Pixie' mandarin trees in February, which was two months before bloom but four months before standard harvest in June, did not increase *FT* expression or the transcript levels of any floral genes downstream from *FT* and neither cultivar flowered the following spring (Chapter 4). The results indicate that the on crop of 'Nules Clementine' and 'Pixie' mandarin fruit needs to be harvested prior to January and February, respectively, to increase the expression of *FT* and the downstream genes sufficiently to increase flowering. How much earlier, without having to harvest as early as August, remains to be determined.

Thus, the overall goal of this research was to identify a harvest period for 'Pixie' mandarin earlier than February but several months later than August that would increase floral intensity at spring bloom following the on-crop year. To meet this goal, on-crop 'Pixie' mandarin trees were harvested in November, December and January. The effects

of early harvest on bud expression of eight genes regulating citrus floral development and on the developmental fate of the buds the following spring were quantified in comparison with off- and on-crop control trees. Floral gene expression analyses included floral timing genes, floral meristem identity genes, and floral organ identity genes. In this experiment, bloom was early, occurring in March instead of April, resulting in the earliest harvest being four months before bloom. Defining the latest time that fruit can be removed and still increase flowering at return bloom is essential for developing management strategies for fruit thinning (by chemical, hand or pruning) with the potential to mitigate AB.

Materials and Methods

Plant materials

Fifteen-year old 'Pixie' mandarin (*C. reticulata* Blanco) trees on 'Troyer' citrange (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) rootstock in a commercial orchard in Ojai Valley, California (34˚27'N, 119˚15'W) were used in this research. There were five blocks (replications) in the orchard, each containing one tree setting an off crop and four nearby trees setting an on crop. Thus, there were five individual tree replicates in each of the five treatments: (i) off-crop control trees harvested on 18 March; (ii) on-crop control trees harvested on 18 March; (iii) on-crop trees with all fruit removed (FR) on 23 November (four months before bloom); (iv) on-crop trees with FR on 13 December (three months before bloom); and (v) on-crop trees with FR on16 January (two months before bloom). Full bloom occurred in March.

Sample collection and gene expression analysis

The apical five buds on nonbearing shoots (NBS) of off-crop control trees, on both NBS and bearing shoots (BS) of on-crop control trees and on BS of FR trees were collected from 20 shoots of each type per five trees for each of the five treatments and used for gene expression analysis. Bud collection for off-crop control trees was initiated in December and continued through February, one month before bloom. Buds could not be collected in March due to > 90% bud break at this time. For on-crop control trees and on-crop trees in the FR treatments, bud collection was started in December or one month after FR, respectively, and continued through March. By March, only a subset of buds on these trees had undergone bud break to produce floral or vegetative shoots. Buds collected in March for floral gene expression analysis were swollen but could not yet be identified as floral or vegetative. At the time of collection, shoots longer than the apical five nodes were excised, leaves removed, and the shoot placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab. In the lab., the shoot was cut just below the fifth node from the apex and the apical five buds were quickly frozen in liquid nitrogen and stored at -80 ˚C until analysis. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 μg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA

Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequences of *A. thaliana* homologs *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). Citrus *FT*, *SOC1*, *LFY* and *AP1* genes analyzed in this research were *CiFT2* (Nishikawa et al., 2007), *CsSOC-like2* (*CsSL2*) (Tan and Swain, 2007), *CsLFY* and *CsAP1* (Pillitteri et al., 2004), *PtAP2* (Song et al., 2010), *CuSEP1* (Nishikawa et al., 2009); each gene was selected based on its demonstrated functional equivalence in its respective *A. thaliana* mutant. In addition, expression of *CiFT2* was related to floral intensity in response to low temperature in *C. unshiu* (Nishikawa et al., 2007) and to crop load in *C. reticulata* (Shalom et al., 2012, 2014); *CsSL2* expression was also related to the effect of crop load on flowering in *C. reticulata* (Shalom et al., 2012). The sequences of *PI* and *AG* chosen in this research share high identity with *A. thaliana PI* and *AG*, respectively; the predicted protein sequences for the putative *PI* and *AG* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com/primerquest/Home/Index) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study

as well as the blast results of PCR product sequence versus target sequence of each gene of interest are listed in Table 5.1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-μL reaction system containing 1.2 μL cDNA (about 40 ng of input RNA), 0.6 μL gene-specific forward and reverse primer mix (10nM), 7.5 μL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 μL PCR-grade water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was run at the end of each qPCR run to confirm that nonspecific products were not formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with the flowers of 'Pixie' mandarin collected from off-crop control trees during spring bloom as the control (expression level of 1) and *β-ACTIN* (*ACT*) as the primary reference gene (endogenous control), and reported herein. The selection of *ACT* as the primary reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Results based on a second reference gene, *ELONGATION FACTOR 1-ALPHA* (*EF1-α*) from *C. unshiu* (Nishikawa et al., 2009), are presented as supplemental results (Tables S5.1). The expression pattern of each floral gene with *ACT* as the endogenous control was strongly correlated with its expression pattern when *EF1-α* served as the reference gene (r = 0.74 to 0.99, $P < 0.001$ for all genes), confirming the consistency and reliability of the results. Gene expression data for

each sampling date were the mean of five biological replicates; each biological replicate was the mean of three qPCR technical replicates.

Effects of fruit on bud development

At full bloom in March, the developmental fate of the apical five buds on each of 20 shoots randomly selected before bloom on NBS and BS of the five trees in each treatment, respectively, was determined. The number of leafless (one to many flowers with no leaves), leafy (one to many flowers with one to many leaves), and total inflorescences (sum of leafless plus leafy inflorescences), vegetative shoots, and inactive (dormant) buds per 20 shoots per tree were counted. Results for the five apical buds on the 20 shoots of each type per tree were averaged for the five individual trees (replications) per treatment and reported as the average value per five apical buds per 20 shoots per five trees per treatment (100 buds/tree).

Yield data

Total yield (kg/tree) for each data tree was recorded in the field during each harvest or FR treatment. For off-crop trees, the number of fruit per tree was counted during harvest. For on-crop trees, a randomly selected sample of 200 fruit per tree, which represented about 5% to 8 % of the average total number of fruit per tree, was collected for each tree and the transverse diameter of each fruit was measured with an electronic caliper. Based on its diameter, each fruit was assigned to a fruit size category. The fruit

weight of a specified number of fruit in each fruit size category was determined in order to estimate the total number of fruit per tree.

Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots and inactive buds per tree and the relative expression levels of each gene obtained by the Pfaffl method (Pfaffl, 2001), using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$. The relative gene expression data are reported as a percentage of the expression of each gene in buds on NBS of off-crop control trees for each month, which was set at 100%, with the level of expression in February also serving as the control (100%) for March, since at this time buds on NBS of off-crop control trees had already produced inflorescences. Pearson's correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $P \leq 0.05$) between gene expression levels and inflorescence number.

Results

Effects of crop load on floral intensity of the return bloom

Off-crop 'Pixie' mandarin control trees had a total yield of 4.8 kg per tree (33 fruit/tree), whereas on-crop control trees harvested in March set a final crop that was 38-fold and 80-fold greater by weight (184.1 kg/tree) and number (2631 fruit/tree),

respectively ($P < 0.0001$ for both) (Table 5.2). With a light crop load, off-crop control trees produced an average of 93.2 inflorescences per NBS (based on 5 apical buds/20 shoots/tree) at return bloom in March, which was composed of 86% leafless and 14% leafy inflorescences, and had only 6.8 buds per tree remaining inactive in spring, with no vegetative shoots (Table 5.2). In contrast, on-crop control trees did not produce any inflorescence on either NBS or BS the following year in March. Instead, for both shoot types of on-crop control trees, almost 100% of the 100 apical buds per tree remained inactive at return bloom. Thus, off-crop control trees had significantly greater number of inflorescences and significantly lower number of inactive buds on NBS compared to both NBS and BS of on-crop control trees ($P < 0.0001$ for both). The results indicate that the presence of the heavy crop load on on-crop 'Pixie' mandarin trees through March led to complete inhibition of spring flowering. For the trees in the data set, floral intensity (inflorescence number) at return bloom was significantly (negatively) correlated with yield per tree as kg ($r = -0.99$, $P < 0.0001$) and fruit number ($r = -0.96$, $P < 0.0001$).

Effects of fruit removal on floral intensity of the return bloom

In November, December and January (four, three and two months before bloom, respectively), a total crop of 137.2, 133.3 and 140.3 kg per tree was removed from different sets of on-crop trees, respectively (Table 5.2). Total yields, as kg per tree, for trees in these FR treatments were all equal but significantly lower than that of on-crop control trees harvested in March ($P < 0.0001$), due to the early harvest. However, the number of fruit removed from on-crop trees in November (3260 fruit/tree), December

(3191 fruit/tree) and January (3147 fruit/tree), respectively, was equal to that of on-crop control trees harvested in March (Table 5.2). More importantly, yield as both kg and fruit number per tree for all FR trees was significantly greater than that of off-crop control trees ($P < 0.0001$), confirming the on-crop status of the trees used for FR treatments in this research.

November FR trees produced 19.4 inflorescences per tree the following March, significantly greater than on-crop control trees ($P < 0.0001$) (Table 5.2). For December FR trees, spring floral intensity was only 8.4 inflorescences per tree, significantly lower than that of November FR trees but still greater than on-crop control trees ($P < 0.0001$). When FR was delayed to January, the trees did not produce any inflorescence the following spring. Compared to NBS and BS of on-crop control trees with fruit persisting through March, FR in November and December resulted in a significantly greater number of leafy inflorescences ($P < 0.0001$), which constituted 85% and 95% of total inflorescences, respectively, but had no effect on leafless inflorescence number. For trees in these two treatments, the number of total inflorescences was only 21% and 9% of that of off-crop control trees, respectively. The results demonstrated that the inhibitory effect of the on crop on floral development is largely irreversible as early as November (four months before bloom).

