EXPRESSION PATTERNS OF FLOWERING GENES DURING FLOWER INDUCTION AND DETERMINATION IN SWEET ORANGE (*Citrus sinensis* L. OSBECK)

By

EDUARDO J. CHICA

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EXPRESSION PATTERNS OF FLOWERING GENES DURING FLOWER INDUCTION AND DETERMINATION IN SWEET ORANGE (*Citrus sinensis* L. OSBECK)

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Eduardo J. Chica

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Chair: Gene Albrigo
Cochair: Christine Chase
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In recent years, several genes putatively involved in the regulation of floral induction have been identified in *C. sinensis*. However, the expression patterns of these genes in response to different treatments known to alter floral induction have not been investigated. As the first level of regulation for the expression of a given phenotype, characterizing transcript levels of *C. sinensis* flowering genes will be useful for developing models that could enable discovery of mechanisms regulating floral induction in *C. sinensis* and other species of subtropical origin. This study investigated patterns of transcript accumulation of putative flowering signal genes *CsFT* and *CsSL1*, and the floral identity genes *CsAP1* and *CsLFY* in response to several drought, low temperature and gibberellin treatments known to alter floral induction in *C. sinensis*. Results supported a role for *CsFT* as an integrator of flowering signals initiated by low temperatures and water deficit whereas *CsSL1* was responsive only to signals initiated by low temperatures. Accumulation of *CsFT* transcripts was proportional to the duration of floral-inductive water deficit and to levels of floral-inductive temperatures. Water deficit reduced *CsAP1* and *CsLFY* transcript accumulation while trees were under water deficit but induced higher levels of *CsAP1* and *CsLFY* transcripts after irrigation was resumed than in well-irrigated trees. The patterns of transcript accumulation of *CsAP1* and *CsLFY* supported a role of these two genes as markers of floral initiation. Based on

the patterns of accumulation *CsAP1* and *CsLFY* transcripts, floral determination occurs right after floral induction and initiation of growth is required for their up-regulation. Accumulation patterns of *CsAP1* and *CsLFY* transcripts corresponded to the basipetal gradient of flowering observed in *C. sinensis* shoots and the initiation of multiple flowering cohorts. Gibberellins and the presence of fruit both had a negative effect on the accumulation of *CsFT* transcripts and exogenous gibberellins also reduce the accumulation of *CsAP1* and *CsLFY* transcripts in buds. Accumulation of *CsFT* transcripts changed diurnally, responded quickly to environmental stimuli, and required alternation of light and dark cycles in order to sustain increasing levels of *CsFT* transcripts accumulation. Results provide initial information about the regulation of flowering in *C. sinensis* at the transcript level and could be helpful to design models of how flowering is induced and regulated in *C. sinensis*, other citrus cultivars and other subtropical species.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Floral induction marks the beginning of a major shift in the developmental program of flowering plants. Through floral induction, shoot meristems of flowering plants stop producing indeterminate vegetative structures and start generating determinate reproductive structures in the form of flowers and inflorescences. Understanding this shift in the developmental program of flowering plants is important for both, biological and horticultural reasons. Biologically, the timely generation of flowers is a major factor determining evolutionary fitness and the balance of the ecosystem to which a species belongs. Horticulturally, flowering is a key factor determining fruit set, development and crop load.

I studied changes in the expression of a set of flowering-related genes in response to factors that affect flowering intensity in sweet orange trees (*Citrus sinensis* Osbeck). The objective was to determine whether bloom characteristics in *C. sinensis* could be modeled from the patterns of transcript accumulation of these flowering-related genes. The starting point of my study was a hypothetical model (section 1.4) describing how flowering is induced in *C. sinensis* shoot meristems. This model was built mostly by merging information about the molecular mechanisms controlling flowering in model species (*A. thaliana*, *Populus sp.* and *Antirrhinum sp.*) with information about environmental regulation of flowering in *C. sinensis*. From this model, a set of hypotheses was selected and tested experimentally. The following three sections review the foundations for the proposed hypothetical model.

1.1 Shoot Meristem Developmental Programs

All aerial plant structures originate from shoot meristems. The type of structure formed in every growth cycle is determined by developmental programs executed in the active shoot meristem and developing primordia. In seed plants, two major developmental programs determine whether new growth will go onto forming indeterminate

vegetative structures or determinate reproductive structures. Both major developmental programs rely heavily on the establishment of spatial domains of expression and activity of several genes, proteins and metabolites within the shoot meristem. Assuming that the generation of indeterminate vegetative structures is the *default* program executed in shoot meristems, shifting to the determinate reproductive program will imply two major developmental changes: first, the shoot meristem must lose its capacity to self-replicate (transition to determinacy), and, second, the morphology of vegetative structures should be modified in order to form reproductive structures (generation of reproductive structures). The following subsections review how indeterminacy and vegetative character is maintained in non-flowering shoot meristems, what changes in spatial domains of expression and activity of genes, proteins and metabolites have been associated with changing developmental programs in shoot meristems and what are the differences between flower and inflorescence development. Unless noted otherwise, the information presented in the following subsections is derived from literature on *Arabidopsis thaliana* because these topics are better understood in this species.

1.1.1 Vegetative Growth: Maintaining Vegetative Indeterminacy

During vegetative growth, leaves, internodes and axillary meristems are produced in a modular, reiterative fashion from the flanks of an active shoot meristem (Sussex, 1989). In order to sustain indeterminate vegetative growth, shoot meristems must (1) maintain a pool of undifferentiated cells to perpetuate the process and (2) actively generate new vegetative structures through cell differentiation (Bowman and Eshed, 2000). The proper execution of these activities depends on the integration of positional information that determines the fate of each cell in the shoot meristem (Laux and Mayer, 1998).

Based on cyto-histological data, the shoot meristem is organized in three zones: (1) a central zone located at the tip of the meristem where cells divide sparingly, (2) a peripheral zone on the flanks of the central zone where cells divide more often and

(3) a medullary zone or pith meristem beneath the central zone and flanked by the peripheral zone with divisions as in the peripheral zone (Gifford and Corson, 1971). The cells from the central zone remain undifferentiated whereas cells in the peripheral zone and pith meristem start differentiating into specific cell types (Laux and Mayer, 1998). Hence, maintenance of the undifferentiated population of cells occurs in the central zone whereas early differentiation/organ initiation occurs in the peripheral zone and pith meristem. In addition to the central, peripheral and medullary zones, the shoot meristem can also be organized in three concentric layers (L1, L2 and L3, the outermost is L1) of cells clonally related to each other and originating from a minimal number of mother cells (Stewart and Dermen, 1970). The boundaries of these zones and layers are apparently defined by activity domains of several proteins and metabolites.

Genetic and molecular evidence indicates that the undifferentiated nature of the cells in the central zone is maintained by the interaction of the proteins encoded by the *SHOOTMERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA1* (*CLV1*), and *CLAVATA3* (*CLV3*) genes[1] . The expression of *STM* and *WUS* in the cells of the central zone promotes cell division and keep these cells undifferentiated (Gallois et al., 2002; Lenhard et al., 2002; Long et al., 1996; Mayer et al., 1998). Shoot meristems of mutants lacking either *STM* or *WUS* are either lost or disorganized and dysfunctional (Barton and Poethig, 1993; Laux et al., 1996). The activity of STM and WUS is antagonized by the activity of CLV1 and CLV3 (Clark et al., 1996, 1995, 1997; Reddy and Meyerowitz, 2005). CLV1 and CLV3 are components of a signaling pathway that maintains meristem size (Brand et al., 2000; Clark et al., 1997; Fletcher et al., 1999; Stone et al., 1998) by

[1] To refer to genes, mutants and proteins I will be following the formats in the Genetic Nomenclature Guide for *Arabidopsis thaliana* published in TRENDS in Genetics (Meinke et al., 1998). Briefly, wild-type gene names will be written in using italic uppercase letters (e.g. *ABC*), mutant alleles using italic lowercase letters (e.g. *abc*) and proteins using non-italic uppercase letters (e.g ABC).

promoting differentiation during organ formation (Laufs et al., 1998; Lenhard and Laux, 1999). Disruption of this signaling pathway in mutants lacking *CLV1* results in over-sized shoot meristems composed of masses of undifferentiated cells (Clark et al., 1993, 1995; Leyser and Furner, 1992). Thus, indeterminacy, enabled by self-regeneration of a functional meristem, is maintained at the genetic level by the interactions between the cell division and stem cell identity promoters STM/WUS and the signals from the CLV1/CLV3 pathway.

The generation of new vegetative structures starts with the initiation of primordia *pre-founder* cells (Carraro et al., 2006). The *pre-founder* cells originate from the central zone of the shoot meristem and show upregulation of primordia initiation gene markers such as *ZWILLE* (Moussian et al., 1998), *PIN1* (Vernoux et al., 2000) and *REVOLUTA* (Otsuga et al., 2001). The *pre-founder* cells then transition to primordia *founder* cells (4-10 cells) located in the peripheral zone of the meristem (Reddy et al., 2004). *Founder* cells show down regulation of *KNOX* genes (that are involved in the maintenance of undifferentiated meristematic cells), and expression of primordia initiation markers such as *AINTEGUMENTA* (*ANT*) (Elliott et al., 1996) or *LEAFY* (*LFY*) (Weigel et al., 1992). *ANT* and *LFY* are also involved in organ identity (Krizek et al., 2000; Weigel et al., 1992). At this stage, a boundary domain for the emerging primordia is established and is defined by the expression of *CUP-SHAPED COTYLEDONS* genes (Aida et al., 1997; Vroemen et al., 2003). The last stage of primordia formation is the establishment of dorso-ventrality, followed by cell differentiation, proliferation and expansion (Carraro et al., 2006). The processes in this last stage are controlled by genetic programs specific to each type of organ formed (Blázquez et al., 2006). Thus, during early organ morphogenesis, meristematic identity is first lost in a group of *founder* cells in the central zone of the meristem and then organ identity is determined. These two processes are under control of several genetic programs integrating signals from within the plant and the environment.

1.1.2 Phase Change: Re-programing the Meristem to Produce Flowers

Floral initiation induces a major change in the shoot meristem's organization and physiology. After the activation of flowering signal integrators *APETALA1* (*AP1*) and *LFY*, the meristem stops producing leaf primordia and initiate floral organ primordia instead (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Floral organ primordia originate in the meristem as a series of concentric whorls with sepal primordia being initiated first in the outermost whorl, followed by the petal primordia, then the stamen primordia and finally the carpel primordia in the innermost whorl (Coen and Meyerowitz, 1991; Smyth et al., 1990). Flowers and shoots show structural homology, and thus flowers can be imagined as shoot systems with minimal internodes, altered phyllotaxy and modified leaves (Coen and Carpenter, 1993; Esau, 1977). An important distinction between floral and shoot meristems is that whereas shoot meristems maintain a population of undifferentiated cells and thus are capable of indeterminate growth, floral meristems eventually lose this population of undifferentiated cells and become incapable of undergoing further growth. Thus, during the phase change from vegetative to reproductive growth, shoot meristems: (1) are re-programed to activate developmental programs that modify the basic morphology of leaves to produce floral organs in the emerging primordia, and (2) lose indeterminacy by failing to maintain a population of undifferentiated cells in the central zone.

AP1 and *LFY* are the targets for flowering signals initiated by different internal and environmental flowering promoter stimuli (Blázquez et al., 1998; Ruiz-Garcia et al., 1997; Wagner et al., 1999). Upregulation of *AP1* and *LFY* establish floral meristem identity in shoot meristems (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Once activated, *AP1* and *LFY* reinforce each other's expression and initiate floral morphogenesis (Liljegren et al., 1999; Parcy et al., 1998; Wagner et al., 1999). Floral morphogenesis in most angiosperms can be explained by the so called *ABC+SEP* model (Jack, 2001). The *ABC+SEP* model considers that genes involved in floral

organ morphogenesis can be classified in four *activity classes* (Coen and Meyerowitz, 1991; Jack, 2001). Genes belonging to each of these *activity classes* are expressed at specific times in specific whorls of the emerging primordia in the floral meristem and the interaction of their products define the type of floral organ to be formed in each whorl (Bowman et al., 1991; Coen and Meyerowitz, 1991; Jack, 2001). *AP1* and *APETALA2* (*AP2*) are *class A* genes and are expressed in the two outermost whorls of the floral meristem (i.e. whorls 1 and 2[2]), *APETALA3* (*AP3*) and *PISTILLATA* are *class B* genes and are expressed in the two middle whorls (i.e. 2 and 3), and *AGAMOUS* (*AG*) is a *class C* gene expressed in the two innermost whorls (i.e. 3 and 4) (Weigel and Meyerowitz, 1994). The *SEPALLATA* genes (*SEP1/2/3*) are expressed in all four whorls (except *SEP3* that is expressed only in whorls 2-4) (Flanagan and Ma, 1994; Mandel and Yanofsky, 1998; Savidge et al., 1995). Then, according to the model, expression of *class A* in the first whorl initiates sepal primordia, joint expression of *class A* and *class B* genes in the second whorl initiates petal primordia, joint expression of *class B* and *class C* genes in the third whorl initiates stamen primordia and finally expression of *class C* gene *AG* in the four whorl initiates carpel primordia and terminates growth by inactivating *WUS* (Mizukami and Ma, 1995; Weigel and Meyerowitz, 1994). The expression of *SEP* genes is required by *class B* and *class C* genes activity (Pelaz et al., 2000).

Determinacy in the floral meristem is achieved primarily by inactivation of *WUS* in the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001; Prunet et al., 2008). Inactivation of *WUS* occurs primarily through a positive-negative feedback loop between *AG* and *WUS* (Lenhard et al., 2001). In early stages of flower development, *AG* expression is activated by *LFY* and *WUS* (Lohmann et al., 2001). Later, expression of *AG* inactivates *WUS* through the action of *KNUCKLES*, that provides temporal

[2] *A. thaliana* floral meristems consists of four whorls

integration for the process. Genetic evidence indicates that other pathways might also be involved in the inactivation of *WUS* (Ming and Ma, 2009). For instance, *SUPERMAN* terminates *WUS* expression independently of *AG* through a pathway mediated by *APETALA3* and *PISTILLATA* (Bowman et al., 1992; Schultz et al., 1991). Other genes involved in floral meristem determinacy include *CRAB CLAW*, possibly acting downstream of *AG*, *APETALA3* and *PISTILLATA* (Bowman and Smyth, 1999; Lee et al., 2005), and the group of *REBELOTE*, *SQUINT* and *ULTRAPETALA* possibly acting upstream of both *SUPERMAN* and *AG* (Carles et al., 2004; Prunet et al., 2008). Regardless of the variety of potential pathways and mechanism, inactivation of *WUS* seems to be a necessary condition for determinacy in floral meristem.

1.1.3 Inflorescences: a Hybrid Program

Flowers can occur singly or in clusters forming an inflorescence. Inflorescences can be determinate or indeterminate depending on whether additional growth is possible through the maintenance of an active meristem. Regardless of the type of inflorescence formed, inflorescence meristems are different from floral meristems in that a population of undifferentiated cells in the central zone is maintained at least until the topology of the inflorescence is established; therefore, certain *vegetative character* is still conserved in inflorescence meristems. Determining whether meristems in an emerging inflorescence will develop into shoot-like or flower structures seems to be regulated (at the molecular level) by the interactions between *AP1*, *LFY* and *TERMINAL FLOWER 1* (*TFL1*)(Shannon and Meeks-Wagner, 1991).

Inflorescences of wild type *Arabidopsis* are indeterminate, and thus maintain a population of undifferentiated cells in their apical meristems (Smyth et al., 1990). In contrast, *Arabidopsis* mutants lacking *TFL1* produce determinate inflorescences in which the apical meristem produces a single flower (Alvarez et al., 1992; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1991), suggesting that *TFL1* is involved in maintaining undifferentiated apical meristems (Bradley et al., 1997). *TFL1* is also

expressed during the vegetative phase, and its mutant shows delayed phase transitions during development (Ratcliffe et al., 1998), suggesting that *TFL1* is a broader regulator of plant development.

In the inflorescence meristem, *TFL1* is expressed primarily below the central zone (Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991). In the central zone, *TFL1* represses the expression of floral identity genes *AP1* and *LFY* by delaying the upregulation of *AP1* and *LFY* and making the meristem less responsive to the activity of *AP1* and *LFY* (Ratcliffe et al., 1999). Thus, *TFL1* keeps the meristem from acquiring floral identity (Shannon and Meeks-Wagner, 1993). In turn, in the peripheral zone, *AP1* and *LFY* inhibit the expression of *TFL1* (Liljegren et al., 1999; Parcy et al., 2002; Shannon and Meeks-Wagner, 1991) and promote floral identity in the axillary meristems (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). *TFL1* expression in the central zone of the inflorescence meristem occurs before the upregulation of *AP1* and *LFY* during flower development and restricts *AP1* and *LFY* to the peripheral zone of the meristem where axillary meristems are forming (Ratcliffe et al., 1999). As these axillary meristems develop, the expression of *AP1* and *LFY* restrict the upregulation of *TFL1* in lateral positions and establish floral identity in these meristems (Ratcliffe et al., 1999). If the axillary meristems form before *AP1* and *LFY* are activated, *TFL1* will be activated first and the axillary meristem will develop into an axillary inflorescence (Ratcliffe et al., 1999). Hence, meristem fate in the inflorescence meristem seems to be determined by the relative timing of upregulation of floral identity genes (*AP1* and *LFY*) and *TFL1* and their mutual inhibition. Besides the *TFL1* – (*AP1*+*LFY*) regulatory loop in *A. thaliana*, other mechanisms controlling the fate of inflorescence meristems have recently been reported in other species but are not as extensively documented as the *TFL1* – (*AP1*+*LFY*) loop (Bull-Hereñu and Claßen-Bockhoff, 2011).

1.2 Floral Induction: a General Overview

Floral development requires the execution of 3 developmental processes in shoot meristems. First, juvenile meristems unable to respond to floral inductive stimuli become competent to flower as the plant ages. Then, floral competent meristems become determined to flower by being exposed to floral inductive stimuli. Finally, floral determined meristems initiate grow and form either flowers or inflorescences (McDaniel et al., 1992)(reviewed in section 1.1). The specifics of each of these developmental processes varies greatly across species and the environment in which each species develops. In this section I review the specifics of the process of floral competence acquisition and floral induction in the model *A. thaliana* and other species in response to different flowering stimuli.

1.2.1 Acquisition of Competence

The acquisition of floral competence is the first developmental transition required to initiate flowering (McDaniel et al., 1992). Most species, either annual or perennial, go through a juvenile phase during their development in which meristems produce only vegetative structures (usually with distinctive characteristics such as thorns, trichome distribution, unique phyllotaxy and leaf shape) and are florally incompetent (Poethig, 1990). The juvenile phase may last from days or weeks in most herbaceous species to several years in most woody species (Poethig, 1990). The principal factor associated to the juvenile-to-adult transition is the developmental age of the plant (Lawson and Poethig, 1995). The specifics of the mechanisms regulating the juvenile-to-adult transition have not been studied as extensively as other phase transitions (Albani and Coupland, 2010; Poethig, 2003). However, the mechanism regulating the juvenile-to-adult transition, and thus the acquisition of floral competence, seems to contain 2 sub-processes: (1) a check process for the developmental age of the plan that initiates or holds the transition to the adult phase, and (2) a developmental program that induces changes in the morphology and physiology of new organs formed

in the adult phase; the latter program includes the acquisition of floral competence in shoot meristems.

The check process for the developmental age of the plant could be controlled by spatial and temporal signals (Brunner and Nilsson, 2004; Day et al., 2002; Lawson and Poethig, 1995). Support for the involvement of a spatial signal comes from works in which plant size rather than age has been correlated with the juvenile-to-adult transition (Greenwood et al., 2010; Longman and Wareing, 1959; Olivera and Browning, 1993). According to this hypothesis, juvenility is maintained by a signal produced in the roots (Greenwood et al., 2010; McDaniel, 1980; Olivera and Browning, 1993; Schwabe and Al-Doori, 1973), then, as the plant grows, the distance between the root and shoot tips increases and the activity of the juvenility signal from roots decreases in distal meristems promoting the transition to the adult phase (Brunner and Nilsson, 2004; Day et al., 2002; Greenwood et al., 2010). This hypotheses, however, is challenged by other works in which the juvenile-to-adult transition is not affected by plant size but by plant age (Lawson and Poethig, 1995; Telfer et al., 1997). Two obstacles for determining the exact mechanism for keeping track of developmental age are (1) confounding effects among processes affected by both plant size and age (Lawson and Poethig, 1995) and (2) the lack of a reliable juvenile-to-adult transition marker other than reproductive competence (Jones, 1999). Still, evidence supports the hypothesis of single central mechanism that keeps track of the developmental age of the plant (Martínez-Zapater et al., 1995; Ratcliffe et al., 1998). For instance, genetic manipulation of genes regulating flowering time in *Arabidopsis* also alter other phase transitions (Ratcliffe et al., 1998; Steynen et al., 2001; Willmann and Poethig, 2011). In woody species, the effect of manipulating flowering gene expression on adult phase transition is more obvious since lengthy juvenile phases of 7-15 years are shortened to 1-2 years when flowering genes such as *AP1*, *LFY* or *FLOWERING LOCUS T* (*FT*) are over-expressed (Endo et al., 2005; Hsu et al., 2006; Peña et al., 2001). Other genes

also involved in timing the transition to the adult phase in *Arabidopsis* are *EARLY FLOWERING1*, *HASTY*, *ZIPPY*, and *SQUINT* (Berardini et al., 2001; Hunter et al., 2003; Scott et al., 1999; Telfer and Poethig, 1998); mutants lacking these genes develop with a shorter juvenile phase compare to wild-type plants. However, even though the juvenile phase in mutants lacking *ZIPPY* is shortened and adult vegetative traits are expressed, floral competence is not immediately acquired, indicating that both processes could be independent (Hunter et al., 2003).

On the other hand, several factors affecting the actual onset of the adult phase have been identified. In *A. thaliana* and maize, transition to the adult phase is controlled by the expression of microRNAs (miRNAs) (Chuck et al., 2007; Peragine et al., 2004; Wu and Poethig, 2006). The signal triggering the transition to the adult phase in *A. thaliana* and maize is the inactivation of miRNA *miR156* (Chuck et al., 2007; Wu et al., 2009; Wu and Poethig, 2006). Expression of *miR156* occurs in leaf primordia and maintains juvenile traits in the developing leaf (Yang et al., 2011). Maintenance of juvenile traits by *miR156* occurs by repression of members of the SBP/SBL family of transcription factors (Chuck et al., 2007; Gandikota et al., 2007; Schwab et al., 2005; Schwarz et al., 2008; Wu and Poethig, 2006). Some members of the SBP/SPL family of transcription factors regulate the expression of several flowering genes such as *AP1* and *LFY* (Wang et al., 2009; Yamaguchi et al., 2009); thus, floral competence could be regulated by this mechanism.

1.2.2 Floral Induction

Floral induction is the process by which florally competent meristems become determined to flower (McDaniel et al., 1992). Floral induction occurs when competent meristems are exposed to stimuli that initiate the development of inflorescences and flowers (Araki, 2001). The specific stimuli inducing flowering vary depending on the species, and are usually signals of developmental and environmental conditions favoring reproductive success (Putterill et al., 2004). In model plants, the most

extensively studied floral promoting stimuli are changes in photoperiod, vernalization, phytohormones and developmental age (Amasino, 2010; Komeda, 2004). Some components of the molecular mechanisms sensing and transmitting flowering signals in *Arabidopsis* seem also to be conserved, at least partially, in other species (Benlloch et al., 2007; Sablowski, 2007).

