Isolation and Characterization of LEAFY and APETALA1 Homologues from Citrus sinensis L. Osbeck ‘Washington’

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ABSTRACT. Homologues of the floral meristem identity genes LEAFY (LFY) and APETALA1 (AP1) were isolated from the hybrid perennial tree crop ‘Washington’ navel orange (Citrus sinensis) and designated CsLFY and CsAP1, respectively. Citrus has an extended juvenile period unlike herbaceous plants and responds to different floral stimuli than herbaceous plants or deciduous tree species. Despite these differences, the deduced amino acid sequences of CsLFY and CsAP1 genes are at least 65% identical with their Arabidopsis thaliana L. Heynh counterparts and share even greater sequence similarity to LFY and AP1 from the deciduous woody perennials, Populus balsamifera Bradshaw and Populus tremuloides Michaux, respectively. Like A. thaliana LFY (AtLFY) and AP1 (AtAP1), CsLFY and CsAP1 expression was restricted almost exclusively to reproductive tissues, but observed expression of CsAP1 in the fourth whorl carpel tissue of mature flowers was distinct from other plant AP1 genes. Transgenic A. thaliana plants ectopically expressing CsLFY or CsAP1 showed early-flowering phenotypes similar to those described for overexpression of AtLFY and AtAP1. In addition, the 35S:CsLFY and 35S:CsAP1 transgenes partially complemented the ify-10 and ap1-3 mutants, respectively. The severity of the overexpression phenotypes correlated with the accumulation of CsLFY or CsAP1 transcripts. LFY is a single-copy gene in flowering plants but consistent with its hybrid origin, the genome of C. sinensis ‘Washington’ has two easily distinguishable CsLFY and CsAP1 alleles derived from it’s parental genotypes, C. maxima L. Osbeck (pummelo) and C. reticulata Blanco (mandarin). Allelic polymorphism at both the CsLFY and CsAP1 loci was restricted to the 5’- and 3’-flanking regions.

In contrast to herbaceous plants, perennial tree crops, such as citrus, flower and produce fruit only after an extensive juvenile phase that can last several years. Although genetic and molecular studies in the herbaceous annual plant A. thaliana have led to the identification of over 40 genes involved in the transition from vegetative to reproductive growth (Araki, 2001), knowledge of specific mechanisms controlling floral initiation in perennial trees is still in its infancy. In A. thaliana, two genes have been shown to be critical positive regulators of floral meristem development: LEAFY (LFY) and APETALA1 (AP1). Both LFY and AP1 are involved in the establishment of floral meristem identity, because 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). A mutation in LFY results in more complete conversion of flowers to shoots and a greater extension of vegetative growth compared to a mutation in AP1 (Bowman et al., 1993; 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; Irish and Sussex, 1990).

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 that there is some conservation of floral regulatory signaling 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 distant related species, including citrus (Pena et al., 2001; Rottmann et al., 2000; Weigel and Nilsson, 1995). However, other studies revealed differences with regard to function or expression patterns (Ahearn et al., 2001; Coen et al., 1990; Kozyuk et al., 1998). Citrus sinensis is a subtropical perennial tree crop with traits that are unlike many other perennial trees studied (Kotoda et al., 2000; Kozyuk et al., 1997; Sung et al., 1999; Walton et al., 2001) in that floral initiation and development occurs within a single growing season without a winter dormancy typical of deciduous tree crops and forest species (Rottmann et al., 2000; Sung et al., 1999; Walton et al., 2001). In addition, C. sinensis (a C. maxima × C. reticulata hybrid) (Barret and Rhodes, 1976; Nicolosi et al., 2000), has relatively high heterozygosity (Federici et al., 1998; 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.
Due to short generation time and high fecundity, annual plant species have been the preferred system for genetic investigation and manipulation of phase transition. However, it is necessary to characterize these genes from additional woody perennial plant species with prominent differences in the length of the juvenile phase, plant architecture, and responsiveness to floral stimuli in order to be able to manipulate these traits in commercially important tree crops such as citrus. To this end *C. sinensis* ‘Washington’ homologues of the floral regulatory genes LEAFY and APETALA1 were isolated and full-length coding sequences from genomic DNA and/or cDNAs for CsLFY and CsAP1 were cloned. The similarity of CsLFY and CsAP1 to LFY and AP1 homologues from other plant species is described. To investigate the functional similarity between CsLFY and CsAP1 and their *A. thaliana* counterparts, the expression patterns of CsLFY and CsAP1 and the phenotypes of *A. thaliana* plants overexpressing the citrus homologues were determined.

**Materials and Methods**

**Plant material and tissue collection.** *Citrus sinensis* ‘Washington’ scions on *C. sinensis* × *Poncirus trifoliata* L. Raf. ‘Carrizo’ citrange rootstock (18 years old) were located at the Agricultural Experimental Station of the Univ. of California, Riverside (UC Riverside). Mature stems that had a high probability to flower were selected using the criteria of Lord and Eckard (1985). Leaves were collected for DNA isolation. Leaves, roots, and stems used in RT-PCR were collected from 5-year-old *C. sinensis* ‘Washington’ trees on ‘Carrizo’ citrange rootstock. Floral organs (receptacle/sepals, petals, stamen and carpels) were separated using forceps. Seeds were collected from fully mature fruit of *C. sinensis* navel cultivar CRC3306A. All tissue was frozen immediately in liquid nitrogen and stored at –80 °C until further use.

