Proc. Intl. Soc. Citricult. IX Congr. 2000. 347–350.

Regulation of Flowering in the 'Washington' Navel Orange: *Floral Genes*

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Additional index words. apetala 1, *Citrus sinensis*, leafy, MADS-box proteins, terminal flower

Abstract. **Transition of a vegetative shoot apex to a floral apex is the first step in fruit production. In the model system *Arabidopsis thaliana*, this transition is controlled, in part, by leafy (LFY), apetala 1 (AP1), and terminal flower (TFL). Floral buds were collected from 'Washington' navel orange [*Citrus sinensis* (L.) Osb.] trees during low-temperature floral induction. Floral bud RNA and degenerate primers were used in a RT-PCR amplification strategy to isolate LFY and AP1 cDNAs. Sequencing of a RT-PCR-generated 446-bp cDNA fragment and its predicted peptide showed 77.5% nucleotide and 89.1% amino acid identity with *Arabidopsis* LFY. In addition, the *C. sinensis* LFY peptide shared 93.2% identity with the *Populus tremuloides* LFY protein. A RT-PCR generated 462-bp *C. sinensis* AP1-like cDNA clone revealed 86.5% and 90.4% amino acid sequence identity with the MADS-box domain of the AP1 protein of *A. thaliana* and *Betula pendula*, respectively. However, the *C. sinensis* AP1-like homologue has a 51-bp in-frame insertion not reported for known AP1-like genes. The insertion is located within the K-box domain of the predicted peptide, reducing the overall amino acid sequence identity with the *A. thaliana* protein to 66.0%. A 1.1-kb TFL-like genomic fragment was amplified from *C. sinensis* using degenerate primers. Presumed introns were excluded, yielding a short 246-bp exon with 77.3% nucleotide and 82.9% amino acid sequence identity to *A. thaliana* TFL. The results suggest that *C. sinensis* and *A. thaliana* floral genes are homologues and that the *Arabidopsis* model system might prove useful for determining the regulation of flowering in *Citrus*.**

Unlike herbaceous annual plants, woody perennials become competent to flower only after a prolonged period of juvenility. In the production of commercial tree crops, including *Citrus*, buds from florally competent adult scions are grafted onto juvenile rootstocks to reduce the time to flowering. Despite grafting, trees still do not flower sufficiently to produce a commercial harvest for 4 to 6 years. An ability to manipulate *Citrus* flowering would enable growers to solve this and many other production problems. Being able to up- or down-regulate floral gene expression would make it possible to maximize yield or optimize fruit size, to eliminate off-season bloom and alternate bearing, and to shift bloom to avoid adverse climatic conditions during flowering and fruit set or to move harvest to a more lucrative marketing window. The ability to regulate flowering with consistent results is highly dependent on understanding the process by which a vegetative shoot apex transitions to a floral apex.

Significant research progress has been made in elucidating the molecular mechanisms controlling the initiation of flowering and development of the inflorescence of the herbaceous annual *Arabidopsis thaliana*. Among the key genes that have been isolated and well characterized are leafy (LFY), apetala 1 (AP1), and terminal flower (TFL) (Irish and Sussex, 1990; Liljegren et al., 1999; Weigel and Nilsson, 1995). Mutations in either LFY or AP1 inhibit or delay development of the inflorescence floral meristem. Mutations at the TFL locus cause early flowering and the production of a terminal flower at the apex of the normally indeterminate floral shoot of *Arabidopsis*. In addition, homologues have been identified in a variety of diverse plant species and, in some cases, have been shown to be functional equivalents (Kotoda et al., 2000; Kyozuka et al., 1997; Molinero-Rosales et al., 1999; Southerton et al., 1998).

In combination, these genes have been proven to be fundamentally important to the regulation of flowering in over 20 species. The *Arabidopsis* LFY gene has been transformed and constitutively expressed in aspen (*Populus tremuloides*) and citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] (Martinez-Zapater, unpublished results; Weigel and Nilsson, 1995), significantly reducing the time to flowering to within the first year in both species. Given the economic importance of *Citrus* production worldwide, identifying homologues of these critical genes in *Citrus* and determining their function and regulation could contribute significantly toward solving yield problems related to flowering. Here, partial homologues to LFY, AP1, and TFL from *C. sinensis* cv. Washington navel orange are described.