Photoperiodic induction of flowering occurs either by extending or shortening day-lengths. Long-day plants (also called short-night plants) flower as the day-length increases whereas short-day plants flower as the night length increases (Amasino, 2010). The mechanisms coupling day-length sensing and floral initiation seem to be similar in both long-day and short-day species (Hayama and Coupland, 2004; Turck et al., 2008). In long-day *Arabidopsis*, increasing day-length is sensed by *CONSTANS* (*CO*) whose expression is controlled by the circadian clock and peaks between 16h and dusk (Suarez-Lopez et al., 2001). CO protein is targeted for degradation by the proteasome under dark conditions (Valverde et al., 2004), so CO is only stable when the day-length is long enough for light to stabilize CO (Hayama and Coupland, 2004; Yanovsky and Kay, 2002). CO acts as a transcription factor for four other genes. Two of these genes, *SUPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *FT* are major integrators of flowering signals (Samach et al., 2000). CO triggers the expression of *FT* in phloem of leaves (An et al., 2004; Mathieu et al., 2007; Takada and Goto, 2003). Then, FT is transported to the shoot meristem through the phloem (Corbesier et al., 2007). In the shoot meristem FT forms a complex with the transcription factor FD (Abe et al., 2005; Wigge et al., 2005) and activates the expression of *AP1*, *LFY* and *SOC1* (Abe et al., 2005; Michaels et al., 2005; Wigge et al., 2005; Yoo et al., 2005). In short day rice, flowering is induced by a similar but reversed photoperiod sensing mechanism (Hayama and Coupland, 2004; Turck et al., 2008). The main difference is that the product of the short-day rice ortholog of *CO* (*Hd1*) not only induces the expression of the *FT* ortholog (*Hd3a*) under inductive short days, but also represses

the expression of *Hd3a* under non-inductive long-days (Kojima et al., 2002; Turck et al., 2008; Yano et al., 2000).

In temperate climates many species require prolonged exposure to cold temperatures to initiate flowering, a process known as vernalization (Kim et al., 2009). In contrast to the effect of changes in photoperiod, vernalization *enables* rather than *induces* flowering (Boss et al., 2004). In *Arabidopsis*, the vernalization response is mostly controlled by the expression of *FLOWERING LOCUS C* (*FLC*) and other members of the *FLC* clade induced by the dominant allele of *FRIGIDA* (*FRI*) (Michaels and Amasino, 1999; Ratcliffe et al., 2003; Scortecci et al., 2001). *FLC* directly represses the expression of floral promoters *FT*, *FD* and *SOC1* and thus block floral initiation (Helliwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006). In turn, the expression of *FLC* is controlled epigenetically by the expression of *VERNALIZATION1* (*VRN1*), *VERNALIZATION2* (*VRN2*) and *VERNALIZATION INSENSITIVE3* (*VIN3*) through histone modifications (Gendall et al., 2001; Levy et al., 2002; Sung and Amasino, 2004). Once the vernalization requirement is met, the expression level of *FLC* becomes and remains low and the plant becomes sensitive to floral inductive signals (Lee et al., 2000; Samach et al., 2000). Interestingly, whereas components of the mechanism for photoperiod-induced flowering are at least partially conserved in many other plant species, the components of the mechanism enabling flowering by vernalization in *Arabidopsis* are not, supporting the hypothesis of vernalization requirements having evolved later than photoperiod-induced flowering (Kim et al., 2009).

In *Arabidopsis*, *FLC* is also repressed (and thus flowering is enabled) by several genes known as *autonomous-pathway* genes (Amasino, 2010). Mutants lacking *autonomous-pathway* genes have delayed flowering and confer a vernalization requirement even in the absence of a dominant *FRI* allele (Michaels and Amasino, 2001). Despite their name, *autonomous-pathway* genes do not appear to belong to a formal *pathway* with a defined topology, but instead they are a set of genes generally

involved in post-transcriptional control of gene expression through several mechanisms (Bäurle and Dean, 2008; He et al., 2003; Macknight et al., 1997; Noh et al., 2004; Schomburg et al., 2001; Wang et al., 2007). Further, most *autonomous-pathway* genes are not exclusively involved in *FLC* repression and flowering control but also in other developmental processes (Veley and Michaels, 2008). The role of *autonomous-pathway* genes in enabling flowering seems to be to maintain *FLC* expression at basal levels (Amasino, 2010).

Other factors either promoting or enabling flowering in *Arabidopsis* are gibberellins (Blazquez and Weigel, 1999; Wilson et al., 1992), non-vernalizing temperatures (i.e. $> 6°C$) (Balasubramanian et al., 2006; Blázquez et al., 2003; Kim et al., 2004) light quality (Halliday et al., 2003) and salinity (Kim et al., 2007). The mechanisms by which the factors just listed regulate flowering time have not been described as thoroughly as those mentioned in the previous paragraphs. However, a common effect of the factors listed at the beginning of this paragraph is the regulation of *FT* either directly or by repression of *FLC*. This indicates that regardless of the triggering stimulus, flowering signals eventually converge to a set of integrator genes that ultimately up-regulate floral identity genes (Araki, 2001).

1.3 Floral Induction in citrus

Citrus trees grown from seed become florally competent only after completing a juvenile phase that may last from 5 to 13 years (Davies and Albrigo, 1994). Then, once the juvenile phase is past, citrus trees flower either continuously or seasonally depending on cultivars and environmental conditions. Only two environmental factors are known to induce flowering in citrus: low ambient temperature (Moss, 1969) and water deficit (Cassin et al., 1969). As with many other perennial species, the specific mechanisms that regulate flowering in citrus has not been identified. However, many components of the mechanisms regulating flowering in model plants (primarily *Arabidopsis*) seem to be conserved in citrus species. In this section, I review the effects of

internal and environmental factors known to affect flowering in citrus, then, I present the putative citrus orthologs of *Arabidopsis* flowering genes.

1.3.1 Factors Regulating Floral Induction in Citrus

Low temperatures and water deficit are the only two factors known to induce flowering in citrus (Cassin et al., 1969). Other factors such as gibberellins, crop load or changes in nitrogen metabolism are also involved in regulating floral induction, but do not properly *induce* flowering; these factors only modify the characteristics of the induced bloom (Krajewsky and Rabe, 1995). The intensity of floral induction in citrus can be inferred from the characteristics of the induced bloom. The two main characteristics of the citrus bloom related to the intensity of floral induction are: the number of inflorescences formed and the type of inflorescence formed (i.e. leafless, leaf abundant and leaf deficient inflorescences) (Moss, 1969; Sauer, 1954).

In general, the intensity of floral induction due to low temperatures and water deficit in citrus depends on both the intensity and time of exposure to these stimuli (Cassin et al., 1969; Moss, 1969; Southwick and Davenport, 1986). Floral induction occurs at temperatures between 5 and 20 °C, with the strongest induction occurring between 10 and 15 °C (García-Luis et al., 1992; Moss, 1969; Valiente and Albrigo, 2004). The exact range of levels of water deficit inducing flowering has not been precisely defined, however, moderate water deficits are more effective in inducing flowering without inducing undesirable leaf loss (Cassin et al., 1969; Southwick and Davenport, 1986). On the other hand, time of exposure to inductive stimuli is apparently additive to the intensity of the floral inductive stimuli (Chica, 2007). Both, low temperatures and water deficit, can induce flowering after exposures of 2 weeks, then the response peaks after 8-9 weeks (Cassin et al., 1969; Chica, 2007; Moss, 1969; Southwick and Davenport, 1986).

Although gibberellins, crop load and changes in nitrogen metabolism regulate the intensity of floral induction in citrus without actually initiating it, application of gibberellins

(Cooper and Peynado, 1958; García-Luis et al., 1986; Monselise et al., 1964) and heavy crops loads (Goldschmidt and Golomb, 1982; Moss, 1971; Valiente and Albrigo, 2004) reduce the level of floral induction whereas applications of nitrogen (in the form of urea) can increase the level of floral induction (Albrigo, 1999; Ali and Lovatt, 1994). It has been proposed that reduced carbohydrate availability or increased gibberellin levels could control the negative effect of crop load on floral induction (Goldschmidt and Golomb, 1982; Koshita et al., 1999). On the other hand, the higher levels of induction after application of foliar urea have been associated with increased concentration of polyamines (Ali and Lovatt, 1995; Lovatt et al., 1992, 1988), which, in other species, have been shown to promote flowering (Havelange et al., 1996; Huang et al., 2004; Wada et al., 1994).

1.3.2 Citrus Orthologs of *Arabidopsis* Flowering Genes

Several (putative) orthologs of *Arabidopsis* flowering-related genes have been identified and characterized in citrus (Endo et al., 2005; Nishikawa et al., 2010, 2009, 2007; Pillitteri et al., 2004a,b; Tan and Swain, 2007). In general, these genes (Table 1-1) show high sequence similarity at the aminoacid level (>60%) with their *Arabidopsis* counterparts, their patterns of expression support their hypothetical involvement in the flowering process in citrus, and they complement the mutant phenotypes of *Arabidopsis* mutants lacking their respective ortholog (Kobayashi et al., 1999; Pillitteri et al., 2004a,b; Tan and Swain, 2007). Also, overexpression of some of these flowering-related genes from citrus or *Arabidopsis* apparently reduce the length of the juvenile phase and promote early flowering in citrus (Endo et al., 2005; Peña et al., 2001). In addition, many of these genes (plus some others) have also been isolated and characterized in a natural early-flowering mutant of a citrus close relative, *Poncirus trifoliata*, and the patterns of expression of these genes in this mutant support their involvement in regulating the flowering process (Li et al., 2010; Zhang et al., 2011, 2008, 2009a,b). However, even though the above evidence supports the hypothesis

of citrus flowering-related genes orthologous to those in *Arabidopsis* being involved in regulating the floral induction, this evidence is insufficient to support the conservation of mechanisms regulating the expression of these genes. In fact, the type of floral inductive stimuli and the time of floral induction support the hypothesis of different mechanisms regulating the expression of flowering genes in citrus and *Arabidopsis*.

1.4 Hypothetical Model for the Transcriptional Regulation of Floral Induction in citrus

Even though several putative orthologs of *Arabidopsis* flowering genes have been identified in citrus, the molecular mechanism that control flowering in both species are likely to be different. Flowering in *Arabidopsis* (and other model species) is induced by changes in photoperiod(Turck et al., 2008) but photoperiod does not seem to influence flowering in citrus (Moss, 1969). Also, exposure to low temperatures enables flowering in *Arabidopsis* through vernalization without properly inducing it (plants either flower or do not) (Kim et al., 2009) whereas in citrus, low temperatures directly induce flowering (trees respond to levels of low temperatures and length of induction quantitatively) (Moss, 1969). Furthermore, in citrus, like in several other perennial species (Albani and Coupland, 2010), gibberellins have a negative effect on floral induction whereas in *Arabidopsis* gibberellins promote flowering under short days (Monselise et al., 1964; Wilson et al., 1992). Nonetheless, the expression patterns of the citrus orthologs of *Arabidopsis* flowering genes (Endo et al., 2005; Nishikawa et al., 2010, 2009, 2007; Pillitteri et al., 2004a,b; Tan and Swain, 2007), the complementation of *Arabidopsis* mutants by inserted citrus flowering genes (Nishikawa et al., 2007; Pillitteri et al., 2004a; Tan and Swain, 2007), and accelerated flowering in citrus when either citrus or *Arabidopsis* flowering genes are over-expressed (Endo et al., 2005; Kobayashi et al., 1999; Nishikawa et al., 2007; Peña et al., 2001; Pillitteri et al., 2004a; Tan and Swain, 2007). This indicates that the individual function of these genes is at least partially conserved in both species. In this section I present an *hypothetical* model (Fig. 1-1)

to explain the transcriptional regulation of flowering in citrus. The model relies on the assumptions of (1) functional orthology between *Arabidopsis* and citrus genes and (2) citrus evolution of regulatory sequences of flowering-related genes that respond to signals generated by low temperature and water deficit.

Flowering signals must be initiated by low temperature and/or low plant water status sensing mechanisms because the only two factors known to induce owering in citrus are low temperatures and water deficit. The signaling pathway initiated by low temperatures could be more specialized to induce flowering than the signaling pathway initiated by water deficit since low temperatures induce flowering more intensely than water deficits (Cassin et al., 1969). Signals initiated by floral-inductive low temperatures eventually activate factors that up-regulate *CsFT* in leaves and stems (Nishikawa et al., 2007). *CsFT* could also be upregulated by signals initiated by water stress, but there is no published evidence supporting this hypothesis.

Signals from either low temperature or water deficit could also be integrated by *CsSL1*, the citrus ortholog of *Arabidopsis*'s *SOC1*. In *Arabidopsis*, *SOC1* is a key integrator of flowering signals from different regulatory pathways (Lee and Lee, 2010). Ectopic expression of *CsSL1* in *Arabidopsis soc* mutants causes early flowering (Tan and Swain, 2007), indicating that *CsSL1* is functionally conserved in both species. However, this is the only evidence supporting a role for *CsSL1* as an integrator of flowering signals in citrus. If *CsSL1* were an integrator of flowering signals from different pathways in *Citrus* as it is in *Arabidopsis*, its expression would likely increase when trees are exposed to inductive low temperatures or water deficit.

If it is assumed that *CsFT* and *CsSL1* are integrators of flowering signals, the increased expression of *CsFT* and *CsSL1* should initiate the expression of floral identity genes *CsAP1* and *CsLFY*. However, expression of *CsAP1* and *CsLFY* is not initiated until the onset of growth-promoting warmer temperatures and non-limiting water availability (Pillitteri et al., 2004a) indicating that activation of *CsAP1* and *CsLFY*

depends also on environmental signals opposite to those that regulate the expression of *CsFT* and *CsSL1*. Ultimately, *CsAP1* and *CsLFY* initiate floral organ organogenesis in shoot meristems. However, citrus blooms are not composed of only single flowers. Instead, citrus blooms are usually a mixture of single flowers, leafless cymes, cymes with varying leaf/flower ratios and also vegetative shoots. The type of new growth formed after floral induction is related to both the intensity of the inductive stimuli and the duration of floral induction (Moss, 1969). Thus, the type of new growth formed after floral induction could be determined in each bud by a balance between factors conferring floral identity and factors conferring vegetative identity. In the model proposed, the factor conferring floral identity is the combined expression of *CsLFY* and *CsAP1*, whereas vegetative identity is conferred by the expression of *CsTFL1*. This hypothesis is supported by the patterns of expression of *CsTFL1* in adult citrus trees after floral induction (Pillitteri et al., 2004a) and the function of the *TFL1* from *Arabidopsis* as a regulator of inflorescence architecture and developmental phase transitions (Conti and Bradley, 2007; Ratcliffe et al., 1998).

The model proposed in Figure 1-1 accounts for the control of floral or inflorescence initiation at the meristem level. However, besides floral/inflorescence initiation at the meristem level, citrus also show responses to floral induction at the shoot level. The shoot level response to floral induction in citrus is twofold: (1) An basipetal gradient of floral intensity (as reported by the type of inflorescence formed) is established in shoots (Sauer, 1954; Valiente and Albrigo, 2004), and (2) multiple flower/inflorescence cohorts are initiated when trees are exposed to intermittent floral induction (Simanton, 1969; Valiente and Albrigo, 2003). Thus, a mechanism should exist for the distribution of flowering signals among meristems on the same shoot so that differential flowering can be expressed. This mechanism is hypothesized to be activated in meristems at more basal positions of the shoot as either meristems in more apical position reach a hypothesized maximal level of induction or during intermittent floral induction.

Table 1-1. Citrus and *Poncirus trifoliata* orthologs of *Arabidopsis* flowering genes

Citrus/*P. trifoliata*	*Arabidopsis*	Function in *Arabidopsis*	References
CiFT	*FT*	Floral signal integrator	Endo et al. (2005); Kobayashi et al. (1999); Matsuda et al. (2009); Nishikawa et al. (2010, 2009, 2007)
CsAP1	*AP1*	Floral identity	Nishikawa et al. (2009, 2007); Peña et al. (2001); Pillitteri et al. (2004a,b)
CsLFY	*LFY*	Floral identity	Nishikawa et al. (2009, 2007); Pillitteri et al. (2004a,b)
CsTFL1	*TFL1*	Floral repressor	Nishikawa et al. (2009, 2007); Pillitteri et al. (2004a,b)
CsWUS	*WUS*	Meristem identity	Tan and Swain (2007)
CsSL1	*SOC1*	Floral signal integrator	Tan and Swain (2007)
CsAp3	*AP3*	Floral homeosis	Tan and Swain (2007)
CuSEP1	*SEP1*	Floral homeosis	Nishikawa et al. (2010)
CuSEP3	*SEP3*	Floral homeosis	Nishikawa et al. (2010)
CuFUL	*FUL*	Floral homeosis	Nishikawa et al. (2010)
PtFT	*FT*	Floral signal integrator	Zhang et al. (2009b)
PtTFL	*TFL1*	Floral repressor	Zhang et al. (2009b)
PtFLC	*FLC*	Floral repressor	Zhang et al. (2009a)
PtSVP	*SVP*	Floral repressor	Li et al. (2010)

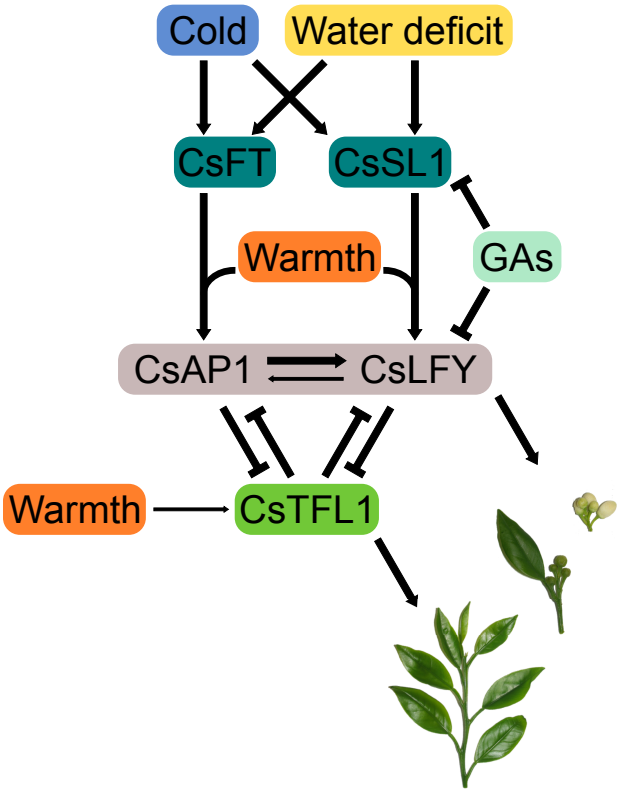


Figure 1-1. Hypothetical model for the transcriptional regulation of floral induction in citrus. Floral inductive signals initiated by the exposure to cold and water deficit are integrated by *CsFT* and *CsSL1*. CsFT and CsSL1 initiate transcription of *CsAP1* and *CsLFY*. Up-regulation of *CsAP1* and *CsLFY* initiates floral organogenesis at growth promoting temperatures and non-limiting water suppply. The type of inflorescence formed depends on the balance between the expression of *CsTFL1* (vegetative character) and floral identity genes (floral character). Arrowheads in lines indicate promotion whereas flat ends indicate inhibition.

CHAPTER 2
EXPRESSION PATTERNS OF FLOWERING GENES IN SWEET ORANGE IN RESPONSE TO FLORAL-INDUCTIVE WATER DEFICITS

Cool ambient temperatures ($<$20 °C) and water deficit are the only factors known to induce flowering in sweet orange (Cassin et al., 1969; Moss, 1969). In recent years, several genes that hypothetically regulate flowering in citrus species have been identified based on their similarity to flowering related genes in the model plant *Arabidopsis* (Nishikawa et al., 2007; Pillitteri et al., 2004a,b; Tan and Swain, 2007). Although changes in transcript levels of these genes have been characterized in response to floral inductive temperatures (Nishikawa et al., 2009, 2007; Pillitteri et al., 2004a), nothing is known about their pattern of expression in response to floral-inductive water deficits. Floral-inductive water deficits are the only source of floral induction of citrus trees growing in regions with tropical climates (Cassin et al., 1969) and an important source of floral induction in regions with humid subtropical climates where they can complement floral-inductive cool temperatures during the Fall and Winter (Albrigo et al., 2006b; Chica, 2007). Water deficit is also the primary source of floral induction for many other species growing in tropical and subtropical climates (Albrigo and Galen-Saúco, 2004).

In this study I investigated the transcript accumulation of citrus flowering genes in response to water deficit. I hypothesized that citrus' *FLOWERING LOCUS T* (*CsFT*) and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*CsSL1*) transcripts accumulate in response to flowering signals initiated by floral-inductive water deficits. *CsFT* is the putative citrus ortholog of *Arabidopsis*'s *FLOWERING LOCUS T* (*FT*) (Kobayashi et al., 1999; Nishikawa et al., 2007). In *Arabidopsis*, the protein encoded by *FT* is a mobile flowering signal originating in leaves in response to floral-inductive photoperiods and transported to the shoot apical meristem where it up-regulates the expression of floral identity genes (Abe et al., 2005; Corbesier et al., 2007; Samach et al., 2000). In *Citrus unshiu*, the expression patterns of the putative *FT* ortholog

(*CiFT*) support the hypothesis of this gene being involved in the regulation of flowering in citrus (Nishikawa et al., 2009, 2007). In addition, constitutive expression of *CiFT* in citrus' close relative *Poncirus trifoliata* resulted in extremely early flowering which provides more support for a role of citrus' *FT* orthologs as regulators of flowering time (Endo et al., 2005). *CsSL1* is the putative ortholog of *Arabidopsis*' *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Tan and Swain, 2007). In *Arabidopsis*, *SOC1* is a key integrator of flowering signals initiated by multiple stimuli (Lee and Lee, 2010). The expression patterns of *CsSL1* in citrus in response to floral inductive stimuli have not been described. However, introducing *CsSL1* in *Arabidopsis soc1* mutants induced early flowering compared to the wildtype and the late-flowering *soc1* mutant (Tan and Swain, 2007), supporting a role for *CsSL1* in the regulation of flowering.

I also investigated whether the pattern of floral identity genes (*CsAP1* and *CsLFY*) transcript accumulation in trees exposed to floral-inductive water deficit induction was similar to the pattern of transcript accumulation of these in trees exposed to floral-inductive cool temperatures. In *Arabidopsis*, up-regulation of *AP1* and *LFY* expression promotes the initiation of floral organs (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Orthologs of *AP1* and *LFY* had been isolated in *C. sinensis* (Pillitteri et al., 2004b) and overexpression of these genes in *C. unshiu* resulted in accelerated flowering, suggesting a role of these in genes in the regulation of flowering in citrus. In *C. sinensis* trees exposed to floral induction by low temperatures, transcript accumulation of *CsAP1* and *CsLFY* remain unchanged from initial levels until the floral-inductive treatment was over and trees were transferred to growth promoting conditions when levels of *CsAP1* and *CsLFY* transcripts increased (Pillitteri et al., 2004a). I hypothesized that a similar pattern of accumulation of *CsAP1* and *CsLFY* transcripts would be induced by exposure to floral-inductive water deficit.

This current work presents evidence that supports a role of *CsFT* as an universal integrator of flowering signals in citrus. In addition to up-regulation of *CsFT*, which is

assumed to promote flowering, water deficit also reduces the sensitivity of buds to other environmental signals promoting flower bud differentiation, which in turn could induce a stronger flowering response if floral induction is continued. These results represent one of the earliest reports characterizing the effects of water-deficit on the expression of flowering genes.

2.1 Materials and Methods

2.1.1 Plant Material

Field experiments were conducted using mature 'Valencia' sweet orange trees grafted on 'Carrizo' citrange in an orchard at the University of Florida's Citrus Research and Education Center in Lake Alfred, Florida (28°5'N, 81°43'W) during 2009 and 2010. The orchard received similar horticultural care as in neighboring commercial groves throughout the experiments. Experiments under controlled environments were conducted using either 2-3 year old potted 'Valencia' trees grafted on 'Swingle' citrumelo or 2-3 year old potted 'Washington Navel' cuttings. All the trees used for experiments were tested for floral competence and were maintained in a shaded greenhouse with natural photoperiods, non-limiting irrigation and standard fertilization when not in use for experiments. The growth rooms in which the controlled conditions experiments were conducted were illuminated with with fluorescent lights (800μmoles$\cdot$m$^{-2}\cdot$s^{-1} at canopy level) with a 11/13h (day/night) photoperiod.