**Nucleic acid extraction from citrus.** *Citrus sinensis* ‘Washington’ genomic DNA was isolated by a modified CTAB-based method (Webb and Knapp, 1990) or CsCl banding (Fischer and Goldberg, 1982). *Citrus reticulata* ‘Fairchild’ and *C. maxima* ‘Chandler’ DNAs were gifts from Virginia Alonzo (UC Riverside) and Drs. Mikeal Roose and Claire Federici (UC Riverside), respectively. RNA was isolated using a LiCl-based method (Puthoff, 1999).

**PCR amplification of CsLFY and CsAP1 from *C. sinensis* ‘Washington’.** The sequences of the forward (LFY F1) and reverse (LFY R2) primers for CsLFY were 5’-AG(A/C)GGGACATCC(A/G)TT(C/T)AT(A/T/C)GT-3’ and 5’-CG(G/A/C)AG(C/T)TTA(G/C)GT(A/G)GG(G/A/C)ACATACCA-3’, respectively. The sequences for the forward (MADS-box) and reverse (AP1 R2) primers for CsAP1 were 5’-GGGTTGAGTTGAAGAGGATAGAGAAC-3’ and 5’-TGGTTGAGTTGAAGAGGATAGAGAAC-3’, respectively. For CsAP1 the cDNA sequence of CsAP1 was amplified using RT-PCR as described above except at 58 °C annealing temperature. The forward (AP1 finalF1) and reverse (AP1 finalR1) primers were 5’-GGGTTGAGTTGAAGAGGATAGAGAAC-3’ and 5’-ATATGGCTTCAG(G/A/C)AG(C/T)TTA(G/C)GTAA-3’, respectively.

**RT-PCR and genomic PCR.** Forward and reverse primer pair for CsLFY cDNA fragment amplification was 5’-CATTCATTGG-GACCGAACCCTGG-3’ (LFY F4) and 5’-GACGGCGTATCATGGCCC-3’ (LFY R1), respectively. These primers produced a 532-bp PCR product that was ligated into pGEM T-Easy vector (pGCsLFY-2). The CsLFY cDNA fragment was excised with EcoRI from pGCSLFY-2, ligated into pBluescript SK+ (pBSCsLFY-1) and used as a positive control for RT-PCR reactions. The forward and reverse primer pair for CsAP1 cDNA fragment amplification was 5’-ACCGCTCTCAAACACATCGAGG-3’ (AP1 sybF1) and 5’-GCAGCCTTCCTCTCTCCCTACT-3’ (AP1 sybR1), respectively. These primers produced a 137-bp PCR product.

**PCR amplification for actin.** The forward and reverse primer pair for actin amplification was 5’-CATCCTCACGCACCTC-3’ and 5’-CCAACCTTAGCCTCTCTC-3’, respectively. The actin primer pair produced a 191-bp product and was designed based on the sequence from an EST clone (accession number BQ623464) identified in GenBank as β-actin *C. sinensis* ‘Ridge Pineapple’. Total RNA (2 μg) was used for first-strand synthesis. PCR reaction conditions were: 30 cycles of 30 s at 94 °C, 30 s at 63 °C (*CsLFY* and *CsAP1*) or 61 °C (*actin*), and 2 min at 72 °C.

Genomic PCR was performed using DNA isolated from *C. sinensis* ‘Washington’, *C. reticulata* ‘Fairchild’ and *C. maxima* ‘Chandler’. The primer pair used for allele-specific CsLFY amplification was LFY finalF1 and LFY finalR1. The primer pair used for allele-specific CsAP1 amplification was GW AP1D6 (5’-GAGCTCGTGACGGAGAGCATCGG-3’ and 5’-GGTGCAGAGAGGAGTCGGG-3’). PCR was performed under the following conditions: 30 cycles of 95 °C for 15 s, 58 °C (*CsLFY*) or 64 °C (*CsAP1*) for 30 s, and 72 °C for 2 min.

**DNA blot analysis.** Citrus genomic DNA (10 μg) was digested completely with a restriction enzyme and electrophoresed for 16 h on a 0.8% agarose gel. Transfer, hybridization and wash procedures were done according to Wahl et al. (1979). Blots were hybridized with a [32P]-α-dCTP-labeled CsLFY probe. The genomic CsLFY fragment used as template for probe production was a PCR product amplified using LFY finalF1 and LFY R5 (5’-GTTGAGTTGAAGAGGATAGAGAAC-3’). The cDNA template preparation and labeling procedures were performed as described below under DNA blot analyses.
**Construction of chimeric CsLFY and CsAP1 transgenes.** The CsLFY genomic DNA and CsAP1 cDNA were ligated into the Xba I site in the plant transformation vector pCL0011 (C. Li and P. Springer, unpublished) in the sense orientation to create pPSCsLFY-1 and pPSCsAP1-1. This Xba I site was located between the CaMV 35S promoter (1338 bp) and octopine synthase 3'-untranslated region (724 bp) in the pCAMBIA3300 binary vector (Cambia, Canberra, Australia) conferring BASTA resistance. The pPSCsLFY-1 and pPSCsAP1-1 constructs were transferred into Agrobacterium tumefaciens strain EHA105.

