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Terminal Flower, Leafy and *Apetala1* Homologues and Their Relationship to Juvenility in *Citrus sinensis* – An overview

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Abstract. **In Arabidopsis and other herbaceous species, *TERMINAL FLOWER* is a key regulator of floral timing, whereas *LEAFY* and *APETALA1* are meristem identity genes that regulate flower formation. Homologues of these genes were isolated from the 'Washington' navel orange (*Citrus sinensis* L. Osbeck). The deduced amino acid sequences of CsTFL, CsLFY and CsAP1 were 65% identical to their Arabidopsis counterparts. Wild-type Arabidopsis plants ectopically expressing *CsTFL* showed late-flowering phenotypes and those ectopically expressing *CsLFY* or *CsAP1* showed early-flowering phenotypes similar to those described for the overexpression of Arabidopsis *TFL*, *LFY* or *AP1*, respectively. The *35S:CsTFL*, *35S:CsLFY* and *35S:CsAP1* transgenes complemented the *tfl1*-2, *lfy-10*, or *ap1-3* mutants, respectively. In each case the severity of the overexpression phenotypes correlated with the amount of transcript that accumulated. Among species studied, *C. sinensis* proved unique in maintaining the heterozygosity of its hybrid origin (*C. maxima* x *C. reticulate*). Two alleles were easily distinguishable for each floral gene. The pattern of *CsTFL* gene expression was distinct from that of most other plant *TFL* genes; *CsTFL* transcripts accumulated in all floral organs but were undetectable in adult vegetative tissues. Results of real-time PCR demonstrated that juvenility in citrus was positively correlated with *CsTFL* transcript accumulation and negatively correlated with *CsLFY* and *CsAP1* RNA levels.**

Introduction

The juvenile phase of plant development is characterized by reproductive incompetence, i.e., an inability to initiate floral development in response to stimuli that promote flowering in reproductively competent adult plants (Hackett, 1985; Poethig, 1990). In citrus, the juvenile phase can last from 2 to 13 years depending on the cultivar (Davies and Albrigo, 1994). Plants that reach the adult phase of vegetative development, although reproductively competent, typically require an appropriate environmental signal to transition from vegetative to reproductive growth. Environmental stimuli necessary to induce flowering are established for many perennial tree crops. However, documentation of the gene activities underlying the vegetative to floral transition is relatively limited. Only *Populus balsamifera*, *Malus* x domestica, and *Eucalyptus globulus* have been extensively investigated (Kyozuka et al., 1997; Southerton et al., 1998; Sung et al., 1999; Kotoda et al., 2000; Rottmann et al., 2000).

In contrast, research over the past decade has resulted in the identification and characterization of numerous genes that disrupt vegetative phase transition or alter meristem identity in the herbaceous annual *Arabidopsis thaliana* (Bowman et al., 1993; Weigel and Nilsson, 1995; Liljegren et al., 1999; Pelaz et al., 2001). Among these genes, *TERMINAL FLOWER* (*TFL*) has been shown to be important for delaying flowering and regulating plant growth through maintenance of indeterminancy of the shoot apex (Shannon and Meeks-Wagner, 1993; Ratcliffe et al., 1998).

Whereas *TFL* maintains the meristem in an indeterminate state, production of determinate floral meristems is accomplished by the cooperative activities of floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Mandel and Yanofsky, 1995; Pelaz et al., 2001). Loss of *LFY* or *AP1* function results in flower-to-shoot conversion along the inflorescence. *LFY* encodes a plant-specific transcription factor and is considered a master regulator of floral meristem development (Weigel et al., 1992). *AP1* is a member of the MADS-box gene family of transcription factors, which play critical roles in developmental processes across the plant, animal, and fungal kingdoms (Schwarz-Sommer et al., 1990). In addition to regulating the establishment and determinacy of the floral meristem, *AP1* plays a role in determining sepal and petal identity (Bowman et al., 1993).

