Summary  To increase our understanding of the genes involved in flower development in citrus, we identified genes differentially expressed between juveniles and adults of an early-flowering mutant of trifoliate orange (precocious trifoliate orange, Poncirus trifoliata L. Raf.) by suppressive subtractive hybridization (SSH) and macroarray hybridization analyses. Based on dot blot analysis, we confirmed that the juvenile subtracted cDNA library comprised genes upregulated during floral induction, inflorescence development and flowering. A total of 190 nonredundant expressed sequence tags were identified that were related flower development. The temporal and spatial expression patterns of four SSH-enriched genes were studied in adult precocious trifoliate orange by real-time PCR. Among these differentially expressed genes, the expression pattern of C5-B16 was closely correlated with inflorescence development and flowering. The full-length cDNA of C5-B16 was isolated by 5′ and 3′ rapid amplification of cDNA ends. Sequence analysis indicated that C5-B16 is a novel gene in citrus.

Keywords: expressed sequence tags, flowering time, precocious trifoliate orange, rapid amplification of cDNA ends (RACE).

Introduction

In higher plants, the transition from the juvenile to the adult stage, which requires several years, and much longer for some species and genotypes, correlates with the development of the ability to initiate flowering. The long juvenile phase presents a major obstacle in fruit crop breeding. If traits could be identified and manipulated to induce flowering, the exploitation of beneficial recessive mutations and the use of introgressive backcrossing to increase rare alleles in breeding populations would be possible. Recently, a homolog-sequence-based approach has been used to isolate flowering genes from apple (Yao et al. 1999, Kotoda et al. 2000), citrus (Pillitteri et al. 2004a, 2004b, Endo et al. 2005, Tan and Swain 2007), grape (Boss et al. 2002, 2006, Carmona et al. 2007), eucalypt (Southerton et al. 1998, Dornelas et al. 2004) and other commercially important perennial crops (Yu et al. 2005, Dornelas and Rodriguez 2006). Most previous studies have concentrated on a single gene, although the complexity of floral induction, inflorescence development and flowering suggests that numerous genes are involved. In addition, the underlying genetics of flower induction and floral organ formation may differ between woody perennials and herbaceous model species (Brunner and Nilsson 2004, Tan and Swain 2006, 2007). Therefore, the identification and characterization of genes involved in flower development in woody perennials are required.

Citrus is one of the most economically important evergreen fruit crops in the world. Flowering is an essential stage for fruit production, and thus, an understanding of the genetic mechanisms underlying the flowering event is important for genetic improvement. Citrus flowering has been the subject of ongoing investigation for many years (Krajewski et al. 1995), but the long juvenile phase in citrus, commonly lasting for 5 to 13 years, has been a limiting factor for traditional breeding and genetic studies. In 1976, a spontaneous mutant with a short juvenile phase (precocious trifoliate orange) derived from Poncirus trifoliata (L.) Raf, was found in Yichang, Hubei province, China. Twenty percent of the seedlings germinated from the mutant seeds flowered in the first year after germination and then flowered twice or three times per year, whereas wild-type trifoliate orange usually has a juvenile period of 5 to 8 years (Liang et al. 1999). Because the mutant and wild-type have nearly the same morphology except flowering habit, and no DNA polymorphism was detected between them, the mutant is speculated to be a direct variant of the wild-type (Pang et al. 2006). Thus, it provides optimal material for studying floral induction, inflorescence development and the molecular mechanisms underlying flowering.

Compared with other methods for analyzing altered gene expression, such as mRNA differential display (Liang and Pardee 1992), serial analyses of gene expression (Velculescu et al. 1997) and cDNA microarray (Chu et al. 1998), suppres-
sive subtractive hybridization (SSH; Diatchenko et al. 1996) is a productive and efficient approach for identifying and characterizing both known and unknown genes involved in complex developmental processes, mutations and stress responses (Namasivayam and Hanke 2006, Fernandez et al. 2007, Tsuwamoto et al. 2007). In our study, a juvenile subtracted cDNA library was successfully constructed by SSH, screened by macroarray analysis, and a gene differentially expressed in the juvenile and adult phases was confirmed by real-time PCR and by in situ hybridization.