**Arabidopsis thaliana seed stocks, transformation and evaluation of transgenic plant phenotypes.** Seed stocks were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, Columbus. The ap1-3 (CS6163) mutant was homozygous recessive in *Landsberg erecta* (Ler) background. The lfy-10 (CS6279) mutant was homozygous recessive in *Columbia* (Col) background. Col, Ler and mutant seeds were washed in 95% ethanol and rinsed three times with distilled water. Seeds were kept in water at 4 °C for 2 to 3 d prior to planting in soil.

Arabidopsis thaliana plants were transformed with *A. tumefaciens* strain EHA105 using the floral dip method described by Clough and Bent (1998). Transformed plants (T0) were selected with BASTA (ammonium-DL-homoalanine-4-yl-(methyl)phosphinate) (AgroEvo, Monvale, N.J.). BASTA was selected with BASTA (ammonium-DL-homoalanine-4-yl-(methyl)phosphinate) (AgroEvo, Monvale, N.J.). BASTA (0.005%) sprays were repeated three to four times at 3-d intervals. For flowering-time experiments, untransformed control seeds were planted at the same time, but did not receive any BASTA applications. Tissue for DNA and RNA analyses was collected from T1 plants when siliques started to form.

**Transgene detection and RNA blot analysis.** To detect the presence of the CsLFY and CsAP1 transgenes, genomic DNA was used in a PCR reaction using a CaMV 35S forward primer (5'-ACCTCCTCGGATTTCCATGCC-3') and a *C. sinensis* 'Washington' gene-specific reverse primer for CsLFY, (GW LFYU4; 5'-CCGCTGGCGGTGATCCTATCCC-3') or CsAP1 (AP1 FinalR1).

For RNA blot analyses, inorescences and leaves were collected from representative transformed plants. Total RNA from all transgenic plants was isolated using the Qiagen RNeasy Isolation kit (Qiagen, Valencia, Calif.) according to manufacturer’s instructions. RNA blots and washes were performed according to Pautot et al. (1991). The full-length coding sequences of the CsAP1 (782 bp) cDNA or partial sequence of the CsLFY cDNA (532 bp) were used as [32P]-α-dCTP labeled probes. Labeling was done using the Prime-a-Gene labeling kit (Promega) according to manufacturer’s instructions. In vitro transcribed RNAs were used as positive controls. Membranes were exposed to Hyper-film-MP (Amersham, Piscataway, N.J.) at −80 °C for at least 2 d.

**Results**

**Isolation of a LFY homologue from Citrus sinensis ‘Washington’.** A partial coding region of CsLFY was amplified by RT-PCR using degenerate primers designed on the basis of a nucleotide alignment of four *LEAFY* homologues. The amplified 443-bp *C. sinensis* ‘Washington’ cDNA fragment shared 76% and 79% nucleotide identity to *AtLFY* and *AmFLO*, respectively. Genome walking primers were designed to this fragment to amplify a genomic segment spanning the CsLFY coding region and portions of the 5'- and 3'-flanking regions. Four overlapping clones spanning 2.2 kb of genomic DNA were isolated and used to amplify a contiguous genomic DNA segment spanning the translational initiation and stop codons. The start and stop codons of CsLFY were deduced based on a comparison to other *LFY* genes. A partial CsLFY cDNA spanning the genomic DNA sequence (nucleotides 1029 to 2091) was obtained, but a full-length cDNA was not cloned.

The genomic organization of the CsLFY gene was similar to that observed for other *LFY* homologues (Fig. 1A). The position of the 3 exons and 2 introns relative to the deduced protein sequence was conserved among distantly related species (Fig. 1B). The 1197-bp open reading frame of the CsLFY gene predicted a 44-kD LFY protein. CsLFY had 68% and 78% amino acid identity with *AtLFY* and *Populus balsamifera* *LFY*, respectively. The CsLFY protein shared two highly conserved regions with all other *LFY* homologues spanning amino acid residues 56 to 162 and 216 to 387, with identities ranging from 91% to 94% (Fig. 1B).

In these conserved regions, nucleic acid identities ranged from 75% to 81% between *A. thaliana* and *C. sinensis* ‘Washington’. In addition, CsLFY shared three conserved motifs outside these conserved regions that are typical of angiosperm *LFY* homologues: a proline-rich region (9 prolines) between amino acids 2 to 53, a basic region spanning amino acids 181 to 194 made up of a core of Arg and Lys residues, and an acidic region composed of Asp and Glu residues from position 197 to 208 of the CsLFY sequence (Carmona et al., 2002; Mouradov et al., 1998).

Genomic DNA blot hybridization using the 3'-end of the CsLFY cDNA as a probe under conditions of high stringency detected two restriction fragments in *C. sinensis* ‘Washington’ (Fig. 2A), which indicated that a gene duplication could have occurred. This was unexpected and in contrast to the observation that *LFY* is a single-copy gene in all other diploid angiosperms studied to date (Frohlich and Parker, 2000). *Citrus sinensis* is believed to be a diploid hybrid between *C. maxima* and *C. reticulata* (Barret and Rhodes, 1976; Pedrosa et al., 2000). *C. sinensis* is thought to maintain heterozygosity due to vegetative propagation via grafting and production of apomictic seedlings through nucellar embryony (Federici et al., 1998; Pedrosa et al., 2000). As an alternative to a duplication event, the two restriction fragments identified by DNA hybridization could represent the two parental alleles of the *LFY* gene at a single locus.