2.1.2 Experimental Conditions

To determine the patterns of *CsFT*, *CsSL1*, *CsAP1* and *CsLFY* transcript accumulation during floral-inductive water deficits, transcript levels of these genes were measured in potted trees kept under water deficit for 60 days in a growth room at 23°C. Water deficit was imposed by withholding irrigation until the desired levels of water deficit was reached; then, the desired level of water deficit was maintained by irrigating the trees daily with a volume of water that matched the daily weight loss of the tree. The water status of the trees was estimated and monitored using the midday stem

water potential (SWP) measured by the pressure chamber method (McCutchan and Shackel, 1992; Scholander et al., 1965). Midday SWP in trees under water deficit was -2.0±0.12MPa whereas midday SWP in well irrigated (control) trees was -1.1±0.1MPa. The desired level of water deficit (-2.0MPa) was reached between day 15 and 20 since the beginning of the experiment. On the day 60 of the experiments, water deficit was interrupted by irrigating the trees until soil saturation to promote growth; irrigation the continued as in the well irrigated control trees. Well irrigated controls were irrigated until soil saturation every 3 days throughout the experiment. Samples were collected every 10-12 days from day 0 until day 74. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of the selected genes between well irrigated and water deficit trees were analyzed using a repeated measurements model. Differences in accumulation of *CsSL1*, *CsAP1* and *CsLFY* transcripts after re-irrigation (day 63 and 74) between trees that had received normal irrigation or water deficit were analyzed using t-test. New growth composition was characterized in all the shoots (6-7 nodes long) formed during the previous year present on the trees. Differences in the composition of the new growth between well irrigated and water deficit trees were analyzed using t-test.

To determine the patterns of *CsFT*, *CsSL1*, *CsAP1* and *CsLFY* transcript accumulation during floral-inductive water deficit at floral-inductive temperatures, transcript levels of these genes were measured in potted trees kept under water deficit for 40 days in a growth room at 12°C. The trees had been kept in a growth room at 23°Cfor about 1 month before transfer to the room at 12°C. Water deficit was imposed and monitored as described before starting 7 7 days before the transfer to the room at 12°C. Well irrigated control trees received irrigation also as indicated before. On day the 40 after the transfer to the room at 12°C, the trees were transferred back to the room at 23°C and the water deficit was interrupted as described before. Samples were collected every 9-10 days from day 0 until day 39 and 3 days after the end of the water

deficit/low temperature treatment. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of the selected genes between well irrigated and water deficit trees were analyzed using a repeated measurements model. Differences in accumulation of *CsSL1*, *CsAP1* and *CsLFY* transcripts after re-irrigation and transfer to 23°C (day 43) between trees that had received normal irrigation or water deficit were analyzed using t-test. New growth composition was characterized in all the shoots (6-7 nodes long) formed during the previous year present on the trees. Differences in the composition of the new growth between well irrigated and water deficit trees were analyzed using t-test.

To determine the patterns of *CsFT*, *CsSL1*, *CsAP1* and *CsLFY* transcript accumulation in mature trees exposed to floral inductive conditions in the field, transcript levels of these genes were measured in mature trees growing in the field under water deficit and normal irrigation during the fall/winter of 2009-2010 and the summer of 2010. Water deficit was induced by completely withholding irrigation for the duration of the experiment and covering the ground beneath the canopy of the trees with a sheet of water-proof material (Tyvek®, DuPont). The water status of the trees was estimated and monitored as indicated before. In the fall/winter experiment, trees were exposed to the water deficit treatment and naturally occurring floral-inductive temperatures from mid-November until late-January. In the summer experiment, trees were exposed to water deficit from June to August. In both experiments, another set of trees received irrigation as in neighboring commercial groves. At the end of both experiments, the sheets of water-proof material were removed and the trees were irrigated overnight for 3 days; then, irrigation continued as in control trees. Samples were collected every 7-10 days for the duration of the experiments. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of the selected genes between well irrigated and water deficit trees were analyzed using a repeated measurements model. Differences in accumulation of

transcripts of the selected genes at specific sampling times of interest between trees that had received normal irrigation or water deficit were analyzed using t-test. New growth composition was characterized in 25 shoots (6-7 nodes long) selected before the begining of the experiment that were formed during the previous year. The shoots selected for new growth characterization were distributed evenly between both sides of the hedgerow. Differences in the composition of the new growth between well irrigated and water deficit trees were analyzed using t-test.

In all the experiments, accumulation of *CsFT* transcripts was quantified in leaves samples whereas accumulation of *CsSL1*, *CsAP1* and *CsLFY* transcripts was quantified in bud samples. The choice of tissues in which transcripts of the selected genes were quantified was made based on the most likely spatial domain of gene expression and protein activity predicted by the hypothetical model in section 1.4. Leaf and buds samples consisted of a pool of at least 6 leaves or buds from separate shoots on each tree replicate. All samples were collected at 15H00 local standard time.

2.1.3 qRT-PCR

Total RNA was extracted using a phenol-chloroform precipitation method and purified using silica membranes with on-column DNase digestion (Qiagen). Leaf samples were used for analysis of *CsFT* expression, whereas bud samples where used for analysis of *CsSL1*, *CsAP1* and *CsLFY* expression. Five hundred nanograms of total RNA were used for cDNA synthesis in a 20μl reaction with oligo dT primers (SuperScriptIII®, Invitrogen). One microliter of the synthesized cDNA was used for two-step (95°C denaturation and 60°C for 1 minute annealing and extension) qPCR in a 20μl reaction (SYBR® Premix ExTaq™II, Takara) on a Applied Biosystems 7500 FAST real-time PCR system (Life Technologies) using optimized qPCR assays (see Appendix). Primers for qPCR were: 5'-CGGCGGAAGGACTATGAC-3' and 5'-TGTGAGAAAGCCAGAGAGGAA-3' (*CsFT*), 5'-CAGCCAGAGAATCTAACAAACG-3' and 5'-TCAGTTTTGTGGTGGTATTGCC-3' (*CsSL1*), 5'-CCCTGGAGTGCAACAACCT-3'

and 5'-CTGATGTGTTTGAGAGCGGT-3' (*CsAP1*), and 5'-TCTTGATCCAGGTCC-AGAACATC-3' and 5'-TAGTCACCTTGGTTGGGCATT-3' (*CsLFY*). *CsGAPDH* was used as reference gene (5'-GGAAGGTCAAGATCGCAATCAA-3' and 5'-CGTCCCT-CTGCAAGATGACTCT-3'). All qPCR assays were validated for specific amplification and optimized for amplification efficiencies between 1.88 and 2.05 with a linear dynamic range of 6 log10 cycles. The sequence of the primers to amplify *CsLFY* was obtained from Nishikawa et al. (2009) whereas all other primer sequences were designed in-house. Relative gene expression was calculated as a fold change ratio using Pfaffl's method (Pfaffl, 2001) with sliding-window efficiencies calculated for each reaction using the `sliwin` function in the `qpcR` R package (Ritz and Spiess, 2008).

2.1.4 Data Analysis

Mean fold change of transcript levels were transformed to a logarithmic scale ($\log_2$) for statistical analysis but data in the graphs represents the untransformed data. Unless noted otherwise, all differences reported are statistically significant ($p<0.05$). All statistical analyses were executed in R (R Development Core Team, 2011).

2.2 Results and Discussion

2.2.1 Floral-inductive Water Deficit Up-regulates *CsFT* but not *CsSL1*.

To test the hypothesis that the floral signal integrator function of *CsFT* and *CsSL1* is conserved in citrus and *Arabidopsis*, I subjected a group of trees to a moderate water deficit for 60 days at 23 °C and sampled leaves and buds every ten days to measure the expression of *CsFT* and *CsSL1*. If either *CsFT* or *CsSL1* were integrators of signals generated by water deficit, their expression would change while the trees remain under water deficit. I assumed that *CsFT* and *CsSL1* are active components of the genetic mechanism regulating flowering in citrus based on *Arabidopsis* mutant complementation studies and experiments with the citrus close relative *P. trifoliata* overexpressing *CiFT* (equivalent to *CsFT*) (Endo et al., 2005; Tan and Swain, 2007).

Figure 2-1 shows that prolonged exposure to water deficit up-regulates the expression of *CsFT* but has no effect on the level of expression of *CsSL1*. After re-irrigating thoroughly at the end of the experiment, trees under water deficit produced a flush of new growth consisting mostly of inflorescences of different leaf to flower ratio as opposed to almost no growth initiated in well-irrigated control trees (Table 2-1). This result is consistent with *CsFT* acting as an integrator of flowering signals initiated by water deficit. Furthermore, this result is consistent with the hypothesis that *CsFT* is an universal integrator of flowering signals in *C. sinensis* since water deficit and low temperatures are the only stimuli known to be floral inductive in *C. sinensis* and up-regulation of citrus *FT* orthologs has been reported in response to low temperatures elsewhere (Nishikawa et al., 2007). However, the lack of an effect of water deficit on the levels of expression of *CsSL1* indicates that *CsSL1* is not a central integrator of flowering signals as opposed to its *Arabidopsis*' ortholog *SOC1* (Lee and Lee, 2010).

In *Arabidopsis*, the protein of *FT* is a mobile flowering signal generated in leaves in response to floral-inductive photoperiods and transported through the phloem to the shoot apical meristem (Corbesier et al., 2007). In the shoot apical meristem FT complexes with FD, a bZIP transcription factor expressed in the meristem (Abe et al., 2005) and activates the transcription of *SOC1* and the floral identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Abe et al., 2005; Wigge et al., 2005; Yoo et al., 2005). *SOC1* is directly regulated by the product of *FT* (Moon et al., 2005; Yoo et al., 2005) and high levels of *FT* mRNA are quickly followed by high levels of *SOC1* mRNA (Yoo et al., 2005). In my experiments, increased transcript levels of *CsFT* did not correspond to increased levels of *CsSL1* during floral induction; *CsSL1* expression only increased slightly after the trees were re-irrigated; at this time, expression of *CsFT* decreased to control levels. Thus, it is possible that in *C. sinensis*, contrary to what occurs in *Arabidopsis*, *CsSL1* is not a target for the product of *CsFT* or that another signal generated by water deficit inhibits the expression of *CsSL1* downstream of *CsFT*.

2.2.2 *CsFT* Transcript Accumulation also Increases in Trees under Water Deficit at Floral-inductive Temperatures

Since both water deficit and floral-inductive temperatures increase the expression of *CsFT* (this work and Nishikawa et al. (2007)), *CsFT* can be a universal integrator of flowering-promoting signals candidate in *C. sinensis*. It has been reported that when floral-inductive temperatures and water deficit are applied simultaneously, more inflorescences are formed than when either stimulus occurs separately (Chica, 2007). This experiment tested the hypothesis that the increase in inflorescence numbers produced by the simultaneous exposure of *C. sinensis* trees to floral-inductive temperatures and water deficit is related to a similar increase in the expression of *CsFT*. The pattern of expression of *CsFT* under water deficit at floral-inductive temperatures (15°C) was compared to that of trees receiving normal irrigation also at floral-inductive temperature of 15°C.

Figure 2-2 shows that the expression of *CsFT* in trees under water deficit at floral-inductive temperatures was markedly higher than that in well-irrigated trees at the same temperature, supporting the hypothesis of *CsFT* being an universal integrator of flowering signals and a factor determining the increase in inflorescence number reported in my previous work (Chica, 2007) and replicated in this project (Table 2-2). The increase in the level of expression of *CsFT* (relative to initial levels) was higher than the increase obtained by floral-inductive temperature and water deficit treatments separately (Figures 2-1 and 2-2). Unfortunately, whether the effects of plant water status and temperature are additive or interact was not investigated due to time constraints and technical difficulties related to applying and defining levels of water stress, particularly when holding plants at different temperatures. Accumulation of *CsSL1* transcripts in trees under water deficit remain at or below initial levels and increased only after the trees were re-irrigated and transferred to the room at growth promoting temperatures

(23°C). However, accumulation of *CsSL1* transcripts increased in well irrigated trees at 12°C.

These results support the hypothesis that *CsFT* can be a universal integrator of flowering signals in *C. sinensis*. Chica (2007); Moss (1969); Southwick and Davenport (1986) have shown that the level of induction of citrus trees (as indicated by the number of inflorescences initiated when growth is resumed) is proportional to the duration of floral-inductive treatments. The level of expression of *CsFT* is also proportional to the duration of the floral inductive treatments in Figures 2-1 and 2-2. Thus, expression of *CsFT* could be an indicator of the level of induction of the buds as it is capable of integrating signals from both floral inductive stimuli, assuming that the levels of expression of *CsFT* correspond to levels of its protein. Also, levels *CsSL1* transcripts at 12°C increased only in well irrigated trees whereas they remained at initial levels in trees under water deficit; therefore, it is possible that water deficit had negatively regulated the expression of *CsSL1* at 12°C. Then, these results indicate that the response of *CsSL1* to water deficit would be opposite to the response of its *Arabidopsis* ortholog (*SOC1*) to floral promoting stimuli.

2.2.3 Water Deficit Reduce the Transcript Accumulation of Floral Identity Genes in Buds during Floral Induction

In *Arabidopsis*, up-regulation of the floral identity genes *AP1* and *LFY* in the shoot apical meristem are early indicators of floral initiation (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995) and follow the up-regulation of *FT* under floral-inductive long days (Wigge et al., 2005). Using samples from the experiments discussed in the previous two subsections, the changes in expression of *CsAP1* and *CsLFY* during water deficit treatments were tested. Based on reports in which over-expression of *AP1* or *LFY* resulted in accelerated flowering (Peña et al., 2001) and complementation of *Arabidopsis* null mutant phenotypes by *CsAP1* and *CsLFY* (Pillitteri et al., 2004b). This experiment tests whether accumulation of *CsAP1* and *CsLFY* transcripts during

and after floral induction by water deficit is similar to the accumulation of these genes' transcripts during and after floral induction by low temperatures.

Figures 2-3 and 2-4 show that water deficit treatments consistently reduced the expression of *CsAP1* and *CsLFY* in the two experiments. Expression of *CsAP1* and *CsLFY* in trees under water deficit was between one-half to three-quarters the expression in control trees. Pillitteri et al. (2004a) reported that expression of *CsAP1* and *CsLFY* during treatments with floral-inductive temperatures remained unchanged when trees were exposed to floral-inductive temperatures and transiently increased when the trees were transferred to growth promoting temperatures. A similar response was observed in the results in Figures 2-3 and 2-4 where expression of *CsAP1* and *CsLFY* increased transiently after re-irrigation and/or transfer to growth promoting temperatures.

In *Arabidopsis*, up-regulation of *CsFT* is followed by up-regulation of floral-identity genes in the shoot meristem (Wigge et al., 2005) and the development of the inflorescence. Figures 2-1, 2-2, 2-3 and 2-4 show that in *C. sinensis*, up-regulation of *CsFT* is not followed by the up-regulation of *CsAP1* or *CsLFY*, but instead, up-regulation of *CsAP1* or *CsLFY* occurs only after the floral inductive stimuli disappear and growth promoting conditions occur. Albrigo et al. (2006a, 2002) reported that periods of warm temperatures during the winter in Florida were good predictors of subsequent bud differentiation in *C. sinensis* trees under field conditions. A rapid reduction in *CsFT* levels after the interruption of floral-inductive treatments (Figures 2-1 and 2-2) was consistent with the hypothesis that bud differentiation occurs only when floral induction is interrupted and growth promoting conditions occur, as opposed to what occurs in *Arabidopsis*, where increased *FT* is followed by floral bud differentiation even when the floral inductive stimuli is still present.

2.2.4 Other Factors Modify the Response of Flowering Genes to Floral-inductive Treatments in Field Trees

The response of *CsFT* in experiments with potted trees and controlled conditions presented in Figures 2-1 and 2-2 was not always consistent with the response observed in experiments in which field grown trees were used (Figures 2-5 and 2-6). In the first field experiment (Figures 2-5), 15 year-old 'Valencia' trees in the field were subjected to water deficit during the Winter of 2009-2010 for 75 days by withholding irrigation and using rain-excluding material under the canopy of the trees. This experiment was designed to be similar to the experiment reported in Figure 2-2. In the second experiment (Figure 2-6) another set of trees from the same grove was subjected to water deficit as before for 60 days during the Summer of 2010. This experiment was designed to be similar to the experiment in Figure 2-1. In both cases, the expression of *CsFT* increased during the course of the experiment, but the magnitude of the increase (fold change) was not as high as the one observed under controlled conditions. Furthermore, in the winter experiment, the expression of *CsFT* in trees under water deficit was not different than the expression of *CsFT* in the well-irrigated control trees, suggesting that the changes in expression of *CsFT* was due to naturally occurring floral-inductive temperatures rather than to the water deficit treatment. Nonetheless, trees under water deficit during the winter produced more inflorescences than the well-irrigated controls (Figure 2-3), indicating that the effect of water deficit in floral induction was still conserved even though no differences were detected in *CsFT* transcript levels. Expression of *CsFT* in trees under water deficit during the summer (Figure 2-6), however, was high early in the experiment and then declined but remained higher than the well-irrigated controls. After irrigation was resumed, the trees in the Summer experiment produced only a minimal flush composed of mainly vegetative shoots and a few inflorescences (data not shown).

The expression of *CsFT* in the Winter experiment (Figure 2-5), shows two periods (early-November to early-December and mid-December to mid-January) in which *CsFT* showed an increasing trend. In these periods, expression of *CsSL1* increased simultaneously with the expression of *CsFT* in the well-irrigated controls but remained unchanged in the trees under water deficit. The lack of response of *CsSL1* expression in trees under water deficit during the treatment is consistent with the results of the experiments using controlled conditions and potted trees (Figures 2-1, 2-2). The level of expression of *CsAP1* and *CsLFY* in trees under water deficit during the Winter was lower in trees under water deficit than in the control, however no statistically significant differences were detected except for *CsLFY* in the Summer experiment. Reduced expression of *CsSL1*, *CsAP1* and *CsLFY* in trees under water deficit relative to well-irrigated controls was also observed when water deficit was applied in the Summer. Expression of these genes was just slightly increased after the trees were re-irrigated. Interestingly, a marked peak in the expression of *CsAP1* and *CsLFY* in well-irrigated trees during the winter was registered at a sampling date that coincided with the end of a 4-day warm spell with average day temperatures higher than 21 °C (Dec. 16 2009). *CsAP1* showed another peak on January 13 (2010) which coincided with a warming trend after 4 days of sub-freezing temperatures. None of these peaks were detected in samples from trees that remained under water deficit.

Nebauer et al. (2006) reported that the competence of buds of 3 citrus species to respond to floral inductive stimuli changes through the seasons, and that sensitivity to floral inductive treatments is lowest during the Summer. Lower sensitivity to floral induction could explain the failure to induce flowering by exposing field grown trees to water deficit during the Summer. In the case of the Summer experiment, it is evident that signals initiated by water deficit produce an up-regulation of *CsFT*. However, since the upregulation of *CsFT* tended to decrease afterwards, even though the trees remain under water deficit, it is possible that *CsFT* is a target for other physiological processes.

On the other hand, even though levels of *CsFT* were reduced later in the experiment, these remained always higher than the levels of well-irrigated trees and still flowering was not successfully induced. If buds during the Summer are less competent to respond to floral inductive treatments, expression of *CsAP1* and *CsLFY* could be indicators of such a status. The expression of *CsAP1* and *CsLFY* did increase slightly after re-irrigation but not to the levels observed under controlled conditions (Figures 2-3 and 2-4 vs. Figures 2-5 and 2-6). Thus, it is possible that the sensitivity of the buds to floral inductive signals could be regulated by other factors in the buds.

In the Winter experiment, levels of *CsFT* expression were not as high as those observed at the beginning of the Summer experiment but were consistent with the proposed environmental regulation of *CsFT*. Results from the Winter experiment also support the hypothesis that flower bud differentiation occurs after and not during floral induction in response to growth promoting conditions. When water deficit is applied during the winter, the expression of the floral identity genes *CsAP1* and *CsLFY* remains constant and does not respond until growth promoting temperatures occur during warm periods. Since levels of *CsFT* in well irrigated trees and trees under water deficit were almost equivalent, it is possible that the increased number of inflorescences formed (Figure 2-3) could have been caused by water deficit keeping the buds dormant during warm periods. *C. sinensis* trees often produce more than one cohort of inflorescences under natural conditions in Florida (Simanton, 1969), usually these cohorts are initiated during discrete warm periods which are common of Florida's mild winters (Albrigo et al., 2006a, 2002) but growth is not apparent until the buds initiate growth in the spring. Once initiated, however, buds will develop shoots or inflorescences depending on the accumulation of inductive hours at the time of the warm period. If water deficit keeps buds from initiating differentiation, induction hours could continue to accumulate and could result in more inflorescences being formed than if water deficit is relieved.

These results show that *CsFT* is an integrator of signals initiated by water deficit and low floral-inductive temperatures in citrus. This could be one of the earliest reports of the regulation of flowering-related genes in response to water deficit. It is interesting that *CsFT* could play a major role in the regulation of flowering in *C. sinensis* since flowering in *C. sinensis*is reportedly insensitive to changes in photoperiod (Cassin et al., 1969; Moss, 1969) which is the most investigated pathway regulating *FT* expression in *Arabidopsis* (Turck et al., 2008). In addition, the complexity of the promoter region of *FT* (Adrian et al., 2010) supports the hypothesis that the regulation of *FT* could involve inputs from processes other than photoperiod and vernalization (Imaizumi and Kay, 2006). In *C. sinensis*, *CsFT* could have evolved to be responsive to environmental stimuli naturally occurring in subtropical climates where citrus could have originated (Gmitter and Hu, 1990). My results also show that *CsSL1*, whose *Arabidopsis* ortholog *SOC1* is a key integrator of flowering signals initiated by multiple stimuli in *Arabidopsis*, is responsive to low temperature but not to water deficit. The expression of *CsSL1* is possibly not only not affected by water deficit but could actually be repressed since expression of *CsSL1* at floral-inductive temperatures increases in well-irrigated controls but remains unchanged in trees under water deficit. Water deficit could also repress the expression of other genes involved the regulation of flowering in *C. sinensis* meristems since the expression of *CsAP1* and *CsLFY* were also reduced in trees under water deficit. Low levels of *CsAP1* and *CsLFY* while floral-inductive stimuli are present, and transient up-regulation of these genes when growth-promoting conditions are re-established, indicate that floral differentiation in *C. sinensis* may not occur simultaneously to floral induction as it does in *Arabidopsis* (Wigge et al., 2005). In addition to regulation of *CsFT*, another flowering-promoting role of water deficit could be to maintain buds from initiating floral differentiation, as happens during warm periods in the Winter humid subtropical climates. In this work, the response of field trees to floral-inductive treatments was not always consistent with results obtained

using potted trees and controlled conditions. Other factors, such as plant growth regulators, carbohydrate balance and products of nitrogen metabolism have been shown to modulate the flowering response of citrus trees (Albrigo, 1999; Ali and Lovatt, 1994; Cooper and Peynado, 1958; Moss, 1971) and are likely to be involved in the regulation of flowering-related genes. In addition, it is also likely that key steps of the regulation of flowering in citrus might not be regulated at the mRNA level but at the protein or metabolite level as it has been shown in some cases in *Arabidopsis* (Blazquez and Weigel, 1999; Corbesier et al., 2007). However, since the transition to flowering involves a major modification of the tree's developmental pattern, characterizing changes at the transcript level could provide insights about the earliest processes being activated or repressed during the transition from vegetative to reproductive growth.

