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 *lfy-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 80 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 distantly 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; Kyozuka 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; Kyozuka 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.

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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 *Cs*LFY and *Cs*AP1 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)GGGAGCATCC(A/G)TT(C/T)AT(A/T/C)GT-3´ and 5´-CG(G/A/C)AG(C/T)TTA/G)GT(A/G)GG(G/A/C)ACATACCA-3´, respectively. The sequence for the forward (MADS-box) and reverse (AP1 R2) primers for *CsAP1* were 5´-GTTGAGTTGAAGAGGATAGAGAAC-3´ and 5´-(G/T/C)T(G/A/T)G(A/T)(G/T)CG(A/G)A(C/T)(G/T)TG(C/T)TT(A/C)AGAG-3´, respectively. For *CsLFY* and *CsAP1*, 3 μg of total bud RNA was used for first-strand cDNA synthesis. PCR reactions were performed using 4 μL of the RT reaction under the following conditions: 40 cycles of 15 s at 95 °C, 30 s at 54 °C (*CsLFY*) or 51 °C (*CsAP1*) and 2 min at 72 °C. A 443-bp (base pairs) cDNA *CsLFY* fragment and a 462-bp cDNA *CsAP1* fragment were ligated into the pGEM T-Easy vector (Promega, Madison, Wis.). All clones were sequenced.

Genome walking. *Citrus sinesis* 'Washington' genomic DNA (5 μg) was digested separately with *Dra* I, *Eco*R V, *Pvu* II, *Sca* I, or *Stu* I and ligated to GenomeWalker adapters obtained from the Universal GenomeWalker kit according to manufacturer's instructions (Clontech, Palo Alto, Calif.). A full description of primer sequences and product sizes can be obtained from Pillitteri (2002). A minimum of 120 bp of overlapping sequence with 100% identity was used as the criteria for identification of overlapping GenomeWalker fragments. The genomic sequences of *CsLFY* and *CsAP1* and cDNA sequence of *CsAP1* are GenBank accessions AY338976, AY338975, and AY338974, respectively.

The genomic DNA of *CsLFY* was amplified under the following conditions: 30 cycles of 15 s at 95 °C, 30 s at 54 °C, and 2 min at 72 °C. The forward (LFY finalF1) and reverse (LFY finalR1) *CsLFY* genomic DNA primers were 5´-GGGGTACCGTACAGTAGCGTTTCAGACTTG-3´ and 5´-GCTCTAGACAACAAGCAAATAGCGACGGCG-3´, respectively. 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) *CsAP1* cDNA primers were 5´-GGGGTACCGATCATACACAAGAATTCATAACAA-3 and 5´-GCTCTAGACTCTCACAGTTCATCCAGCAAAGC-3´, respectively.

RT-PCR and genomic PCR. Forward and reverse primer pair for *CsLFY* cDNA fragment amplification was 5´-CATTCATTGTGACCGAACCTGG-3´ (LFY F4) and 5´-GACGGCGTATCAGTATGGCTTC-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 *Eco*R I 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´-ACCGCTCTCAAACACATCAG-3´ (AP1 sybrF1) and 5´-GCAGCCTTCTCTCTCTCC-3´ (AP1 sybrR1), respectively. These primers produced a 137-bp PCR product.

The forward and reverse primer pair for actin amplification was 5´-CATCCCTCAGCACCTTCC-3´ and 5´-CCAACCTTAGCACTTCTCC-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´-GCCATGTATGCATTCATAACAATTTGTGG-3´) and GW AP1U5(5´-GCTCAGAGATGGACTCTTGCATGAGTTG-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 to completion 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 [^{32}P]-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´-GTGCAGCAAGATAAGGAGGC-3´). The cDNA template preparation and labeling procedures were performed as described below under RNA 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 (T_1) were 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 T_1 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´-ACCTCCTCGGATTCCATTGCC-3´) and a *C. sinensis* 'Washington' gene-specific reverse primer for *CsLFY,* (GW LFYU4; 5´-CTTCGCTGCCGTGTGGTATCTTATCCC-3´) or *CsAP1* (AP1 FinalR1).