Materials and methods

Plant material

Adult and juvenile precocious trifoliate orange samples were collected in the experiment fields of the National Citrus Breeding Center (30°28′ N, 114°21′ E, 30 m a.s.l.) at Huazhong Agricultural University. The adult trees were 3- to 5-year-old precocious mutants (which flowered 12 to 24 months after germination), and all adult trees had flowered several times. The juvenile material was obtained from seedlings germinated from seeds of the adult mother plants. Because the embryo originates from a nucellar cell in trifoliate orange, the seedlings have the same genetic background as the mother plants. In January 2005, seeds of precocious trifoliate orange were sown in 20-cm pots containing commercial potting mix and perlite (3:1, v/v). Following germination, the seedlings were watered regularly with nutrient solution.

Several plant organs (lateral buds, terminal buds and shoot meristem) at different developmental stages were sampled and immediately frozen in liquid nitrogen and stored at –80 °C until analyzed. In citrus, there are three flushes during the growing season, and the spring flush is the most important one for growth and flower formation. We sampled the terminal bud and the following five buds (the major node position for flower formation) from spring flushes every 2 months in the year after bud swelling. Shoot meristems of the juvenile plants were collected in March, June, September and December. To prepare a representative sample of total RNA from the adult and juvenile tissues for SSH, different developmental stages of plant organs from roughly equal numbers of adult and juvenile plants were pooled for each plant type. For spatial expression analysis of differentially expressed genes at the mRNA level, total RNA was isolated from terminal meristems of spring flushes, leaves, flowers at full bloom, whole fruits at 30 days after flowering and roots of three plants.

Total RNA and mRNA extraction

Total RNA was extracted as described by Hu et al. (2002) with modifications. Modified extraction buffer contained: 2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 30 mM EDTA, 2.0 M NaCl, 100 µg ml−1 proteinase K, 0.05% spermidine trihydrochloride and 4% 2-mercaptoethanol (added just before use). Messenger RNA (mRNA) was isolated from total RNA using Oligotex (Qiagen, Holland) according to the manufacturer’s instructions. The total RNA and mRNA were quantified spectrophotometrically at wavelengths of 230, 260 and 280 nm, and mRNA was adjusted to a final concentration of 1 µg µl−1. The integrity of the total RNA and mRNA was verified by subjecting samples to electrophoresis on 1.2% agarose gels.

SSH cDNA library construction

An SSH library was constructed using the PCR Select cDNA Subtraction kit (Clontech, USA) according to the manufacturer’s instructions. The final amplified products were purified using QIAquick PCR purification kit (Qiagen, USA). The fragments were cloned into the PGEM-T vector (Promega, USA) and used to transform Escherichia coli DH5α. Individual clones from the library were randomly chosen and stored in 384-well plates; 1920 cDNA clones were collected. The inserts were amplified in a 25-µl reaction mix containing 1 µl of single clone bacterial culture as PCR template, 5 µmol primer 2R, 20 nmol primer 1, 0.25 µl Taq DNA polymerase (Shanghai Sangon, China), 2.5 µl 10x DNA polymerase buffer, 5 mmol dNTPs and 37.5 mmol MgCl2. Conditions for the PC were 30 cycles of 94 °C for 5 min, 94 °C for 30 s, 68 °C for 35 s and 72 °C for 1.5 min followed by a 5-min final extension at 72 °C.

Differential screening of SSH library

The PCR products (1 µl) from each of the recovered clones of the subtractive library were printed onto Hybond-N+ nylon membranes (Amersham, U.K.). After air drying, membranes were denatured in 0.6 M NaOH for 2 min, neutralized with 0.5 M Tris-HCl (pH 7.5) for 2 min and rinsed in distilled water for 30 s. Samples were cross-linked to membranes by baking for 2 h at 80 °C and then stored at –20 °C until analyzed. β-Actin cDNA was also printed on each membrane as an internal control. The PCR-amplified cDNA was digested with Rsal to remove the adaptor sequences, and digests were purified with the QIAquick PCR purification kit (Qiagen). Digoxigenin-labeled probes of the SSH tester and SSH driver were synthesized using a DIG DNA labeling kit (Roche, Switzerland) according to the manufacturer’s instructions. The membranes were pre-hybridized for 3 h at 42 °C and then hybridized overnight at 42 °C with probes of the SSH tester and SSH driver. The results from the two hybridizations were compared for each clone by UVP with PDQuest 7.4 (Bio-Rad, USA) and those showing the greatest differential expression were selected for sequencing.