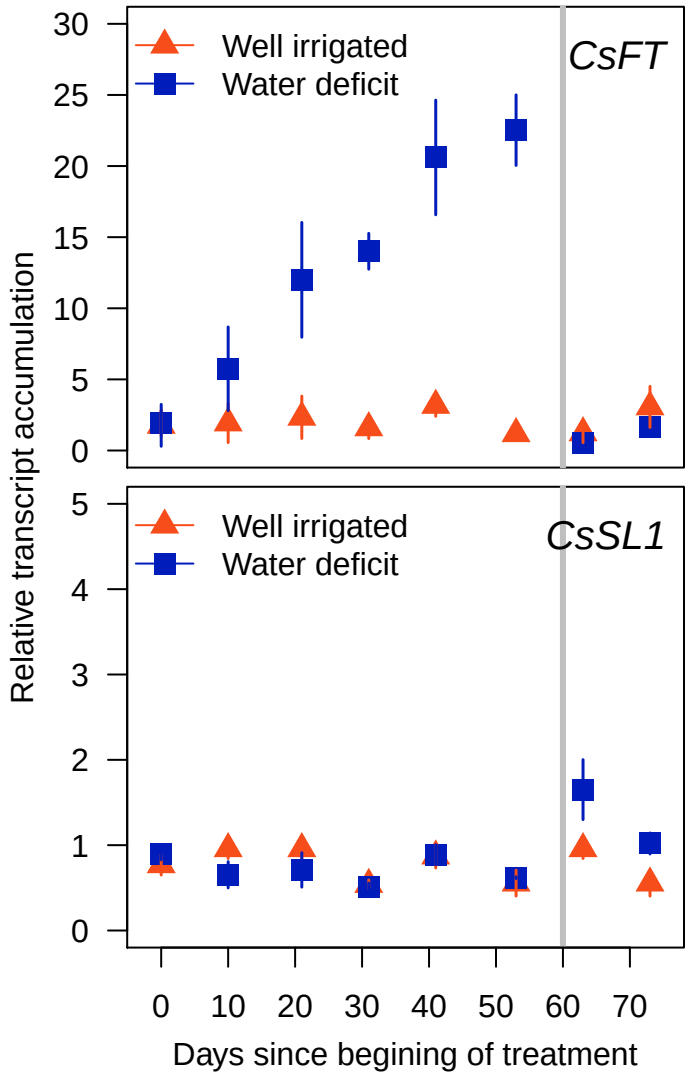


Figure 2-1. Expression of *CsFT* and *CsSL1* in 2 year old 'Navel' trees exposed to floral-inductive water deficit. "Water deficit" trees stopped receiving irrigation on day 0; then, when the midday stem water potential reached about -2MPa (day 15 to 20), the trees started to receive daily irrigation to match the daily weight loss of the tree. On day 60 (grey line), water deficit was interrupted by irrigating the soil to saturation. "Well irrigated" trees were irrigated to saturation every 3 days throughout the experiment. Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on day 0. Tree water status was monitored by measuring stem water potential and stomatal conductance.

Table 2-1. Flowering characteristics of 'Washington Navel' citrus trees exposed to water deficit. "La" and "Ld" refer respectively to "leaf abundant" and "leaf deficient" inflorescences based on their leaf/flower ratios (La $\geq$ 1, Ld $<$1). "Single" refers to single flowers without leaves. Figures are means per shoot ± S.E. The average number of nodes per shoot was 6.77±0.31

	New growth	Inflorescences	La	Ld	Leafless	Single	Vegetative	Flowers
Irrigated	0.2 ±0.09	0.2 ±0.09	0.11 ±0.07	0.04 ±0.04	0.02 ±0.02	0.04 ±0.03	0 ±0	0.39 ±0.19
Water deficit	3.16 ±0.46	2.04 ±0.51	1.68 ±0.48	0.04 ±0.04	0.16 ±0.09	0.16 ±0.07	1.12 ±0.23	3.24 ±0.86

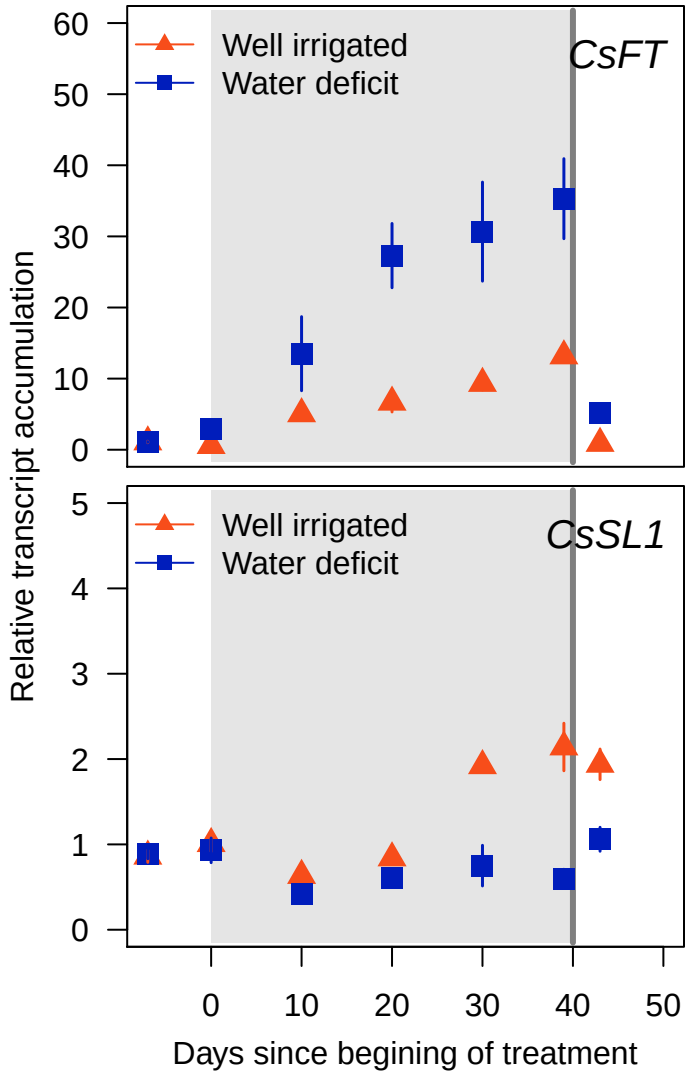


Figure 2-2. Expression of *CsFT* and *CsSL1* in 2 year old 'Navel' trees exposed to floral-inductive water deficit at floral-inductive temperatures (15°C). "Water deficit" trees stopped receiving irrigation on day -7 and kept in a chamber at 23°C until day 0. On day 0 the trees were transferred to chamber at 15°C along with a set of "Well irrigated" control trees for 40 days (light grey area). "Water deficit" trees did not receive irrigation until the midday stem water potential reached about -2MPa (day 15 to 20), after this point "Water deficit" trees were irrigated daily to match the daily weight loss of the tree. On day 40 (dark grey line), water deficit was interrupted by irrigating the soil to saturation and both groups of trees were transferred to the 23°C chamber to promote growth. "Well irrigated" trees were irrigated to saturation every 3 days throughout the experiment. Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on day -7. Tree water status was monitored by measuring stem water potential and stomatal conductance.

Table 2-2. Flowering characteristics of 'Washington Navel' citrus trees exposed to water deficit at floral inductive temperatures (15°C). "La" and "Ld" refer respectively to "leaf abundant" and "leaf deficient" inflorescences based on their leaf/flower ratios (La $\geq$ 1, Ld $<$1). "Single" refers to single flowers without leaves. Figures are means per shoot ± S.E. The average number of nodes per shoot was 6.16±0.27

	NewGrowth	Inflorescences	La	Ld	Leafless	Single	Vegetative	Flowers
Irrigated	2.21 ±0.07	1.47 ±0.08	1.08 ±0.05	0.07 ±0.03	0.25 ±0.03	0.07 ±0.04	0.55 ±0.11	3.35 ±0.12
Water deficit	3.64 ±0.16	2.73 ±0.45	2.25 ±0.36	0.05 ±0.07	0.21 ±0.09	0.21 ±0.06	0.91 ±0.31	4.24 ±0.73

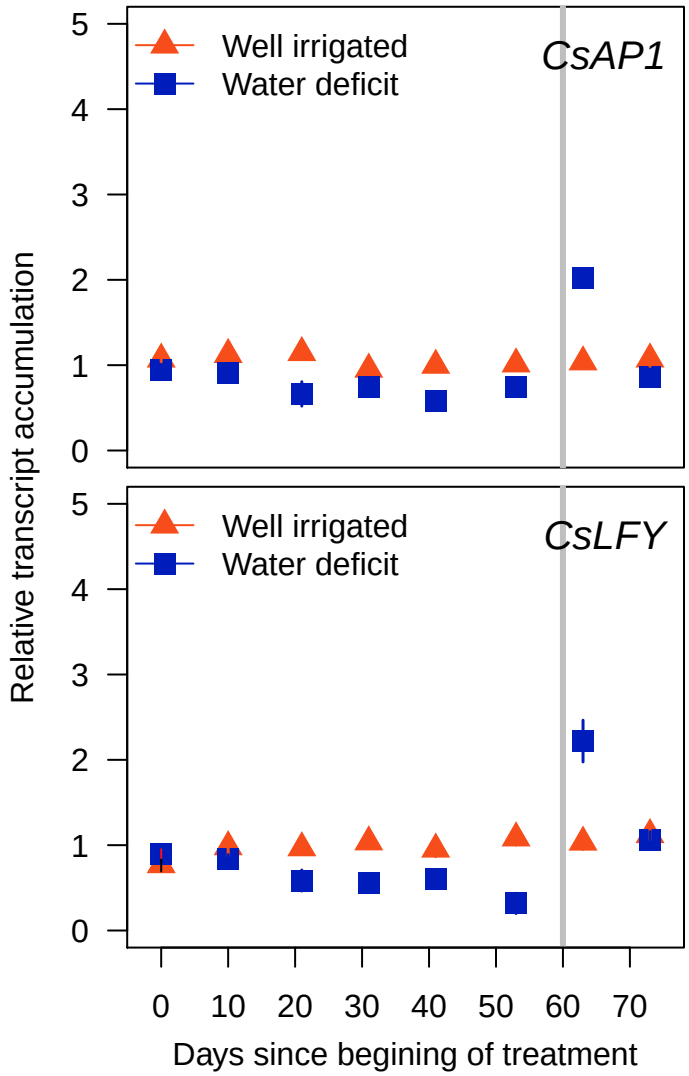


Figure 2-3. Expression of *CsAP1* and *CsLFY* in 2 year old 'Navel' trees exposed to floral-inductive water deficit. "Water deficit" trees stopped receiving irrigation on day 0; then, when the midday stem water potential reached about -2MPa (day 15 to 20), the trees started to receive daily irrigation to match the daily weight loss of the tree. On day 60 (grey line), water deficit was interrupted by irrigating the soil to saturation. "Well irrigated" trees were irrigated to saturation every 3 days throughout the experiment. Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on day 0. Tree water status was monitored by measuring stem water potential and stomatal conductance.

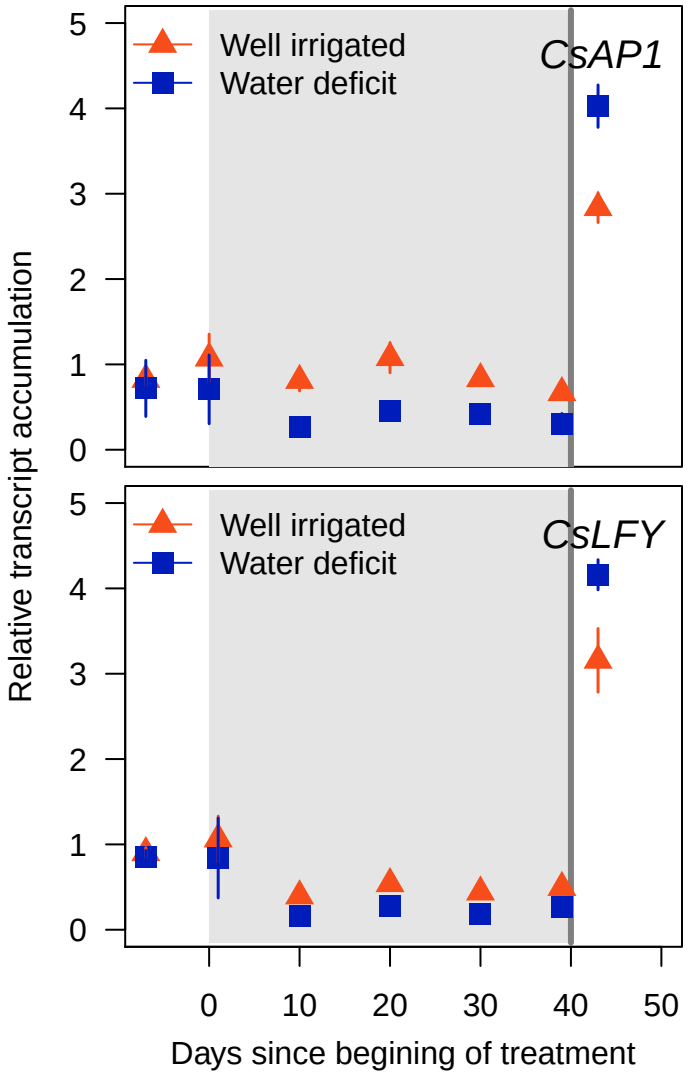


Figure 2-4. Expression of *CsAP1* and *CsLFY* in 2 year old 'Navel' trees exposed to floral inductive water deficit at floral-inductive temperatures (15°C). "Water deficit" trees stopped receiving irrigation on day -7 and kept in a chamber at 23°C until day 0. On day 0 the trees were transferred to chamber at 15°C along with a set of "Well irrigated" control trees for 40 days (light grey area). "Water deficit" trees did not receive irrigation until the midday stem water potential reached about -2MPa (day 15 to 20), after this point "Water deficit" trees were irrigated daily to match the daily weight loss of the tree. On day 40 (dark grey line), water deficit was interrupted by irrigating the soil to saturation and both groups of trees were transferred to the 23°C chamber to promote growth. "Well irrigated" trees were irrigated to saturation every 3 days throughout the experiment. Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on day -7. Tree water status was monitored by measuring stem water potential and stomatal conductance.

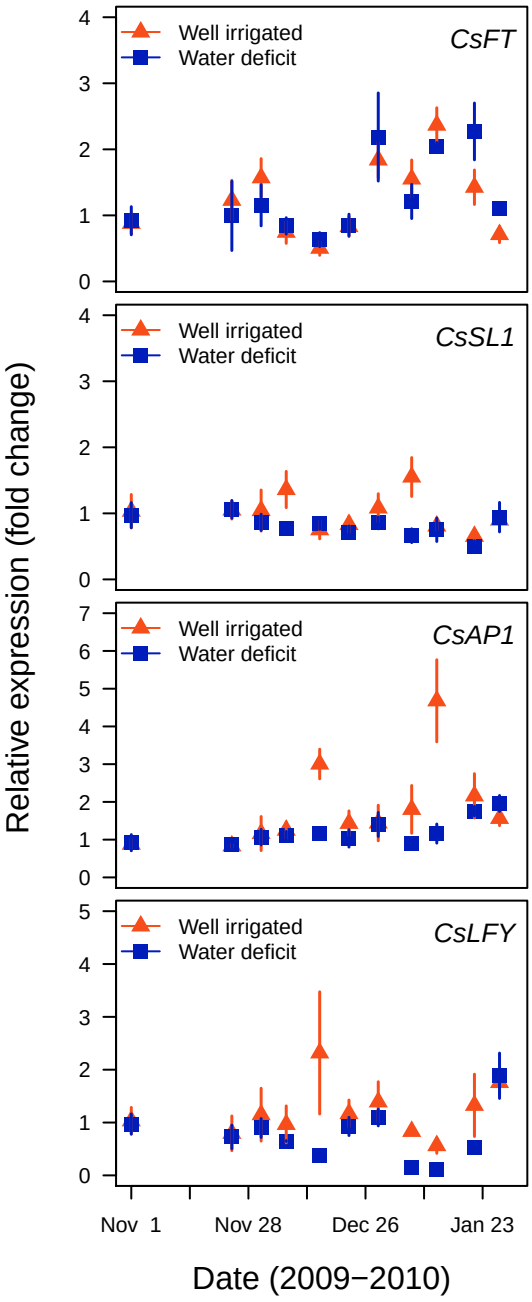


Figure 2-5. Expression of *CsFT*, *CsSL1*, *CsAP1* and *CsLFY* in 15 year-old field-grown 'Valencia' trees under water deficit during Winter. "Water deficit" trees received no irrigation from November 15 to January 28 and had the soil under their canopy covered by a sheet of impermeable material for the same time-period. "Well irrigated" trees received irrigation as neighboring commercial groves throughout the experiment. On January 28, the impermeable sheets were removed and "Water deficit" trees were irrigated overnight for 3 consecutive days. Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on November 1st.

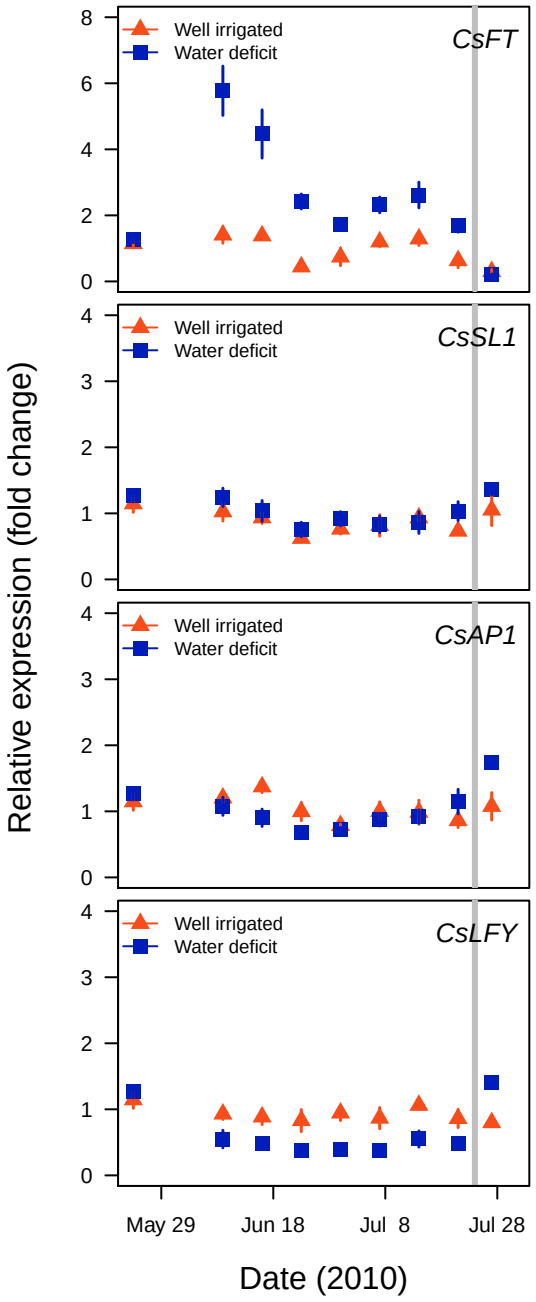


Figure 2-6. Expression of *CsFT*, *CsSL1*, *CsAP1* and *CsLFY* in 15 year-old field-grown 'Valencia' trees under water deficit during Summer. "Water deficit" trees received no irrigation from May 24 to July 23 and had the soil under their canopy covered by a sheet of impermeable material for the same time-period. "Well irrigated" trees received irrigation as neighboring commercial groves throughout the experiment. After 60 days (grey line), the impermeable sheets were removed and "Water deficit" trees were irrigated overnight for 3 consecutive days. Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in "Well irrigated" trees on day 0.

Table 2-3. Flowering characteristics of field grown 'Valencia' trees exposed to water deficit during Winter. "La" and "Ld" refer respectively to "leaf abundant" and "leaf deficient" inflorescences based on their leaf/flower ratios (La $\geq$ 1, Ld $<$1). "Single" refers to single flowers without leaves. Figures are means per shoot of 4 tree-replicates± S.E. The average number of nodes per shoot was 6.19±0.17

	NewGrowth	Inflorescences	La	Ld	Leafless	Single	Vegetative	Flowers
Irrigation	2.6±0.27	2.15±0.39	0.33±0.07	0.46±0.14	0.77±0.13	0.25±0.08	0.41±0.16	7.06±1.22
Water deficit	3.74±0.25	3.46±0.28	0.79±0.06	0.82±0.18	1.13±0.1	0.33±0.08	0.28±0.06	12.42±1.25

CHAPTER 3
RELATIONSHIP BETWEEN EXPRESSION PATTERNS OF FLOWERING GENES, FLOWERING INTENSITY GRADIENTS AND FLOWERING COHORTS IN SWEET ORANGE SHOOTS

The Spring flush of sweet orange trees growing in subtropical climates is composed of a mixture of inflorescences, flowers and vegetative shoots formed in a basipetal gradient in one year-old terminal shoots (Sauer, 1954; Valiente and Albrigo, 2004)(Figure 3-1A and 3-1B). In humid subtropical climates, the Spring flush of sweet orange trees is often made up of discrete cohorts of new growth (inflorescences or vegetative shoots) initiated at different times in the same terminal shoots during the preceding Winter (Valiente and Albrigo, 2003)(Figure 3-1C). These observations led to the development of the hypothesis that the initiation of multiple inflorescence cohorts and the flowering gradient observed in sweet orange shoots could be an indication of dynamically changing patterns in the distribution and activity of flowering signals at the shoot level in response to changing environmental conditions.

Several putative orthologs of *Arabidopsis* flowering genes have been identified in citrus in the last ten years; among these are the citrus orthologs of *Arabidopsis*'s floral identity genes *APETALA1* (*CsAP1*) and *LEAFY* (*CsLFY*) (Pillitteri et al., 2004b) and the flowering repressor *TERMINAL FLOWER 1* (*CsTFL1*) (Pillitteri et al., 2004a). In *Arabidopsis*, increased expression of *LEAFY* (*LFY*) in the shoot meristem is one of the earliest signs of the initiation of flower development and the increased expression of *APETALA1* (*AP1*) is an indicator of floral determination (Hempel et al., 1997; Simon et al., 1996). In contrast, the product of *Arabidopsis*' *TERMINAL FLOWER 1* (*TFL1*) is a strong suppressor of *AP1* and *LFY* activity (Ratcliffe et al., 1999) and prevents acquisition of floral identity (Shannon and Meeks-Wagner, 1993). The balance between the activities of *AP1*, *LFY* and *TFL1* products probably control the determination of shoot meristems to flower (Hempel et al., 2000). Thus, assuming that *CsAP1*, *CsLFY* and *CsTFL1* are also determinants of floral identity in citrus as they are in

Arabidopsis, expression patterns of these genes could help understand the dynamic of the establishment of the flowering gradient and the initiation of inflorescence cohorts in sweet orange.

The establishment of flowering gradients and the initiation of inflorescence cohorts could be regulated by changing production and distribution patterns of flowering signals or changing sensitivity to those signals in the meristems. *Arabidopsis*' *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) are integrators of flowering signals initiated by different stimuli (Lee and Lee, 2010; Samach et al., 2000). FT itself, is a mobile flowering signal that integrates information from photoperiod-sensing systems and promote flowering by up-regulation of *LFY* and *AP1* (Corbesier et al., 2007). Putative orthologs of *Arabidopsis*' *FT* (*CsFT*) and *SOC1* (*CsSL1*) have also been identified in citrus (Nishikawa et al., 2007; Tan and Swain, 2007). Expression patterns of *CsFT* and *CsSL1* could indicate whether differential production of flowering signals could be a factor in establishing the flowering gradient and the initiation of inflorescence cohorts in sweet orange shoots.