For RNA blot analyses, inflorescences 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 [^{32}P]-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 *At*LFY 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 apopmictic 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, *Hin*d 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

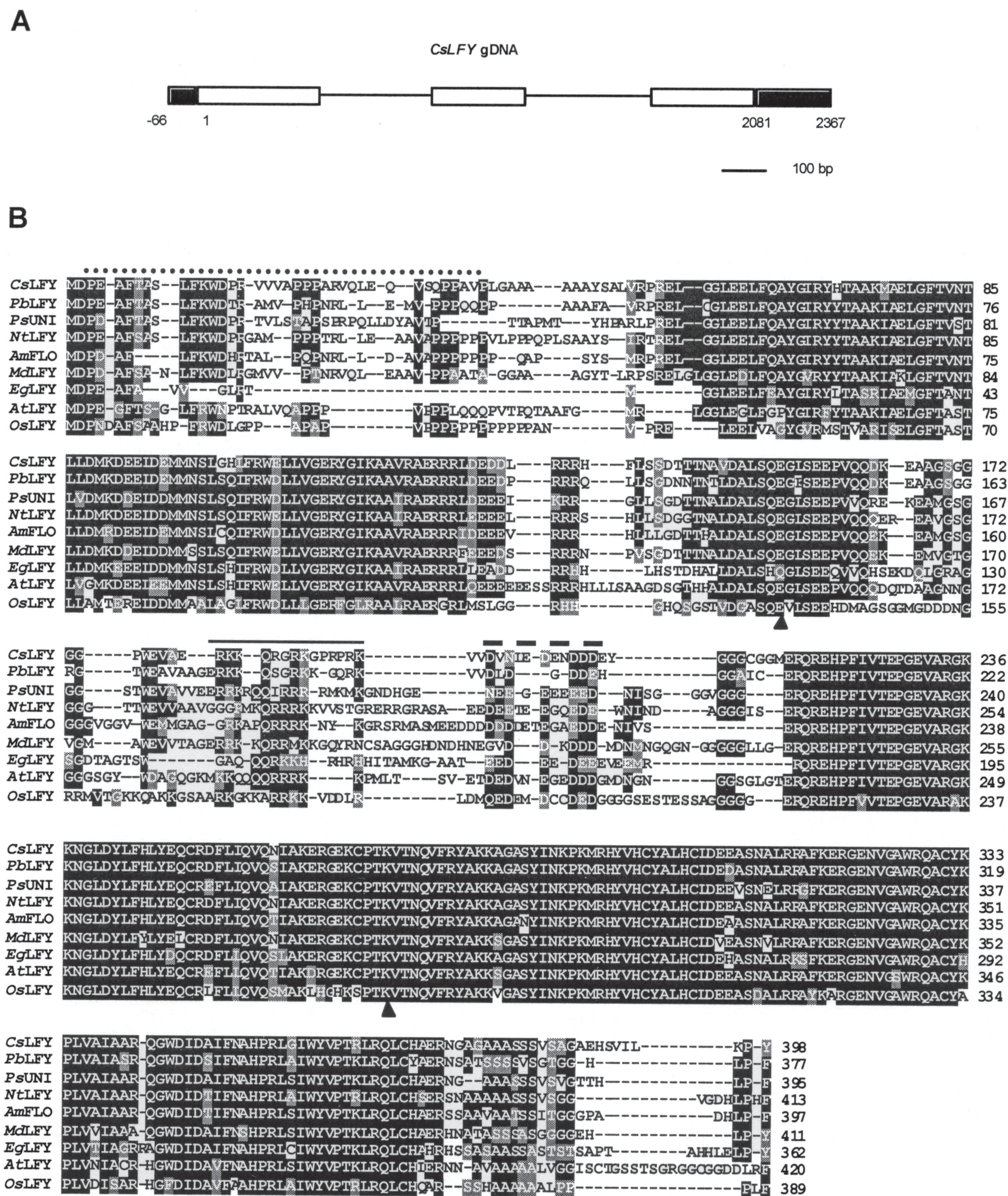

Fig. 1. Genomic organization and sequences of *LFY*-like gene products are highly conserved among diverse plant species. (**A**) The organization of the *CsLFY* gene (AY338976), flanking regions (black boxes), coding regions (white boxes), and introns (thin lines) are illustrated. Number 1 indicates the location of the translational start codon. (**B**) Comparison of the deduced amino acid sequence of *CsLFY* with LFY proteins of *Populus balsamifera* (*Pb*LFY, AAB51533), *Pisum sativum* UNIFOLIATA (*Ps*UNI, AAC49782), *Nicotiana tabacum* (*Nt*LFY, T03240), *Malus* (*Md*LFY, BAB83096), *Eucalyptus globulus* (*Eg*LFY, AAC31359), *Arabidopsis thaliana* (*At*LFY, NP200993), *Oryza sativa* (*Os*LFY, AAC96098) and *Antirrhinum majus* FLORICAULA (*Am*FLO, A36339). The 