Both *LFY* and *AP1* genes have been identified in members of diverse plant families, and in some cases, have been shown to be functionally equivalent, indicating some degree of conservation of floral regulatory pathways among plant families (Kelly et al., 1995; Mena et al., 1995). Consistent with this, overexpression of either *AtLFY* or *AtAP1* is sufficient to promote precocious flowering in distantly related species (Rottmann et al., 2000; Weigel and Nilsson, 1995), including *Citrus* (Pena et al., 2001). However, in other cases, differences in function and expression patterns were identified (Ahearn et al., 2001; Kyozuka et al., 1998).

Recently, details of the molecular interactions among floral regulatory genes revealed that the opposing activities of *TFL* and *LFY* and *AP1* are spatially separated within the meristem and are maintained through a mutual inhibition mechanism (Liljegren et al., 1999; Samach et al., 2000; Pelaz et al., 2001). TFL prevents floral development by blocking both the expression and activities of LFY and AP1 in the central dome of the shoot apex (Liljegren et al., 1999). Reciprocally, both LFY and AP1 have roles in the negative regulation of *TFL* along the flanks of the inflorescence to promote floral development (Ratcliffe et al., 1999). Loss-of-function mutations in *TFL* result in *LFY* and *AP1* expression

in the shoot apex; *TFL* is expressed in the meristem of *LFY* or *AP1* mutants. *TFL* expression is reduced when *LFY* or *AP1* are constitutively expressed. Evidence suggests that the ratio of *LFY:TFL* transcript accumulation determines meristem fate. A higher ratio results in shortening of the vegetative phase and production of floral meristems (Ratcliffe et al., 1999).

Citrus sinensis has traits that are unlike many other perennial trees studied thus far. As a subtropical perennial, floral initiation and development occur within a single growing season without the winter dormancy typical of deciduous tree crops and forest species. Molecular and phytochemical data in the literature predict that *C. sinensis* is a pummelo (*C. maxima*) x mandarin (*C. reticulata*) hybrid (Nicolosi et al., 2000) that maintains relatively high heterozygosity (Federici et al., 1998) due to vegetative propagation through grafting and the production of apopmictic seedlings through nucellar embryony (Pedrosa et al., 2000). These attributes make citrus a novel perennial tree crop to study and a potentially useful model for broadening our understanding of floral development.

Presented herein is an overview of the results of research under taken to investigate molecular mechanisms underlying juvenility and flower production in *C. sinensis. TFL, LFY* and *AP1* homologues (*CsTFL, CsLFY* and *CsAP1*) from 'Washington' navel orange were isolated and their structural and functional similarities to TFL, LFY and AP1 homologues from other plant species were determined. In addition, the accumulation of *CsTFL, CsLFY* and *CsAP1* transcripts was compared in juvenile (florally incompetent) and adult (florally competent) citrus trees using real-time PCR. The details of this research are reported in Pillitteri (2002) and Pillitteri et al. (2004).

Materials and Methods

Plant material and tissue collection. 'Washington' navel orange (*Citrus sinensis* L. Osbeck) scions on 'Carrizo' citrange (*C. sinensis* x *Poncirus trifoliata* L. Raf.) rootstock (18 years old) located at the University of California, Riverside (UCR) Agricultural Experimental Station were used in the research. Adult stems with a high probability of producing floral shoots were selected using the criteria of Lord and Eckard (1985). For RNA isolation, the apical four buds from 14 stems were excised from the leaf axil. For real-time PCR, 5-year-old 'Washington' navel orange trees (adult) and 4-month-old seedlings (variety CRC3306A, juvenile) were maintained at 15 °C day/10 °C night temperatures for 8 weeks (low-temperature floral-induction treatment) followed by 3 weeks at 24 °C day/19 °C night temperatures (warm temperature control conditions) under 16-h days/8-h nights.

Nucleic acid extraction. Citrus genomic DNA used in PCR and genome walking was isolated by a CTAB-based method (Webb and Knapp, 1990). *C. sinensis* genomic DNA used for restriction enzyme digests was isolated by CsCl banding (Fischer and Goldberg, 1982). RNA was isolated using a LiCl method (Puthoff, 1999).