To distinguish between these hypotheses, representatives of the parental genotypes (*C. maxima* ‘Chandler’ and *C. reticulata* ‘Fairchild’) were digested with the same restriction enzymes and hybridized to the CsLFY cDNA probe (Fig. 2A). Both *C. maxima* ‘Chandler’ and *C. reticulata* ‘Fairchild’ DNA blot hybridizations detected a single restriction fragment, each of which matched the size of one of the restriction fragments detected in *C. sinensis* ‘Washington’ (Fig. 2A). These data suggest that *C. sinensis* ‘Washington’ has maintained two distinct alleles of the *LFY* gene derived from its parental genotypes, *C. reticulata* and *C. maxima*. Based on the predicted restriction fragment patterns (Fig. 2B) using enzymes that cut at least once outside the coding region (Dra I, Hind III, and Eco R I), the polymorphisms between the two alleles were located in the 5'- and 3'-flanking regions of the CsLFY gene.

To determine which CsLFY allele was isolated in these experiments, PCR was performed using primers designed to anneal in the 5'- and 3'-flanking regions of CsLFY (nucleotides 39 to 2102). These data confirmed that the CsLFY alleles are distinguishable from each other and are divergent in the flanking regions. Based on these results, the gene described here was most likely derived from an ancestral *C. maxima* allele (Fig. 2C).

**Isolation of an AP1-like gene from *C. sinensis* ‘Washington’.** RT-PCR was used to isolate *CsAP1* using primers designed on
the basis of an mRNA alignment of four AP1 homologues. The amplified 462-bp cDNA fragment was used to design primers for genome walking to obtain genomic sequences spanning the CsAP1 coding region and partial 5'- and 3'-flanking regions.

Eight overlapping fragments that spanned 5.5 kb of citrus genomic DNA were assembled and the location of the start and stop codons were deduced based on comparison with other AP1 homologues. Primers were designed outside the coding region to isolate sequence spanning the entire coding region of the CsAP1 cDNA. Because of the large size of the CsAP1 gene (5.2 kb), the sequence of the CsAP1 gene was derived from the eight overlapping fragments isolated from genome walking (see below). Comparison of the CsAP1 gene with the CsAP1 cDNA sequences determined the location of intron/exon borders.

These data showed that the number and location of introns in CsAP1 was identical to AtAP1 and other MADS-box genes in the API/SQUAMOSA subfamily, having eight exons and seven introns. (Fig. 3A). Translation of the 732-bp CsAP1 open reading frame predicted a 28-kD protein (Fig. 3B). The deduced CsAP1 sequence revealed that CsAP1 had higher amino acid identity with the API/SQUAMOSA subfamily of genes (63% to 70% identity) than to any other MADS-box gene family (Fig. 3B).
Fig. 2. Citrus LFY genomic DNA blot analyses revealed heterozygosity in flanking regions. (A) Genomic DNA blot. *C. sinensis ‘Washington’, C. maxima ‘Chandler’, and C. reticulata ‘Fairchild’* DNA were digested to completion with the indicated restriction enzymes. *Dra*I, *Bgl*I and *Acc*I blots were hybridized with a 32P-labeled *CsLFY* genomic DNA probe spanning nucleotides –39 to 909. *Eco*RI and *Hind*III DNA blots were hybridized with a *CsLFY* cDNA probe that spans nucleotides 1079 to 2090. Size markers (kb) are shown to the left of the blots. (B) Schematic diagram of the location of *Dra*I, *Acc*I, *Bgl*I, *Hind*III, and *Eco*RI restriction sites in the *CsLFY* gene and 5’-flanking region are shown. The position of restriction sites is indicated in parentheses with the ATG start codon as +1. Blunt-ended lines represent expected restriction fragment size for each enzyme. Hatched marks and arrowheads indicate restriction fragments of unknown length. Location of the genomic and cDNA probes is indicated by a bold and thin line, respectively, under the gene. *CsLFY* introns are indicated by striped boxes, exons are indicated by gray boxes. (C) PCR amplification of *CsLFY* and *CsAP1* from *C. sinensis ‘Washington’, *C. reticulata ‘Fairchild’* or *C. maxima ‘Chandler’* genomic DNA. PCR products were separated on a 0.8% agarose gel and stained with ethidium bromide for size determination. Size markers (kb) are given to the right of the gel.

Fig. 3. Genomic organization and sequence of AP1-like gene products are highly conserved among diverse plant species. (A) The organization of the *CsAP1* gene (AY338975), flanking regions (black boxes), coding regions (white boxes), and introns (thin lines) are illustrated. Hatched marks indicate that a feature is not to scale. Number 1 indicates the location of the translational start codon. (B) Comparison of the deduced amino acid sequence of *CsAP1* with AP1-like proteins of *Sinapis alba* (*SaAP1*, Q41276), *Arabidopsis thaliana* (*AtAP1*, P35631), *Nicotiana tabacum* (*NtAP1*, AAD01422), *Antirrhinum majus* SQUAMOSA (*AmSQUA*, CAA45228), *Populus tremuloides* (*PtAP1*, AAF12700), *Eucalyptus globulus* (*EgAP1*, AAG24909), and *A. thaliana CAULIFLOWER* (*AtCAL*, NP_564243). The MADS-box (solid heavy), I region (dotted heavy), K box (solid thin), and C-terminal (dotted thin) domains are indicated (Ma et al., 1991; Purugganan et al., 1995; Schwarz-Sommer et al., 1990). Conserved hydrophobic residues characteristic of the K box (Munster et al., 1997) are indicated with asterisks. ClustalW program was used to make the alignment. Identical residues are shaded in black; conserved residues are shaded in gray. Dashed lines indicate gaps introduced to achieve maximum alignment. Intron positions are indicated by black arrowheads below the protein sequence.
AtAP1 is a member of a large gene family with high amino acid identity (76%) with a functionally redundant gene called CAULIFLOWER (AtCAL) (Kempin et al., 1995). CsAP1 showed similar degrees of relatedness to AtAP1 (66%) and AtCAL (63%) (Fig. 3B). The regulatory regions important for DNA binding (MADS-box) and dimerization (I- and K-domains) shared higher sequence identity with ArAP1 than to other members of MADS-box transcription factors. The MADS-box domain, I-domain, and K-domain were 92%, 76%, and 73% identical, respectively, to AtAP1 domains. This was compared to 87%, 72%, and 70% identity, respectively, to the respective AtCAL domains.