In this chapter, the patterns of expression of putative citrus floral identity genes and floral signal integrators under conditions that alter the flowering gradient and the initiation of flowering cohorts in sweet orange shoots were investigated. The hypotheses tested were that transcript accumulation of floral identity genes occurs in a basipetal gradient within shoots and that exposure to growth-promoting conditions and then re-exposure to floral-inductive conditions increases the accumulation of floral identity genes transcripts in buds at a more basal position. Another hypothesis tested in this chapter was that the establishment of the flowering gradient in shoots could be disrupted by treatments with 1,2,3 tri-iodo benzoic acid (TIBA), a known auxin transport inhibitor (Niedergang-Kamien and Skoog, 1956). TIBA inhibits auxin transport by blocking the auxin efflux carriers (Geldner et al., 2001) and releases apical dominance in axillary buds (Snyder, 1949). Auxin transport within shoots has been

related to the establishment of apical dominance (Everat-Bourbouloux and Bonnemain, 1980; Thimann and Skoog, 1934). Since the flowering gradient observed in citrus shoots resembles responses controlled by apical dominance, it is possible that this two phenomena be related.

3.1 Materials and Methods

3.1.1 Plant Material

Experiments were conducted under controlled environments were conducted using either 2-3 year old potted 'Valencia' trees grafted on 'Swingle' citrumelo or 2-3 year old potted 'Washington Navel' cuttings or in the field using mature 'Valencia' trees grafted on 'Carrizo' citrange. All the potted trees used for experiments were tested for floral competence and were maintained in a shaded greenhouse with natural photoperiods, non-limiting irrigation and standard fertilization when not in use for experiments. The growth rooms in which the controlled conditions experiments were conducted were illuminated with with fluorescent lights (800μmoles$\cdot$m$^{-2}\cdot$s^{-1} at canopy level) with a 11/13h (day/night) photoperiod.

3.1.2 Experimental Conditions

To determine whether *CsFT* transcripts accumulate in a gradient in leaves at different positions within shoots, *CsFT* transcripts were quantified in leaves from shoots of field-grown and potted trees under floral inductive and non floral-inductive conditions. Levels of *CsFT* in leaves under non floral-inductive conditions were determined in field-grown trees in October (temperatures continuously above 20°C) and in potted trees kept in a growth room at 23°C. Levels of *CsFT* in leaves under floral-inductive conditions were determined in field-grown trees in December (trees had been exposed to naturally occurring floral-inductive temperatures since early November) and in potted trees kept in a growth room at 12°Cfor 1 month. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of the

selected genes between well irrigated and water deficit trees were analyzed using a general linear model.

To determine whether transcripts of floral identity genes (*CsAP1* and *CsLFY*) accumulate in a gradient in buds at different positions within shoots, *CsAP1* and *CsLFY* transcripts were quantified in buds on shoots of potted trees during and after floral induction by low temperatures. Trees that had been kept at 23°C for at least one month were transferred to a growth room at 12°C and kept at this temperatures for 30 days to induce flowering. Then, trees were transferred back to the room at 23°C to promote growth. Bud samples (stratified by position) were collected before transfer the transfer to the room at 12°C, on the last day of the floral inductive treatment and 3 days after the final transfer to 23°C. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of *CsAP1* and *CsLFY* in buds at different position were analyzed using a general linear model. Differences in transcript accumulation of *CsAP1* and *CsLFY* in buds at the same position between days 29 and 33 were analyzed using t-test.

To determine whether the auxin transport inhibitor TIBA disrupts the establishment of flowering gradients in shoots, a lanolin paste (about 30mg) that contained 2,3,5-triiodo benzoic acid (TIBA, Sigma) a 0.5% (w/w) concentration was applied as a ring around the midsection of each internode of selected shoots in potted trees. Another set of shoots in the same trees were treated similarly with a lanolin paste without TIBA as a control. The trees had been kept at 23°C for at least one month before the application of the TIBA-containing paste and were transferred to a growth room at 12°C after the application. The trees were kept at 12°C for 30 days and then transferred back to a room at 23°C to promote growth. The number of buds initiating floral development in TIBA-treated and control shoots was recorded. The distribution of buds initiating floral development by bud position in TIBA-treated shoots was compared to the distribution of buds initiating floral development in control shoots using the chi-square test.

To test whether pattern of accumulation of floral identity genes transcripts were related to the initiation of inflorescence cohorts, *CsAP1* and *CsLFY* transcripts were quantified in buds of potted trees exposed to intermittent floral induction. Trees that had been kept at 23°C for at least one month were transferred to a growth room at 12°C and kept at this temperatures for 15 days. Then, the trees were transferred back to the room at 23°C for 4 days and the transferred again to the room at 12°C for another 10 days. Then, the trees were finally transferred to the room at 23°C to promote growth. This temperature regime was designed to mimic conditions known to induce the formation of inflorescence cohorts in the field in humid subtropical climates (Valiente and Albrigo, 2003). Bud samples (stratified by position) were collected before transfer the initial transfer to the room at 12°C, on the last day of each temperature period, and 3 days after the final transfer to the room at 23°C. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in transcript accumulation of *CsAP1* and *CsLFY* in buds at different position were analyzed using a general linear model. Differences in transcript accumulation of *CsAP1* and *CsLFY* in buds at the same position between day 14-18 and day 29-33 were analyzed using t-test. The number of inflorescences formed by bud position was recorded and compared using t-tests for each bud position between trees exposed to intermittent induction or continuous induction in all the shoots (6-7 nodes long) formed during the previous year present on the trees.

In all the experiments, accumulation of *CsFT* transcripts was quantified in leaves samples whereas accumulation of *CsSL1*, *CsAP1* and *CsLFY* transcripts was quantified in bud samples. The choice of tissues in which transcripts of the selected genes were quantified was made based on the most likely spatial domain of gene expression and protein activity predicted by the hypothetical model in section 1.4. Leaf and buds samples consisted of a pool of at least 6 leaves or buds from separate shoots on each tree replicate. All samples were collected at 15H00 local standard time.

3.1.3 qRT-PCR

Total RNA was extracted using a phenol-chloroform precipitation method and purified using silica membranes with on-column DNase digestion (Qiagen). Leaf samples were used for analysis of *CsFT* expression, whereas bud samples where used for analysis of *CsSL1*, *CsAP1* and *CsLFY* expression. Five hundred nanograms of total RNA were used for cDNA synthesis in a 20μl reaction with oligo dT primers (SuperScriptIII®, Invitrogen). One microliter of the synthesized cDNA was used for two-step (95 °C denaturation and 60 °C for 1 minute annealing and extension) qPCR in a 20μl reaction (SYBR® Premix ExTaq™II, Takara) on a Applied Biosystems 7500 FAST real-time PCR system (Life Technologies) using optimized qPCR assays (see Appendix). Primers for qPCR were: 5'-CGGCGGAAGGACTATGAC-3' and 5'-TGTGAGAAAGCCAGAGAGGAA-3' (*CsFT*), 5'-CCCTGGAGTGCAACAACCT-3' and 5'-CTGATGTGTTTGAGAGCGGT-3' (*CsAP1*), and 5'-TCTTGATCCAGGTCCAGAACATC-3' and 5'-TAGTCACCTTGGTTGGGCATT-3' (*CsLFY*). *CsGAPDH* was used as reference gene (5'-GGAAGGTCAAGATCGCAATCAA-3' and 5'-CGTCCCTCTGCAAGATGACTCT-3'). All qPCR assays were validated for specific amplification and optimized for amplification efficiencies between 1.88 and 2.05 with a linear dynamic range of 6 log10 cycles. The sequence of the primers to amplify *CsLFY* was obtained from Nishikawa et al. (2009) whereas all other primer sequences were designed in-house. Relative gene expression was calculated as a fold change ratio using Pfaffl's method (Pfaffl, 2001) with sliding-window efficiencies calculated for each reaction using the `sliwin` function in the `qpcR` R package (Ritz and Spiess, 2008).

3.1.4 Data Analysis

Mean fold change of transcript levels were transformed to a logarithmic scale ($\log_2$) for statistical analysis but data in the graphs represents the untransformed data. Unless noted otherwise, all differences reported are statistically significant ($p<0.05$). All statistical analyses were executed in R (R Development Core Team, 2011).

3.2 Results and Discussion

3.2.1 *CsFT* Transcripts Accumulate at Equal Levels in Leaves Regardless of Their Position in the Shoot.

Under natural conditions, inflorescences in *C. sinensis* shoots are formed in a basipetal gradient in shoots formed during the previous years (Sauer, 1954; Valiente and Albrigo, 2004)(Figure 3-2).To test the hypothesis that there is gradient in the transcript levels of the putative flowering-signal gene CsFT in leaves at different positions, samples of leaves borne at different positions in selected shoots (7-8 nodes long formed in the previous summer or spring) were collected before the onset of floral inductive weather and during floral induction and expression of *CsFT* was measured.

Figure 3-3 shows that the levels of expression of *CsFT* in leaves of 7-8 nodes long shoots formed during the previous year was not significantly different among leaves at different positions within the shoot. In general, the level of expression of *CsFT* tended to be lower at more basal positions (4-7) but this trend was not significant. No differences in the level of expression of *CsFT* could be detected in either samples collected before or during floral induction. Assuming that *CsFT* encodes a mobile flowering signal in *C. sinensis*, similar to *FT* in *Arabidopsis*, and that transcript levels of *CsFT* correspond to levels of the CsFT protein, these results are against the hypothesis that flowering gradients are established by differential production of flowering signals in leaves.

3.2.2 Accumulation of *CsAP1* and *CsLFY* transcripts is Higher at Nodes Closer to the Apex.

Given that the level of transcription transcription of CsFT is not different in leaves at different positions within shoots, it was hypothesized that the establishment of flowering gradients could be related to differential accumulation of transcripts of floral identity genes in buds at different positions. To test this hypothesis, the expression of floral identity genes CsAP1 and CsLFY was measured in buds at different positions after floral induction.

Figure 3-4 shows that the expression of floral identity genes *CsAP1* and *CsLFY* after floral induction (Day 33) is lower in basal buds than in the apical bud. Basal levels of expression of *CsAP1* and *CsLFY* (Day 0) were not statistically different between buds at different positions. Levels of *CsAP1* and *CsLFY* at the end of floral induction (Day 29) were also not different in buds at different positions and were slightly reduced (not statistically significant) from basal levels. The levels of expression of the putative flowering repressor *CsTFL1* were also measured but not analyzed as *CsAP1* and *CsLFY* because of some replicates having levels of *CsTFL* extending below the detection range of the qPCR assay. In general, levels of expression of *CsTFL1* were higher (more reactions in which *CsTFL1* were detected) in buds at positions 7 and 5 than in positions 1 and 3 (data not shown). Together, these results supports the hypothesis that flowering gradients in *C. sinensis* are related to a basipetal gradient of expression of floral identity genes at different bud positions when growth is resumed after floral induction.

3.2.3 TIBA Disrupts the Establishment of Flowering Gradients.

Results in the previous subsection (3.2.1) showed that expression of CsAP1 and CsLFY and flowering occurs as a basipetal gradient in shoots after floral induction. Since apical dominance is partly mediated by the basipetal transport of auxins synthesized in younger and actively growing organs near the apex (Chatfield et al., 2000; Tanaka et al., 2006; Thimann and Skoog, 1934), it was hypothesized that the auxin distribution within shoots is related to the establishment of flowering gradients in *C. sinensis*. To test this hypothesis, apical dominance was disrupted in selected shoots using TIBA, an auxin transport inhibitor (Niedergang-Kamien and Skoog, 1956). TIBA has been reported to release apical dominance in axillary buds (Snyder, 1949). A lanolin paste containing 0.5% TIBA was applied in all the internodes in selected shoots and the shoots were exposed to floral-inductive temperatures and then transferred to growth promoting temperatures to measure flowering.

The TIBA treatment changed the flowering response of buds under the conditions tested (Figure 3-5). TIBA-treated buds produced inflorescences at all bud positions, whereas non-TIBA-treated buds produced inflorescences primarily in positions 1 to 3 with no inflorescences being formed at position 6 and 7. Furthermore, the type of inflorescences formed in TIBA-treated buds was exclusively leafless, whereas non-TIBA-treated shoots produced a mixture of inflorescences types (primarily of the leafy type, data not shown). Moss (1969) reported that more inflorescences and higher proportion of leafless inflorescences are usually associated with higher levels of floral induction. Thus, the TIBA treatment applied to shoots in this experiment altered the mechanism controlling floral induction in a way that increased the level of floral induction sensed by buds. Buds at basal positions in *C. sinensis* shoots do not normally flower under natural conditions, however, if apical buds are removed basal buds respond to floral induction and produce inflorescences when growth is re-started (Chica and Albrigo, 2011). Pillitteri et al. (2004a), and Chapter 2 of this dissertation contain evidence supporting the hypothesis that floral initiation (as reported by up-regulation of floral identity genes) occurs only right after the onset of growth-promoting conditions. TIBA inhibits auxin transport by blocking the auxin efflux carriers (Geldner et al., 2001) and releases apical dominance in axillary buds (Snyder, 1949). Since apical dominance inhibit the growth of basal buds (Thimann and Skoog, 1933) and up-regulation of *CsAP1* and *CsLFY* only occurs when growth is resumed, it is possible that the TIBA treatments in this experiment released apical dominance in axillary buds and thus, allowed all the buds in the shoot to initiate growth as inflorescences. Another possible explanation is that the movement of the CsFT protein was controlled by auxin or auxin-related mechanisms. Auxin-mediated apical dominance has previously been related to the distribution of assimilates and nutrients (Davies and Wareing, 1965-06-01; Patrick and Steains, 1987). Thus, it is possible that TIBA treatments induced the accumulation of

CsFT in basal axillary buds which was produced in the leaves associated with these buds instead of CsFT being transported and accumulated near the apex.

3.2.4 Transcript Accumulation of Floral Identity Genes after Intermittent Induction is Related to Initiation of Flowering Cohorts.

Under Florida's humid subtropical conditions, multiple cohorts of inflorescences are often initiated by sporadic periods of warmer weather during the fall/winter (Valiente and Albrigo, 2003). Since expression of *CsAP1* and *CsLFY* was correlated to the initiation of flowering, it was hypothesized that levels of expression of these two genes could also be related to the initiation of multiple flowering cohorts. To test this hypothesis, the expression of *CsAP1* and *CsLFY* was measured at different positions within the shoot in potted trees subjected to intermittent floral induction.

Figure 3-6 shows that when trees were transferred from cool floral-inductive temperatures to warmer growth-promoting temperatures after 15 days of induction, the expression of *CsAP1* and *CsLFY* increased as reported in subsection 3.2.2 (Day 18). Transferring trees back to floral-inductive temperatures reduced the level of expression of *CsAP1* and *CsLFY*. Expression of *CsAP1* and *CsLFY* remained low until the trees were permanently transferred to growth-promoting temperatures (Day 33). After the final transfer, the gradient in the expression of *CsAP1* and *CsLFY* was reversed from bud 1 to 5 whereas expression in bud 7 was out of the trend but still higher than basal levels. More inflorescences were formed in buds 5 and 7 of trees exposed to intermittent induction than in control trees that were exposed to un-interrupted induction (Figure 3-7), supporting the hypothesis that flowering cohorts are initiated when floral induction is interrupted by periods of warmer weather which up-regulates the expression of *CsAP1* and *CsLFY*. These results also show that basal buds are capable of responding to floral induction after initiation of flowering in buds at more apical positions (which is assumed to have occurred by the up-regulation of *CsAP1* and *CsLFY*).

To summarize, results from the experiments of this chapter show that the establishment of flowering gradients within *C. sinensis* shoots and the initiation of multiple flowering cohorts is more related to the pattern of expression of floral identity genes *CsAP1* and *CsLFY* than to the pattern of expression of the putative flowering signal gene *CsFT*. Factors associated with auxin transport within shoots were involved in regulating the establishment of flowering gradients in *C. sinensis* shoots. The involvement of auxin-related mechanisms in the regulation of flowering in *C. sinensis* is new and will require further investigation to determine its exact role in the establishment of flowering gradients. The results presented in this chapter provide useful information for modeling the dynamic of floral initiation in shoots of *C. sinensis*.

A

B

C

Figure 3-1. Inflorescence gradient, type of new growth and inflorescence cohorts in *C. sinensis* spring flush. A) Inflorescence gradient, note that inflorescences were formed in the 4 more-apical nodes only. B) Types of new growth in the spring flush. From left to right: new vegetative shoots, mixed inflorescences and leafless inflorescences. Mixed inflorescences can be further divided in leaf-abundant and leaf-deficient inflorescences depending on whether the inflorescences has more leaves than flower or vice versa. C) Inflorescence cohorts. Note inflorescences with fully-developed open flowers in more apical positions and developing inflorescences at more basal positions in the same shoot (red squares).

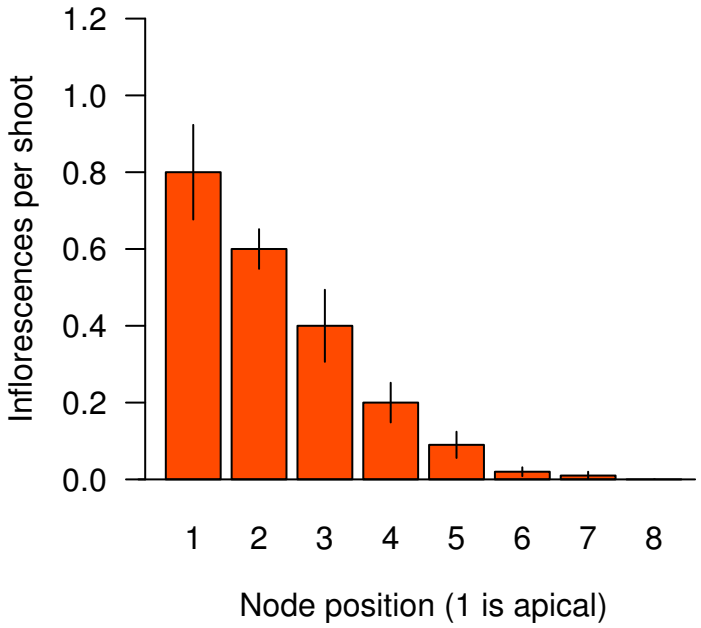
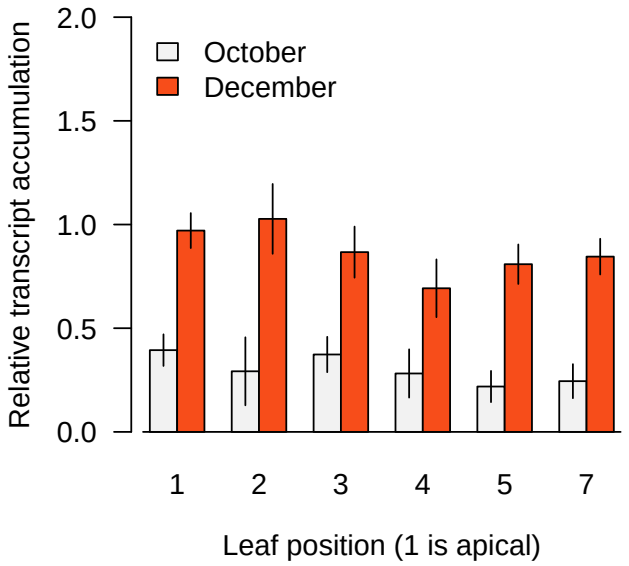
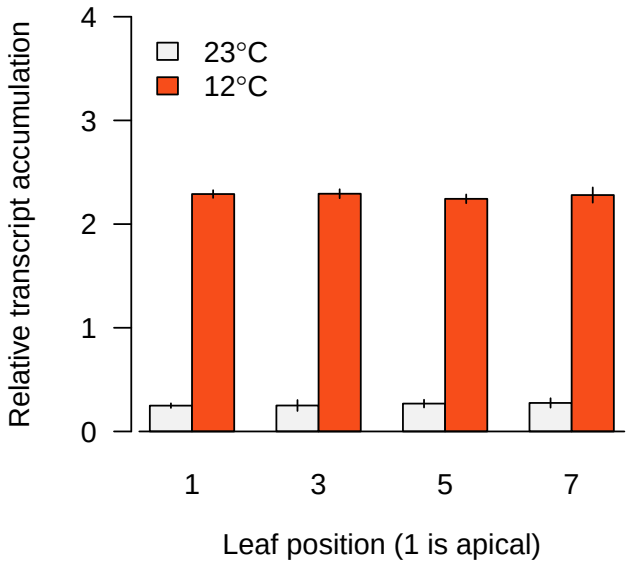


Figure 3-2. Number of inflorescences formed by node position in field-grown 15 year-old ‘Valencia’ tree in the spring of 2010. Data are means ± S.E. of 4 tree-replicates (25 shoot-sub-replicates per tree).



A



B

Figure 3-3. Expression of *CsFT* in leaves at different positions in shoots of field-grown 'Valencia' trees in October and December of 2010 (A) and potted trees at 23°C or 12°C (B). The level of expression of *CsFT* in leaves in October and at 23°C is representative of the levels of *CsFT* in leaves not exposed to floral-induction whereas the level of expression of *CsFT* in December and at 12°C is representative of levels of *CsFT* of leaves under floral-induction. Data are means ± S.E. of 4 tree-replicates (6 shoot-sub-replicates per tree).

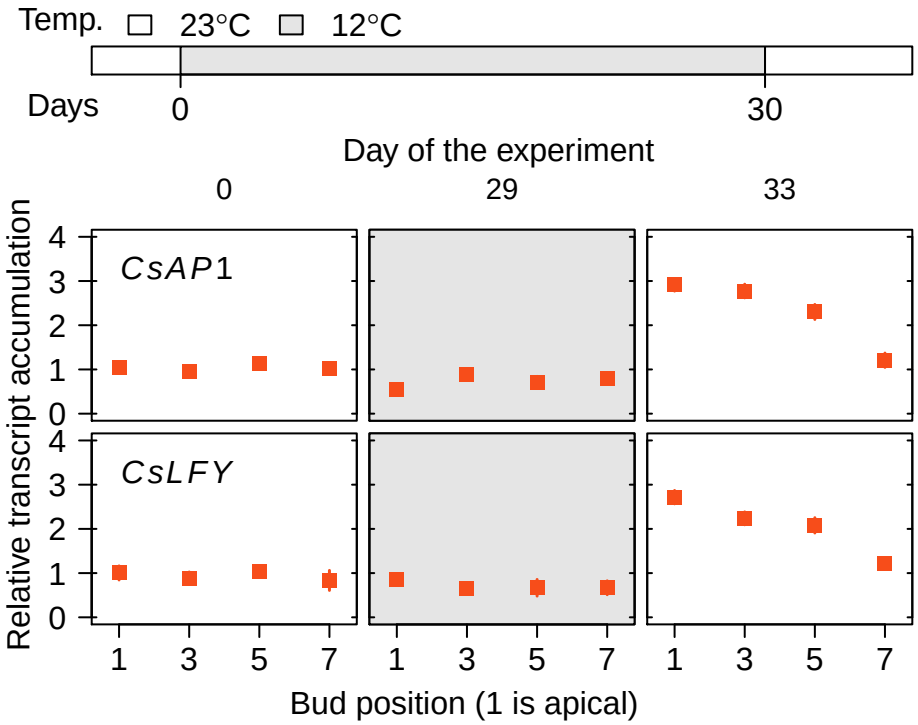


Figure 3-4. Expression of *CsAP1* and *CsLFY* in buds at different position in shoots of 3-year-old potted 'Valencia' trees. Trees were transferred from a room at 23° C to a room at 12°C(Day 1). After 29 days, the trees were transferred back to the room at 23°C to promote growth. Data are means ± S.E. of 4 tree-replicates (3 shoot-sub-replicates per tree).