Removal of fruit in November, December and January also resulted in 39.8, 39.2 and 39.4 vegetative shoots per tree in March, respectively, a number significantly greater than that of NBS and BS of off- and on-crop control trees ($P < 0.0001$) (Table 5.2). The development of vegetative shoots instead of inflorescences in response to FR suggests

that the persistence of the on crop through November (or later) prevented this population of buds (approximately 40%) from becoming determined. The progressively later FR treatments in December and January had no effect on vegetative shoot number, but reduced inflorescence number and increased the number of inactive buds compared to off-crop control trees ($P < 0.0001$) (Table 5.2), indicating that reversion of floral buds to vegetative buds did not occur and suggesting inactive buds were potential floral buds. Further, the results demonstrate that the presence of the on crop of fruit on the tree through March inhibited both floral and vegetative buds from undergoing spring bud break.

Effects of fruit removal on the expression of citrus floral timing genes

For buds of off-crop control trees, maximum *FT* expression occurred in December, but the transcript level was significantly lower (89%) by January and remained low in February ($P < 0.01$) (Table 5.3). In contrast, for on-crop control trees, *FT* transcripts were only at the limit of detection in buds on NBS in December through March but not detected in buds on BS during this period. Removal of the on crop of fruit in November, December and January (four, three and two months before bloom, respectively) increased *FT* transcripts in buds on BS to a detectable level the month after the treatment through February (Table 5.3). By March, bud *FT* expression for November and December FR trees was equal to 101% and 71% of the level attained by off-crop control trees in February, respectively; in contrast, bud *FT* expression for January FR trees remained at the limit of detection through March. Across treatments, total inflorescence number was

strongly correlated with bud expression of *FT* in February, the month before bloom (r = 0.81, $P < 0.001$).

Interestingly, *SOC1* was consistently expressed in buds of trees in all treatments on all sampling dates. Buds on NBS of off-crop control trees had greater expression levels of *SOC1* in December and January than February ($P < 0.01$) (Table 5.3). For on-crop control trees, expression of *SOC1* in buds on NBS did not change across sampling dates, whereas *SOC1* expression in buds on BS increased from December through March ($P <$ 0.01). Moreover, bud *SOC1* expression for off-crop control trees was never greater than that of NBS or BS of on-crop control trees at any time except December, during which *SOC1* expression levels were greater in buds on NBS of off-crop and on-crop control trees than BS of on-crop trees ($P < 0.05$). When fruit were removed from on-crop trees in November, *SOC1* expression increased to 97% of the level of off-crop control trees by December, a level greater than buds on BS of on-crop control trees ($P < 0.05$) (Table 5.3). Thereafter, *SOC1* transcripts continued to increase in buds of November FR trees to values equal to or greater than that of off-crop control trees. When the on crop was removed in December or January, bud *SOC1* expression equaled that of off-crop control trees in February. It is of interest that the relationship between total inflorescence number and bud *SOC1* expression was equally negative in both February (r = -0.47, P = 0.008) and in March (r = -0.42, P = 0.025), suggesting that fruit regulate floral development in citrus independently of *SOC1*.

For buds on NBS of off-crop control trees, *LFY* transcripts were below the limit of detection in December and January, but increased to a significant level in February, the

month before bloom (Table 5.3). Transcripts of *LFY* in buds on NBS and BS of on-crop control tees were also below the limit of detection in December and January, with *LFY* expression increasing in February, but to a level significantly lower in buds of on-crop trees than off-crop control trees ($P < 0.0001$). By March, bud *LFY* transcripts were below the limit of detection for NBS of on-crop control trees but for BS at a low level equal to that attained by off-crop control trees in February. Removal of fruit in November did not result in detectable levels of *LFY* expression the month after FR but *LFY* expression increased in January through March (Table 5.3). Buds of December and January FR trees expressed *LFY* the month following each treatment through March. For buds on BS of on-crop control trees and on-crop trees in all FR treatments, *LFY* expression was significantly lower than that of off-crop control trees in February ($P < 0.0001$), but not significantly different by March. There was a significant relationship between inflorescence number and bud expression of *LFY* in February, the month before bloom ($r = 0.82$, $P < 0.001$).

Effects of fruit removal on the expression of citrus genes having class A activity

All buds from trees in all treatments expressed *AP1* on all sampling dates. For buds on NBS of off-crop control trees, *AP1* expression remained similar in December through February (Table 5.4). For on-crop control trees, buds on NBS expressed *AP1* at low levels from December through February with an increase in the transcript level in March ($P < 0.05$), whereas for buds on BS, *AP1* expression levels fluctuated across the four sampling dates ($P < 0.05$). Levels of *AP1* expression in buds on both NBS and BS of on-

crop control trees were significantly lower than those on NBS of off-crop control trees in December ($P < 0.0001$), January ($P < 0.05$) and February ($P < 0.05$). There were no significant differences in *AP1* transcript levels between buds on NBS and BS of on-crop control trees on any sampling date. Compared to on-crop control trees, removal of fruit in November and December did not affect *AP1* transcript accumulation in December or January but by February, bud *AP1* expression was 89% and 57% of that of off-crop control trees, respectively, a level of expression that was not significantly different from that of off-crop control trees ($P < 0.05$) (Table 5.4). In February, *AP1* expression in buds on BS of January FR trees was significantly lower than that of off-crop control trees ($P < 0.05$). However, bud *AP1* transcript levels continued to increase through March to a level equal to that attained by off-crop control trees in February for buds on NBS and BS of on-crop control trees and BS of on-crop trees in all three fruit removal treatments. Total inflorescence number at spring bloom was correlated with bud *AP1* expression across treatments in February, the month before bloom ($r = 0.57$, $P = 0.001$).

Like *AP1*, buds from trees in all treatments expressed *AP2* on all sampling dates, but *AP1* expression levels were 5- to 26-fold greater than those of *AP2* (compare 2.4, 3.7 and 3.7 for *AP1* expression in buds of off-crop control trees in December, January and February, respectively, to 0.2, 0.1 and 0.7 for *AP2*, respectively). Surprisingly, transcript levels of *AP2* were approximately 2-fold greater in buds on both NBS and BS of on-crop control trees than those on NBS of off-crop control trees in December ($P < 0.001$) and January ($P < 0.0001$). However, for buds of off-crop control trees, *AP2* expression increased 7-fold from January to February, whereas for buds on NBS and BS of on-crop

control trees, *AP2* expression was 18% and 31% lower in February than in January, respectively, and remained at these low levels in March ($P < 0.0001$ and $P < 0.05$ for the two bud types, respectively). As a result, in February and March, *AP2* transcript levels in buds on NBS and BS of on-crop control trees were significantly lower than the value for off-crop control trees observed in February ($P < 0.0001$ for both bud types for each month). For buds on NBS and BS of on-crop control trees, there was no significant difference in bud *AP2* transcription at any time except December, when *AP2* expression was significantly lower in buds on BS. Removal of the on crop in November, December or January failed to increase *AP2* expression to levels greater than buds on BS of on-crop control trees on any sampling date (Table 5.4). Further, expression of *AP2* in buds of December and January FR trees decreased from February to March ($P < 0.05$ for both FR treatments). For all three FR treatments, bud *AP2* expression was lower in February and March than the level of *AP2* expression attained by off-crop control trees in February ($P < 0.0001$ for the three FR treatments). The number of inflorescences at return bloom was strongly correlated with the expression of *AP2* in buds across treatments in February ($r = 0.84$, $P < 0.001$) and March ($r = 0.85$, $P < 0.001$).

Effects of fruit removal on the expression of citrus floral organ identity genes downstream from AP2

For buds on NBS of off-crop control trees, *SEP1* transcripts were at the limit of detection in December, but increased in January and February (Table 5.5). For buds on NBS of on-crop control trees, *SEP1* transcripts were at the limit of detection from

December through March. In contrast, *SEP1* expression in buds on BS increased from December to the maximum level in January that was equal to that of off-crop control trees ($P < 0.05$), but decreased significantly in February to 30% of off-crop control trees and remained at this low level in March ($P < 0.05$). Thus, *SEP1* expression was greater in buds of off-crop control trees than buds on both shoot types of on-crop control trees in February ($P < 0.001$) and March ($P < 0.001$). For trees with the on crop removed in November, December and January, respectively, bud *SEP1* transcripts were at the limit of detection for trees in all treatments on all sampling dates, with the exception that *SEP1* was expressed in buds of November FR trees in March at a level equal to 17% of off-crop control trees, which was significantly lower than that of off-crop control trees and equal to that of on-crop control trees ($P < 0.001$) (Table 5.5). Total inflorescence number at spring bloom was strongly correlated with *SEP1* expression in March ($r = 0.94$, $P < 0.001$).

For buds on NBS of off-crop control trees, transcripts of *PI* were at the limit of detection in December and January; *AG* transcripts were not detected in December but were at the limit of detection in January (Table 5.5). For both genes, bud expression increased to significant levels by February, one month before bloom. In contrast, buds on NBS and BS of on-crop control trees never expressed *PI* or *AG* above the limit of detection from December through March. Removal of the on crop in November increased bud *PI* and *AG* expression to 33% and 30% of off-crop control trees, respectively, by March (Table 5.5). For buds of November and December FR trees, transcript levels of *PI* in March were significantly lower than that observed in buds of off-crop control trees in

February ($P < 0.05$), whereas the transcript levels of *AG* in March were equal to that for off-crop control trees in February (Table 5.5). Delaying FR to January did not result in expression of either gene above the limit of detection in March. The number of inflorescences at return bloom was strongly correlated with bud expression of *PI* ($r = 0.81$, $P < 0.001$) and *AG* ($r = 0.71$, $P < 0.001$) in March.