Isolation of CsTFL, CsLFY and CsAP1 genomic clones and cDNAs. Degenerate primer pairs were designed for *TFL, LFY* and *AP1* based on conserved coding sequence among homologues identified in Genbank. Forward and reverse primer pairs and conditions for genomic PCR are found in Pillitteri (2002). Overlapping clones were isolated using Universal GenomeWalker kit (Clontech, Palo Alto, Calif.). A full description of primer sequences and product sizes are given in Pillitteri (2002). All PCR products were sequenced at the UCR Genomics Institute Core Facility. The genomic sequences of *CsTFL, CsLFY* and *CsAP1* are in GenBank accessions AY344245, AY338976 and AY338975, respectively. *CsTFL* and *CsAP1* cDNA sequences are reported in Genbank accessions AY344244 and AY33894, respectively.

Citrus DNA blots and PCRs to evaluate parentage. Citrus genomic DNA was digested to completion with a restriction enzyme and electrophoresed on a 0.8% agarose gel. Transfer, hybridization and wash procedures were according to Wahl et al. (1979). Blots were hybridized with a ^{32}P-labeled *CsTFL* full-length cDNA probe. The *CsTFL* cDNA was PCR amplified, gel purified and ^{32}P-labeled using a Prime-a-Gene labeling kit (Promega) and α-[^{32}P]-dCTP (PerkinElmer Life Sciences Inc, Boston, Mass.). Membranes were exposed to Hyper-film-MP (Amersham) at –80 °C for at least 2 days. Similar strategies were used to evaluate *CsLFY* and *CsAP1*. Primer pairs used in PCR for allele-specific *CsTFL, CsLFY* and *CsAP1* amplification are given in Pillitteri (2002). PCR products were separated on a 1% agarose gel and stained with ethidium bromide for size determination.

RT-PCR. CsTFL, CsLFY and *CsAP1* RNAs were detected in citrus tissues using RT-PCR. Total RNA (2 μg) was used for first-strand synthesis using an oligo-dT primer (20-mer). A citrus β-actin gene (accession number BQ623464) was used as a positive control for PCR. PCR conditions for each gene are given in Pillitteri et al. (2004).

Construction of chimeric CsTFL, CsLFY and CsAP1 transgenes. The complete coding region of *CsTFL* cDNA, *CsAP1* cDNA and *CsLFY* genomic DNA were ligated into pCL0011 (C. Li and P. Springer, unpublished) to create pPSCsTFL-1, pPSCsAP1-1 and pPSCsLFY-1, respectively. Details of their construction are given in Pillitteri (2002). All constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method of Gelvin and Schilperoot (1995).

Arabidopsis seed stocks, transformation and evaluation of transgenic plant phenotypes. Seed stocks were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, Ohio). The *tfl1-2* mutant (CS3091) and *ap1-3* mutant (CS6163) were both homozygous recessive in Landsberg *erecta* (Ler) background. The *lfy-10* (CS6279) mutant was homozygous recessive in Columbia (Col) background. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transformed seeds (T_1) were planted in soil and selected with BASTA (0.005% ammonium-DL-homoalanine-4-yl-(methyl) phosphinate) (AgroEvo, Monvale, N.J.). For flowering experiments, plants were kept under long-day (LD) conditions (16-h day/8-h night) at 22 °C. Days to flowering and rosette leaves were counted when plants had a 1-cm long inflorescence.

Transgene detection and RNA blot analyses. To detect the presence of the *CsTFL, CsLFY* or *CsAP1* transgene, genomic DNA was used in a PCR reaction using a CaMV 35S forward primer and a gene-specific primer for *CsTFL, CsLFY* or *CsAP1*. Total RNA from transgenic plants was isolated using the Qiagen RNAeasy Isolation kit (Qiagen, Valencia, Calif.). RNA blots and washes were performed according to Pautot et al. (1991). *In vitro* transcribed RNAs were used as positive

controls and were produced using the T3 MAXIscript transcription kit (Ambion, Austin, Texas).