Similar to the data described for CsLFY, CsAP1 genomic blot hybridization revealed allelic variation at the CsAP1 locus (data not shown). The CsAP1 locus also showed 5'- and 3'-flanking regions were more polymorphic than the coding region. However, based on the restriction enzymes used in this study, the CsAP1 locus was less polymorphic than the CsLFY locus. To determine which CsAP1 allele was isolated in these experiments, PCR was performed using primers designed to anneal to the C. sinensis ‘Washington’ CsAP1 first intron and fifth exon. A 1.9-kb fragment was amplified from C. sinensis ‘Washington’ and C. maxima ‘Chandler’ only (Fig. 2C). These data confirmed that the two CsAP1 alleles could be distinguished from one another using primers that anneal to noncoding regions and that the CsAP1 gene described here was derived from C. maxima. The C. maxima derivation of the CsAP1 gene was confirmed using four other sets of primer pairs that spanned the entire coding region. With all primer pairs, AP1 was amplified solely from C. sinensis ‘Washington’ and C. maxima ‘Chandler’ (data not shown).

Expression of CsLFY and CsAP1. To determine where CsLFY and CsAP1 transcripts were expressed in citrus, LFY and AP1 RNA levels were examined in vegetative and floral C. sinensis ‘Washington’ tissues using RT-PCR. The coding-region CsLFY and CsAP1 primers amplified LFY and AP1 genomic fragments from C. sinensis ‘Washington’, as well as C. maxima ‘Chandler’ and C. reticulata ‘Fairchild’. Therefore, these primers monitored levels of both CsLFY and CsAP1 alleles (Fig. 4A).

The CsLFY transcripts were not detected in most vegetative tissues (seed, root and leaf) of the plant except for whole stems (Fig. 4B). The CsLFY transcripts were readily detectable in fully open flowers. In all experiments, the CsLFY RNAs were detected in the fourth whorl carpel tissue. However, occasionally CsLFY transcripts were detected at low levels in the other floral whorls (petal and stamen). This may reflect the extremely low level of CsLFY RNAs in these tissues or, although unlikely, inadvertent contamination of the first, second or third whorl tissues with fourth whorl material might have occurred.

As has been described for other plant AP1 homologues, CsAP1 transcript was only detected in flowers and not in any of the adult vegetative tissues (stem and leaf) sampled (Fig. 4B) (Berbel et al., 2001; Kyozuka et al., 1997; Sung et al., 1999). In A. thaliana, AP1 is expressed throughout the floral meristem, but is restricted to the first and second whorl floral organs at later stages of development (Mandel et al., 1992). In contrast, CsAP1 transcripts were consistently detected in all four floral whorls in mature citrus flowers. CsAP1 transcript accumulation was distinct relative to other plant AP1 homologues and may reflect an alternative regulatory program in citrus. Alternatively, the CsAP1 gene may be a more diverged member of the MADS box gene family homologue despite its high sequence identity with the AP1/SQUA subfamily members or, although unlikely, third and fourth whorl samples might have been contaminated by minor amounts of sepal and petal tissue during dissection.

33S:CsLFY caused an increase in floral meristem conversion. In A. thaliana, LFY is responsible for the establishment of the floral meristem and is an upstream regulator of AP1 (Blazquez et al., 1997; Weigel et al., 1992). Based on this, ectopic expression of CsLFY should cause early flowering and shoot-to-flower conversion along the inflorescence stem. A chimeric 33S:CsLFY:ocs construct (Fig. 5A) was introduced into wild-type Columbia and Ify-10 mutant plants. Ectopic expression of the CsLFY gene (nucleotides –39 to 2021) in wild-type A. thaliana plants produced phenotypes similar to those that were previously noted for the 35S:AtLFY cDNA construct in A. thaliana (Weigel and Nilsson, 1995). Twenty of 36 independent, Basta-resistant T1 plants were placed into three classes based on phenotypic variation.

Class I plants had the most severe early flowering phenotype. Only three plants (8%) were categorized in this class and all were early flowering, which correlated with a significant decrease in rosette leaf production (Table 1). All secondary and cossflorescences were converted to solitary flowers. However, solitary flowers produced in place of secondary inflorescences remained closed and never produced seed (Fig. 6A–B).