Discussion

Comparing the expression of floral timing genes (*FT*, *SOC1* and *LFY*), floral meristem identity genes (*LFY* and *AP1*) and floral organ identity genes (*AP1*, *AP2*, *SEP1*, *PI* and *AG*) in buds from off- and on-crop 'Pixie' mandarin trees and on-crop trees harvested several months before bloom provided insights into the regulation of floral development in citrus. Additionally, these comparisons provided important information on the effect of the on crop of citrus fruit on bud expression of specific genes related to floral intensity the following spring. The results documented the critical role of *FT* in citrus flowering. Trees in treatments for which bud *FT* expression failed to exceed the limit of detection in this research did not flower. This included buds on NBS and BS of on-crop control trees and BS of on-crop trees with all fruit removed in January. In contrast, buds on NBS of off-crop control trees and on BS of on-crop trees in the November and December fruit removal treatments expressed *FT* significantly above the limit of detection by March and flowered. These results confirmed that the on crop of fruit exerted an inhibitory effect on *FT* transcription that inhibited floral development as reported previously (Muñoz-Fambuena et al., 2012; Shalom et al., 2012; Chapter 4). In

addition, the results of this research demonstrated that mitigation of the inhibitory effect of the on crop of fruit by FR in November and December, four and three months before bloom, respectively, was mediated through *FT* transcription, with November FR trees having greater bud *FT* expression and return bloom than trees with fruit removed in December.

The results presented herein confirmed that the expression of the floral timing gene *SOC1* was not related to crop load or to floral intensity at return bloom, providing additional evidence that fruit inhibit citrus flowering independently of *SOC1* (Muñoz-Fambuena et al., 2012; Shalom et al., 2012; Chapter 4). Further, the results demonstrated that the significantly greater expression of *SOC1* in buds on NBS and BS of on-crop control trees than NBS of off-crop control trees (respectively, 80% and 164% greater in February and respectively, 92% and 194% greater in March) did not overcome the inhibition of *FT* expression or promote flowering through an alternate pathway in citrus whereby *SOC1* upregulates *LFY* as occurs in *A. thaliana* (Lee and Lee 2010; Lee et al., 2008; Samach et al., 2000).

In *A. thaliana*, *FT* targets both *LFY* and *AP1*, each of which can upregulate the other (Adrian et al., 2009; Liljegren et al., 1999; Pajoro et al, 2014; Parcy, 2005). The expression of *LFY* was first observed in January in buds on BS of on-crop trees with all fruit removed in November and December, but not in buds of off-crop control trees until February, one month before bloom. In contrast, *AP1* was expressed in all buds on all sampling dates. It should also be noted that in buds of off-crop control trees, *AP1* expression increased 54% from December to January and one month later *LFY*

expression changed from not detected to maximum expression in February. These results taken together suggest that *FT* might target *AP1,* which then activates *LFY*.

Activities of both class A genes, *AP1* and *AP2,* are necessary for sepal formation in *A. thaliana* (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). In citrus, sepal formation is the developmental marker associated with bud determinacy (irreversible commitment to floral development) (Lord and Eckard, 1987). For on-crop trees, the results of the fruit removal treatments presented herein provided strong evidence that floral development occurred only in trees for which bud transcript levels of *AP1* reached that of off-crop control trees by February, i.e., the trees with the on crop of fruit removed in November and December. Only these trees went on to express *PI* and *AG* and to produce inflorescences the following spring. In contrast, expression of *AP2* in buds of these trees did not reach the level of off-crop control trees in February or March, suggesting that the level of *AP2* expression was sufficient for *AP2* to act in concert with *AP1* and activate the downstream floral organ identity genes or that *AP1* activity alone was adequate for the upregulation of *PI* and *AG* expression. Taken together, the results of this research established that a critical level of *FT* transcription (above the limit of detection in this research) is an essential prerequisite for the upregulation of *AP1* expression to a level sufficient to activate the downstream floral organ identity genes *PI* and *AG*.

In earlier research, comparison of the number of inflorescences produced at return bloom by buds of NBS and BS on off- and on-crop 'Pixie' mandarin trees provided evidence of the inhibitory effect associated with total fruit number produced by the tree

(crop load), i.e., whole-tree effect, and the localized effect of individual fruit borne on a shoot (Verreynne and Lovatt, 2009). In this comparison, maximum floral intensity was observed for buds on NBS of off-crop trees, with the least flowering for buds of BS of on-crop trees, which reflected both the localized effect of fruit set on a shoot and the whole-tree effect of the on-crop. In the prior study, buds on NBS of on-crop trees produced a small number of inflorescences documenting the inhibitory effect of the on crop of fruit only (no localized effect). In the current research, 100% of the buds on both NBS and BS of on-crop 'Pixie' mandarin control trees failed to flower, suggesting the possibility that more fruit were produced by the on-crop trees in the research presented herein. Consistent with the failure to flower, buds on both NBS and BS of on-crop control trees in this study failed to express *FT* above the limit of detection on any sampling date. Bud *AP1* transcript levels for both NBS and BS of on-crop control trees were significantly lower than that of NBS off-crop control trees in February, but increased to 67% and 60%, respectively, of the transcript level of off-crop trees by March. Although this level of *AP1* expression was not significantly different from that of off-crop control trees, it was likely insufficient to activate the downstream floral organ identity genes *PI* and *AG*. Transcripts of *PI* and *AG* never increased above the limit of detection in buds on NBS and BS of on-crop trees. Taken together, the results provide further evidence of the critical role of *FT* in regulating citrus floral development and the strong inhibitory effect of the on crop of 'Pixie' mandarin fruit on *FT* expression.

Results of the FR treatments established that the inhibitory effect of the on crop of 'Pixie' mandarin fruit on return bloom increased with the length of time the fruit

remained on the tree. The effect was two-fold, reduced transcription of key genes in the citrus floral development pathway and inhibition of spring bud break. The earlier the on crop of fruit was removed between November and January, the fewer inactive buds and the more inflorescences that developed. Interestingly, FR in November, December and January increased the number of vegetative shoots that developed during spring bloom equally (approximately 40/100 buds /tree). This result was also obtained with Satsuma mandarin trees. Fruit removal from September through January resulted in an increasingly lower inflorescence number and percent bud break, but no changes in vegetative shoot number (Garcia-Luis et al., 1986). Taken together, the results of the two studies demonstrate that floral buds are not converted to vegetative shoots and support the interpretation that the majority of inactive buds are failed floral buds. This interpretation is also consistent with the increasingly negative effect of the on crop on *FT*, *AP1*, *PI* and *AG* expression that results when on-crop 'Pixie' mandarin trees are harvested progressively later than November. The November FR treatment provided evidence that only 20% of the buds were still viable floral buds at this time, indicating that FR needs to be carried out significantly earlier than November to dramatically increase inflorescence number at return bloom. Removal of the on crop of 'Pixie' mandarin fruit in July (Verryenne and Lovatt, 2009) and 'Murcott' mandarin in August (Shalom et al., 2014) restored inflorescence number to the floral intensity of the spring bloom following the off-crop year. The results suggest that partial removal of the on crop this early in the season might have a positive effect on return bloom. The degree of FR

necessary to significantly increase return bloom and yield to an acceptable commercial value remains to be determined.

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Table 5.1 Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence BLAST against target gene sequence	
				E-value	Identity
FT	AB027456.1 (*C. unshiu*)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
SOC1	EU032532.1 (*C. sinensis*)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
LFY	AY338976.1 (*C. sinensis*)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
AP1	AY338974.1 (*C. sinensis*)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
AP2	EU883665.1 (*C. trifoliata*)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEP1	AB329715.1 (*C. unshiu*)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PI	XM_ 006472790.1 (*C. sinensis*)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AG	HM246523.1 (*C. sinensis*)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov)

Table 5.2 Crop load and developmental fate of buds, as number of total, leafless and leafy inflorescences, vegetative shoots or inactive (dormant) buds, on nonbearing shoots (NBS) of off-crop trees (off-crop control), NBS and bearing shoots (BS) of on-crop trees (on-crop control), and BS of on-crop trees with all fruit removed (FR) in November, December and January, respectively, in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest or FR time	Total yield[y] (kg/tree)	Total yield (no./tree)	Inflorescences[z] (no./tree)			Vegetative shoots (no./tree)	Inactive buds (no./tree)
					Total	Leafless	Leafy		
Off-crop	NBS	Mar	4.8 c	33 b	93.2 a	80.6 a	12.6 ab	0.0 b	6.8 e
On-crop	NBS	Mar			0.0 d	0.0 b	0.0 c	0.2 b	99.8 a
On-crop	BS	Mar	184.1 a	2631 a	0.0 d	0.0 b	0.0 c	0.0 b	100.0 a
On-crop	BS	Nov FR	137.2 b	3260 a	19.4 b	2.6 b	16.8 a	39.8 a	40.8 d
On-crop	BS	Dec FR	133.3 b	3191 a	8.4 c	0.4 b	8.0 b	39.2 a	52.4 c
On-crop	BS	Jan FR	140.3 b	3147 a	0.0 d	0.0 b	0.0 c	39.4 a	60.6 b
P-value			****	****	****	****	****	****	****

[x] Means followed by different letters within a vertical column are significantly different according to Fisher's least significant difference (LSD) test in which **** refers to a significant effect at $P < 0.0001$.

[y] For crop load, data are the means of five trees per treatment.

[z] For numbers of inflorescences (total, leafless, leafy), vegetative shoots and inactive buds, data are the average values per five apical buds per 20 shoots per five trees per treatment (100 buds/tree).