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. Angiosperm conserved proline-rich, basic, and acidic regions are indicated by a dotted, solid, and dashed lines, respectively, above the peptide sequence (Carmona et al., 2002; Mouradov et al., 1998).

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 *AP1/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 *Cs*AP1 sequence revealed that *Cs*AP1 had higher amino acid identity with the AP1/SQUAMOSA subfamily of genes (63% to 70% identity) than to any other MADS-box gene family (Fig. 3B).

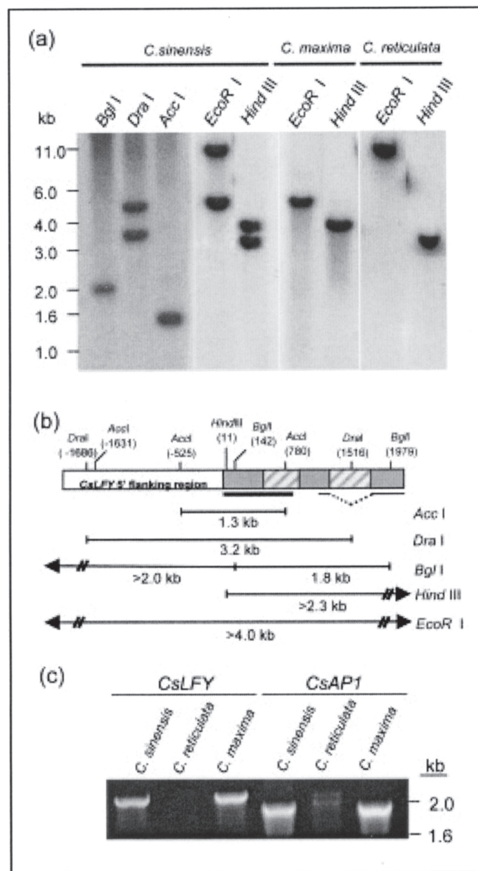

Fig. 2. Citrus *LFY* genomic DNA blot analyses revealed heterozygosity in flanking regions. (**A**) Genomic DNA blot. *C. sinensis* 'Washington', *C. maxima* 'Chandler', and *C. reticulata* 'Fairchild' DNA were digested to completion with the indicated restriction enzymes. *Dra* I, *Bgl* I and *Acc* I blots were hybridized with a ^{32}P-labeled *CsLFY* genomic DNA probe spanning nucleotides –39 to 909. *Eco*R I and *Hin*d 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, *Hin*d III, and *Eco*R I 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.