Real-time PCR. Total RNA (3 µg) from adult and juvenile stem tissue was treated with 3 units of RQ1 DNase (Promega) and used in first-strand synthesis using an oligo-dT primer (20-mer) and ImProm-II reverse transcriptase (Promega). Sequences for forward and reverse primers for *CsTFL*, *CsLFY* and *CsAP1* and PCR conditions are given in Pillitteri (2002). Reactions were run on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, Calif.). Confirmation of specific product amplification was done by melting temperature analysis using Dissociation Curve 1.0 program (PE Applied Biosystems). To establish a standard curve for quantification, sense-strand RNAs for *CsTFL*, *CsLFY*, and *CsAP1* were synthesized *in vitro* using the MAXIscript T3 transcription kit (Ambion). First-strand cDNAs were produced using gene-specific primers (Pillitteri, 2002), serially diluted ranging from 5 x 10-4 ng to 5 x 10-9 ng and used as template in parallel reactions for all real-time PCR experiments. Threshold cycle (Ct) value is the cycle number at which a significant increase in product amplification can be detected. The Ct value for each serial cDNA dilution was plotted against the log of the cDNA concentration to determine the concentrations of target-gene transcript in unknown samples.

Statistical analysis of real-time PCR data. At each weekly collection, 3 stems with leaves removed (biological replicates) were collected from both adult and juvenile citrus plants. RNA isolated from each stem was used in 3 independent RT-real-time PCR reactions (technical replicate). *Csβ-actin* was amplified from one technical replicate from each biological replicate. Ct values from the three independent technical replications were averaged and statistical analyses were done across biological replicates. The main effects of age (adult or juvenile) and time (and their interaction) were included in an analysis of covariance using *Csβ-actin* as a covariate to control for sample variation. Least squared means were compared at each time point between the different age groups. These analyses were done using JMP statistical software version 4.0.3 (Statistical Analysis Software, SAS).

Results

Isolation of TFL, LFY and AP1 homologues from C. sinensis. Comparison of the *CsTFL* gene with the *CsTFL* cDNA sequence identified the intron/exon borders. *CsTFL* exons and introns have a conserved location among *TFL* homologues relative to the protein sequence. The 522-bp open reading frame of the *CsTFL* gene translated into a 19-kD protein. The coding region showed 74% and 80% amino acid identity to the Arabidopsis TFL and *Oryza sativa* TFL homologues, respectively. Genomic organization of the *CsLFY* gene was similar to that observed for other *LFY* homologues with the position of exons and introns relative to the deduced protein sequence conserved among distantly related species. The 1197-bp open reading frame of the *CsLFY* gene predicted a 44-kD LFY protein. *Cs*LFY had 68% and 78% identity with *At*LFY and *P. balsamifera* LFY, respectively. The *Cs*LFY protein shared two highly conserved regions with all other LFY homologues that had 75% to 81% identity with *AtLFY*, respectively. A full-length *CsLFY* cDNA was not cloned. Comparison of the *CsAP1* gene with the *CsAP1* cDNA sequence determined the location of intron/exon borders. The number and location of introns in *CsAP1* were identical to *AtAP1* and other MADS-box genes. Translation of the 732-bp *CsAP1* open reading frame predicted a 28-kD protein that had higher amino acid identity with the AP1/SQUAMOSA subfamily of genes (63% to 70% identity) than to any other MADS-box gene family. *Cs*AP1 showed 66% similarity to *At*AP1. The MADS-box domain, I-domain, and K-domain of *Cs*AP1 were 92%, 76%, and 73% identical to *At*AP1, respectively.