Seven 33S:CsLFYT plants (19%) were categorized as Class II. Secondary inflorescences on Class II plants often terminated growth with the production of compound or abnormal flowers (Fig. 6C–D). Class II plants produced flowers instead of shoots at either axillary or accessory bud meristems (Fig. 6E–F). However, solitary flowers at accessory bud positions were most common. Floral conversion occurred sporadically along the inflorescence stem and in many instances, a normal inflorescence was directly adjacent to a node converted to a flower. Two solitary flowers were never produced from the axillary and accessory bud at the same node. Class II plants showed no significant change in flowering time or the number of rosette leaves produced prior to bolting compared to wild-type plants (Table 1).
All representative plants from 35S::CsLFY Class II and III plants tested positive for the presence of the CsLFY transgene (Fig. 5B). The level of CsLFY transcripts was determined for all three 35S::CsLFY classes. Mature CsLFY RNAs were detected in Class I, II, and III transgenic A. thaliana plants indicating that the citrus LFY primary transcript was accurately spliced in A. thaliana to yield mature mRNAs of 1.4 kb (Fig. 5C). Based on RNA blot analyses, there was a strong correlation of the 35S::CsLFY phenotypic classes with CsLFY transcript accumulation. Classes I and II, which produced flowers in place of shoots, accumulated CsLFY transcripts at higher levels than Class III transgenic plants (Fig. 5C). In addition, Class I plants, which had the more complete shoot-to-flower conversion, accumulated CsLFY RNAs at the highest levels. Shoot-to-flower conversion in plants ectopically expressing CsLFY indicated that a functional gene product was produced. No viable seeds were produced from Class I plants. However, the floral conversion phenotype of Class II T1 plants was inherited by T2 generation plants.

To assess if CsLFY was a functional homologue of AtLFY, transgenic plants ectopically expressing CsLFY in the ify-10 background were constructed and characterized. The ify-10 mutation is a weak allele that causes flower-to-shoot conversion along the inflorescence (Page et al., 1999). In this mutant, early arising flowers have more complete flower-to-shoot conversion than flowers produced towards the inflorescence apex. The ify-10 allele did not affect flowering time or rosette leaf production under the conditions used in this study (Table 1).

The 35S::CsLFY ify-10 T1 plants showed a reduction in branching compared to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I).

Ten Class III plants (27%) were indistinguishable from non-transformed wild-type plants in both flowering time and overall morphology (Table 1). The remaining sixteen plants (44%) produced a variety of abnormal growth characteristics including increased collorescence production, abnormal floral morphology, compound flower production, reduced bracts, or alterations in internode length along the inflorescence. These characteristics were not quantified during evaluation because of the high degree of phenotypic variation among these plants.

All representative plants from 35S::CsLFY Class II and III plants tested positive for the presence of the CsLFY transgene (Fig. 5B). The level of CsLFY transcripts was determined for all three 35S::CsLFY classes. Mature CsLFY RNAs were detected in Class I, II, and III transgenic A. thaliana plants indicating that the citrus LFY primary transcript was accurately spliced in A. thaliana to yield mature mRNAs of 1.4 kb (Fig. 5C). Based on RNA blot analyses, there was a strong correlation of the 35S::CsLFY phenotypic classes with CsLFY transcript accumulation. Classes I and II, which produced flowers in place of shoots, accumulated CsLFY transcripts at higher levels than Class III transgenic plants (Fig. 5C). In addition, Class I plants, which had the more complete shoot-to-flower conversion, accumulated CsLFY RNAs at the highest levels. Shoot-to-flower conversion in plants ectopically expressing CsLFY indicated that a functional gene product was produced. No viable seeds were produced from Class I plants. However, the floral conversion phenotype of Class II T1 plants was inherited by T2 generation plants.

To assess if CsLFY was a functional homologue of AtLFY, transgenic plants ectopically expressing CsLFY in the ify-10 background were constructed and characterized. The ify-10 mutation is a weak allele that causes flower-to-shoot conversion along the inflorescence (Page et al., 1999). In this mutant, early arising flowers have more complete flower-to-shoot conversion than flowers produced towards the inflorescence apex. The ify-10 allele did not affect flowering time or rosette leaf production under the conditions used in this study (Table 1).

The 35S::CsLFY ify-10 T1 plants showed a reduction in branching compared to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H). These transgenic plants were small relative to non-transformed ify-10 plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to ify-10 plants (Table 1). The 35S::CsLFY ify-10 plants with the largest reduction in branching also produced solitary flowers from both accessory and axillary bud meristems (Fig. 6G–H).
Fig. 6. Ectopic expression of CsLFY in wild-type and lfy-10 mutant A. thaliana caused early flowering. Transgenic wild-type Columbia (A–F) and lfy-10 mutant (G–I) plants expressing the 35S:CsLFY construct are shown. (A) Class I 35S:CsLFY plant approximately 18 days after planting. All secondary inflorescences were converted to solitary flowers (white arrowhead). (B) Close-up of panel A. (C) Abnormal flower produced on Class II 35S:CsLFY plants showing increased number of petals. (D) Wild-type Columbia flower. (E–F) Shoot-to-flower conversion in 35S:CsLFY Class II plants. Solitary flowers were produced in place of inflorescences from the accessory (e) or axillary (f) buds. (G) Shoot-to-flower conversion of axillary bud in 35S:CsLFY lfy-10 plants. (H) Representative 35S:CsLFY lfy-10 plant =30 d after planting showing shoot-to-flower conversion of accessory bud (white arrowhead). (I) A representative lfy-10 plant, showing typical highly branched phenotype. Scale bars are 1 mm (B–F, H) and 1 cm (A, G, I).