DNA sequencing, sequence analysis and accession numbers

Sequencing of differentially expressed clones was performed with an Applied Biosystems ABI3730 sequencer. Vector and adaptor sequences were trimmed using VecScreen (http://www.ncbi.nlm.nih.gov/VectScreen/VectScreen.html) before further analysis. Homology searches of all sequences were queried in the GenBank database with BLASTn (homology) and BLASTx (translation homology) available at the NCBI network service (http://www.ncbi.nlm.nih.gov/BLAST). The cDNAs were named according to homologous sequences in the database, and cDNAs with BLAST scores < 45 bits (no
homologous stretch > 50 bp) were designated as having no significant similarity. To classify genes into functional groups, the Arabidopsis database at the MIPS website (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp) was searched for each sequence. Sequence data reported in this paper have been deposited with the GenBank data libraries under Accession nos. EU325893–EU325872.

Analysis of differential expressed sequence tags by real-time PCR

Total RNA (3 mg) from the adult and juvenile samples, prepared as described previously for subtractive hybridization, was treated with 3 units of DNase (Promega) and used in first-strand synthesis with an oligo (dT) primer (20-mer) and reverse transcriptase according to the manufacturer’s instructions. Differential expressed sequence tag (EST) expression levels were analyzed by real-time quantitative PCR kinetics using SYBR Green I and primers designed with the Primer Express software (PE Applied Biosystems, Foster City, CA) and tested to ensure amplification of single discrete bands with no primer dimers. The PCR product sizes were 160–200 bp. Real-time PCR products were amplified with 1 µl of the RT reaction mixture as template, 10 µl 2× SYBR Green Master Mix (PE Applied Biosystems) and 5 µmol forward and reverse primers in a final volume of 20 µl. Gene expression was quantified with ABI 7500 Sequence Detection System software (PE Applied Biosystems) and normalized against the expression of β-actin. Real-time quantitative RT-PCR was performed for four replicates per sample, and data represent means ± SD (n = 4).

Molecular cloning and bioinformatics analysis of the full-length candidate genes

Because C5-B16 was isolated from the 5′ untranslated region by SSH, 3′ rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNA. The adult plant mRNA, prepared by the procedures described previously for subtractive hybridization, was used to prepare RACE-Ready cDNA with a SMART-RACE cDNA Amplification kit (Clontech) following the user manual. Open reading frame (ORF) cDNA fragments of C1-C16, C3-L11 and C5-E3 were isolated by SSH. The 3′ and 5′ RACE were performed with gene-specific primer designed based on the respective sequences of C1-C16, C3-L11 and C5-3. The obtained cDNA ends were ligated into PGEM-T vector (Promega) and sequenced. The full-length cDNA sequence was analyzed with the DNAStar LASERGENE programs. For protein domain analysis, we used the translated amino acid sequence of C5-B16 as the query in the InterPro database (http://www.ebi.ac.uk/interpro/index.html) and SWISS-PROT (http://us.expasy.org/ExpasyHunt/). Sequence data from this study have been deposited with the GenBank data libraries under Accession nos. EU325896–EU325897.

RNA in situ hybridization and detection

Shoot apical meristems at different development phases were fixed overnight in 4% formaldehyde and 0.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.0) at 4 °C. Fixed tissues were dehydrated and embedded in Tissue Prep (Fisher Scientific) and sectioned at a thickness of 10 µm with a rotary microtome. Sections were placed on slides coated with poly-D-lysine overnight at 45 °C, as described by Drews (1998). To make probes for in situ hybridization, digoxigenin-labeled sense and antisense probes, cDNA fragments were cloned into PGEM-T (Promega) using digoxigenin-11-UTP and the SP6 or T7 Ribozyme In Vitro Transcription kit (Roche) according to the manufacturer’s instructions. The DIG-labeled RNA probes were degraded to about 150 µl in 0.2 M sodium carbonate buffer (pH 10.2) before hybridization. For hybridization, the sectioned samples were deparaffinized in xylene and rehydrated in a graded ethanol series. Samples were then treated for 30 min at 37 °C with proteinase K (1 µg µl–1). Prehybridization, hybridization, washing and detection were carried out using the Digoxigenin Nucleic Acid Detection kit (Roche) as described by the manufacturer.