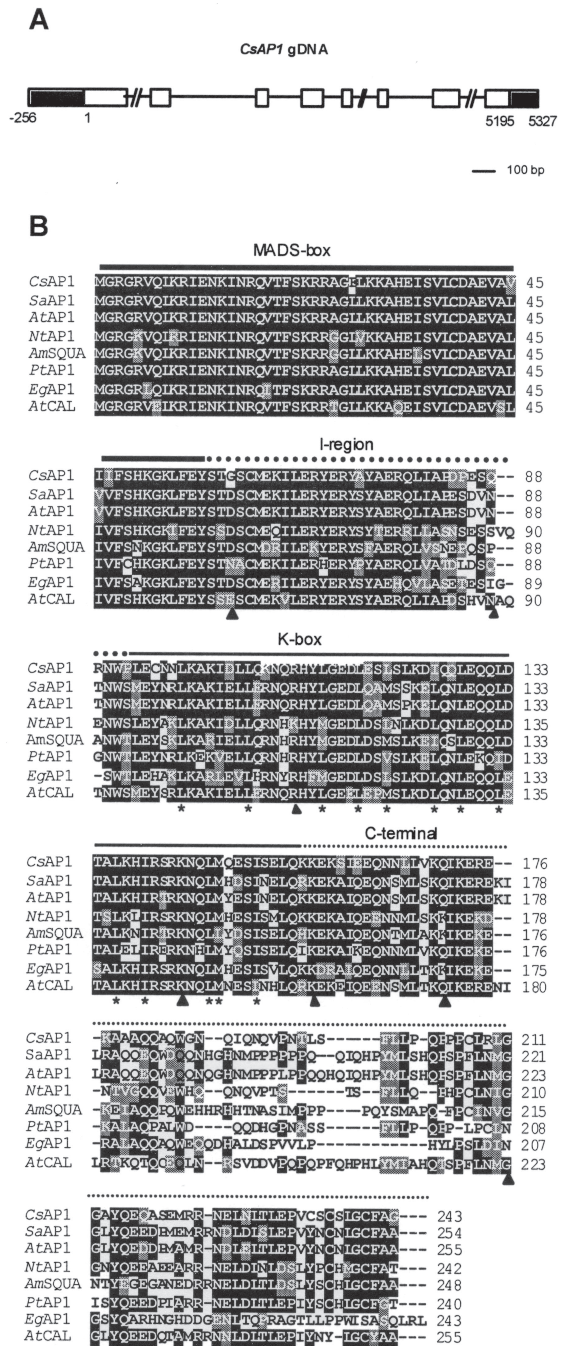

Fig. 3. Genomic organization and sequence of AP1-like gene products are highly conserved among diverse plant species. (**A**) The organization of the *CsAP1* gene (AY338975), flanking regions (black boxes), coding regions (white boxes), and introns (thin lines) are illustrated. Hatched marks indicate 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 *Sinapsis alba* (*Sa*AP1, Q41276), *Arabidopsis thaliana* (*At*AP1, P35631), *Nicotiana tabacum* (*Nt*AP1, AAD01422), *Antirrhinum majus* SQUAMOSA (*Am*SQUA, CAA45228), *Populus tremuloides* (*Pt*AP1, AAF12700), *Eucalyptus globulus* (*Eg*AP1, AAG24909), and *A. thaliana* CAULIFLOWER (*At*CAL, 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.

*At*AP1 is a member of large gene family with high amino acid identity (76%) with a functionally redundant gene called *CAULIFLOWER* (*AtCAL*) (Kempin et al., 1995). *Cs*AP1 showed similar degrees of relatedness to *At*AP1 (66%) and *At*CAL (63%) (Fig. 3B). The regulatory regions important for DNA binding (MADS-box) and dimerization (I- and K-domains) shared higher sequence identity with *At*AP1 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 *At*AP1 domains. This was compared to 87%, 72%, and 70% identity, respectively, to the respective *At*CAL 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 (sepal and stamens). 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.

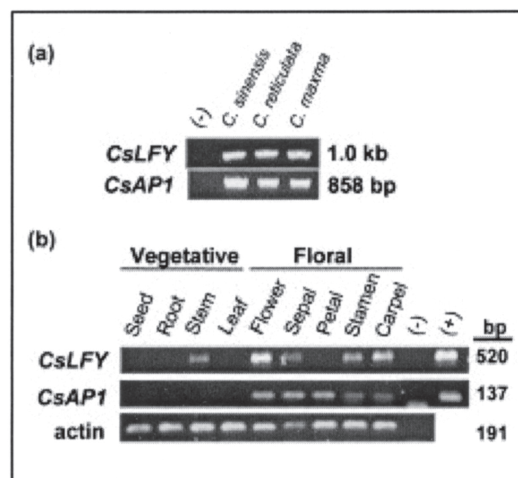

Fig. 4. *CsLFY* and *CsAP1* mRNA levels in vegetative and floral tissues revealed differences in gene expression. (**A**) Genomic DNA PCR showing product amplification from *C. sinensis* 'Washington', *C. maxima* 'Chandler', and *C. reticulata* 'Fairchild'. No DNA was added to the negative control lane (–). (**B**) Total RNA (4 μg) was used in the RT reactions. All tissue was collected from *C. sinensis* 'Washington' with the exception of seeds that were from navel variety CRC3306A. No RNA was added to the negative control lane (–). The *CsLFY* and *CsAP1* positive controls (+) were pBSCsLFY-1 vector (10 ng) and pPSCsAP1-1 vector (10 ng), respectively.