Previous studies demonstrated that *C. sinensis* maintains a relatively heterozygous genome due to its hybrid origin (*C. maxima* x *C. reticulata*) (Federici et al., 1998; Pedrosa et al., 2000). 'Chandler' pummelo and 'Fairchild' mandarin were used as representatives of the parental genotypes, respectively. The *CsTFL* genomic region was investigated using primer pairs that spanned the *CsTFL* gene. These primers amplified the *C. sinensis* and *C. maxima TFL* genes; a product from *C. reticulata* was not detected. These data indicated that the two *C. sinensis* parental alleles could be readily distinguished from each other using allele-specific primers. Restriction digests indicated that sweet orange had limited allelic variation in the flanking region at the *TFL* locus in marked contrast to the heterozygosity detected at the *CsLFY* and *CsAP1* loci. Genomic digests of *C. maxima* x *C. reticulata* were hybridized to *CsLFY* or *CsAP1* cDNA probes. Both pummelo and mandarin DNA blot hybridizations detected a single restriction fragment for each probe, each of which matched the size of one of the restriction fragments detected in 'Washington' navel orange. The data indicated that 'Washington' navel orange has maintained two distinct alleles for both the *LFY* and *AP1* genes derived from its parental genotypes, mandarin and pummelo. For the *CsLFY* and *CsAP1* genes, polymorphisms between the two alleles were located in the 5'- and 3'-flanking regions, with *CsAP1* less polymorphic than *CsLFY*. The *CsLFY* and *CsAP1* genes cloned were both derived from an ancestral pummelo allele.

Expression of CsTFL, CsLFY and CsAP1 in mature citrus tissues. To determine if *CsTFL* transcripts accumulated in citrus vegetative and floral tissues, *CsTFL* RNAs were detected by RT-PCR using primers that amplified a single *TFL* gene segment from navel orange, mandarin and pummelo genomic DNA and, thus, monitored the accumulation of both *CsTFL* allele RNAs. The *CsTFL* transcript was not detected in any adult vegetative tissues tested, including root, stem, leaf, and seed, but was detectable in all four floral whorls of fully open flowers. In contrast, other *TFL* homologues are expressed in a variety of vegetative tissues in addition to floral organs (Nakagawa et al., 2002). *CsLFY* and *CsAP1* RNA levels were examined in vegetative and floral citrus tissues using RT-PCR. The *CsLFY* and *CsAP1* primers that were used amplified *LFY* and *AP1* genomic sequences from *C. sinensis*, as well as mandarin and pummelo and, therefore, monitored the RNA levels of both alleles of *CsLFY* and *CsAP1*, respectively. *CsLFY* transcripts were not detected in vegetative tissues (seed, root and leaf) except whole stems, but were readily detected in fourth whorl carpel tissue of fully open flowers. *CsAP1* transcript also was not detected in adult vegetative tissues sampled but was detected in all four whorls of mature citrus flowers. In Arabidopsis, *AP1* is not expressed in vegetative tissues but is expressed throughout the floral meristem, although restricted at later stages to the first and second whorl floral organs (Mandel et al., 1992).

Results of over-expression and complemetation experiments. Ectopic expression of TFL, a repressor of flowering, extends the vegetative phase and delays flowering in wild type Arabidopsis. In addition, flowers are at least partially converted to shoots (Bradley et al., 1997). A chimeric *35S:CsTFL:ocs* gene was introduced into both wild type and *tfl1-2* Arabidopsis plants. Ectopic expression of *CsTFL* cDNA produced phenotypes similar to those described for other *TFL* homologues (Ratcliffe et al., 1998; Nakagawa et al., 2002). All 32 independent BASTA-resistant T_1 plants showed a 10-day delay in flowering compared to wild type plants. *LFY* is responsible for the establishment of the floral meristem and is an upstream regulator of *AP1* in Arabidopsis (Blazquez et al., 1997; Weigel et al., 1992). A chimeric *35S:CsLFY:ocs* gene was introduced into wild type Columbia and *lfy-10* mutant plants. Ectopic expression of the *CsLFY* gene in wild type Arabidopsis plants resulted in early flowering and, to varying degrees, shoot-to-flower conversion along the inflorescence stem. *35S:CsLFY lfy-10* T_1 plants had reduced branching compared to non-transformed *lfy-10* plants. Those with the greatest reduction in branching produced more flowers and accumulated higher levels of *CsLFY* transcripts. Wild type Arabidopsis plants were transformed with the *35S:CsAP1:ocs* cDNA construct. In 15 of the 36 T_1 plants examined, ectopic expression of *CsAP1* cDNA caused an extreme early flowering phenotype. Similar phenotypes were obtained when *AtAP1* was over-expressed in wild type Arabidopsis (Pelaz et al., 2001). *CsAP1* cDNA was over-expressed in *ap1-3* Arabidopsis plants. All BASTA-resistant *35S:CsAP1 ap1-3* plants had reduced height, less branching and fewer rosettes and flowered early. BASTA-resistant *35S:CsAP1 ap1-3* plants accumulated varying levels of *CsAP1* RNAs that did not strictly correlate with phenotype.