Materials and Methods

Plant material. In October, shoots were selected on 18-year-old 'Washington' navel orange [*Citrus sinensis* (L.) Osb.] scions on Carrizo citrange [*C. sinensis* (L.) Osb. x *P. trifoliata* (L.) Raf.] rootstock using the following criteria: shoot length ≤8 cm, leaf area per leaf ≤18 cm^2, node number per shoot ≤7, and thorn number per shoot ≤1. Shoots selected by these criteria have a high probability to produce predominantly floral shoots in the spring (Lord and Eckard, 1985). The research trees were located in a commercially bearing orchard at the Agricultural Experimental Station of the University of California, Riverside.

RNA isolation. The apical four buds from the selected shoots were collected and pooled (≈0.8 g fresh weight tissue), frozen in liquid nitrogen and ground to a fine powder. Total RNA was isolated using the LiCl-based procedure according to Gu et al. (1996).

Gene isolation. Homologous cDNA fragments of LFY and AP1 were amplified using RT-PCR. Total RNA was used to synthesize first strand cDNA using Superscript II RNAse H reverse transcriptase (Gibco BRL, Rockville, Md.) according to manufacturers recommendations. cDNAs were synthesized in a 50-µL reaction containing 0.4 µM degenerate gene-specific primers, 1 unit Taq DNA polymerase, 0.2 µM dNTPs, and 1 × PCR buffer A (Promega, Madison, Wis.). For LFY, amplification was carried out under the following conditions: 32 cycles of 1 min at 95 °C, 30 s at 56 °C, and 2 min at 72 °C. Amplification conditions for AP1 were 30 cycles of 1 min at 95 °C, 30 s at 52 °C, and 2 min at 72 °C. A TFL genomic fragment was amplified in a 50-µL reaction containing 40 ng leaf genomic DNA under the following conditions: 30 cycles of 1 min at 95 °C, 30 s at 54 °C, and 2.5 min at 72 °C.

Nucleotide sequencing and sequence analysis. A 470-bp cDNA fragment of LFY, 462-bp cDNA fragment of an AP1-like gene, and 1.14-kb genomic fragment of TFL were cloned using the pGEM T-Easy Vector System (Promega, Madison, Wis.) and sequenced. Nucleotide sequencing was conducted using universal primers with the *fmol* DNA Cycle Sequencing System (Promega, Madison, Wis.) on a GenomyxLR GX100 DNA sequencer. For sequence analysis, full-length cDNA sequences of LFY and AP1 and AP1-like genes obtained from GenBank were aligned, respectively, using Clustal X (Jeanmougin et al., 1998). The *C. sinensis* TFL genomic fragment was aligned with TFL cDNA sequences imported from GenBank and presumed introns were removed from the *C. sinensis* TFL sequence.

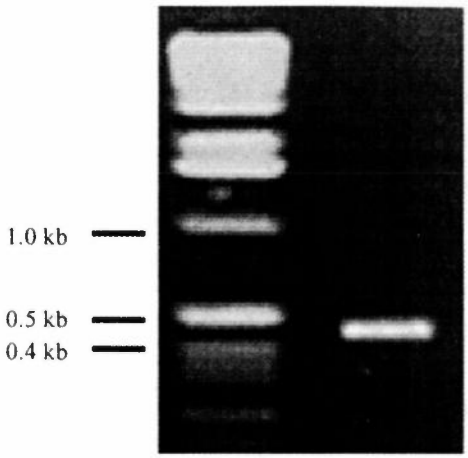

Fig. 1. *Citrus sinensis* LFY homologue isolated by RT-PCR amplification from apical bud RNA of 'Washington' navel orange.

Results and Discussion

Leafy. Buds collected during the low-temperature floral-induction period for use in RT-PCR amplification were consistently floral for all years of the study. The developmental fate of a parallel set of selected buds was quantified. Of the buds selected, 60% to 84% sprouted and 100% of sprouted buds produced inflorescences. Based on the primers selected, the *C. sinensis* LFY cDNA fragment amplified by PCR from these buds was of the anticipated size relative to other sequences in GenBank (Fig. 1). *Citrus sinensis* LFY appears to be highly conserved both at the nucleotide and amino acid level. The nucleotide sequence used for alignment representing 446-bp from the 'Washington' navel orange cDNA fragment had 77.5% identity with *Arabidopsis* LFY. A computer-assisted translation of the *C. sinensis* LFY fragment was aligned at the amino acid level with LFY peptides representing a wide range of plant species. The predicted 'Washington' navel orange peptide shares an 89.1% amino acid sequence identity to the *Arabidopsis* LFY protein (Fig. 2). This degree of identity is high, but typical among LFY proteins. *Citrus sinensis* LFY shares 78.2% and 93.2% aa identity with *Pinus radiata* and *P. tremuloides*, respectively. Taken together, the substantial degree of sequence identity between *Arabidopsis* and *C. sinensis* LFY peptide sequences and the ability of *Arabidopsis* LFY to promote precocious flowering in transformed citrange (Martinez-Zapater, unpublished results) suggests that these homologues may play the same role in promoting floral meristem identity.