***35S: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 *35S:CsLFY:ocs* construct (Fig. 5A) was introduced into wild-type Columbia and *lfy-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 T_1 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 coflorescences were converted to solitary flowers. However, solitary flowers produced in place of secondary inflorescences remained closed and never produced seed (Fig. 6A–B).

Seven *35S:CsLFY* T_1 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).

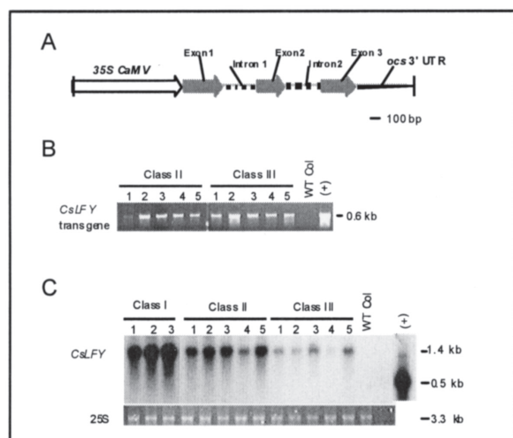

Fig. 5. *35S:CsLFY* transgene expression is correlated with shoot-to-flower conversion. (**A**) Diagram of the *CsLFY* transgene in pPSCsLFY-1. The chimeric *CsLFY* construct contained the *CaMV 35S* promoter (1.33 kb; white arrow), complete coding region of the *CsLFY* genomic DNA (2.13 kb) (exons grey arrows, intron hatched lines), and octopine synthase 3´-untranslated region (*ocs* 3´-UTR, 724 bp; heavy black line). (**B**) Detection of the *35S:CsLFY* transgene using PCR. Wild-type Columbia and representatives of Class II and III *35S:CsLFY* T_1 plants were analyzed. Class I *35S:CsLFY* plants were not tested for the presence of their transgene due to their extremely small size (<50 mg fresh weight), which limited material for analysis. Negative control for transgene detection was wild-type Columbia genomic DNA (WT Col). The positive control (+) was pPSCsLFY-1. (**C**) RNA blot analysis of *35S:CsLFY* T_1 plants in wild-type Columbia background. RNA was isolated from a mixture of inflorescence and rosette leaves. Total RNA (1 μg) was loaded per lane, blotted and hybridized with a P^{32}-labeled *CsLFY* cDNA probe (pBSCsLFY-1). The positive control (+) was 0.1 ng of in vitro-transcribed sense *CsLFY* RNA (0.5 kb) from pBSCsLFY-1. The negative control (–) was wild-type Columbia RNA. 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 blots.

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 coflorescence 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 mRNA 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 T_1 plants was inherited by T_2 generation plants.

To assess if *CsLFY* was a functional homologue of *AtLFY,* transgenic plants ectopically expressing *CsLFY* in the *lfy-10* background were constructed and characterized. The *lfy-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 *lfy-10* allele did not affect flowering time or rosette leaf production under the conditions used in this study (Table 1).

The *35S:CsLFY lfy-10* T_1 plants showed a reduction in branching compared to non-transformed *lfy-10* plants (Fig. 6H-I). However, no significant difference in flowering time was noted compared to *lfy-10* plants (Table 1). The *35S:CsLFY lfy-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 *lfy-10* plants (Fig. 6H) and inflorescence development was terminated with a flower. RNA blot analyses determined that *35S:CsLFY lfy-10* plants with the most reduced branching and/or increased shoot-to-flower conversion accumulated *CsLFY* transcripts at the highest levels (Fig. 7B). The introduction and ectopic expression of *CsLFY* complemented the nontransformed *lfy-10* phenotype by decreasing the number of shoots produced and increasing flower production.

Table 1. Flowering time of CsLFY and CsAP1 transgenic *A. thaliana* plants under long-day conditions.