Table 5.3 Expression of *FT*, *SOC1* and *LFY* in buds on nonbearing shoots (NBS) of off-crop trees, NBS and bearing shoots (BS) of on-crop trees, and BS of on-crop trees with all fruit removed (FR) in November, December and January, respectively, in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest or FR time	Relative expression				
			Dec	Jan	Feb	Mar	*P*-value
FT expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 ^{A}	100.0 ^{B}	100.0 ^{B}	100.0 a	**
On-crop	NBS	Mar	< 0.05[y]	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Mar	ND[z]	ND	ND	ND	--
On-crop	BS	Nov FR	< 0.05	< 0.05	< 0.05	100.9 a	--
On-crop	BS	Dec FR		< 0.05	< 0.05	71.4 a	--
On-crop	BS	Jan FR			< 0.05	< 0.05	--
P-value			--	--	--	ns	
SOC1 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 a^{A}	100.0 a^{A}	100.0 c^{B}	100.0 a	**
On-crop	NBS	Mar	102.4 a^{A}	94.0 a^{A}	179.7 b^{A}	191.9 a^{A}	ns
On-crop	BS	Mar	72.2 b^{B}	89.7 a^{B}	264.4 a^{A}	293.7 a^{A}	**
On-crop	BS	Nov FR	96.7 a^{A}	100.5 a^{A}	202.8 ab^{A}	231.3 a^{A}	ns
On-crop	BS	Dec FR		102.9 a^{A}	153.7 bc^{A}	206.0 a^{A}	ns
On-crop	BS	Jan FR			164.5 bc^{A}	154.4 a^{A}	ns
P-value			*	ns	**	ns	
LFY expression (% off-crop control)							
Off-crop	NBS	Mar	ND	ND	100.0 a	100.0 a	--
On-crop	NBS	Mar	ND	ND	10.8 b	ND	--
On-crop	BS	Mar	ND	ND	16.5 b^{A}	36.2 a^{A}	ns
On-crop	BS	Nov FR	ND	10.4 a^{A}	28.2 b^{A}	57.6 a^{A}	ns
On-crop	BS	Dec FR		17.8 a^{A}	40.2 b^{A}	29.3 a^{A}	ns
On-crop	BS	Jan FR			11.6 b^{A}	36.9 a^{A}	ns
P-value			--	ns	****	ns	

[x] Data are means for five trees (replications) per treatment presented as a percent of the control value set at 100%. The expression level of each gene in buds on NBS of off-crop control trees served as the control for each month except March, for which the February expression level was used as the control (100%) (buds could not be collected from off-crop trees in March). For buds on NBS of off-crop control trees, the relative expression

levels in December, January and February for *FT* were 0.9, 0.1 and 0.1, respectively, and for *SOC1* were 5.0, 5.0 and 3.1, respectively. Transcripts of *LFY* were only at levels that could be quantified in February, thus, the expression level obtained in February (2.7) was used as the control (100%) value in January and March. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which ** refers to a significant effect at $P < 0.01$, **** at $P < 0.0001$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table 5.4 Expression of *AP1* and *AP2* in buds on nonbearing shoots (NBS) of off-crop trees, NBS and bearing shoots (BS) of on-crop trees, and BS of on-crop trees with all fruit removed (FR) in November, December and January, respectively, in 'Pixie' mandarin[z].

Tree status	Shoot status	Harvest or FR time	Relative expression				
			Dec	Jan	Feb	Mar	*P*-value
AP1 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 a^{A}	100.0 a^{A}	100.0 a^{A}	100.0 a	ns
On-crop	NBS	Mar	65.0 b^{B}	46.4 b^{B}	40.2 c^{B}	66.7 a^{A}	*
On-crop	BS	Mar	61.7 bc^{B}	58.0 b^{A}	45.5 bc^{AB}	60.0 a^{A}	*
On-crop	BS	Nov FR	49.9 c^{B}	69.4 b^{AB}	89.0 ab^{A}	100.4 a^{A}	*
On-crop	BS	Dec FR		54.7 b^{B}	57.4 abc^{B}	92.4 a^{A}	*
On-crop	BS	Jan FR			46.5 bc^{A}	55.0 a^{A}	ns
P-value			****	*	*	ns	
AP2 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 c^{B}	100.0 b^{B}	100.0 a^{A}	100.0 a	***
On-crop	NBS	Mar	223.3 a^{A}	212.3 a^{B}	29.7 b^{C}	23.0 b^{C}	****
On-crop	BS	Mar	166.4 b^{A}	191.3 a^{B}	31.5 b^{B}	35.0 b^{B}	*
On-crop	BS	Nov FR	124.1 bc^{A}	126.1 b^{A}	24.2 b^{A}	30.9 b^{A}	ns
On-crop	BS	Dec FR		107.2 b^{AB}	25.5 b^{A}	18.2 b^{B}	*
On-crop	BS	Jan FR			21.6 b^{A}	13.4 b^{B}	*
P-value			***	****	****	****	

[z] Data are means for five trees (replications) per treatment presented as a percent of the control value set at 100%. The expression level of each gene in buds on NBS of off-crop control trees served as the control for each month except March, for which the February expression level was used as the control (100%) (buds could not be collected from off-crop trees in March). For buds on NBS of off-crop control trees, the relative expression levels in December, January and February for *AP1* were 2.4, 3.7 and 3.7, respectively, and for *AP2* were 0.2, 0.1 and 0.7, respectively. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

Table 5.5 Expression of *SEP1*, *PI* and *AG* in buds on nonbearing shoots (NBS) of off-crop trees, NBS and bearing shoots (BS) of on-crop trees, and BS of on-crop trees with all fruit removed (FR) in November, December and January, respectively, in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest or FR time	Relative expression				
			Dec	Jan	Feb	Mar	*P*-value
SEP1 expression (% off-crop control)							
Off-crop	NBS	Mar	< 0.05[y]	100.0 a[A]	100.0 a[A]	100.0 a	ns
On-crop	NBS	Mar	< 0.05	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Mar	< 0.05	84.3 a[A]	29.7 b[B]	22.4 b[B]	*
On-crop	BS	Nov FR	< 0.05	< 0.05	< 0.05	17.0 b	--
On-crop	BS	Dec FR		< 0.05	< 0.05	< 0.05	--
On-crop	BS	Jan FR			< 0.05	< 0.05	--
P-value			--	ns	***	***	
PI expression (% off-crop control)							
Off-crop	NBS	Mar	< 0.05	< 0.05	100.0	100.0 a	--
On-crop	NBS	Mar	ND[z]	ND	ND	ND	--
On-crop	BS	Mar	ND	ND	< 0.05	ND	--
On-crop	BS	Nov FR	ND	ND	< 0.05	32.8 b	--
On-crop	BS	Dec FR		ND	< 0.05	18.9 b	--
On-crop	BS	Jan FR			ND	< 0.05	--
P-value			--	--	--	*	
AG expression (% off-crop control)							
Off-crop	NBS	Mar	ND	< 0.05	100.0	100.0 a	--
On-crop	NBS	Mar	ND	ND	< 0.05	ND	--
On-crop	BS	Mar	< 0.05	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Nov FR	< 0.05	ND	< 0.05	30.4 a	--
On-crop	BS	Dec FR		< 0.05	< 0.05	15.5 a	--
On-crop	BS	Jan FR			< 0.05	< 0.05	--
P-value			--	--	--	ns	

[x] Data are means for five trees (replications) per treatment presented as a percent of the control value set at 100%. The expression level of each gene in buds on NBS of off-crop control trees served as the control for each month except March, for which the February expression level was used as the control (100%) (buds could not be collected from off-crop trees in March). For buds on NBS of off-crop control trees, transcripts of *SEP1* were

at the limit of detection in December but the relative expression levels in January and February for *SEP1* were 0.6 and 0.4, respectively. Transcripts of *PI* and *AG* were only at levels above detectable in February, during which the relative expression levels were 0.5 and 0.5, respectively. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, *** at $P < 0.001$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Supplemental Results to Chapter 5

Table S5.1 Expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI* and *AG* relative to *EF1-α* expression in buds on nonbearing shoots (NBS) of off-crop trees harvested in March, NBS and bearing shoots (BS) of on-crop trees harvested in March, and BS of on-crop trees with all fruit removed (FR) in November, December and January, respectively, in 'Pixie' mandarin[x].

Tree status	Shoot status	Harvest or FR time	Relative expression				
			Dec	Jan	Feb	Mar	*P*-value
FT expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 a^{A}	100.0 a^{B}	100.0 a^{B}	100.0 a	**
On-crop	NBS	Mar	< 0.05[y]	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Mar	ND[z]	ND	ND	ND	--
On-crop	BS	Nov FR	2.2 b^{B}	< 0.05	48.1 b^{AB}	96.7 a^{A}	*
On-crop	BS	Dec FR		< 0.05	11.3 b^{B}	119.8 a^{A}	*
On-crop	BS	Jan FR			< 0.05	40.1 a	--
P-value			*	--	*	ns	
SOC1 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 a^{A}	100.0 a^{B}	100.0 b^{A}	100.0 a	*
On-crop	NBS	Mar	64.0 b^{A}	59.1 b^{A}	110.5 b^{A}	123.1 a^{A}	ns
On-crop	BS	Mar	44.5 b^{B}	62.6 b^{B}	106.2 b^{B}	357.5 a^{A}	**
On-crop	BS	Nov FR	61.9 b^{B}	108.0 a^{B}	343.6 a^{A}	213.8 a^{AB}	**
On-crop	BS	Dec FR		116.7 b^{B}	87.1 b^{B}	307.1 a^{A}	*
On-crop	BS	Jan FR			81.2 b^{A}	104.2 a^{A}	ns
P-value			**	****	***	ns	
LFY expression (% off-crop control)							
Off-crop	NBS	Mar	ND	ND	100.0 a^{A}	100.0 a	--
On-crop	NBS	Mar	ND	ND	7.7 b^{A}	ND	--
On-crop	BS	Mar	ND	ND	8.3 b^{A}	29.7 b^{A}	ns
On-crop	BS	Nov FR	ND	4.7 a^{A}	33.4 b^{A}	38.7 b^{A}	ns
On-crop	BS	Dec FR		7.2 a^{A}	16.2 b^{B}	50.1 ab^{A}	*
On-crop	BS	Jan FR			5.4 b^{B}	23.7 b^{A}	*
P-value			--	ns	****	*	

Table S5.1 continued.