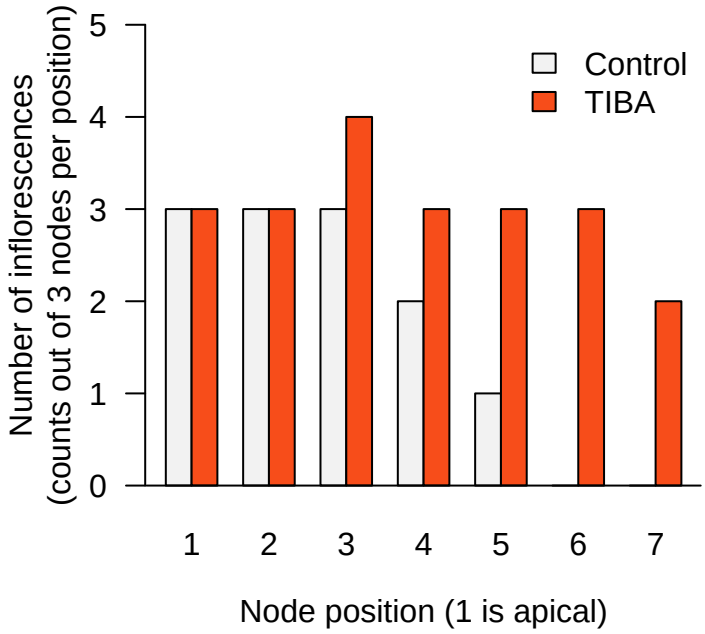


Figure 3-5. Number of inflorescences formed by position in shoots of potted 3 year-old ‘Valencia’ trees treated with 2,3,5-triiodobenzoic acid (TIBA). TIBA-treated shoots had a lanolin paste containing 0.5% (w/w) of TIBA applied as a ring around each internode, halfway between nodes. Control trees had a lanolin paste without TIBA applied as before. Both sets of trees were transferred to a room at 12°C for 30 days and then transferred to another room at 23°C to promote growth. Each group consisted of 3 trees. Data are sums of the number of inflorescences formed in each position. The distributions of inflorescences formed by bud position between TIBA-treated and control shoots were statistically different (chi-square test).

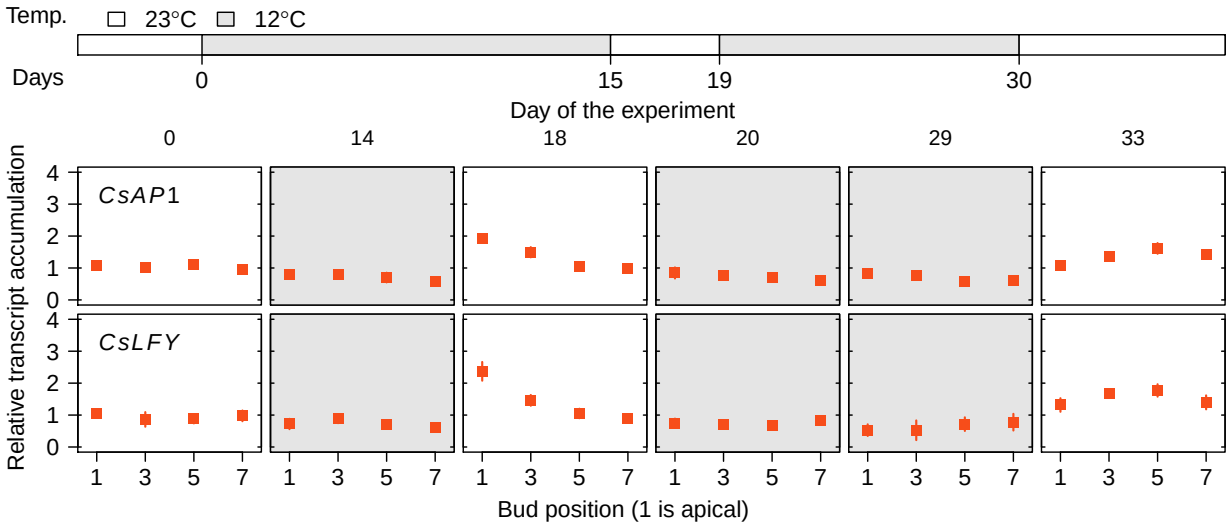


Figure 3-6. Expression of *CsAP1* and *CsLFY* in buds at different position in shoots of 3-year-old potted 'Valencia' trees under intermittent floral induction. Trees were transferred from a room at 23°C to a room at 12°C(Day 1). On day 15, the trees were transferred to the room at 23°C and kept there for 3 days. On day 19 the trees were transferred again to the room at 12°C until day 30 when the trees were permanently transferred to 23°C. Data are means ± S.E. of 4 tree-replicates (3 shoot-sub-replicates per tree).

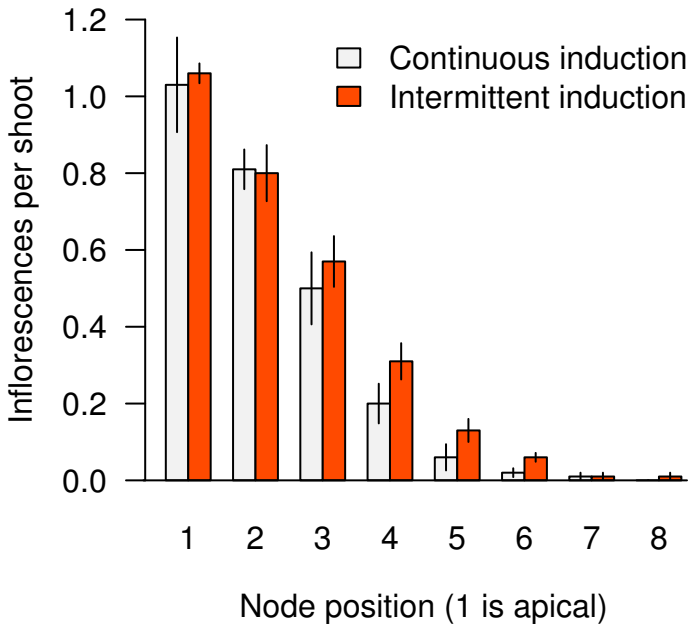


Figure 3-7. Number of inflorescences formed by position in 3 year-old 'Valencia' trees exposed to intermittent induction. Trees were transferred from a room at 23° C to a room at 12°C(Day 1). On day 15, the trees were transferred to the room at 23°C and kept there for 3 days. On day 19 the trees were transferred again to the room at 12°C until day 30 when the trees were permanently transferred to 23°C. Data are means ± S.E. of 4 tree-replicates (3 shoot-sub-replicates per tree).

CHAPTER 4
OTHER FACTORS ALTERING THE EXPRESSION OF SWEET ORANGE FLOWERING GENES DURING FLORAL INDUCTION

Although low temperatures and water deficit are the only factors known to induce flowering in *C. sinensis*, other factors like crop load, carbohydrate levels, gibberellins and products of nitrogen metabolism can also alter the level of floral induction (Albrigo and Galen-Saúco, 2004; Krajewsky and Rabe, 1995). Since these other factors (crop load, etc.) modify the level of floral induction in *C. sinensis* trees, it was hypothesized that signals initiated by these factors integrate with the regulatory pathway that controls the expression of citrus flowering genes. Even though *C. sinensis* flowering genes are probably orthologous to *Arabidopsis* flowering genes (Endo et al., 2005; Nishikawa et al., 2010, 2009, 2007; Pillitteri et al., 2004a,b; Tan and Swain, 2007), the mechanisms controlling floral induction are apparently different in both species. For instance, although flowering in *Arabidopsis* is induced primarily by changes in photoperiod (Valverde et al., 2004), changes in photoperiod do not seem to have any effect on floral induction in citrus (Moss, 1969). Furthermore, gibberellins promote flowering in *Arabidopsis* (Blázquez et al., 1998) but they produce the opposite effect in citrus (Monselise et al., 1964). The effects of low temperatures and water deficit on the expression of *C. sinensis* flowering genes have been reviewed and reported earlier in Chapter 2 of this dissertation and in the published literature (Nishikawa et al., 2007; Pillitteri et al., 2004a).

In this chapter I present a collection of experiments designed to determine the effects of crop load, gibberellins and light on the expression of flowering genes in *C. sinensis*. Also, I present experiments that describe the changes in the level of transcript accumulation of flowering genes early in the floral induction process. Results from these experiments provide information about the molecular mechanism underlying the effects of crop load and gibberellins on flowering in *C. sinensis*. In addition, these results show that flowering genes respond rapidly and are highly sensitive to changes

in environmental conditions. Together, these results add more details to the proposed hypothetical mechanism by which flowering is induced in *C. sinensis*.

4.1 Materials and Methods

4.1.1 Plant Material

Field experiments were conducted using mature 'Valencia' sweet orange trees grafted on 'Carrizo' citrange in an orchard at the University of Florida's Citrus Research and Education Center in Lake Alfred, Florida (28°5'N, 81°43'W) during 2009 and 2010. The orchard received similar horticultural care as in neighboring commercial groves throughout the experiments. Experiments under controlled environments were conducted using either 2-3 year old potted 'Valencia' trees grafted on 'Swingle' citrumelo or 2-3 year old potted 'Washington Navel' cuttings. All the trees used for experiments were tested for floral competence and were maintained in a shaded greenhouse with natural photoperiods, non-limiting irrigation and standard fertilization when not in use for experiments. The growth rooms in which the controlled conditions experiments were conducted were illuminated with with fluorescent lights (800μmoles·m^{-2}·s^{-1} at canopy level) with a 11/13h (day/night) photoperiod.

4.1.2 Experimental Conditions

To determine the effect of gibberellins on the accumulation of *CsAP1*, *CsLFY*, *CsSL1* and *CsWUS* transcripts in buds previously exposed to water deficit, transcripts of these genes were quantified in buds of potted trees following a floral-inductive treatment by low temperatures. Trees that had been kept at 23°C for at least one month were transferred to a growth room at 12°C and kept at this temperatures for 30 days to induce flowering. On the last day of the floral-inductive treatment, a 2.5μl drop of a 90ppm aqueous solution of gibberellic acid (GA, Acros organics) and 0.5% Tween 80 (Fisher) was applied to buds of previously selected shoots. Then trees were transferred to a room at 23°C to promote bud growth. One day after the transfer to the room at 23°C, a drop of the same gibberellic acid solution described before was applied to the buds of

another set of previously selected shoots. Another set of shoots was treated with a 0.5% Tween 80 (Fisher) aqueous solution as a control. Three days after the final transfer to 23°C, the treated buds were sampled for transcript quantification. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in levels of transcript accumulation of *CsAP1*, *CsLFY*, *CsSL1* and *CsWUS* in buds treated with gibberellin and control buds were analyzed using analysis of variance.

To test determine the effect of gibberellins on the accumulation of *CsFT* transcripts, leaves from the same trees of the experiment described in the previous paragraph were treated with the same gibberellic acid solution described before after 25 days at 12°C. The gibberellic acid solution was applied using cotton swabs and spreading it on both the adaxial and abaxial surface of the leaves. Another group of leaves were treated with the same solution without gibberellic acid as control. One day after the treatments were applied the leaves were sampled for transcript quantification. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in levels of transcript accumulation of *CsFT* in leaves of gibberellin-treated and control leaves were analyzed using a t-test.

To determine the effect of factors associated with fruit on the accumulation of *CsFT* transcripts, leaves located at different distances from fruit on the same limb unit were sampled in field-grown trees during the winter of 2009. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in levels of transcript accumulation of *CsFT* in leaves at different position relative to the nearest fruit were analyzed using a general linear model. In the following spring, the number of inflorescences formed in the shoots from which the leaves were sampled were recorded and analyzed also using a general linear model.

To test whether cycles of light and dark alter the accumulation of *CsFT* transcripts, *CsFT* transcripts were quantified in trees exposed to normal day/night conditions (11/13h day/night), constant darkness or constant light in growth rooms at 12°C. Trees

that had been kept at 23°C for at least one month with a constant photoperiod (11/13h day/night) were transferred to another room at 12°Cunder the 3 light regimes previously indicated. Before the transfer, leaves were sampled to determine reference initial levels of *CsFT* transcripts. Three days after the beginning of the treatments, leaf samples were collected and *CsFT* transcripts quantified. This experiment was conducted using a completely randomized design with 4 tree replicates. For each treatment, differences in transcript levels between samples collected before the transfer and 3 days after the transfer were analyzed using t-tests. Differences between the 3 light regimes on samples collected 3 days after the transfer were analyzed using analysis of variance.

To determine whether the accumulation of *CsFT* transcripts changes throughout the day, leaves samples were collected at different times under different conditions known to modify floral induction in *C. sinensis*. To determine levels of *CsFT* transcripts at non floral-inductive 23°C and floral-inductive 12°C, potted trees exposed to these temperatures for 15 days were sampled every 2 hours from 06H00 until 18H00 for 3 consecutive days. To determine levels of *CsFT* in trees exposed to floral-inductive water deficits and naturally occurring floral inductive temperatures, field-grown trees were sampled during the summer of 2010 and the winter of 2010 at 08H00, 15H00, 18H00 and 21H00 for 3 consecutive days. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in accumulation of *CsFT* transcripts were analyzed using analysis of variance. To investigate whether the time at which floral inductive temperatures occur has an effect on the accumulation of *CsFT* transcripts, transcripts of *CsFT* of trees transferred from 23°C to 12°C at either 07H00 or 15H00 were quantified before the transfer, 2 hours after the transfer, and at 07H00 and 15H00 on the next day. Differences in *CsFT* transcript accumulation between trees transferred in the morning or in the afternoon were analyzed using analysis of variance. To investigate the short-term response of *CsFT* transcript accumulation to naturally occurring reductions in temperature under field conditions,

trees were sampled at 08H00 and 15H00 during the fall of 2010 2 days before and after the passing of a cold front that reduced night temperatures below 20°C. Differences in *CsFT* transcript accumulation in leaves before and after the passing of the cold front were analyzed by t-test.

To determine the effect of levels of floral-inductive temperature on the accumulation of *CsFT* transcripts, *CsFT* transcripts were quantified on leaves of trees after 3 days of exposure to either 5, 10, 15, 20 or 23°C. Trees that had been kept at 23°C for at least one month before the transfer to rooms at each temperature of the indicated temperatures. Leaves were sampled before transfer and on day 3 after the transfer. This experiment was conducted using a completely randomized design with 4 tree replicates. Differences in *CsFT* transcript accumulation in leaves at the 5 temperatures were analyzed by analysis of variance.

4.1.3 qRT-PCR

Total RNA was extracted using a phenol-chloroform precipitation method and purified using silica membranes with on-column DNase digestion (Qiagen). Leaf samples were used for analysis of *CsFT* expression, whereas bud samples where used for analysis of *CsSL1*, *CsAP1* and *CsLFY* expression. Five hundred nanograms of total RNA were used for cDNA synthesis in a 20μl reaction with oligo dT primers (SuperScriptIII®, Invitrogen). One microliter of the synthesized cDNA was used for two-step (95°C denaturation and 60°C for 1 minute annealing and extension) qPCR in a 20μl reaction (SYBR® Premix ExTaq™II, Takara) on a Applied Biosystems 7500 FAST real-time PCR system (Life Technologies) using optimized qPCR assays (see Appendix). Primers for qPCR were: 5'-CGGCGGAAGGACTATGAC-3' and 5'-TGTGAGAAAGCCAGAGAGGAA-3' (*CsFT*), 5'-CAGCCAGAGAATCTAACAAACG-3' and 5'-TCAGTTTTGTGGTGGTATTGCC-3' (*CsSL1*), 5'-CCCTGGAGTGCAACAACCT-3' and 5'-CTGATGTGTTTGAGAGCGGT-3' (*CsAP1*), 5'-TCTTGATCCAGGTCCAGAACATC-3' and 5'-TAGTCACCTTGGTTGGGCATT-3' (*CsLFY*), and 5'-CCATGCACCAGAGACCAG-3'

and 5'-GTCTCCCATTTGACCACCA-3' (*CsWUS*). *CsGAPDH* was used as reference gene (5'-GGAAGGTCAAGATCGCAATCAA-3' and 5'-CGTCCCTCTGCAAGATGACTCT-3'). All qPCR assays were validated for specific amplification and optimized for amplification efficiencies between 1.88 and 2.05 with a linear dynamic range of 6 log10 cycles. The sequence of the primers to amplify *CsLFY* was obtained from Nishikawa et al. (2009) whereas all other primer sequences were designed in-house. Relative gene expression was calculated as a fold change ratio using Pfaffl's method (Pfaffl, 2001) with sliding-window efficiencies calculated for each reaction using the `sliwin` function in the `qpcR` R package (Ritz and Spiess, 2008).

4.1.4 Data Analysis

Mean fold change of transcript levels were transformed to a logarithmic scale ($\log_2$) for statistical analysis but data in the graphs represents the untransformed data. Unless noted otherwise, all differences reported are statistically significant ($p<0.05$). All statistical analyses were executed in R (R Development Core Team, 2011).

4.2 Results and Discussion

4.2.1 Gibberellins Down-regulate the Accumulation of Putative Flowering Signals and Floral Identity Genes Transcripts

In *Arabidopsis*, gibberellins (GA) promote flowering by directly up-regulating the floral identity gene *LFY* under short day conditions (Blázquez et al., 1998). In citrus however, exogenous GA inhibit flowering when applied during floral induction (Cooper and Peynado, 1958; García-Luis et al., 1986; Monselise et al., 1964). To test the hypothesis that GA regulates the expression of floral identity genes (*CsAP1* and *CsLFY*) in *C. sinensis* and *Arabidopsis* although with opposite effects, 2.5μl of 90ppm solution of GA were applied directly to buds of shoots 6-7 nodes long of potted *C. sinensis* trees. Pillitteri et al. (2004a) reported that the expression of floral identity genes is kept at basal levels during floral induction and their expression is only up-regulated after transfer to growth promoting conditions. Similar results are also reported in Chapter

2 of this dissertation. Thus, it was assumed that the hypothetical effect of GA on the expression would be easier to determine during the transition from floral-inductive to growth-promoting conditions.

Figure 4-1 shows that application of GA consistently reduced the expression of *CsSL1*, *CsAP1* and *CsLFY*. These results support the hypothesis that GA signals regulate the expression of the same genes in *C. sinensis* and *Arabidopsis* but in opposite ways. Decreased expression of *CsAP1* and *CsLFY* occurred both when GA was applied before and after the transfer to growth-promoting conditions, however, the reduction was greatest when GA was applied before the transfer. Besides *CsSL1*, *CsAP1* and *CsLFY*, the expression of the meristem identity gene *CsWUS* was also measured and similarly reduced by GA application.

Guardiola et al. (1982) suggested that the application of exogenous GA during floral induction could induce reversion of floral meristems to vegetative meristems. Lord and Eckard (1987) reported that hypothetical inflorescence reversion did not occur if GA was applied when sepals had already differentiated. Whereas there is no conclusive evidence on whether actual inflorescence reversion occurs in *C. sinensis*, it is possible that rather than inducing inflorescence reversion, GA disrupt floral identity determination by down-regulating *CsAP1* and *CsLFY* (reversion would not occur since no floral identity has not been determined). Assuming that floral-bud differentiation occurs at the onset of growth-promoting conditions, greater reduction of *CsAP1* and *CsLFY* expression in buds when GA is applied before the transfer to growth-promoting conditions than when it is applied one day after the transfer supports the hypothesis that of GA disrupts acquisition of floral identity. Since the samples used in the experiment reported here consisted of a pool of bud tissue, higher expression of *CsAP1* and *CsLFY* when GA was applied after transfer than before transfer could indicate that some of the buds in the pool had already acquired floral identity and thus were less affected by GA signals.

Application of GA also reduced the expression of *CsWUS*. In *Arabidopsis*, the product of *WUS*, along with the products of *STM* and the *CLAVATA* clade, are key components of the mechanism that maintains meristems undifferentiated and organized (Gallois et al., 2002; Lenhard et al., 2002; Long et al., 1996; Mayer et al., 1998). Furthermore, *WUS* and *LFY* activate the expression of *AG* (Lenhard et al., 2001; Lohmann et al., 2001) which is required for floral organ identity in whorls 3 and 4 (Weigel and Meyerowitz, 1994). Thus, reduced expression of *CsWUS* provides further support to the hypothesis of GA disrupting the acquisition of floral meristem identity.

Besides reduced expression of floral and meristem identity genes, GA application to leaves during induction also reduced the expression of floral signal integrator *CsFT* (Figure 4-2). In *Arabidopsis*, application of GA is key to induce flowering under non-floral-inductive short days (Blázquez et al., 1998). The role of GA on *Arabidopsis* floral induction under long days is less clear (Mutasa-Göttgens and Hedden, 2009), but apparently GA signals are also required for up-regulating *CsFT* (Hisamatsu and King, 2008). Thus, the down-regulation of *CsFT* in response to GA also supports the hypothesis of GA inducing opposite effects on the expression of flowering genes in *C. sinensis* and *Arabidopsis*.

4.2.2 Fruit Proximity

In *C. sinensis*, fruit inhibits floral induction in nearby shoots (Moss, 1971) and trees carrying heavier crops usually flower less profusely than trees with lighter crops (Valiente and Albrigo, 2004). The exact mechanism by which fruit inhibits flowering in *C. sinensis* has not been elucidated. Fruit-induced reduction in carbohydrate levels Goldschmidt et al. (1985), higher concentration of gibberellins in fruit-bearing branches (Koshita et al., 1999) and changes in nitrogen metabolism in fruit-bearing branches (Martínez-Fuentes et al., 2010) have been related to the inhibition of flowering. In this experiment, the hypothesis that fruit inhibits the expression of the flowering signal integrator gene *CsFT* was tested. Based on genetic transformation and gene expression

reports (Endo et al., 2005; Kobayashi et al., 1999; Nishikawa et al., 2007), it was assumed that *CsFT* is in fact a flowering signal, similar to the role of its ortholog in *Arabidopsis* (Corbesier et al., 2007), and thus reductions in the level of expression of this gene in leaves nearby fruit would indicate disruption in the mechanism producing flowering signals.

The expression of *CsFT* was proportional to the distance between fruit and leaves, indicating that the presence of fruit probably reduces the production of flowering signals (assuming that protein expression is proportional to transcript accumulation for this gene, Figure 4-3. The inhibitory effect of fruit on *CsFT* occurs in a gradient and was present at least as far as 30cm away from the fruit. It is possible that expression of floral identity genes were also affected by nearby fruit, however this possibility was not tested. Nonetheless, these results indicated that the flower-inhibiting factor associated with fruit at least disrupts the production of flowering signals in nearby leaves and thus, could partially explain the negative effect of fruit on flowering. At the whole tree level, reduced flowering in trees carrying a heavy crop could be explained by overall reduced production of flowering signals since heavier crops imply shorter average distances between fruit and nearby leaves than lighter crops.

4.2.3 Effect of Light/Dark Cycles on *CsFT* Transcript Accumulation

Photoperiod is a key regulator of the transcription of *FT* in *Arabidopsis* (Turck et al., 2008). In *Arabidopsis*, expression of *FT* is up-regulated by *CONSTANS* (*CO*) in the phloem of leaves (An et al., 2004) and the *FT* protein is transported to the shoot apical meristem (Corbesier et al., 2007) where, along with the product of *FD* (Abe et al., 2005), activates the expression of floral identity genes *LFY* and *AP1* (Samach et al., 2000). Expression of *CO* is controlled by the plant's circadian clock and peaks late in the day (Suarez-Lopez et al., 2001). *CO* transcription is followed by translation of the CO protein, however, in the absence of light, the CO protein is targeted for degradation in the proteosome (Valverde et al., 2004) and does not

up-regulate *FT*. Conversely, under floral-inductive long days, CO is stabilized by light and effectively up-regulates *FT* and promote flowering (Valverde et al., 2004). Since *CsFT* is apparently the citrus ortholog of *Arabidopsis FT*, it was hypothesized that light/dark cycles could also regulate expression of *CsFT* even though floral induction in *C. sinensis* is considered to be photoperiod-insensitive (Moss, 1969). In this experiment trees were exposed to 2 extreme photoperiods for 3 days at floral-inductive temperatures to test whether disrupting light/dark cycles altered the expression of *CsFT* compared to an intermediate photoperiod.