Fig. 7. lfy-10 plants ectopically expressing the 35S:CsLFY transgene showed reduction in branching. 35S:CsLFY/lfy-10 transgenic plants with shoot-to-flower transformations are shown, with the most noted decrease in branching (left) to those with no significant deviation in phenotype from non-transformed lfy-10 plants (right). (A) Detection of the 35S:CsLFY transgene in representative 35S:CsLFY lfy-10 plants. Negative control (−) was lfy-10 genomic DNA. The positive control (+) was pBSKCsLFY-1. (B) RNA blot analyses of 35S:LFY lfy-10 plants. RNA was isolated from a mixture of inflorescence and rosette leaves. Total RNA (1 µg) was loaded in each lane, blotted, and hybridized with a 32P-labeled CsLFY cDNA probe (pBSKCALFY-1). As a control for equal RNA loading, a picture of the 25S ribosomal subunit from the gel stained with ethidium bromide is shown under the blot.

CsAP1 caused early flowering in A. thaliana. AP1 is a floral meristem and floral organ identity gene. When AtAP1 is overexpressed in wild-type A. thaliana, plants flower earlier, have shoot-to-flower conversion, and produce abnormal flowers with chimeric organs (Pelaz et al., 2001). Wild-type A. thaliana plants were transformed with the 35S:CsAP1:ocs cDNA construct (Fig. 8A). In 15 of the 36 T1 plants examined (41%, Class I), ectopic expression of CsAP1 cDNA caused an extreme early flowering phenotype, which was correlated with reduced rosette leaf production (Table 1). Generally, early-flowering 35S:CsAP1 plants produced a single primary inflorescence with few or no inflorescences or secondary inflorescences (Fig. 9A). Similar
is a weak allele that causes slightly late flowering. Compared to the homeotic conversion of the sepals and petals to chimeric petaloid organs (Pelaz et al., 2001). Compared to the ap1-3 mutant, all BASTA-resistant 35S:CsAPI ap1-3 plants had reduced height, less branching and terminated growth prematurely with an ap1-3-like flower (Fig. 9D–E). Flowering time and rosette leaf number were significantly reduced in the transgenic 35S:CsAPI ap1-3 plants compared with non-transformed ap1-3 plants (Table 1). BASTA-resistant 35S:CsAPI ap1-3 plants accumulated CsAPI RNAs at varying levels, which did not strictly correlate with phenotype (data not shown). These results showed that even moderate overexpression of CsAPI caused early flowering.

While the flowering-time phenotype was complemented by the expression of CsAPI, floral organ morphology defects were not. Although AP1 establishes sepal and petal identity in A. thaliana, ectopic expression of CsAPI did not noticeably influence floral organ identity in the ap1-3 mutant background (Fig. 9E–F). The ap1-3 mutant allele is weak and ap1-3 plants produce petals to varying degrees.

**Discussion**

One of the major goals of perennial crop improvement is to reduce the juvenile phase and hasten floral production to decrease the time between generations. The promotive roles of LEAFY and APETALA1 in vegetative phase transition and flower development in herbaceous dicot species is well established. However, the specific molecular mechanisms controlling these processes in woody perennial tree crops are still relatively poorly understood due to difficulties in genetic analysis and transgenic approaches in many of these species. One strategy for developing a means for accelerating flowering time in citrus is to identify and determine factors which affect endogenous genes that regulate these developmental processes. As a first step to understanding the mechanisms controlling reproductive competence in citrus, C. sinensis ‘Washington’ LFY and AP1 homologues were isolated and characterized, their tissue-specific transcript accumulation was monitored using RT-PCR, and their function evaluated by ectopic expression in wild-type and mutant A. thaliana plants.

**Protein coding regions of CsLFY and CsAPI have high identity to known homologues.** CsLFY shares 68% identity at the amino acid level with its A. thaliana counterpart. DNA blot analysis indicated that CsLFY was a single-copy gene as has been reported for other angiosperm LFY homologues described in the literature (Frohlich and Parker, 2000). However, two distinct alleles have been maintained within the C. sinensis ‘Washington’ genome. Whereas DNA blot analyses indicated that the alleles were conserved in the protein coding regions, the 5′- and 3′-flanking regions had detectable polymorphisms. Heterozygosity in the flanking regions of C. sinensis ‘Washington’ was additionally confirmed using a gene isolated from mandarin coding for a prenyltransferase-stimulating protein (V. Alonzo and L. Walling, data not shown). Allelic heterozygosity in gene regulatory regions may result in alterations in expression level or pattern of the C. maxima- and C. reticulata-derived CsLFY alleles; however, flower initiation phenotypes among C. sinensis, C. maxima, and C. reticulata are very similar (Davenport, 1990). There are at least 82 MADS-box genes present in the A. thaliana genome (Ratcliffe and Riechmann, 2002; Riechmann et al., 2000), which can be separated into at least nine different subfamilies (Theissen et al., 2000). The sequence identity among subfamily members is based on gene duplication events and is reflected in some instances with redundant function (Kempin et al., 1995). However, based on overall sequence identity, and more specifically within the MADS-domain, I-domain, and K-
domain, CsAP1 is most similar to AtAP1 and is likely to represent a homologue of this gene.