Plant genotypes[z]	Time to flowering (days)[y]	Leaves (no.)[x]	Plants (no.)
Ler	21.2 ± 0.39	6.2 ± 0.16	15
Col	24.0 ± 0.56	8.7 ± 0.28	20
35S:CsLFY WT			
Class I	<18*	5.3 ± 0.34*	3
Class II	23.5 ± 0.28	8.1 ± 0.15	7
Class III	24.2 ± 0.20	8.9 ± 0.10	10
lfy-10	22.7 ± 0.50	8.0 ± 0.22	18
35S:CsLFY lfy-10	23.1 ± 0.53	7.7 ± 0.37	18
35S:CsAP1 WT			
Class I	<18*	4.9 ± 0.21*	15
Class II	26.3 ± 0.5*	13.5 ± 0.44*	6
Class III	23.4 ± 0.4	9.0 ± 0.38	13
ap1-3	21.9 ± 0.35	7.3 ± 0.21*	15
35S:CsAP1 ap1-3	20.1 ± 0.5*	5.9 ± 0.23*	9

[z]Plants were classified as Classes I-III based on severity of phenotype imparted by the *35S:CsLFY* or *35S:CsAP1* transgene. Plants were kept at 22 °C on a 16-h day/8-h night light cycle. Wild-type and *lfy-10* plants were in Columbia (Col) background. *ap1-3* plants were in *Landsberg erecta* (Ler) background.
[y]Days from sowing to a 1-cm inflorescence ± SE.
[x]Number of rosette leaves on plants with a 1-cm inflorescence ± SE.
*Indicates that values are significantly different from control phenotype using Student's *t* test ($P < 0.01$).

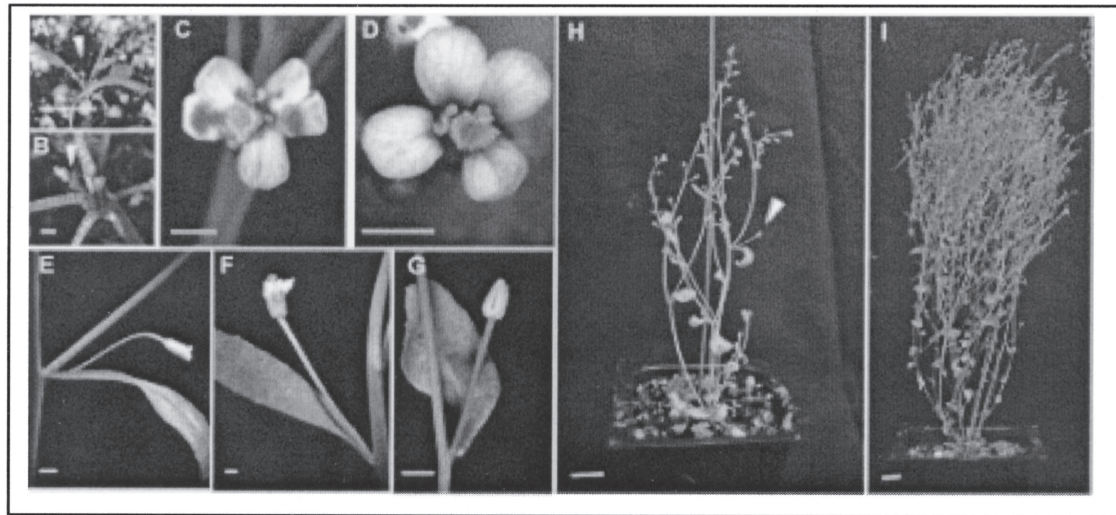

Fig. 6. Ectopic expression of *CsLFY* in wild-type and *lfy-10* mutant *A. thaliana* caused early flowering. Transgenic wild-type Columbia (A–F) and *lfy-10* mutant (G–I) plants expressing the *35S:CsLFY* construct are shown. (**A**) Class I *35S:CsLFY* plant approximately 18 days after planting. All 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 coflorescences 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).