Tree status	Shoot status	Harvest or FR time	Relative expression Dec	Jan	Feb	Mar	*P*-value
AP1 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 a^{B}	100.0 a^{B}	100.0 b^{A}	100.0 bc	**
On-crop	NBS	Mar	41.1 b^{A}	34.4 b^{A}	33.0 c^{A}	50.5 d^{A}	ns
On-crop	BS	Mar	37.9 b^{B}	42.8 b^{B}	33.4 c^{B}	72.4 cd^{A}	****
On-crop	BS	Nov FR	32.8 b^{C}	93.5 a^{BC}	182.1 a^{A}	111.9 ab^{AB}	**
On-crop	BS	Dec FR		55.6 b^{B}	44.4 bc^{B}	145.4 a^{A}	**
On-crop	BS	Jan FR			36.3 bc^{A}	66.4 cd^{A}	ns
P-value			****	**	***	***	
AP2 expression (% off-crop control)							
Off-crop	NBS	Mar	100.0 ab^{B}	100.0 a^{B}	100.0 a^{A}	100.0 a	**
On-crop	NBS	Mar	141.4 a^{A}	143.1 a^{B}	17.8 b^{B}	10.6 b^{B}	**
On-crop	BS	Mar	103.5 ab^{AB}	145.9 a^{B}	17.5 b^{B}	34.3 b^{A}	*
On-crop	BS	Nov FR	76.5 b^{B}	150.6 a^{B}	39.7 b^{A}	25.5 b^{AB}	*
On-crop	BS	Dec FR		114.6 a^{B}	13.0 b^{B}	31.5 b^{A}	*
On-crop	BS	Jan FR			12.4 b^{A}	9.2 b^{A}	ns
P-value			*	ns	****	**	
SEP1 expression (% off-crop control)							
Off-crop	NBS	Mar	< 0.05	100.0 a^{A}	100.0 a^{A}	100.0 a	ns
On-crop	NBS	Mar	< 0.05	< 0.05	3.9 b^{A}	4.8 b^{A}	ns
On-crop	BS	Mar	< 0.05	65.5 a^{A}	16.2 b^{A}	25.6 b^{A}	ns
On-crop	BS	Nov FR	< 0.05	6.7 b^{B}	12.9 b^{A}	15.5 b^{A}	ns
On-crop	BS	Dec FR		6.6 b^{A}	4.3 b^{A}	10.3 b^{A}	ns
On-crop	BS	Jan FR			7.2 b^{A}	4.5 b^{A}	ns
P-value			--	**	****	****	
PI expression (% off-crop control)							
Off-crop	NBS	Mar	< 0.05	< 0.05	100.0	100.0 a	--
On-crop	NBS	Mar	ND	ND	ND	ND	--
On-crop	BS	Mar	< 0.05	< 0.05	< 0.05	ND	--
On-crop	BS	Nov FR	ND	ND	< 0.05	8.9 b	--
On-crop	BS	Dec FR		< 0.05	< 0.05	4.1 b	--
On-crop	BS	Jan FR			ND	< 0.05	--
P-value			--	--	--	***	

Table S5.1 continued.

Tree status	Shoot status	Harvest or FR time	Relative expression				
			Dec	Jan	Feb	Mar	*P*-value
AG expression (% off-crop control)							
Off-crop	NBS	Mar	ND	< 0.05	100.0	100.0 a	--
On-crop	NBS	Mar	ND	ND	< 0.05	ND	--
On-crop	BS	Mar	< 0.05	< 0.05	< 0.05	< 0.05	--
On-crop	BS	Nov FR	< 0.05	< 0.05	< 0.05	6.9 b	--
On-crop	BS	Dec FR		< 0.05	< 0.05	3.0 b	--
On-crop	BS	Jan FR			< 0.05	< 0.05	--
P-value			--	--	--	*	

[x] Data are means for five trees (replications) per treatment presented as a percent of the control value of 100%. The expression level of each gene in buds on NBS of off-crop control trees served as the control for each month except March, for which the February expression level was used as the control (100%) (buds were not collected from off-crop trees March). For buds of NBS of off-crop control trees, the relative expression levels in December, January and February for *FT* were 2.8, 0.2 and 0.5, respectively; for *SOC1* were 13.5, 9.1 and 12.0, respectively; for *AP1* were 7.5, 7.2 and 12.1, respectively; for *AP2* were 0.7, 0.3 and 3.0, respectively. Transcripts of *LFY* were only at levels that could be quantified in February, and thus, the expression level obtained in February (14.4) was used as the control (100%) value January and March. Transcripts of *SEP1* were at the limit of detection in December but the relative expression levels in January and February for *SEP1* were 1.3 and 1.5, respectively. Transcripts of *PI* and *AG* were only at levels above the limit of detection in February, during which the relative expression levels were 2.1 and 2.1, respectively. Means followed by different lower-case letters within a vertical column are significantly different for the same month and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which ** refers to a significant effect at $P < 0.01$, **** at $P < 0.0001$; ns, not significant.

[y] < 0.05, detected, the mean relative expression level of the five biological replications for the target gene was greater than 0 but less than 0.05 and thus was not included in statistical analysis.

[z] ND, not detected, the expression level of the target gene in each of the five biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Chapter 6

Conclusions to the Dissertation

For alternate bearing (AB) citrus (*Citrus* spp.), yields alternate between a heavy on crop composed of small fruit with reduced commercial value (on-crop year) and a light off crop of large fruit, many with undesirable fruit quality (off-crop year). After initiation, typically in response to an adverse climate event resulting in reduced yield, cycles of on and off crops are perpetuated by internal factors brought about by the number of fruit per tree (crop load) that negatively impacts floral intensity of the return bloom the following spring (Monselise and Glodschmidt, 1982). In this research, the economic problem of AB was addressed. 'Pixie' and 'Nules Clementine' mandarin trees (*C. reticulata* Blanco) producing an off crop (185 and 203 fruit/tree, respectively) flowered profusely (96 and 43 inflorescences/5 buds/20 shoots/tree, respectively) in April the following year. In contrast, 'Pixie' and 'Nules Clementine' mandarin trees setting a heavy on crop (3381 and 1262 fruit/tree) did not flower the next spring (0 inflorescence/tree for both cultivars). In preparation for the study of the effect of the on crop of fruit on floral development in AB cultivars, floral gene transcription in buds of 'Washington' navel orange (*C. sinensis* L. Osbeck) under floral-promoting conditions, low temperature (LT) (15/10 ˚C, day/night) and water deficit (WD) (stem water potential ≤ -2.40 MPa), was analyzed. The results of the dissertation research provided new insights into the role of specific genes in the regulation of citrus floral development, which are summarized in the model depicted in Figure 6.1.

For ‘Washington’ navel orange, floral development was initiated in buds by September. At this time, *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *APETALA2* (*AP2*) were already expressed in buds collected from mature adult ‘Washington’ navel orange trees maintained in non-floral-promoting well irrigated (WI) and warm (WC) condition for previous five months. Trees remained under WI and WC for an additional 11 weeks, but never expressed *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) and did not flower. In contrast, starting in September, a subset of trees was subjected to LT or WD, respectively. At the end of eight weeks of treatment, these trees had increased bud expression of *AP1* and *AP2*, which subsequently activated *SEP1*, *PI* and *AG*, and flowered intensely (63 and 51 inflorescences/75 buds, respectively). Interestingly, upregulation of the above floral genes only occurred after transfer of the trees from LT and WD to WC and WI condition. This suggests that both treatments promote floral development but also prevent flower formation until temperature and water are optimal. In contrast, LT or WD did not affect bud transcript levels of *FT*, *SOC1* and *LFY*. In *Arabidopsis thaliana*, the activity of class A genes *AP1* and *AP2* is required for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). It is noteworthy that in ‘Washington’ navel orange, sepal formation coincided with irreversible commitment of the shoot apical meristem (SAM) to floral development (determinacy) (Lord and Eckard, 1987). The results of this research provide the first evidence of the essential role of *AP1* and *AP2* in bud

determinacy and activation of the downstream floral organ identity genes in citrus (Figure 6.1).

To identify when induced citrus buds are irreversibly committed to floral development, gibberellic acid (GA_3), which inhibits citrus flowering only when applied before buds are determined (Lord and Eckard, 1987), was applied to both 8-week LT- and WD-treated trees during weeks 2 through 8. The GA_3 treatment had no effect on bud *FT*, *SOC1* and *LFY* transcription, but reduced bud *AP1* and *AP2* expression to levels equal to those of non-flowering WC- and WI-treated trees, and completely repressed *SEP1*, *PI* and *AG* expression with a concomitant decrease in floral intensity. These results provide additional evidence that *AP1* and *AP2* activity regulate bud determinacy (Figure 6.1) This is the first research documenting the pattern of transcript accumulation for floral organ identity genes *AP2*, *SEP1*, *PI* and *AG* in citrus. Whether *AP1* activates *AP2* and the genes downstream from *AP2* or whether *AP1* and *AP2* must act in concert to upregulate *SEP1*, *PI* and *AG* remain unresolved (Figure 6.1). The current results also leave open the possibility that LT, WD and GA_3 might regulate each of the floral organ identity genes independently (Figure 6.1).

With this new basic understanding of the regulation of floral determinacy in citrus, floral gene expression was compared in buds of off- and on-crop Pixie and Nules Clementine mandarin trees, late- and early-maturing cultivars, respectively, grown in climatically different areas in Southern California. The 'Pixie' mandarin orchard was located in a valley near the coast with annual maximum and minimum temperatures of 26 and 7 ˚C, respectively, whereas 'Nules Clementine' mandarin trees were grown in an

inland orchard with annual maximum and minimum temperatures of 23 and 13 ˚C, respectively. Consistent with the interpretation that floral induction had occurred before September in 'Washington' navel orange, *FT*, *SOC1*, *LFY*, *AP1* and *AP2* were expressed in buds collected in October from off-crop 'Pixie' and 'Nules Clementine' mandarin trees. Buds of these trees did not express *SEP1*, *PI* and *AG* until March, one month before bloom in April, suggesting floral organs differentiate shortly before flower opening.