Figure 4-4 shows that either constant light or constant darkness inhibited the expression of *CsFT* compared to the intermediate photoperiod. This indicated that the expression of *CsFT* requires alternation of periods of light and darkness. Moss (1969) reported that flowering was induced in *C. sinensis* trees exposed to floral-inductive temperatures at daylengths ranging from 8 to 16 hours, proposing that floral induction in *C. sinensis* is probably insensitive to photoperiod. Furthermore, Cassin et al. (1969) reported that *C. sinensis* trees are capable of flowering and produce commercial crops even at tropical locations where seasonal changes in daylength are negligible. The results in Figure 4-4 support a role for photoperiod regulating the expression of *CsFT* and floral induction (assuming that *CsFT* is a floral-promoting signal as in *Arabidopsis*). Since flowering in *C. sinensis* can be induced in a wide range of photoperiods, floral-induction in *C. sinensis* may not be as sensitive as floral-induction in *Arabidopsis* but still requires alternation of periods of light and darkness.

4.2.4 Accumulation of *CsFT* Transcripts Changes Throughout the Day

In *Arabidopsis*, the photoperiodic induction of flowering relies on the translation of *CO* protein during periods of light (Turck et al., 2008). The expression of *FT* usually follows the expression of *CO* under floral-inductive long days (Suarez-Lopez et al., 2001). The transcription of *CO* is regulated by components of the circadian clock (Suarez-Lopez et al., 2001) but the activity of *CO* is regulated after translation (Valverde

et al., 2004). Whereas no *C. sinensis* ortholog of *CO* has been characterized, the genome of *C. sinensis* contains several sequences with high degree of sequence similarity to *CO* and *CO-LIKE* genes in other species (data not shown). Furthermore, as reported in the previous section (4.2.3), the up-regulation of *CsFT* by floral-inductive temperatures requires the alternation of periods of light and darkness, a response that could be mediated by a putative *CsCO* ortholog. In this experiment the hypothesis was tested that the expression of *CsFT* changes throughout the day, similar to the expression of *FT* in *Arabidopsis* (Suarez-Lopez et al., 2001).

Figure 4-5 shows that expression of *CsFT* changes throughout the day in *C. sinensis* under the conditions evaluated. Expression of *CsFT* was highest between 13H00 and 15H00 local standard time (LST, UTC-5) and then decreased sharply. The expression of *CsFT* oscillated also under non-floral-inductive conditions (23 °C in growth rooms and irrigated trees in Summer 2010) but the amplitude of the peak was significantly smaller than at floral-inductive temperatures. Expression of *CsFT* started to decrease before the onset of periods of dark suggesting that diurnal changes in expression might be regulated by some other factor besides light. The up-regulation of *CsFT* did not occur under either constant light or darkness (Subsection 4.2.3) so light/dark cycles could still be involved in the regulation of *CsFT*, though less directly. Complete inhibition of *CsFT* up-regulation under constant light or darkness conditions did not support the hypothesis of direct regulation of *CsFT* expression by the tree's internal clock. Since diurnal changes in the expression of *CsFT* could be induced by various factors, the diurnal cycling supports the hypothesis that *CsFT* could be regulated by a putative *CO* ortholog.

4.2.5 Early Changes in Transcript Levels of *CsFT* in Response to Floral Inductive Temperatures

The expression of *CsFT* has been shown to change seasonally in trees growing in the field and in response to artificial floral inductive treatments (Nishikawa et al.

2007, 2009 and Chapter 2 of this dissertation). However, details about the earliest changes in transcription of *CsFT* after initial exposure to floral inductive stimuli are lacking. Assuming that *CsFT* is a flowering signal as in *Arabidopsis*, describing the earliest responses of *CsFT* could help elucidate characteristics of the mechanism regulating *CsFT* expression and floral induction. In this set of experiments changes in the expression of *CsFT* taking place within the first 3 days of exposure to floral-inductive conditions are described.

Figure 4-6 shows the expression of *CsFT* within 24 hours of transfer to floral-inductive temperatures. In this experiment trees were transferred either in the morning or in the afternoon to test whether processes occurring early in the day (or during the evening/night) could be related to immediate up-regulation of *CsFT*. Expression of *CsFT* was significantly up-regulated regardless of the time at which trees were transferred, indicating that the temperature-sensing mechanism regulating *CsFT* expression is active throughout the day. However, the magnitude of the up-regulation of *CsFT* 2 hours after the transfer was higher in trees transferred at 15H00 LST, indicating that *CsFT* sensitivity to changes in temperature is higher at this time than earlier in the morning. Furthermore, at 17H00 LST the level of expression *CsFT* in trees transferred at 07H00 was equivalent to the level of expression of *CsFT* in trees transferred at 15H00 LST, indicating that the effect of the exposure to floral-inductive temperatures for 10h through most of the day on *CsFT* was equivalent to the effect of exposing the trees for 2h to floral-inductive temperatures in the afternoon.

On the next day, the level of expression of *CsFT* in trees transferred at 08H00 LST the day before was no different than the level of *CsFT* expression in trees transferred at 15H00 LST the day before at any of the times sampled. Diurnal changes in *CsFT* expression also occurred in this experiment as reported before (Figure 4-5). The level of expression of *CsFT* in morning samples on the day after the transfer were equivalent to the level of expression of *CsFT* at 17H00 on the day of the transfer. This

may indicate that floral induction is intensified with time by maintaining the levels of expression of *CsFT* reached by the end of the day overnight and re-setting the diurnal cycle at continuously increasing levels. This response was also observed in field-grown trees during the winter of 2010 before and after the passing of cold fronts that reduced ambient temperatures below the hypothetical floral-inductive threshold of 20°C (Figure 4-7). In the latter case, cooling also occurred by the end of the day and the lowest level of the diurnal oscillation in *CsFT* expression was continuously shifted in the two days following the drop in ambient temperatures.

In *C. sinensis*, temperatures ranging from 5°C to about 20°C are considered to be floral-inductive (García-Luis et al., 1992; Moss, 1969; Valiente and Albrigo, 2004). Within this range, temperatures between 10°C and 15°C induce flowering most intensely. To test whether transcript accumulation of *CsFT* is sensitive to levels of temperature, trees were kept at growth-promoting temperatures and then transferred to rooms at different floral-inductive temperatures and expression of *CsFT* was quantified three days later. Figure 4-8 shows that the expression of *CsFT* was sensitive to levels of floral-inductive temperatures. Consistent with earlier reports of floral induction (Moss, 1969), transcript accumulation of *CsFT* was highest after transfer to 15°C. At temperatures lower than 15°C transcript accumulation of *CsFT* was also increased whereas transcript accumulation at 20°C was higher but not statistically different than transcript accumulation at 23°C (growth-promoting non-floral-inductive). These results support the hypothesis of *CsFT* acting as a quantitative and qualitative integrator of temperature-regulated flowering signals.

Results of the experiments reported in this chapter show that the negative regulation of flowering by gibberellins (Monselise et al., 1964) could be a consequence of reduced levels of expression of *CsFT* (assumed to be a floral-promoting signal) and reduced levels of expression of floral identity genes in buds. Also, these results show that, different from *Arabidopsis* (Blázquez et al., 1998), gibberellins negatively regulate

the expression of *CsLFY* and *CsAP1* in *C. sinensis* which supports a hypothesis for diverging evolution of mechanisms regulating the response of flowering genes to gibberellin signals. It was also shown that reduced levels of floral induction associated with the presence of fruit and crop load (Moss, 1971; Valiente and Albrigo, 2004), could be a consequence of reduced expression of *CsFT* in the proximity of fruit. Even though gibberellin levels were not quantified in this study, higher concentration of endogenous gibberellins near fruit (Koshita et al., 1999) could be related to the reduced level of expression of *CsFT* since application of exogenous gibberellin reduce the expression of *CsFT*. Other factors that have been proposed as possible factors that reduce the level of floral induction in fruit-bearing branches such as carbohydrates (García-Luis et al., 1988) and nitrogen metabolism (Martínez-Fuentes et al., 2010) were not tested in this study. The results presented in this chapter also show that *C. sinensis* requires alternation of cycles of light and darkness for floral induction, indicating that floral induction in *C. sinensis* is not insensitive to photoperiod signals, but instead sensitivity to photoperiod is much reduced compared to other species. Furthermore, the mechanism regulating floral induction in *C. sinensis* seems also to be regulated by diurnal cycles with sensitivity to floral inductive signals being highest in the afternoon-evening. Continuous increase in the level of floral induction could be explained by continuously shifting the lowest level of the oscillation overnight. Together, the results presented in this Chapter offer details about the regulation of flowering-related genes in *C. sinensis* by factors known to modify the level of floral induction. Some of these factors (i.e. gibberellins and light) induce critical diverging responses in *C. sinensis* and the model *Arabidopsis*. Thus, characterizing the response of flowering-related genes to these factors could help elucidate where the differences in the mechanisms regulating flowering in *C. sinensis* and *Arabidopsis* could be. In addition, the earliest changes in transcript accumulation of *CsFT* (assumed to be the floral-promoting signal in *C. sinensis*) in response to floral-inductive temperature, were

also described and provide insights about the mechanism regulating floral induction in *C. sinensis*.

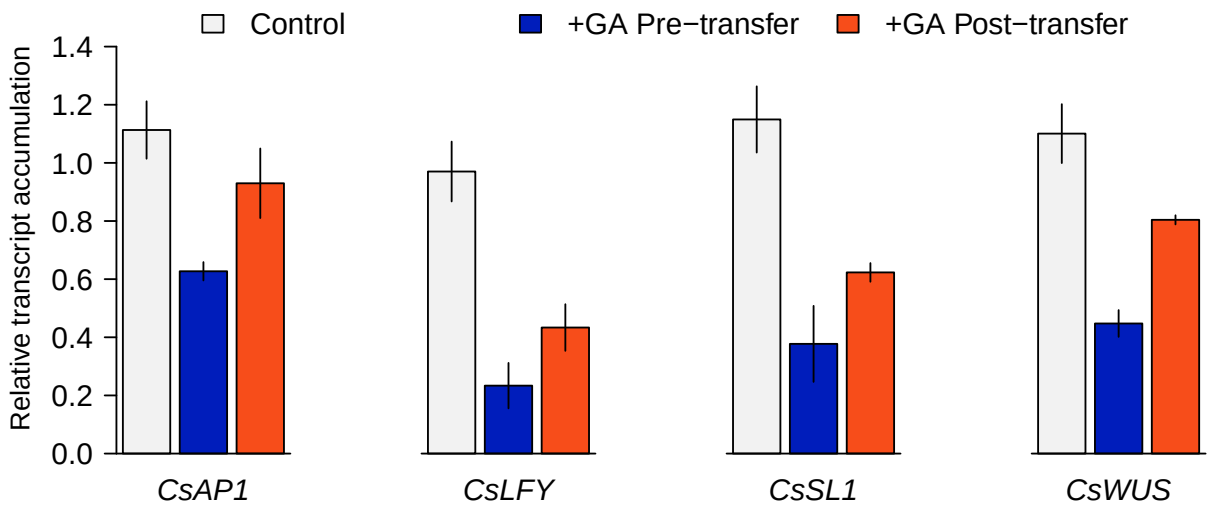


Figure 4-1. Expression of *CsAP1*, *CsLFY*, *CsSL1* and *CsWUS* in buds of 2 year old 'Valencia' trees treated with gibberellic acid one day before or one day after transfer from floral-inductive to growth-promoting conditions. Three sets of 3 trees each were exposed to floral-inductive temperatures (12°C) for 30 days. On the 30th day of the experiment ("pre-transfer") the buds of 4 shoots in one set of trees were treated with 2.5μl of a 90ppm gibberellic acid 0.5% Tween 80 solution using a micropipette. The 3 sets of trees were then transferred to a chamber at growth-promoting temperatures (23°C). On the 31st day of the experiment ("post-transfer"), the buds of 4 shoots in another set of trees were treated with gibberellic acid as before. The buds on the shoots of the trees in the last set were treated with the same solution used before except that the solution did not contain gibberellic acid ("Untreated"). Bud samples were collected on the 33rd day of the experiment (3 days after transfer). Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of each gene in the "Untreated" buds.

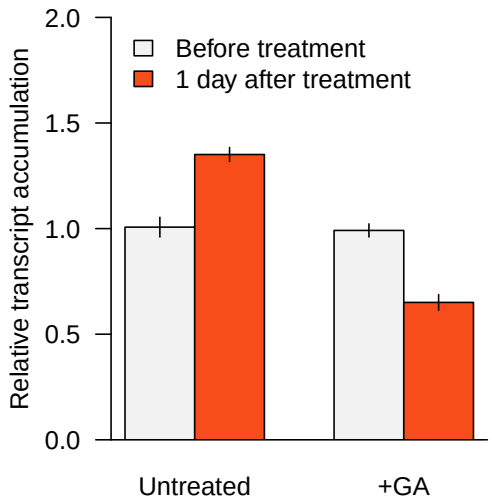


Figure 4-2. Expression of *CsFT* in leaves of 2 year old 'Valencia' trees treated with gibberellic acid during floral induction. Two sets of 4 trees were exposed to floral-inductive temperatures (12°C) for 25 days. On day 25, the leaves of 4 shoots in one set of trees were treated with a 90ppm gibberellic acid 0.5% Tween 80 solution using a cotton swab ("+GA"). The leaves of 4 shoots in the other set of trees were treated using a similar solution without gibberellic acid ("Untreated"). Leaf discs from shoots in both set of trees were collected before application of the treatments and on the next day. Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of *CsFT* in "Untreated" leaves before treatments were applied.

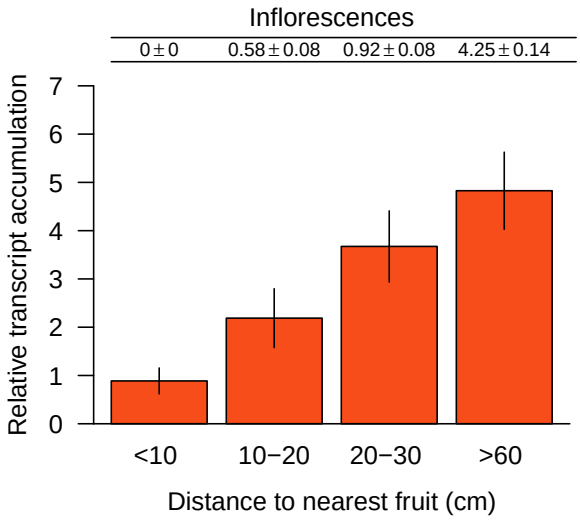


Figure 4-3. Expression of *CsFT* in leaves of 15 year old 'Valencia' in the field located at different distances from single fruit. Leaves at located at different distances from single fruit in the same branch were sampled in December of 2009 on 4 branches of three different trees from the same grove. The leaves sampled were located in shoots formed during the previous year. The distance to the nearest fruit was measured adding up the length of individual shoot segments (formed in preceding growth cycles) separating the fruit from the sampled leaf. Table on top of the figure indicates the number of inflorescences that formed in the sampled shoots in the spring. Figures are means of 3 tree-replicates ± S.E. (4 shoot-subreplicates). Gene expression is relative to the levels of *CsFT* in leaves located in shoots at $<$10cm from a fruit.

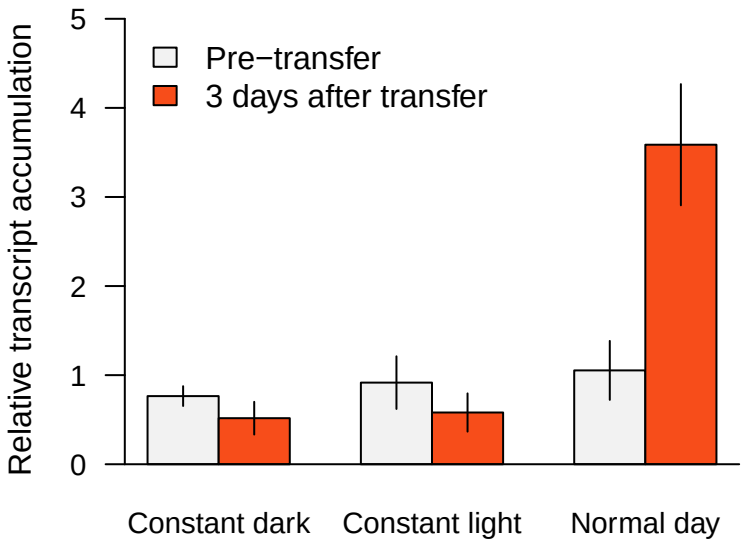


Figure 4-4. Expression of *CsFT* in leaves of 2 year old 'Valencia' trees under constant light, constant darkness and an intermediate photoperiod ("Normal day", 11h light / 13h darkness) at floral-inductive conditions (12°C). Three sets of 3 trees were exposed for 3 days to each of the light regimes. Leaves were sampled before transfer to the growth rooms and 3 days after. Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of *CsFT* before the transfer.

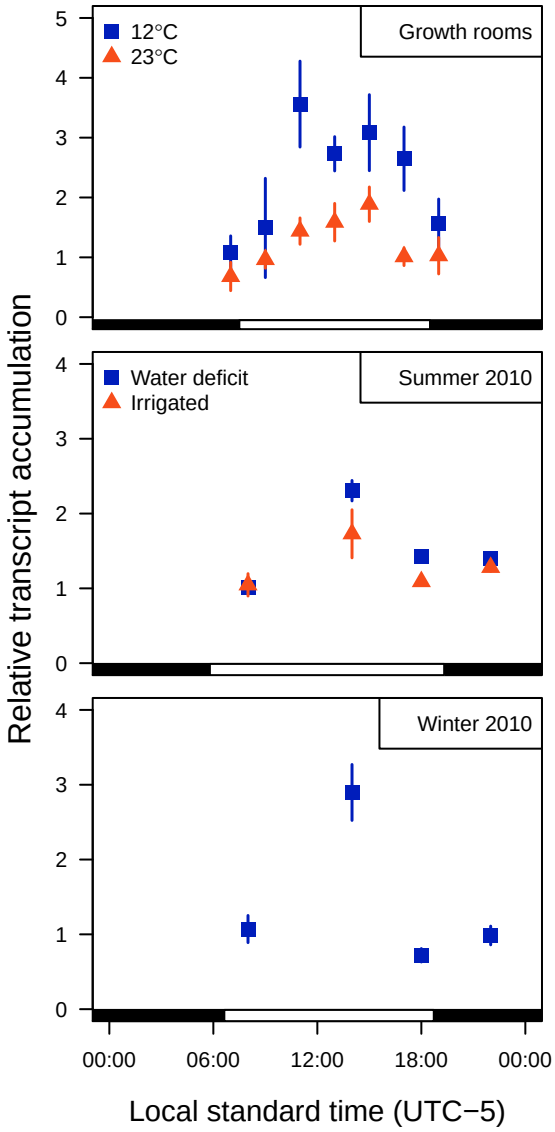


Figure 4-5. Expression of *CsFT* in leaves of potted 2 year old 'Valencia' trees (Growth rooms) and field-grown 15 year old 'Valencia' trees (Summer 2010 and Winter 2010) at different times during the day. Leaf samples were collected at different times over 2 days from 3 different trees for each condition evaluated. In the growth room experiment trees were exposed to either 12 or 23 °C constantly, in the Summer 2010 (July) experiment, trees were exposed to either water deficit or received normal irrigation, in the Winter 2010 (December) experiment trees received normal irrigation and were exposed to naturally occurring floral-inductive temperatures. Data are means of 6 tree-replicates ± S.E. (2 consecutive days, 3 samples per time-point). Gene expression is relative to the levels of *CsFT* in the first samples collected in the morning (07H00 or 08H00). Black/white horizontal bars at the bottom of each graph represents the photoperiod.

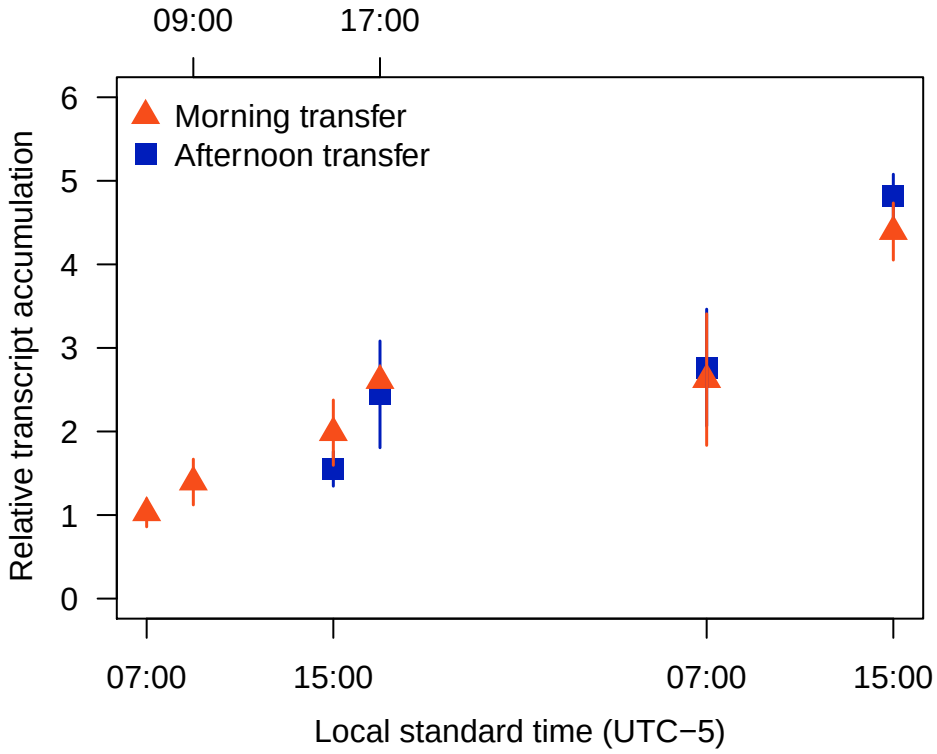


Figure 4-6. Expression of *CsFT* in leaves of 3 year old 'Valencia' trees after transfer to floral-inductive temperatures (12°C). Two sets of 3 trees maintained at 23°C were transferred either at 07H00 or 15H00 to a growth room at 12°C (floral-inductive). Leaves were sampled before transfer, two hours after the transfer and on the next day. Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of *CsFT* before the 08H00 transfer.

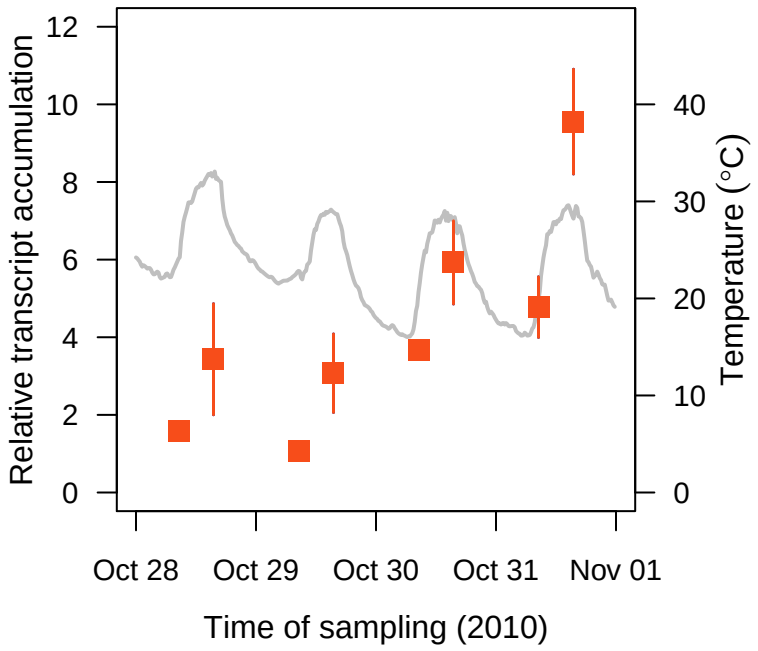


Figure 4-7. Expression of *CsFT* in leaves of field-grown 15 year old 'Valencia' trees during the passing of a cold front in the Fall of 2010 (filled squares). Based on weather forecasts, leaves were sampled two days before and two days after the passing of a cold front that caused a reduction in night temperatures below 20°C (grey line). Leaves were sampled at 08H00 and 15H00 local standard time (UTC-5). Figures are means of 4 tree-replicates ± S.E. Gene expression is relative to the levels of *CsFT* before at 08H00 on Oct. 28.

