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. reticulata). 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 necessary to induce flowering are established for many perennial tree crops. However, documentation of the gene activities underlying the vegetative to floral transition is relative limited. Only Populus balsamifera, Malus x domestica, and Eucalyptus globulus have been extensively investigated (Kyozuaka 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 indeterminacy 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 AAp1 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; Kyozuaka 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 (Nicoli et al., 2000) that maintains relatively high heterozygosity (Federici et al., 1998) due to vegetative propagation through grafting and the production of apomictic seedlings through nucellar embryo (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 underlyong 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 ‘Carriço’ 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 Ratcliffe et al. (1995). Blots were hybridized with a 32P-labeled CsTFL full-length cDNA probe. The CsTFL cDNA was PCR amplified, gel purified and 32P-labeled using a Prime-a-Gene labeling kit (Promega) and α-[32P]dCTP (PerkinElmer Life Sciences Inc, Boston, Mass.). Membranes were exposed to Hyper-film-MP (Amsersham) 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 pCL0111 (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 ffl-1-2 mutant (CsS091) and ap1-3 mutant (CsS6163) were both homozygous recessive in Landsberg erecta (Ler) background. The fll-10 (CsS6279) mutant was homozygous recessive in Columbia (Col) background. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transformed seeds (T1) 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 roseate 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. CsLFY had 68% and 78% identity with *AtLFY* and *P. balsamifera* LFY, respectively. The CsLFY 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. CsAP1 showed 66% similarity to *AtAP1*. The MADS-box domain, I-domain, and K-domain of CsAP1 were 92%, 76%, and 73% identical to *AtAP1*, 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 digestions 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 complementation 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 T1 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 ify-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 ify-10 T1 plants had reduced branching compared to non-transformed ify-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 T1 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 CsTFL 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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Literature Cited