The results documented for the first time the critical role of persistent *FT* transcription in the expression of *AP1* at sufficiently high levels to activate the downstream floral organ identity genes for successful citrus flowering. For buds of on-crop 'Pixie' and 'Nules Clementine' mandarin trees, *FT* was never expressed from October through March and, by March, the month before bloom, bud transcript levels of *LFY*, *AP1*, *AP2* were lower than those off-crop trees, with no expression of *SEP1*, *PI* and *AG*; no inflorescences developed in April. Removing the on crop of 'Pixie' mandarin fruit four months before bloom resulted in flowering equal to 21% of the inflorescence number of off-crop trees, indicating that only 21% of the bud population were viable floral buds at the time of fruit removal. This treatment also increased bud *FT* transcripts from undetectable to expressed shortly before bloom, increased *AP1* expression to levels equal to off-crop trees the month before bloom, and resulted in expression of *PI* and *AG* just before flowering, but did not increase *AP2* transcript levels. In *A. thaliana*, sustained bud *FT* expression is essential for the SAM to become committed to floral development (Müller-Xing et al., 2014). This developmental event was demonstrated to require *AP1* and *AP2* expression in 'Washington' navel orange. Taken together, the results of this

research provided evidence suggesting that *AP1* is the main target of *FT*. Further, the results of obtained by removing the on crop in November suggested that activity of *AP1* alone may be adequate for bud determinacy, but left open the possibility that the low level of *AP2* expression was sufficient to function in concert with *AP1* to confer bud determinacy and activate the downstream floral organ identity genes *PI* and *AG* (Figure 6.1). In *A. thaliana*, *AP1* acts as a major coordinator of floral development by regulating the switch from floral induction to flower formation (Wellmer and Riechmann, 2010), lending further support to the proposal that *AP1* plays a dominant role in citrus floral development. Repression of *FT* by the on crop of fruit as early as October did not inhibit floral induction. In the absence of *FT* expression, *SOC1*, *LFY*, *AP1*, *AP2* and *SEP1* were still expressed in buds of on-crop 'Pixie' and 'Nules Clementine' mandarin trees. In *A. thaliana*, expression of *LFY* and *AP1* is one the first signs indicating that floral induction has taken place (Melzer et al., 1999). Thus, it is evident that floral induction had successfully occurred in on-crop trees. Taken together, the results provided evidence documenting for the first time that the on crop of fruit does not prevent floral induction, but prevents floral determinacy, through the downregulation of *FT* and its downstream target *AP1*, leading to reduced return bloom.

Based on the results of this research, which documented the expression of *FT*, *SOC1*, *LFY*, *AP1*, *AP2* and *SEP1* in September, and an earlier report of *FT* and *LFY* expression in buds collected in August from off-crop 'Murcott' mandarin trees (*C. reticulata* Blanco) (Shalom et al., 2014), citrus floral induction appears to occur as early as summer, suggesting that buds of adult citrus trees undergo transition from vegetative to floral

development (floral induction) annually in response to autonomous (age-dependent) signals or changes in photoperiod. This is in contrast to the current idea that low temperature in fall/winter induce flowering in citrus (Figure 6.1). This proposal is supported by the fact that other subtropical evergreen perennial tree crops, such as avocado (*Persea americana* Mill.) (Salazar-García et al., 1998) and olive (*Olea europaea* L.) (Cuevas et al., 1999), transition from vegetative to floral development during the summer (July). In olive, floral and vegetative buds can only be distinguished by an increase in floral bud size in November; no differences in the SAM were observed until the initiation of the sepals at bud break in February (Cuevas et al., 1999). Similarly, for 'Washington' navel orange, floral and vegetative buds remained indistinguishable until November when floral buds underwent microscopic bud break compared to December for those buds ultimately producing vegetative shoots. The SAM of each bud remained indistinguishable until sepals formed in January (Lord and Eckard, 1985). However, it must be noted that there remains the possibility that all buds of adult citrus trees are not only competent, but also have been induced to flower as a result of phase transition from juvenile to adult (Tan and Swain, 2006) (Figure 6.1).

The research results presented in this dissertation provided new information on the genetic regulation of citrus floral development, especially regarding the regulation of bud determinacy, a key event in flowering physiology. The results did not clarify when floral induction occurs in citrus, but were consistent with an annual transition (in summer) much earlier than the generally accepted low temperature induction in fall/winter, but leaving open the possibility of induction occurring with phase transition of buds from

juvenile to adult. Resolution of this question should be a high priority in future research on citrus floral development. First, knowing when induction occurs is critical to avoid applying GA_3 at a time that might prevent induction. Second, this information is essential to determine whether the on crop of citrus fruit inhibits floral induction at *FT*, or an upstream gene, in order to mitigate the negative effect of the on crop on return bloom and yield.

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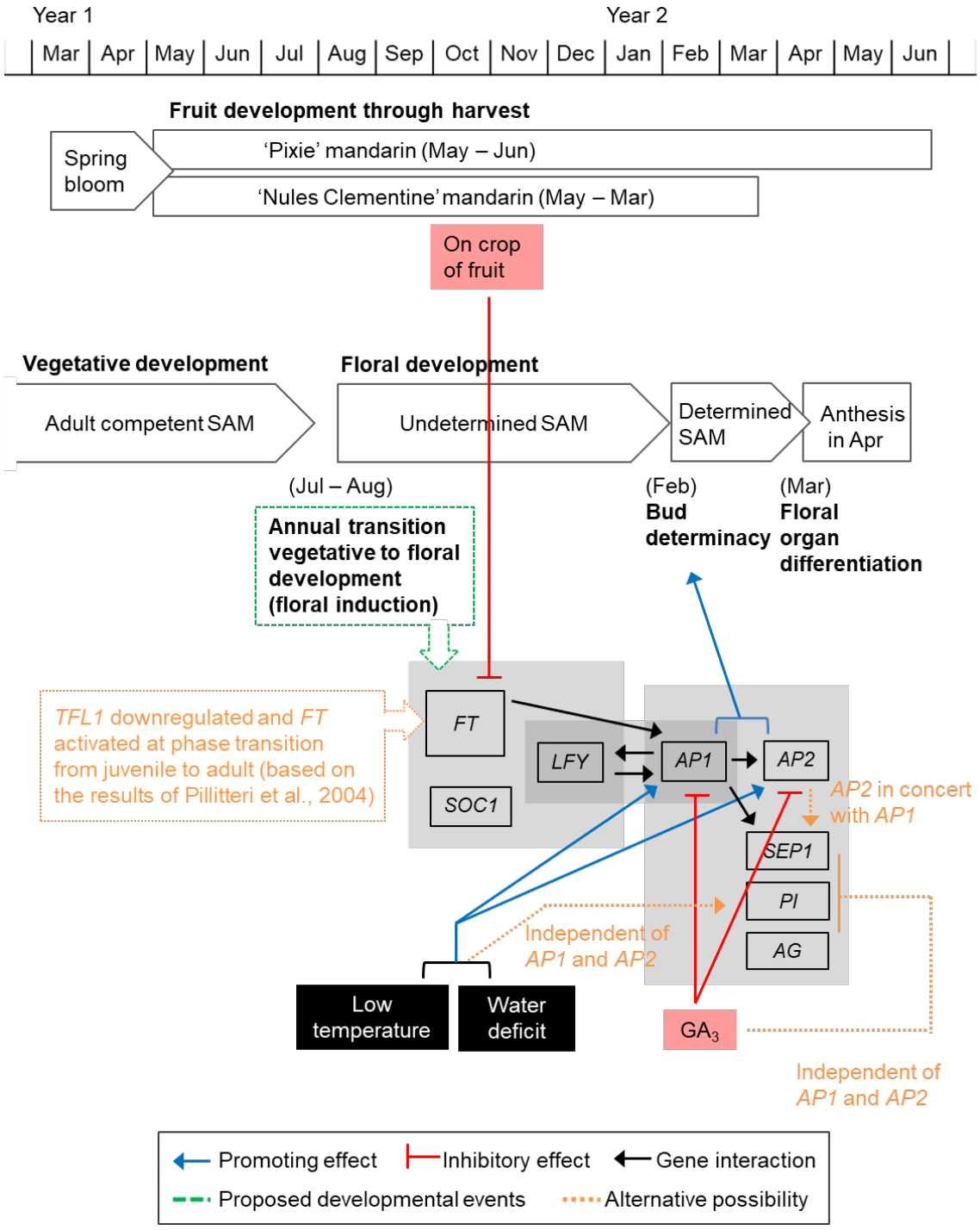

Figure 6.1 Proposed model of floral regulation in buds of *Citrus* spp. with associated changes in the shoot apical meristem (SAM) based on the results of the dissertation research.

Appendix A

Effects of low temperature, water deficit and gibberellic acid on bud expression of hormone-metabolizing genes in 'Washington' navel orange

Introduction

In citrus (*Citrus* spp.), the relationship between endogenous hormone homeostasis and flowering has been demonstrated (Koshita and Takahara, 2004; Koshita et al., 1999; Shalom et al., 2014; Verreynne, 2005). The results of Verreynne (2005) provided evidence that the changes in hormone ratios brought about by the presence of fruit inhibit bud break at two stages in the phenology of alternate bearing 'Pixie' mandarin (*C. reticulata* Blanco) and thereby reduce floral intensity at return bloom. First, the auxin indole-3-aetic acid (IAA) accumulated and the concentration of the cytokinin isopentenyladenine (2iP) decreased in apical buds of vegetative shoots of on-crop trees, corresponding to reduced growth of summer vegetative shoots compared to off-crop trees. The summer vegetative shoots have the potential to produce 40% of total inflorescences developing the following spring. Removal of the on crop in July restored hormone homeostasis by August and floral intensity at spring bloom. Second, for buds of on-crop trees, abscisic acid (ABA) and IAA accumulated starting in January, resulting in higher ratio of both ABA and IAA to 2iP in comparison with off-crop trees, with the lower bud break rate and floral intensity in spring (Arbona and Lovatt, unpublished results; Verreynne, 2005). When bud concentrations of ABA and IAA decreased and 2iP increased in response to removal of the on crop in December or January, percent spring

bud break, as well as inflorescence number, increased compared to on-crop trees without fruit removed. Similarly, on-crop 'Murcott' mandarin trees (*C. reticulata* Blanco) had greater levels of IAA and ABA in buds by August compared to off-crop trees, with a reduction in floral intensity the following April (Shalom et al., 2014).

Despite the negative impact of endogenous ABA accumulation on flowering observed in *C. reticulata* cultivars, the increase in leaf ABA concentration correlated with elevated floral intensity in Satsuma mandarin (*C. unshiu* Marc.) in response to a water-deficit (WD) treatment (Koshita and Takahara, 2004). Thus, the role of ABA in citrus flowering remains unclear. Low-temperature (LT) and WD treatments increased flowering of 'Washington' navel orange (WNO) (*C. sinenesis* L Osbeck) (Chapters 2 and 3). Applying GA_3 (50 mg L^{-1}) to LT- and WD-treated trees dramatically decreased floral intensity compared to trees receiving LT and WD not treated with GA_3. Whether LT, WD and GA_3 brought about changes in bud hormone concentrations related to inflorescence number at bloom remains unknown.