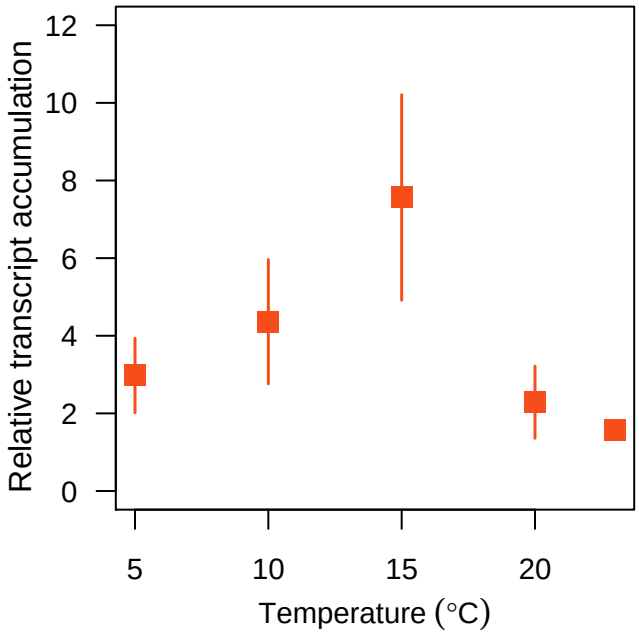


Figure 4-8. Expression of *CsFT* in leaves of 3 year old 'Valencia' trees after transfer to 3 floral-inductive temperatures. Four sets of 3 trees each kept at 23°C were transferred to growth rooms at either 5, 10 or 15°C or kept at 23°C. Leaves were sampled before transfer to the growth rooms at 15H00 and 3 days after the transfer at the same time. Figures are means of 3 tree-replicates ± S.E. Gene expression is relative to the levels of *CsFT* before the transfer.

CHAPTER 5
CONCLUSIONS

The objective of my study was to characterize changes in transcript accumulation of *C. sinensis* flowering-related genes in response to treatments known to alter floral induction and determination. Figure 5-1 shows a graphic summary of the conclusions reached from the results of the experiments reported in previous chapters.

I found that the expression of *CsFT* is up-regulated by both known floral-inductive stimuli of *C. sinensis*, water deficit and low temperatures. Up-regulation of *CsFT* after exposure to low-temperature had been reported earlier (Nishikawa et al., 2007) but there had been no reports about the response of *CsFT* (or its putative orthologs in other species) in response to floral-inductive water deficit. Furthermore, when low temperature and water deficit were present at the same time, accumulation of *CsFT* transcripts was induced above the levels observed when each stimulus was present separately. This, suggests that *CsFT* could be an integrator of flowering signals initiated by both flowering stimuli in *C. sinensis*. Furthermore, I also reported that expression of *CsFT* was increased as the time under floral-inductive conditions increased and was sensitive to levels of temperature. Another effect of water deficit was to repress up-regulation of floral identity genes *CsAP1* and *CsLFY* during periods of warmer weather during the winter. This repression of *CsAP1* and *CsLFY* could keep buds undifferentiated and responsive to additional floral-inductive signals when temperatures decrease again later in the winter. Flowering in *C. sinensis* could be considered as a quantitative response to levels of floral induction intensity. My results support a role for *CsFT* as a quantitative marker of floral induction intensity.

In the model species *Arabidopsis*, *CsFT*'s ortholog, *FT*, is a key component in the mechanism that controls flowering (Turck et al., 2008). However, flowering responses of each *Arabidopsis* and *C. sinensis* to floral-inductive stimuli in the other species do not support the existence of an exact common mechanism regulating flowering in both

species (Amasino, 2010; Krajewsky and Rabe, 1995). Consequently, it is proposed that regulation of expression of *CsFT* in *C. sinensis* and *FT* in *Arabidopsis* have evolved to respond to different environmental stimuli. Evolution of flowering control mechanism involving *FT* orthologs has been reported in short-day rice (Hayama and Coupland, 2004). Even if *FT*-mediated control of flowering in *C. sinensis* and *Arabidopsis* evolved to respond to different environmental stimuli, some characteristics of the mechanism related with *FT* are conserved such as diurnal changes in the level of expression of *CsFT* and the dependence on light to enable up-regulation of *CsFT*. *CsFT* expression dependence of light/dark cycles indicated that although not as sensitive as *Arabidopsis* and other species induced to flower by specific photoperiods, floral induction in *C. sinensis* is not totally insensitive to photoperiod.

In addition to *CsFT*, I also found that the response of *CsAP1* and *CsLFY* to exogenous gibberellins was opposite to the response of *Arabidopsis*' *AP1* and *LFY* to similar treatments. Consistent with reduced formation of inflorescences after the application of gibberellins (Monselise et al., 1964), expression of *CsAP1* and *CsLFY* (whose expression is assumed to determine floral identity and initiate floral differentiation) was significantly reduced by the application of gibberellins to buds. However, gibberellin application in *Arabidopsis* up-regulates the expression of these genes and promote flowering under non-floral-inductive short days (Blázquez et al., 1998). My results also show that the inhibitory effect of gibberellins on the transcript accumulation of *CsAP1* and *CsLFY* is greater before growth has been stimulated by warmer temperatures, suggesting that floral determination and initiation might not occur while under floral-inductive conditions and require the re-initiation of growth. The pattern of expression of *CsAP1* and *CsLFY* was more related to the establishment of a flowering gradient and the initiation of multiple flowering cohorts than the pattern of expression of *CsFT*. This suggested that flowering gradients are not a consequence of biased production of flowering signals near the apex but maybe a consequence of

biased distribution of flowering signals and/or a gradient in the sensitivity of buds at different position within the shoot to flowering signals.

A major limitation of this study was that it was restricted to characterize the expression of flowering-related genes at the transcript level and did not explore other levels of regulation of the expression of these genes. Regulation of the activity of flowering genes before or after transcription is a key component of the flowering regulatory mechanisms in *Arabidopsis* and other species in which these mechanisms have been more thoroughly investigated (Adrian et al., 2010; Dennis and Peacock, 2007; Valverde et al., 2004). However, regulation of transcription is still a major factor in the mechanisms controlling flowering in model species, and comparing those patterns of transcript accumulation of flowering-related genes with those in *C. sinensis* and model species can help identify where mechanisms regulating flowering in these species differ. Another potential limitation of this study is that it relies on the assumption of orthology between genes in *C. sinensis* and model species. Although reports of transgenic over-expression, *Arabidopsis* mutant complementation, and expression patterns data support this assumption (Endo et al., 2005; Kobayashi et al., 1999; Nishikawa et al., 2007; Peña et al., 2001; Pillitteri et al., 2004a; Tan and Swain, 2007), the activity and function of the genes used in this study has not been confirmed in citrus. Nonetheless, this study provides useful information for developing models of how floral induction and initiation is regulated at the genetic level in *C. sinensis*. Some of the findings of this study could also be relevant to elucidating the flowering regulatory mechanisms in other citrus cultivars and species originated in subtropical climates.

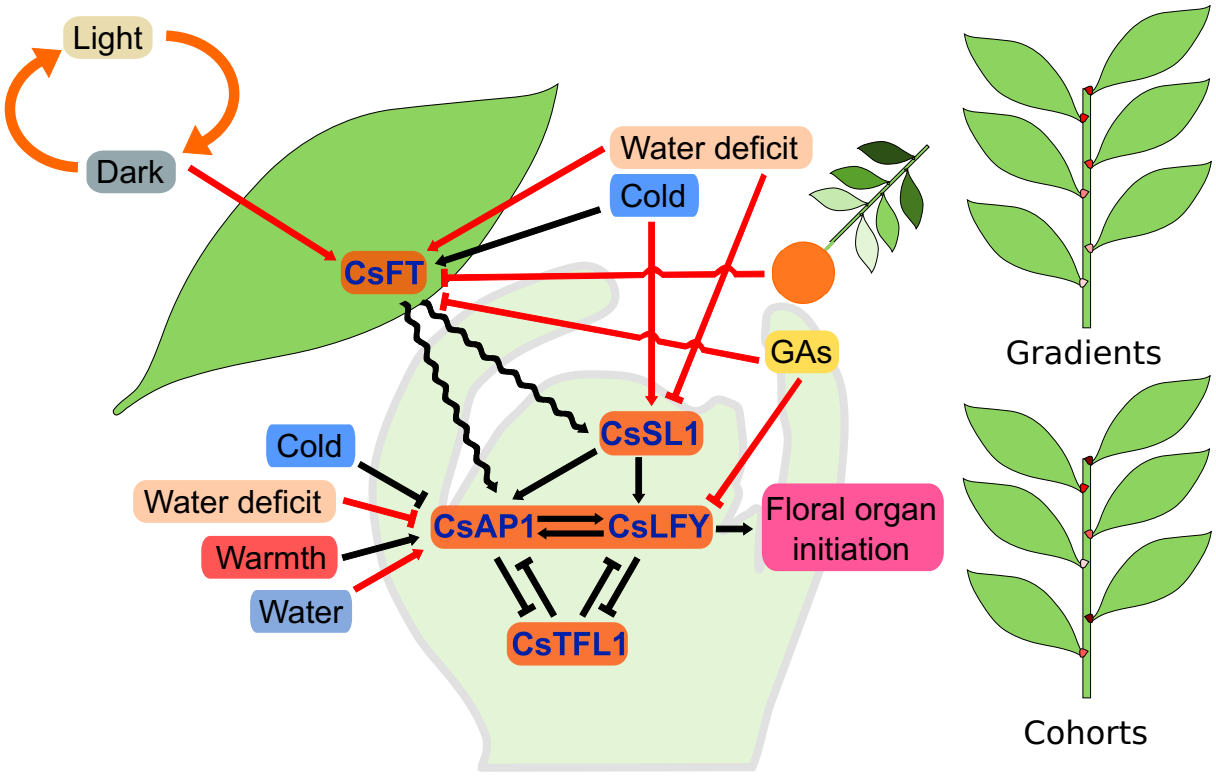


Figure 5-1. Graphical summary of the conclusions of this dissertation.

APPENDIX
DESIGN, VALIDATION AND OPTIMIZATION OF qPCR ASSAYS

qPCR assays to quantify *CsFT*, *CsSL1*, *CsAP1*, *CsLFY* and *CsWUS* transcripts in *C. sinensis* were designed from *C. sinensis* and *C. unshiu* sequences of these genes available in the NCBI's Nucleotide database and optimized using the algorithm in Figure A-2.

For each assay, primer pairs that amplified products between 60 and 200 base pairs (bp) were selected (Table A-1). Amplification specificity of these primers was checked by agarose gel electrophoresis (Figure A-3A) and by generating dissociation curves of the amplified products on a Applied Biosystems 7500 FAST real-time PCR system (Life Technologies) (Figure A-3B). The amplified products were then cloned into a vector and sequenced to confirm each amplicon's identity. Amplification efficiencies for each assay were calculated for each reaction using the `sli.win` function (Ritz and Spiess, 2008) and by the dilution curve method (Figure A-4). The linear dynamic range of the qPCR assays were determined by the dilution curve method (Figure A-4).

RNA was extracted as indicated in the Materials and Methods section of each chapter in batches of 30 to 50 samples. From each batch, 6 samples were randomly selected to check RNA integrity by electrophoresis of glyoxylated RNA. When degradation of RNA was suspected, the rest of the samples of the batch were checked as already indicated and the suspected degraded RNA samples discarded. Reverse transcription reactions were run as indicated in the Materials and Methods section of each chapter in batches of 30 samples. In each batch, control reactions that did not contain reverse transcriptase in the reaction mix were run using 5 randomly selected samples to check for potential DNA contamination after subsequent amplification by qPCR. No amplification products were detected in any of these control reactions.

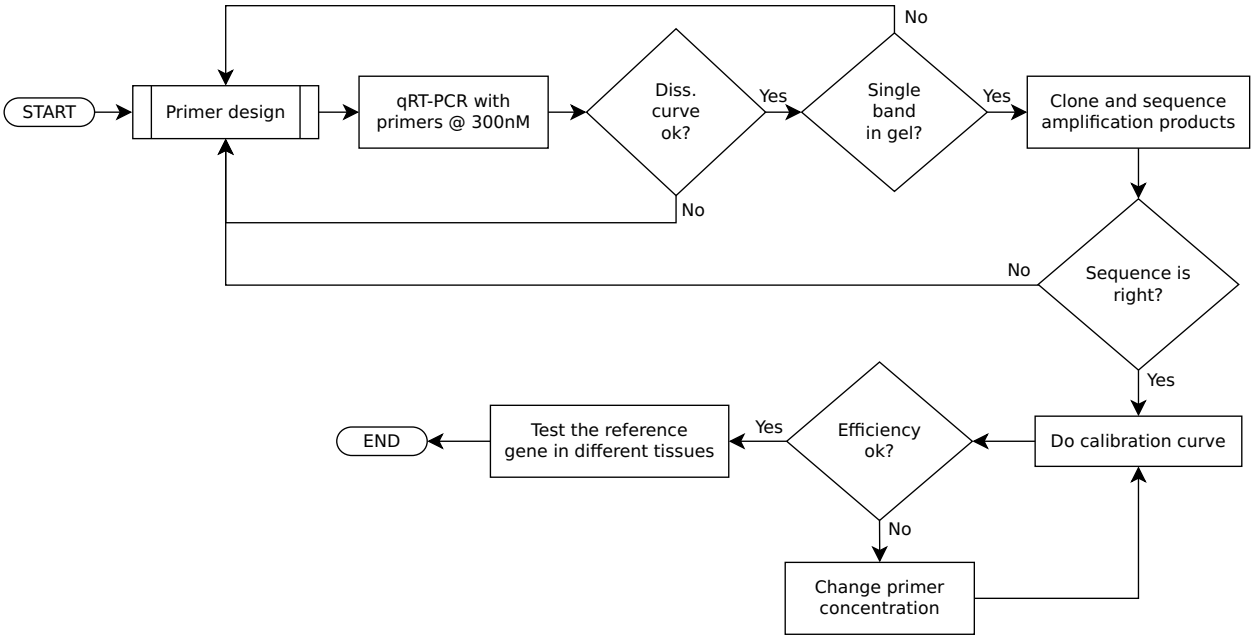


Figure A-2. Algorithm for the design, validation and optimization of qPCR assays.

Table A-1. Primer pairs used for transcript quantification assays by qPCR.

Target	Amplicon length (bp)	Primers (5'→ 3')	Reference
CsFT	173	CGGCGGAAGGACTATGAC	This dissertation
		TGTGAGAAAGCCAGAGAGGAA	This dissertation
CsSL1	120	CAGCCAGAGAATCTAACAAACG	Tan and Swain (2007)
		TCAGTTTTGTGGTGGTATTGCC	This dissertation
CsAP1	145	CCCTGGAGTGCAACAACCT	This dissertation
		CTGATGTGTTTGAGAGCGGT	This dissertation
CsLFY	63	TCTTGATCCAGGTCCAGAACATC	Nishikawa et al. (2009)
		TAGTCACCTTGGTTGGGCATT	Nishikawa et al. (2009)
CsWUS	143	CCATGCACCAGAGACCAG	This dissertation
		GTCTCCCATTTGACCACCA	This dissertation
CsGAPDH	75	GGAAGGTCAAGATCGCAATCAA	Alferez et al. (2008)
		CGTCCCTCTGCAAGATGACTCT	Alferez et al. (2008)

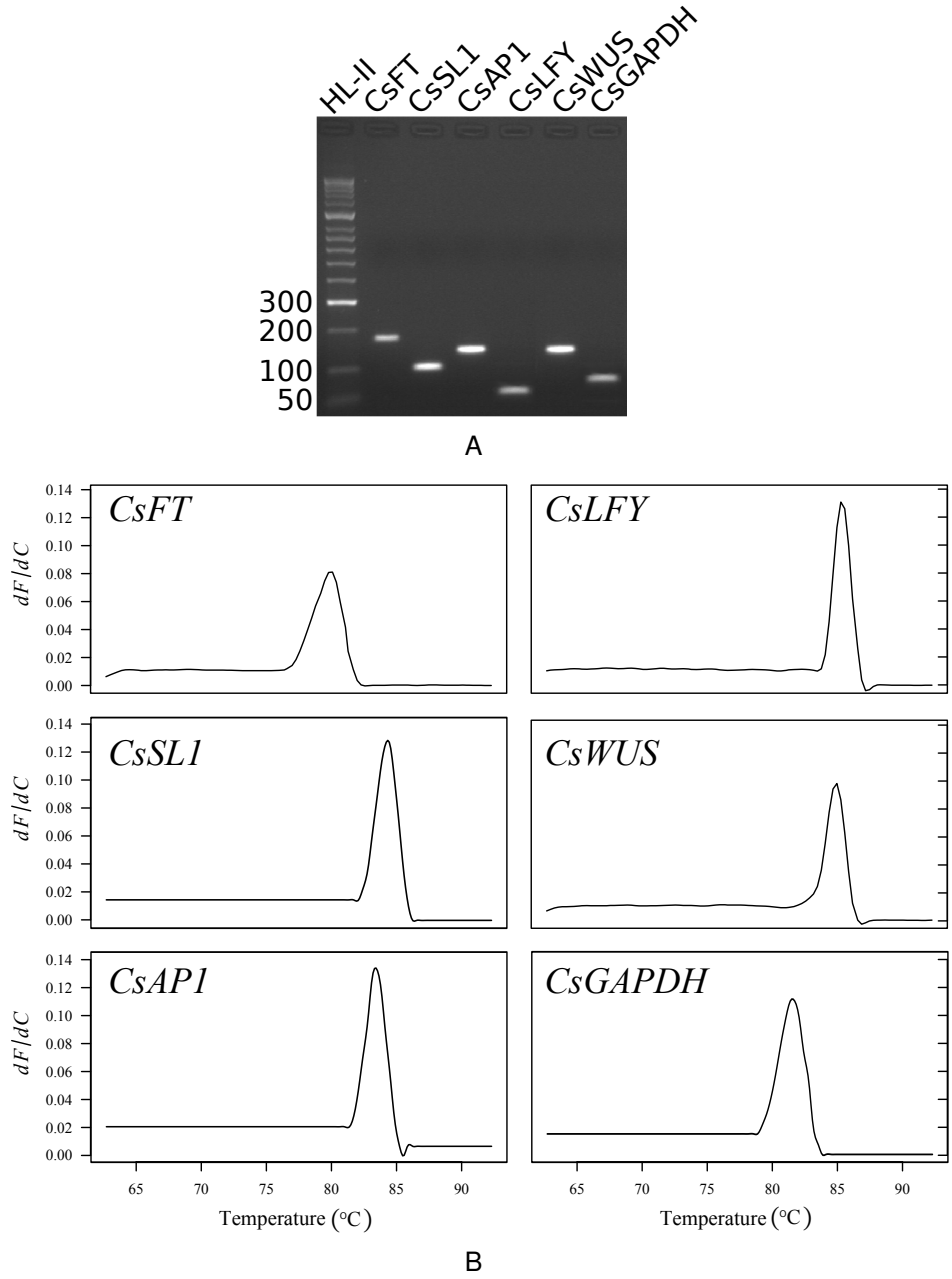


Figure A-3. Amplification specificity of qPCR assays. A) Agarose gel electrophoresis of qPCR products of each of the assays. B) Dissociation curves of qPCR products of each assay.

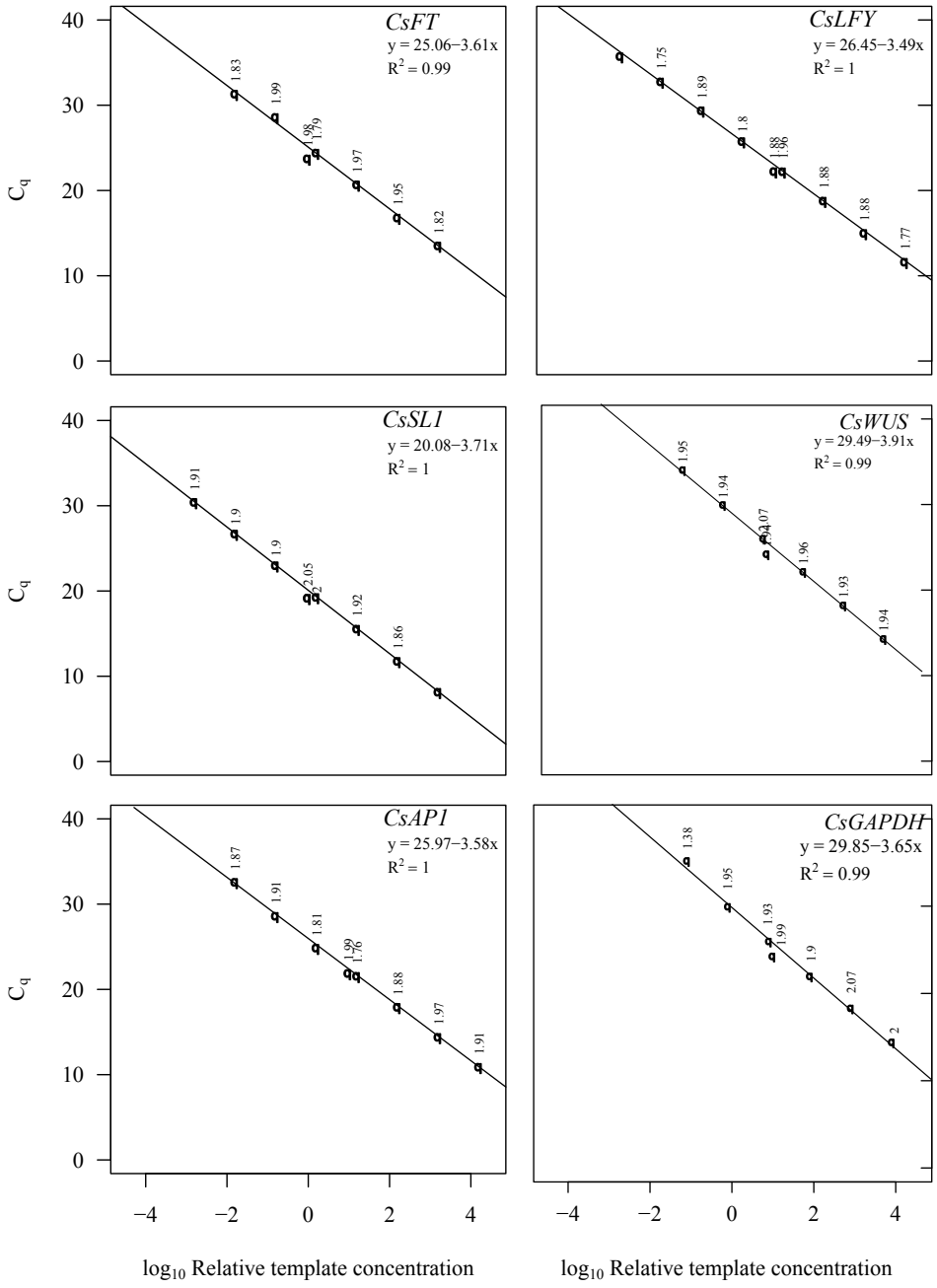


Figure A-4. Linear dynamic range and amplification efficiency of qPCR assays. Numbers above each symbol represent individual reaction efficiency calculated using the `sli.win` function in the `qpcR` R package (Ritz and Spiess, 2008)

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BIOGRAPHICAL SKETCH

Eduardo José Chica Martínez was born in 1982 in Guayaquil, Ecuador. In 2005, he obtained the degree of Ingeniero Agropecuario from the Escuela Superior Politécnica del Litoral in Guayaquil. In 2007, he obtained his M.Sc. degree from the Horticultural Science Department at the University of Florida. In 2011, he obtained his Ph.D. degree from the Horticultural Science Department at the University of Florida.