Apetala 1. The RT-PCR amplified 462-bp AP1-like cDNA fragment from 'Washington' navel orange was slightly larger than other plant AP1-like sequences in GenBank (Fig. 3). Sequencing the *C. sinensis* fragment revealed a 51-bp in-frame insertion not present in AP1 and AP1-like genes from any of the plant species used for this analysis. The origin of the insertion is unknown and shows no similarity to any sequence currently in GenBank.

AP1 is part of a family of transcription factors called MADS-box proteins that are comprised of four characteristic domains: 1) the MADS-box; 2) the I region, a variable intervening region; 3) the K-box; and 4) the highly variable C-domain. The MADS-box is responsible for DNA binding, but also performs dimerization and accessory factor binding functions (Ma, 1994). This region is highly conserved (86.5% aa identity with *Arabidopsis* AP1) in the predicted *C. sinensis* AP1-like peptide, including the phosphorylation site within this domain (Fig. 4). Similarly, amino acid sequence identity of *C. sinensis* with *Betula pendula* and *P. radiata* AP1 is 90.4% and 78.8% within the MADS-box region.

The K-box resembles the structure of keratin (Ma et al., 1991) and is characterized by the regular spacing of hydrophobic residues (Munster et al., 1997). This domain is believed to be involved in protein-protein interactions including the production of homo- or hetero-dimers. The unique insertion in the *C. sinensis* AP1-like fragment is located within the K-box domain of the predicted peptide. Even without the insertion,