Real-time expression pattern of CsTFL, CsLFY, and CsAP1 in juvenile and adult citrus in response to a floral-induction treatment. To begin to understand the endogenous roles played by *TFL*, *LFY* and *AP1* in juvenility and phase transition in citrus, *CsTFL*, *CsLFY*, and *CsAP1* RNA levels were compared in adult and juvenile navel orange trees in response to floral-inductive conditions. For adult navel orange trees, 100% of the branches produced inflorescences in response to 8 weeks of low temperature treatment. In contrast, juvenile navel orange trees produce only vegetative shoots in response to this treatment. Real-time PCR was used to quantify the *CsLFY*, *CsAP1* and *CsTFL* RNA levels that accumulated in adult and juvenile tissues under the floral-inductive condition. *CsTFL* transcripts accumulated to higher levels in juvenile stem tissue compared to adult tissue. During the 8-week low-temperature treatment, *CsTFL* RNAs were 7- to 32-fold more abundant in juvenile versus adult plants, but decreased in juvenile plants after transfer to the warm-temperature control conditions. In adult tissues a small increase (3-fold) in *CsTFL* transcript level was observed under warm temperature conditions. In contrast, *CsAP1* and *CsLFY* transcripts were present at low levels in juvenile tissue with *CsAP1* RNA more abundant in juvenile plants than *CsLFY* RNA. *CsLFY* and *CsAP1* RNA levels in juvenile navel orange trees did not change after transfer to warm temperature. This is distinct from patterns of *CsLFY* and *CsAP1* RNA accumulation in adult tissues. *CsLFY* and *CsAP1* transcripts accumulated to higher levels in adult tissues relative to juvenile tissues towards the end of the low-temperature induction period and after transfer to warm temperature. Although the concentration of *CsAP1* RNA was approximately 6 times that of *CsLFY* RNA in mature stems, both transcripts increased 6-fold in mature stems after week 7 of low-temperature treatment. Two to three weeks after transfer to warm temperature, both *CsLFY* and *CsAP1* transcript levels declined. This pattern was expected, since it correlated with fruit set, where flowers were senescing and ovaries were expanding.

Discussion

Citrus sinensis expresses floral regulatory gene homologues of *TERMINAL FLOWER*, *LEAFY*, and *APETALA1*. The deduced amino acid sequences of *CsTFL*, *CsLFY* and *CsAP1* share high identity with their *Arabidopsis thaliana* counterparts. Moreover, the phenotypes of Arabidopsis plants overexpressing the *CsTFL*, *CsLFY* and *CsAP1* transcripts provide evidence that citrus genes are also functionally similar to *AtTFL*, *AtLFY* and *AtAP1*. *CsTFL* RNA accumulated at high levels in florally-incompetent juvenile navel orange trees compared to florally-competent adult trees, suggesting that *Cs*TFL activity might be inhibiting *CsLFY* and *CsAP1* expression as occurs in Arabidopsis. Accumulation of *CsLFY* and *CsAP1* RNA at higher levels in florally-competent adult navel orange trees than juvenile plants when each was exposed to low-temperature floral-inductive conditions is consistent with the ability of these genes to promote early flowering in Arabidopsis. The different expression pattern observed for juvenile and adult 'Washington' navel orange trees with regard to *CsTFL* versus *CsLFY* or *CsAP1* identifies the possible roles these genes might play in regulating the transition from floral incompetence to floral competence in *C. sinensis*.

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