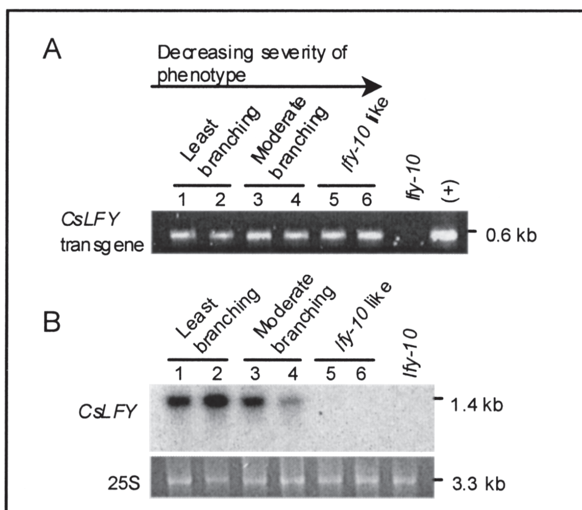

Fig. 7. *lfy-10* plants ectopically expressing the *35S:CsLFY* transgene showed reduction in branching. *35S:CsLFY lfy-10* transgenic plants with shoot-to-flower transformations are shown, with the most noted decrease in branching (left) to those with no significant deviation in phenotype from non-transformed *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 P32-labeled *CsLFY* cDNA probe (pBSKCsLFY-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.

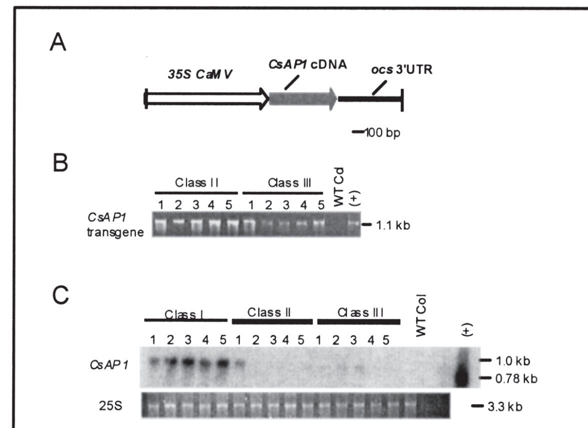

Fig. 8. *35S:CsAP1* transgene expression was correlated with early flowering. (**A**) Diagram of the *CsAP1* transgene in pPSCsAP1-1. The chimeric *CsAP1* construct contained the *CaMV 35S* promoter (1.33 kb; white arrow), full coding region of the *CsAP1* cDNA (782 bp; grey arrow), and octopine synthase 3´-untranslated region (*ocs* 3´-UTR, 724 bp, heavy black line). (**B**) Detection of the *35S:CsAP1* transgene using PCR. Wild-type Columbia and representatives of Class II or III plants were analyzed. Class I *35S:CsAP1* plants were not tested for the presence of their transgene due to their extremely small size (<50 mg fresh weight), which limited material for analysis. Negative control was wild-type Columbia genomic DNA (WT Col). The positive control (+) was pPSCsAP1-1. C. RNA blot analysis of *35S:CsAP1* T_1 plants in wild-type Columbia background. For RNA blot analyses, RNA was isolated from a mixture of inflorescence and rosette leaves. Total RNA (1 µg) was loaded per lane, blotted and hybridized with a P32-labeled *CsAP1* cDNA probe (pBSCsAP1-1). The positive control (+) was 0.1 ng of in vitro-transcribed sense *CsAP1* RNA from pBSCsAP1-1. The negative control was wild-type Columbia RNA. 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 blots.

***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 T_1 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 coflorescences or secondary inflorescences (Fig. 9A). Similar