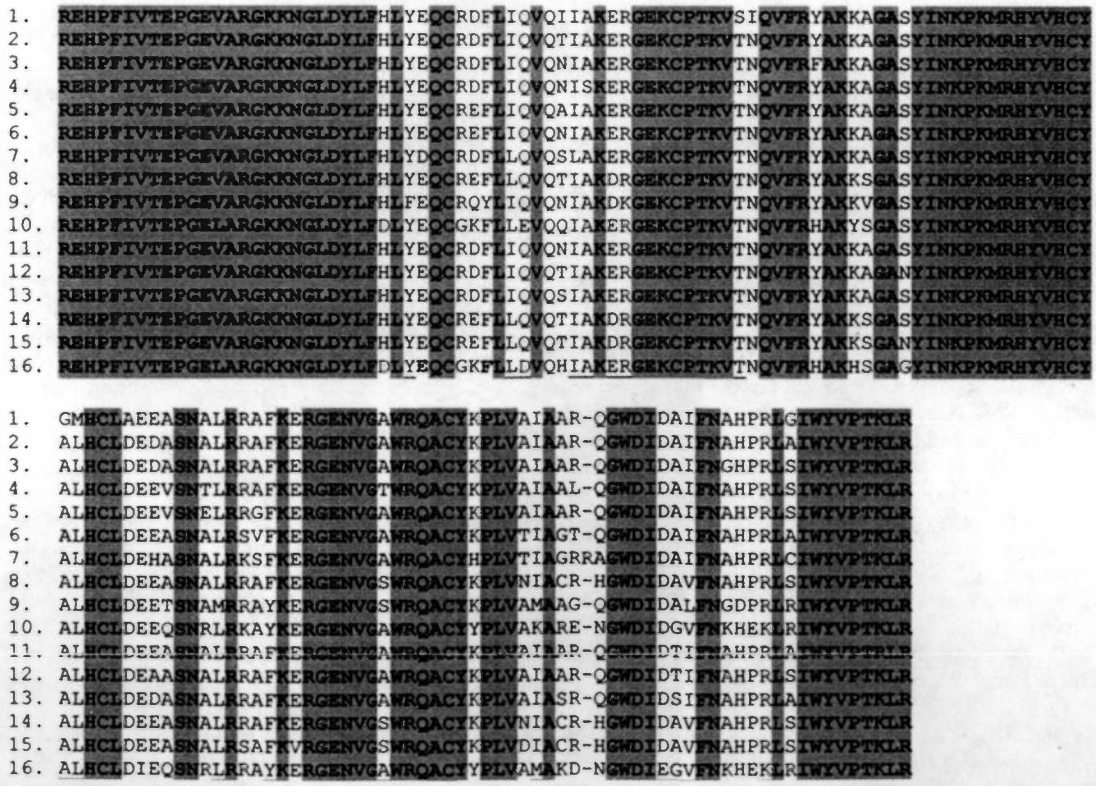

Fig. 2. Sequence comparison of LFY proteins. Sequences with accession numbers include: 1) *Citrus sinensis*; 2) *Lycopersicon esculentum* (AF197934); 3) *Petunia ×hybrida* (AF030171); 4) *Trochodendron aralioides* (AF230078); 5) *Pisum sativum* (AF035163); 6) *Cucumis sativus* (AF059320); 7) *Eucalyptus globulus* (AF034806); 8) *Jonopsidium acaule* (AF184589); 9) *Peperomia* HBG70537 (AF106843); 10) *Welwitschia mirabilis* (AF072369); 11) *Nicotiana tobacum* (AH006598); 12) *Antirrhinum majus* (M55525); 13) *Populus tremuloides* (U93196); 14) *Arabidopsis thaliana* (M91208); 15) *Brassica oleracea* (Z18362); 16) *Pinus radiata* (U92008). Conserved amino acids are shaded; similar amino acid substitutions are underlined.

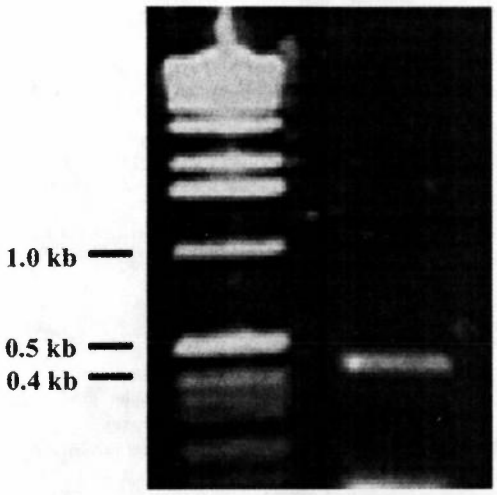

Fig. 3. *Citrus sinensis* AP1-like homologue isolated by RT-PCR from apical bud RNA of 'Washington' navel orange.

the predicted amino acid sequence shares only 56.8% sequence identity with the *Arabidopsis* AP1 peptide. The lower similarity in the K-box was seen in other species as well. *Betula pendula* and *P. radiata* were 68.6% and 46.0% identical to the *C. sinensis* K-box (without the insertion). The effect of the insertion on protein function has not yet been determined. However, the deletion of a single hydrophobic lysine residue in the K-box of the deficiens (DEF A) gene in *Antirrhinum majus* caused a homeotic conversion of petals to sepals and stamens to carpels (Schwarz-Sommer et al., 1992). The MADS-box domain of the *Citrus* AP1-like peptide is more highly conserved than the K-box, consistent with other plant species.

Terminal flower. A 1.14-kb *C. sinensis* genomic fragment was isolated and when compared to the genomic and cDNA *Arabidopsis* TFL sequences, the *C. sinensis* genomic fragment spanned exons 1 though 4. Removal of predicted introns yielded only a 246-bp open reading frame sharing 77.3% nucleotide and 82.9% amino acid identity with the *Arabidopsis* TFL homologue (Fig. 5). Comparing the *C. sinensis* TFL peptide with the other sequences used in the alignment, aa identity ranged from 72.0% to 87.3%. Exon length is similar between