**CsLFY and CsAP1 can function as flower meristem identity genes in A. thaliana.** The observed phenotypes of A. thaliana plants overexpressing CsLFY and CsAP1 indicated that both genes were able to promote floral transition in a manner similar to the endogenous A. thaliana genes (Irish and Sussex, 1990; Weigel et al., 1992). These data are consistent with those of Peña et al. (2001), demonstrating that overexpression of a 35S:AtLFY or 35S:AtAP1 construct in carrot was sufficient to cause precocious flowering. However, undesirable effects such as curled leaves, thin stems, and a weepy growth habit were also produced using the AtLFY homologue. Undesirable vegetative effects were also observed in Populus tremuloides using 35S:AtLFY or 35S:PTFL constructs (Rottmann et al., 2000). In contrast, Peña observed that AtAP1 not only promoted flowering, but also reduced juvenile characteristics such as thorniness. In A. thaliana, AP1 also specifies the fate of first and second whorl floral organs (Mandel et al., 1992). However, unlike AtAP1, ectopic expression of CsAP1 did not affect ap1-3 floral morphology to a noticeable extent. This could be the result of using the weak ap1-3 allele instead of a null allele like ap1-1. Complementation of AtAP1 function in plants with a strong null allele (ap1-1) that produces no petals showed clear restoration of petals (Berbel et al., 2001). It may have been more difficult to see complementation of AP1 function in the weak ap1-3 plants, which produce some petals.

Alternatively, CsAP1 may not have retained or assumed the role for specifying first and second whorl organs during citrus evolution as is the case for the AP1 homologue of Antirrhinum majus L., SQUAMOSA (SQUA) (Bradley et al., 1993; Huijser et al., 1992). Theissen et al. (2000) suggested that the lack of genes with AP1-like function might reflect the more recent evolutionary origin of sepal and petals relative to stamen and carpels. The Malus xdomestica Borkh. (apple) AP1-like gene (MdMADS2) showed a phenotype similar to that described for CsAP1, where overexpression caused early flowering but no notable change in floral organ development (Sung et al., 1999).

Another possibility is that CsAP1 was not able to efficiently participate in the gene interactions necessary for floral organ development in an A. thaliana background. MADS-box gene products are known to form homo- and heterodimers and ternary complexes with many unrelated proteins (Lamb and McKnight, 1991; Pelaz et al., 2001). The 34% difference in amino acid sequence between the C. sinensis ‘Washington’ and A. thaliana AP1’s may reflect the functional divergence between these proteins. The inability of CsAP1 to interact and function properly in A. thaliana may also explain the late-flowering phenotype of CsAP1 Class II plants. There was no example in the literature of an AP1-like gene producing a significantly late-flowering phenotype as was described for CsAP1 Class II plants. This suggested that CsAP1 might interact with other A. thaliana floral regulatory genes in a novel manner compared to other AP1 genes. Transcript accumulation in 35S:CsAP1 Class II and III plants was similar, therefore the reason for a significant difference in flowering-time phenotype is unclear. The possibility that CsAP1 caused suppression of A. thaliana AP1 or some other floral-timing gene in Class II plants was not investigated, but could also provide an explanation for the difference in the flowering-time phenotype. These results demonstrate that CsLFY and CsAP1 have at least partial functional conservation and can be considered useful tools for reducing the time to flowering in citrus.

**MRNA expression of CsLFY and CsAP1 is comparable to other homologues.** The CsLFY and CsAP1 transcripts in citrus were at low or undetectable levels in vegetative tissues, including leaves and roots. Consistent with other studies, CsLFY expression was restricted to floral organs and whole stems, which included early developing floral inflorescences. However, some LFY homologues have been detected in early developing vegetative leaf primordia using in situ hybridization (Kelly et al., 1995; Melérovicz et al., 1998; Mouradov et al., 1998; Walton et al., 2001). The CsAP1 transcript was detected in all four floral organ whorls. This is a broader expression pattern than observed in A. thaliana, but is similar to what was described for the AP1 homologues from Malus xdomestica (MdMADS2), Brassica oleracea L. var. italicca Plenk (broccoli, BotiAP1), and Lycopersicon esculentum L. Mill. (tomato, TM4) when plants were grown under low temperatures (Carr and Irish, 1997; Lozano et al., 1998; Sung et al., 1999). However, the MdMADS2 protein levels were not correlated with RNA levels, since the MdMADS2 protein was not detectable in stamens and carpel at later stages of development, despite high levels of MdMADS2 RNA (Sung et al., 1999). A similar mechanism involving translational or post-translational regulation may exist in C. sinensis.

The phenotypes described here for overexpression of CsLFY and CsAP1 in A. thaliana suggested they participated as functional components in floral development pathways. Using traditional breeding, new citrus cultivars can require up to 20 years to be released into the consumer market. The long juvenile phase is a major factor in limiting genetic manipulation in citrus and many other perennial tree crops. Transgenic approaches in both poplar (Populus L.) and citrus have indicated that overexpression of genes such as AtLFY and AtAP1 can reduce generation time and juvenile trait characteristics in perennial tree crops. The work presented here described the characterization of the floral regulatory genes CsLFY and CsAP1. Key floral regulatory genes isolated from important agricultural crops provide the basis for further investigation into the precise mechanisms that regulate expression of these genes and broaden the tools available for floral manipulation and juvenile phase reduction in citrus.

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