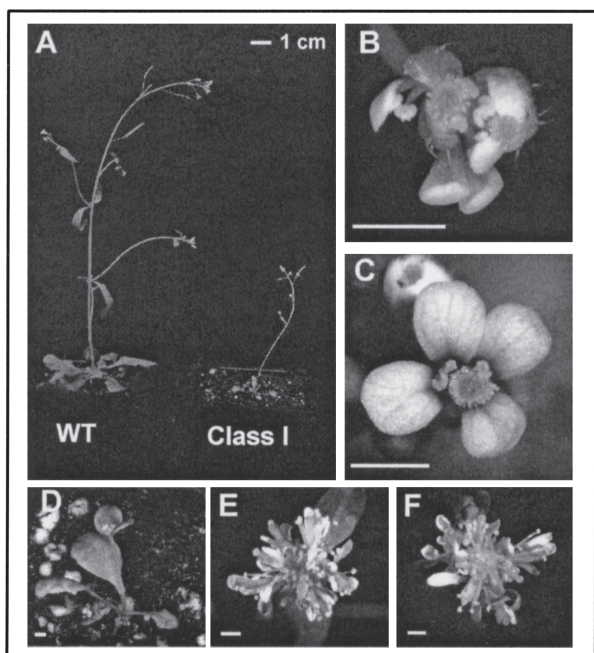

Fig. 9. Ectopic expression of *CsAP1* in wild-type Columbia and *ap1-3 A. thaliana* caused early flowering. Transgenic wild-type Columbia (A–C) and *ap1-3* mutants (D–F) expressing the *35S:CsAP1* transgene are shown. (**A**) Comparison of a wild-type Columbia (left) and a Class I *35S:CsAP1* (right) plant at 27 d after planting. (**B**) Typical compound flower observed on primary inflorescence of Class I *35S:CsAP1* plants. (**C**) Wild-type Columbia flower. (**D**) *35S:CsAP1 ap1-3* plant 18 d after planting showing reduced leaf production. (**E**) *35S:CsAP1 ap1-3* plant primary inflorescence. (**F**) *ap1-3* mutant primary inflorescence. Scale bars are 1 mm for panels B–F.

to the phenotype described for the *tfl1* mutation (Bradley et al., 1997; Shannon and Meeks-Wagner, 1993), compound flowers were produced at the apex of *35S:CsAP1* plants (Fig. 9B–C). Compound flowers occurred only on Class I *35S:CsAP1* plants and were not produced on any nontransformed wild-type plants. Many of *35S:CsAP1* plants had rosette leaves that curled tightly toward the adaxial side of the leaf (data not shown). The remaining plants had less severe phenotypes. Class II *35S:CsAP1* plants that produced flowers with shortened petals were late flowering and produced significantly more rosette leaves than non-transformed wild-type plants (Table I). Class III *35S:CsAP1* plants were indistinguishable from non-transformed wild-type plants in both morphology and flowering time (Table 1).

A representative sample of BASTA-resistant plants from Classes II and III tested positive for the presence of the *35S:CsAP1* transgene by RT-PCR (Fig. 8B). RNA blot analysis revealed that flowering time and compound flower production were correlated with *CsAP1* transcript accumulation (Fig. 8C). Class I *35S:CsAP1* plants accumulated the highest levels of *CsAP1* RNA, whereas Class II and III plants had low or undetectable levels of the *CsAP1* transcript. The extreme early-flowering phenotype of *CsAP1* Class I T_1 plants was inherited by T_2 plants. The data suggested that *CsAP1* expression was sufficient to cause early flowering and convert the vegetative apex to a flower in *A. thaliana*.

To further test the function of *CsAP1*, *CsAP1* cDNA was overexpressed in *ap1-3 A. thaliana* plants. In the Ler background, *ap1-3* is a weak allele that causes slightly late flowering and 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:CsAP1 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:CsAP1 ap1-3* plants compared with non-transformed *ap1-3* plants (Table 1). BASTA-resistant *35S:CsAP1 ap1-3* plants accumulated *CsAP1* RNAs at varying levels, which did not strictly correlate with phenotype (data not shown). These results showed that even moderate overexpression of *CsAP1* caused early flowering.

While the flowering-time phenotype was complemented by the expression of *CsAP1*, floral organ morphology defects were not. Although *AP1* establishes sepal and petal identity in *A. thaliana*, ectopic expression of *CsAP1* 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 *CsAP1* 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 bot 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, *Cs*AP1 is most similar to *At*AP1 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 citrange 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:PTLF* 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 sepals and petals relative to stamen and carpels. The *Malus* ×*domestica* Borkh. (apple) AP1-like gene (*MdMADSS2*) 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* AP1s 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; Mellerowicz 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* ×*domestica* (*MdMADS2*), *Brassica oleracea* L. var. *italica* Plenck (broccoli, *BoiAP1*), 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 *Md*MADS2 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 broadens the tools available for floral manipulation and juvenile phase reduction in citrus.

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