MADS-box I-region

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1.  VELKRIENKINRQVTFSKRRAGFLKKAHEISLLCDAEVAVIIFSHKGKLFEYSTDSCMEKILERYERYAYAERQLIAPDPE
2.  VQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYERYSYAERQLIAPESD
3.  VQLKRIENKINRQVTFSKRRAGLMKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYERYSYAERQLIAPESD
4.  VQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYERYSYAERQLIAPESD
5.  VQLKRIENKINRQVTFSKRRGGLLKKAHEISVLCDAEVAVIVFSHKGKLFEYATDSSMEKILERYERYSYAEAQLVAADSE
6.  VQLRRIENKINRQVTFSKRRGGLVKKAHEISVLCDAEVALIVFSHKGKIFEYSSDSCMEQILERYERYSYTERRLLASNSE
7.  -----IENKINRQVTFSKRRSGLLKKANXISVLCDAEVALIVFSHRGKLFEYSTDACMEKILERYERYSYAERELVNDPET
8.  VQLRRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVGLHCFSSKGKLFEYSTDSCMERILERYERYSYAERQLNATDVE
9.  VQLKRIENKINRQVTFSKRRSGLLKKANEISVLCDAEVALIIFSTKGKLYEYATDSCMDKILERYERYSYAEKVLISAESD
10. VQLKRIENKINRQVTFSKRRNGLLKKAHEISVLCDAEVAVIVFSPKGKLYEYATDSRMDKILERYERYSYAEKALISAESE
11. LQLKRIENKINRQITFSKRRAGLLKKAHEISVLCDAEVALIVFSAKGKLFEYSTDSCMERILERYERYSYAEHQVLASETE
12. VQLKRIENKINRQVTFSKRRSGLMKKAHEISVLCDAEVALIIFSTKGKLFEYSNDSCMERILERYERYSYTERQLLANDNE
13. VQMKRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVGLIVFSTKGKLFEYATDSCMERILERYERYSYAERQLVSTDHS
14. VQLKRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVGLIVFSTKGKLFEYANDSCMERILERYERYSFAEKQLVPTDHT
15. VELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSSSSMMKTIEKYQKCSYGSLETNCSINE
16. ------ENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSTSNMLKTLERYQKCSYGAEEVNKPSKE
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K-box

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1.  ----SQRNWPLECNNLKAKIDLLQKNQRCSSKSTFYTGSQIDLFRHYLGEDLESLSLKDIQQLEQQLDTALKQVRTK
2.  ----VNTNWSMEYNRLKAKIELLERN----------QRHYLGEDLQAMSSKELQNLEQQLDTALKHIRSR
3.  ----SNTNWSMEYNRLKAKIELLERN----------QRHYLGEDLQAMSPKELQNLEQQLDTALKHIRSR
4.  ----VNTNWSMEYNRLKARIELLERN----------QRHYLGEDLNAMSPKELQNLEQQLDTALKNIRTR
5.  ----GQGSWTMEFARLKGKVELLQRN----------HRHYLGDDLESLSHKELQNLEQQLDTALKHVRTR
6.  --SSVQENWSLEYAKLKAKIDLLQRN----------HKHYMGEDLDSLNLKDLQNLEQQLDTSLKLIRSR
7.  PFLNVQKNWSLECTRLGEKIELLQRN----------IGHYMGQHLDSLSLKDLQHFEQQL----------
8.  ----TPGSWTLEHAKLKARLEVLQRN----------QRHYAGEDLDSLSMKELQNLEQQLDSALKHIRSR
9.  ----TQGNWCHEYRKLKAKVETIQKC----------QKHLMGEDLESLNLKELQQLEQQLENSLKHIRSR
10. ----SEGNWCHEYRKLKAKIETIQKC----------HKHLMGEDLESLNPKELQQLEQQLDSSLKHIRSR
11. ----SIGSWTLEHAKLKARLEVLHRN----------YRHFMGEDLDSLSLKDLQNLEQQLESALKHIRSR
12. ----STGSWTLEHAKLKARVEVLQRN----------QRHYMGEDLQSLSLKELQNLEQQLDSALKHIRSR
13. ----SPGSWNLEHAKLKARIEVVQRN----------QRHYMGEDLDSLSMKDLQNLEQQLDSSLKHIRSR
14. ----SPVSWTLEHRKLKARLEVLQRN----------QKHYVGEDLESLSMKELQNLEHQLDSALKHIRSR
15. ----MQNSY-QDYLELKARVEVLQRS----------QRNLLGEELGPLNSKELEQLEHQLENSLKQIRSA
16. ----LENSY-REYLKVKARFEALQRT----------QRNLLGEDLGPLNTKILQLERQLLESSLNQVRST
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Fig. 4. Sequence comparison of AP1 and AP1-like proteins. Sequences used with accession numbers include: 1) *Citrus sinensis*; 2) *Sinapsis alba* (X81480); 3) *Arabidopsis thaliana* (Z16421); 4) *Brassica oleracea* (U67451); 5) *Betula pendula* (X99653); 6) *Nicotiana tobacum* (AF009126); 7) *Actinidia deliciosa* (AF181664); 8) *Capsicum annuum* (AF130118); 9) *Oryza sativa* (AB041020); 10) *Zea mays* (Z46400); 11) *Eucalyptus globulus* (AF305076); 12) *Malus* ×*domesica* (U78948); 13) *Petunia* ×*hybrida* (AF176782); 14) *Lycopersicon esculentum* (X60757); 15) *Pinus radiata* (U42399); 16) *Populus tremuloides* (AF034095). MADS-box domain is shaded with the phosphorylation site in bold and underlined. K-box domain is shown in the inverted box with conserved hydrophobic residues indicated with an asterisk.