The results of analysis of the expression of genes regulating hormone metabolism would provide insight into the potential roles of plant hormones in citrus flowering. In this regard, the objective of this study was to compare bud expression of hormone-metabolizing genes in WNO trees subjected to 11 weeks of non-floral-promoting well irrigated (WI) and warm (WC) conditions, 8 weeks of floral-promoting LT and WD conditions, and 8 weeks of LT- and WD with weekly applications of GA_3 to inhibit flowering (Chapters 2 and 3). Expression of the following genes was quantified: *ISOPENTENYLTRANSFERASE* (*IPT*) encoding isopentenyl transferase for cytokinin

biosynthesis (Hwang et al., 2012; Takei et al., 2001), *YUCCA2* (*YUC2*) encoding flavin monooxygenase for IAA biosynthesis (Zhao, 2012; Zhao et al., 2001), *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) for 9-*cis*-epoxycarotenoid dioxygenase in ABA biosynthesis in response to water-deficit signals (Agustí et al., 2007; Rodrigo et al., 2006), and *PIN-FORMED 1* (*PIN1*), which encodes a cellular auxin efflux carrier protein (Adamowski and Friml, 2015; Gälweiler et al., 1998; Geldner et al., 2001).

Materials and Methods

Plant material and treatment conditions

Five-year-old mature WNO scions on 'Carrizo' citrange rootstock (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) grown in 56-liter pots containing steam-sterilized University of California soil mix I (Baker, 1957) were used in this study. All trees used in this research were grown in a glasshouse under warm condition (WC) (16-hr day [500 μmol m^{-2} s^{-1}] at 24˚C/8-hr night at 19˚C) and well irrigated (WI) (stem water potential [SWP] around -1.00 MPa) for five months prior to the start of the experiment in September. All fruit were removed from the trees before the initiation of the experiment. The research used a complete randomized design with four WNO trees (replications) per treatment and seven treatments. In treatment 1, trees were maintained in the WC for 11 weeks with midday SWP kept around -1.00 MPa for the duration of the experiment by daily irrigation in comparison with trees exposed to LT (treatments 2 through 4) and WD (treatments 5 and 6) not receiving GA_3 and treated with GA_3 in this study.

For the first experiment, trees in treatments 2 through 4 were exposed to LT (16-hr day [500 μmol m^{-2} s^{-1}] at 15 ˚C/8-hr night at 10˚C) (Percival PGW growth chamber; 2.3 x 1.5 x 2.0 m; Percival, Boone, IA) for 8 weeks and then transferred to the WC for the remainder of the experiment culminating with bloom in week 11. In treatments 3 and 4, LT-treated trees were sprayed weekly with 50 mg L^{-1} GA_3 (ProGibb 40%, Valent BioScience Corporation, Libertyville, IL), containing 0.01% Silwet L77 surfactant (Helena Chemical Company, Collierville, TN), in weeks 2 through 8 and weeks 4 through 8, respectively, of the LT treatment. With the exception of temperature, all trees in treatments 1 through 4 were treated the same, including irrigation time and amount, fertilization, and relative humidity (~ 80%).

For the second experiment, trees in treatments 5 and 6 were maintained at an SWP less than or equal to -2.40 MPa by deficit irrigation for 8 weeks (day 0 to day 54) (Southwick and Davenport 1986); on day 55, the WD trees were re-irrigated and by day 60, tree SWP had recovered to a non-stress level (> -1.00 MPa). In treatment 6, WD-treated trees were sprayed weekly with 50 mg L^{-1} GA_3 containing 0.01% Silwet L77 surfactant in weeks 2 through 8 of the WD treatment. With the exception of irrigation regime, all trees in treatments 1, 5 and 6 were treated the same, including temperature (24/19 ˚C, day/night), day length (16-hr day [500 μmol m^{-2} s^{-1}]/8-hr night), fertilization, and relative humidity (~ 80%).

Sample collection and gene expression analysis

The apical five buds from 15 nonbearing shoots per tree were collected at weeks 2, 4, 6, 8, 9, and 10 from each of the four trees (four replications) in all treatments, with the exception that sample collection for the GA_3-treated trees was delayed until 2 weeks after the first GA_3 application. Collected buds were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab. Bud samples were quickly frozen in liquid nitrogen and stored at -80 ˚C until analyzed. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequence of *Arabidopsis thaliana* homologs *IPT*, *YUC2*, *PIN1*, *NCED* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). The sequences of *IPT*, *YUC2* and *PIN1* chosen in this research share high identity with *A. thaliana IPT*, *YUC2* and *PIN1*, respectively; the predicted protein sequences for the putative *IPT*, *YUC2* and *PIN1* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Citrus *NCED* analyzed in this

research was *CsNCED1*, a homolog of *A. thaliana NCED3*, for which the leaf expression was correlated with water deficit and dehydration-induced ABA accumulation in leaves (Rodrigo et al., 2006). Moreover, bud expression of this *NCED* gene, renamed *NCED3*, was related to spring floral intensity in response to crop load (Shalom et al., 2014). The gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study as well as the BLAST results of PCR product sequence versus target sequence of each gene of interest are listed in Table A1.1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-µL reaction volume containing 1.2 µL cDNA (about 40 ng of input RNA), 0.6 µL gene-specific forward and reverse primer mix (10nM), 7.5 µL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 µL PCR-grade water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was performed at the end of each qPCR run to confirm that nonspecific products were not formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with WNO flowers collected from orchard trees at spring bloom used as the control (expression level of 1) and *β-ACTIN* (*ACT*) as the reference gene

(endogenous control). The selection of *ACT* as the reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Gene expression data for each treatment and sample date were the mean of four biological replicates; each biological replicate was the mean of three qPCR technical replicates.

Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the relative expression levels of genes using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Fisher's Least Significant Difference (LSD) procedure with a family error rate of $\alpha \leq 0.05$.

Results and Discussion

Effects of low temperature on the expression of citrus hormone-metabolizing genes

Buds of trees that were kept under WC for 11 weeks and failed to produce inflorescences (Chapter 2) expressed *IPT*, *YUC2*, *PIN1* and *NCED* as early as week 2 (Table A1.2). Whereas *YUC2* and *NCED* were expressed on all sampling dates and fluctuated over time ($P < 0.05$ and $P < 0.001$, respectively), *IPT* and *PIN1* transcripts decreased below the limit of detection during weeks 6 and 8, with expression of both genes restored at weeks 9 and 10. For buds of trees exposed to 8 weeks of LT, the pattern of bud *IPT* transcript accumulation was similar to that of WC-treated trees, with the notable exception that *IPT* expression was restored at week 8, indicating upregulated bud

2iP biosynthesis in response to 8 weeks of LT (Table A1.2). Buds of LT-treated trees had *YUC2*, *PIN1* and *NCED* expression on all sampling dates, with the maximum values present at week 9, a week after transfer to the WC ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). Thus, between the trees subjected to WC and those exposed to LT, only the latter had *PIN1* expression at weeks 6 and 8, suggesting enhanced IAA efflux from the shoot apex and therefore reduced IAA levels in buds. Six weeks of LT also resulted in a significant reduction in bud *YUC2* and *NCED* expression levels that were 80% and 71% lower than that of trees subjected to WC for the same week, respectively ($P < 0.05$ for the two genes), indicating downregulation of IAA and ABA biosynthesis in buds at week 6.

The above results suggest that the ratios of IAA and ABA to 2iP concentration in buds were lower in LT-treated trees than trees maintained under WC, respectively, at weeks 6 and 8 and week 6, as a result of the ostensible increase in 2iP concentration (at week 8) and reduction in the levels of IAA (at weeks 6 and 8) and ABA (at week 6). The putative shift in bud hormone ratios likely increased bud break as the number of inflorescences was greater and the number of inactive buds was reduced at week 11 (Chapter 2). The results of this study are in agreement with those of Verreynne (2005), which demonstrated an increase in floral intensity at return bloom in April following reduced bud ratios of IAA and ABA to 2iP by January as a result of removal of the on crop of fruit in December. The results confirmed that fruit were the source of IAA and the sink for 2iP.

Effects of water deficit on the expression of citrus hormone-metabolizing genes

For buds of trees subjected to 8 weeks of WD, *IPT* transcripts were detected at weeks 2 and 4, decreased below the limit of detected during weeks 6 and 8, and increased to the maximum levels after re-irrigation at weeks 9 and 10 ($P < 0.01$) (Table A1.3). Bud *YUC2* and *NCED* expression occurred consistently on all sampling dates in WD-treated trees, with the maximum expression levels in week 9, one week after removal of the stress ($P < 0.001$ for the two genes). Between buds of WD-treated trees and WI trees, there was no significant difference in *IPT*, *YUC2* or *NCED* expression at any time. Similar to trees maintained under WI conditions for 11 weeks, transcripts of *PIN1* were detected at weeks 2 and 4 but not detected at week 6 in buds of trees exposed to 8 weeks of WD. Nevertheless, buds of WD-treated trees expressed *PIN1* at week 8 with maximum expression of *PIN1* at week 10, whereas *PIN1* expression was not elevated in buds of WI trees until week 9.

Both LT and WD for 8 weeks increased floral intensity and reduced inactive bud number (Chapter 3). However, in contrast to LT, WD did not result in significant changes in bud *IPT*, *YUC2* or *PIN1* expression compared to WI trees, except for the earlier restoration of *PIN1* expression. In the study reported here, increased flowering in response to WD was not accompanied by increased bud *NCED* expression during the stress or after re-irrigation relative to 11-week WI trees (Table A1.3). For Satsuma mandarin, the WD treatmentthat increased flowering caused leaf ABA accumulation (Koshita and Takahara, 2004) and in 'Nules Clementine' mandarin (*C. reticulata*), increased leaf ABA concentrations corresponded with inreased leaf *NCED* expression

following a 24-hour water-deficit period (Agusti et al., 2007). The different results are likely due to differences in the tissues used for gene expression analysis It should be noted that the results of the present study suggest ABA biosynthesis in the bud is not affected by WD, but did not preclude the possibility that WD brings about the changes in bud ABA levels through other mechanisms, such as translocation.