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1. TNGHEFFPSTVV-SKPRVEIQGG-DMRSFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDATFGRELVSYEIPRPNIGIHR
2. SNGHEFLPLAVS-SKPRVEIHDG-DLRSFFTLVMTDPDVPNPSDPFLKERLHWLVMNIPGTTDATFGKEVVSYELPKPNIGIHR
3. SNGHELFPLAVS-SKPRVEIHDG-DLRSFFTLVMTDPDVPNPSDPFLKERLHWLVMNIPGTTDATFGKEVVSYELPKPNIGIHR
4. SNGHELFPSSVS-SKPRVEIHGG-DLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSIGIHR
5. FNGHELMPA-VIAAKPRVEI-GGEDMRSAYTLIMTDPDVPGPSDPYLREHLHWIVTDIPGSTDSSFGREIVSYESPKPVIGIHR
6. FNGHELYPSAVVS-KPRVEVQGG-DLRSFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDASFGREVISYESPKPNIGIHR
7. YNGHEFFPSSVTS-KPRVEVHGG-DLRSFFTLIMIDPDVPGPSDPYLREHLHWIVTDIPGTTDCSFGREVVGYEMPRPNIGIHR
8. YNGHELFPSAVTS-TPRVEVHGG-DMRSFFTLIMTDPDVPGPSDPYLREHLHWIVTDIPGTTDSSFGKEVVSYEMPRPNIGIHR
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Fig. 5. Sequence comparison of TFL proteins. Sequences used with accession numbers include: 1) *Citrus sinensis*; 2) *Brassica oleracea* (3650424); 3) *Brassica napus* (3650418); 4) *Arabidopsis thaliana* (U77674); 5) *Nicotiana tobacum* (AF145259); 6) *Oryza sativa* (AF159882); 7) *Lycopersicon esculentum* (U84140); 8) *Antirrhinum majus* (S81193). Conserved or similar aa substitutions

the species. There is, however, a noticeable difference between the *Arabidopsis* and *C. sinensis* TFL intron length. The second intron of the *C. sinensis* homologue is over three times longer than that of *Arabidopsis*. Whether this has any effect on expression remains to be determined. However, at this time we have not yet detected the *C. sinensis* TFL transcript in any of the total mRNA populations screened thus far using RT-PCR or RNase-protection assay.

Conclusions

The sequence similarities, both at the nucleotide and amino acid level, as well as the lengths of the *C. sinensis* fragments correspond well with sequences of GenBank accessions and highly suggest that the amplified fragments are derived from LFY, AP1, and TFL homologues in *Citrus* . The putative LFY protein has a very high similarity to other LFY proteins in GenBank, including the gymnosperm, *P. radiata.* The predicted *C. sinensis* AP1-like protein has a 17-residue insertion in the protein-protein binding domain (K-box). The significance of this insertion on protein interaction is not clear at this time and merits investigation. The small amount of aa residues predicted from the *C. sinensis* TFL homologue shows high identity with other TFL genes. Given the high degree of aa identity between *C. sinensis* and *A. thaliana* floral genes, ongoing research using the *A. thaliana* floral model should facilitate future attempts to determine regulation of flower initiation and development in *C. sinensis.*

Acknowledgments

This research was supported in part by the Citrus Research Center and Agricultural Experiment Station of the Univ. of California, Riverside. This paper represents a portion of the dissertation submitted by L.J. Pillitteri in partial fulfillment of requirements for the PhD in Botany at the Univ. of California.

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