Effects of GA_3 on the expression of citrus hormone-metabolizing genes

The results of Chapter 2 demonstrated that weekly applications of GA_3 in weeks 2 through 8 and weeks 4 through 8 of the LT period reduced inflorescence number with a reciprocal increase in vegetative shoot number compared to LT-treated trees not receiving GA_3. The two GA_3 treatments did not result in any change in bud expression of *IPT*, *YUC2* or *PIN1* (Table A1.2), suggesting the inhibitory effect of GA_3 on citrus flowering is likely independent of 2iP and IAA balance in buds of WNO trees, at least under LT condition. Similar to the effect of foliar-applied GA_3 in LT-treated trees, weekly application of GA_3 in weeks 2 through 8 during the WD period resulted in a concomitant decrease in floral intensity with an increase in vegetative shoots in week 11 (Chapter 3) and had no influence on bud *YUC2* or *PIN1* expression compared to trees subjected to WD but not treated with GA_3 (Table A1.3). However, for WD-treated trees treated with the GA_3, bud *IPT* transcript levels were significantly greater at week 4 ($P < 0.05$) than WD-treated trees without GA_3 and 11-week WI trees (Table A1.3).

Weekly GA_3 applications in weeks 2 to 8 of the LT or WD period had no effect on *NCED* expression (Table A1.2, A1.3), indicating exogenous GA_3 applications that

reduced flowering did not affect ABA biosynthesis in buds of WNO trees. In contrast, delaying GA_3 applications to weeks 4 through 8 under LT resulted in an increase in *NCED* transcripts at week 8 to a level greater than LT-treated trees not receiving GA_3 ($P < 0.05$) (Table A1.2). This result suggests that bud ABA biosynthesis was transiently upregulated by GA_3, contradicting the well-established antagonistic relationship between endogenous GA and ABA during multiple developmental processes in many species (Iglesias et al., 2007; Weiss and Ori, 2007). It also remains unclear why bud *NCED* transcription only responded to the GA_3 treatment initiated in week 4, but not earlierin the LT period.

It should be noted that across all treatment in the two experiments in this study, there was no significant correlation between the number of inflorescences, vegetative shoots and inactive buds and the expression pattern of *IPT*, *YUC2*, *PIN1* or *NCED*, suggesting the changes in the expression of these hormone-metabolizing genes in response to LT, WD and GA_3 might not be strongly related to flower formation or bud break, but associated with other physiological and/or developmental events. Nevertheless, since hormone homeostasis in particular organs at particular times could change due to the degradation or translocation of endogenous hormones, the results of this study do not rule out the possibility that plant hormones have a direct role in modulating floral development and/or bud break in citrus. In other words, corresponding endogenous hormone concentrations are needed to fully understand the role of hormones in regulating floral gene expression and flowering, specifically analyses of the genes regulating

biosynthesis, degradation and transport of endogenous hormones combined with quantification of endogenous hormone concentrations are required.

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Table A1.1 Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence blast against target gene sequence	
				E-value	Identity
IPT	XM_ 006469022.1 (*C. sinensis*)	GCCCGAGTTCGAGCGGTATTTCAA TTCCTGGTCTCCGTTCACCAACAT	199	4E-90	99%
YUC2	XM_ 006469022.1 (*C. sinensis*)	ACACAAACCATTTCGGCTTGGACC TCTCCTCTTGTTTGAGCCCTTGCT	117	3E-30	97%
PIN1	XM_ 006482003.1 (*C. sinensis*)	AGGAGAACCCAAGCCAACTGCTAT GCAATTATCGCCGGCATCTCAACA	172	2E-42	93%
NCED	DQ028471.1 (*C. sinensis*)	TTTGGACAAGAATGCCACCGATGC AATGGAGTCGGCAGGAGTCATACA	148	8E-40	94%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov).

Table A1.2 Expression of *IPT*, *YUC*, *PIN1*, and *NCED* in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 ˚C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WC) (24/19 ˚C, day/night)[y].

LT	WC	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
IPT expression									
0 wks	11 wks	No GA_3	0.4 a^A	0.8 a^A	ND[z]	ND	38.5 a^A	104.7 a^A	ns
8 wks	3 wks	No GA_3	0.2 a^B	24.0 a^B	ND	17.8 a^B	235.3 a^A	29.4 a^B	*
8 wks	3 wks	Wk 2 to 8		31.1 a^A	5.8 a^A	17.8 a^A	513.6 a^A	26.1 a^A	ns
8 wks	3 wks	Wk 4 to 8			ND	162.4 a^A	39.5 a^A	63.7 a^A	ns
P-value			ns	ns	--	ns	ns	ns	
YUC2 expression									
0 wks	11 wks	No GA_3	0.7 a^C	1.1 a^C	500.2 a^{AB}	135.3 a^{BC}	542.4 a^A	343.2 a^{BC}	*
8 wks	3 wks	No GA_3	0.8 a^B	202.7 a^B	102.3 b^B	486.4 a^B	1490.0 a^A	148.4 a^B	*
8 wks	3 wks	Wk 2 to 8		240.8 a^A	159.6 b^A	178.4 a^A	2005.3 a^A	215.1 a^A	ns
8 wks	3 wks	Wk 4 to 8			40.6 b^B	1235.7 a^A	253.3 a^B	309.9 a^B	***
P-value			ns	ns	*	ns	ns	ns	
PIN1 expression									
0 wks	11 wks	No GA_3	1.1 a^B	1.6 a^B	ND	ND	6.1 a^A	4.7 a^A	***
8 wks	3 wks	No GA_3	0.3 b^B	2.9 a^B	2.7 a^B	3.1 a^B	30.7 a^A	4.0 a^B	**
8 wks	3 wks	Wk 2 to 8		7.3 a^B	5.8 a^B	2.8 a^B	92.8 a^A	3.8 a^B	*
8 wks	3 wks	Wk 4 to 8			4.5 a^A	23.8 a^A	6.8 a^A	6.7 a^A	ns
P-value			ns	ns	ns	ns	ns	ns	
NCED expression									
0 wks	11 wks	No GA_3	1.4 a^B	1.0 a^B	5.6 a^A	1.0 b^B	7.9 a^A	6.9 a^A	***
8 wks	3 wks	No GA_3	0.6 b^C	2.8 a^C	1.6 b^C	5.0 b^B	18.5 a^A	5.3 a^B	**
8 wks	3 wks	Wk 2 to 8		2.4 a^B	0.8 b^B	1.7 b^B	26.5 a^A	8.0 a^B	*
8 wks	3 wks	Wk 4 to 8			1.0 b^C	14.6 a^A	6.2 a^B	6.8 a^B	**
P-value			*	ns	**	*	ns	ns	

[y] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least

significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).

Table A1.3 Expression of *IPT*, *YUC*, *PIN1*, and *NCED* in buds of 'Washington' navel orange trees subjected to 8 weeks of water deficit (WD) (midday stem water potential ≤ -2.40 MPa), 8 weeks of WD plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8, or well-irrigated for 11 weeks (WI) (SWP > -1.00 MPa)[y].

WD	WI	GA_3	Relative expression						
			Week 2	Week 4	Week 6	Week 8	Week 9	Week 10	*P*-value
IPT expression									
0 wks	11 wks	No GA_3	0.4 a^{A}	0.8 b^{A}	ND[z]	ND	38.5 a^{A}	104.7 a^{A}	ns
8 wks	3 wks	No GA_3	0.8 a^{B}	0.3 b^{B}	ND	ND	81.2 a^{A}	58.1 a^{A}	**
8 wks	3 wks	Wk 2 to 8		40.8 a^{AB}	11.5 a^{B}	22.9 a^{B}	93.3 a^{A}	26.3 a^{B}	*
P-value			ns	*	--	--	ns	ns	
YUC2 expression									
0 wks	11 wks	No GA_3	0.7 a^{C}	1.1 a^{C}	500.2 a^{AB}	135.3 a^{BC}	542.4 a^{A}	343.2 a^{BC}	*
8 wks	3 wks	No GA_3	0.6 a^{B}	1.3 a^{B}	136.4 a^{B}	192.5 a^{B}	580.7 a^{A}	763.2 a^{A}	***
8 wks	3 wks	Wk 2 to 8		174.2 a^{B}	13.2 a^{B}	153.6 a^{B}	774.2 a^{A}	152.8 a^{B}	**
P-value			ns	ns	ns	ns	ns	ns	
PIN1 expression									
0 wks	11 wks	No GA_3	1.1 a^{B}	1.6 a^{B}	ND	ND	6.1 a^{A}	4.7 a^{A}	***
8 wks	3 wks	No GA_3	0.5 b^{C}	0.6 a^{C}	ND	2.7 a^{BC}	7.1 a^{AB}	9.3 a^{A}	**
8 wks	3 wks	Wk 2 to 8		1.8 a^{C}	1.1 a^{C}	2.3 a^{C}	9.0 a^{A}	4.5 a^{B}	****
P-value			ns	ns	--	ns	ns	ns	
NCED expression									
0 wks	11 wks	No GA_3	1.4 a^{B}	1.0 a^{B}	5.6 a^{A}	1.0 a^{B}	7.9 a^{A}	6.9 a^{A}	***
8 wks	3 wks	No GA_3	4.6 a^{B}	1.7 a^{C}	8.8 a^{AB}	0.7 a^{C}	9.5 a^{A}	9.9 a^{A}	***
8 wks	3 wks	Wk 2 to 8		3.7 a^{C}	8.9 a^{AB}	1.3 a^{D}	11.1 a^{A}	6.8 a^{BC}	*
P-value			ns	ns	ns	ns	ns	ns	

[y] Data are the means for four trees (replications) per treatment. Means followed by different lower-case letters within a vertical column are significantly different for the same week and means followed by different upper-case letters within a horizontal row are significantly different over time for the same treatment according to Fisher's least significant difference (LSD) test in which * refers to a significant effect at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, **** at $P < 0.0001$; ns, not significant.

[z] ND, not detected, the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35).