Results

Construction of suppression-subtracted cDNA library

Messenger RNA isolated from the adult and juvenile precocious trifoliate orange was used to construct a subtractive cDNA library by applying SSH. The adult mRNA was used as the tester and the juvenile mRNA was used as the driver, and 2 µg of mRNA was collected and used to construct an SSH library according to the manufacturer’s instructions (Clontech). To evaluate the success of the subtraction, β-actin gene expression was compared between subtracted and unsubtracted cDNA by PCR. In subtracted cDNA, PCR products were first detected after 30 cycles, whereas the PCR products of unsubtracted cDNA were first observed after 21 cycles, indicating that the β-actin gene was reduced up to 29-fold by subtractive hybridization, suggesting that the subtraction procedure was successful (Figure 1). The subtractive hybridization and differential screening approach resulted in the isolation of 190 nonredundant ESTs (FE896593–FE896782).

<table>
<thead>
<tr>
<th>Number of PCR cycles</th>
<th>Subtracted</th>
<th>Unsubtracted</th>
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</thead>
<tbody>
<tr>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
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<tr>
<td>24</td>
<td></td>
<td></td>
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<tr>
<td>27</td>
<td></td>
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<tr>
<td>30</td>
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</table>

Figure 1. Analysis of subtraction efficiency by polymerase chain reaction (PCR). The unsubtracted and subtracted pools of cDNA were amplified with primers for the constitutively expressed β-actin gene. Aliquots of the samples were taken after 21, 24, 27 and 30 cycles of PCR amplification, and the products were analyzed on a 2% agarose gel.
Differential screening of SSH library and analysis of SSH cDNA sequences

To exclude false-positive clones and provide additional data on the expression levels of the cloned cDNAs, we performed further cDNA differential screening. The cDNA clones of the differentially expressed genes were first identified by successive screening with the subtracted tester and driver as probes. We chose those clones that showed a >1.5-fold differential hybridization. We identified 812 positive clones, which expressed only or significantly higher expression levels (>1.5-fold) with tester compared with driver (Figure 2). To further reduce false-positive clones, these 812 clones were also tested with the unsubtracted tester and driver cDNA as probes in the second differential screening. Finally, 467 cDNAs were identified.

The 467 differentially expressed clones were sequenced, and 439 (94%) produced good results. BLAST analysis against the GenBank database revealed that these genes share homology with diverse classes of genes from plants such as apple, tomato, citrus, Arabidopsis, and cotton. Of the 467 clones, 162 were singletons, and the remaining 274 were assembled into 28 groups with occurrence ranging from 2 to 69 times. Because synthesized cDNA was digested by Rsal, we obtained only partial sequences of full-length cDNA; thus, different parts of the same differentially expressed transcript might be enriched in the SSH library. As a result, several clones having the same putative function were classified as different sequences. We identified 190 nonredundant sequences. Classification of the 190 clones based on a BLASTx homology search revealed that 111 clones (58%) were homologous to known genes, 40 clones (21%) were homologous to genes of unknown function and 39 clones (21%) had no matches in the database. We identified or assigned putative functions to about two-thirds of the genes in the cDNA collection. The flower-development-related cDNAs of known functions were sorted into seven potential functional categories according to gene annotations from the MIPS Arabidopsis database. The largest set of genes (17%) was assigned to the transcription and post-transcriptional category. Genes involved in disease defense and metabolism formed the second (10%) and the third largest groups (9%), respectively, whereas the remaining categories, especially signal transduction, protein kinase and transport facilitation, comprised a small number of ESTs (Figure 3).

Differential EST expression analysis by real-time PCR

Of the 190 identified ESTs, most showed similarity to genes encoding proteins in transcriptional and post-transcriptional regulation, signal transduction, protein kinase and unknown proteins. To verify whether the genes corresponding to the ESTs generated by SSH were differentially expressed during floral induction, inflorescence development and the flowering process, we analyzed their expression in adult precocious trifoliate orange by semiquantitative RT-PCR (data not shown). Expression of most of the genes was correlated with flower development patterns in the adult plant. Among these genes, the expression patterns of four genes closely paralleled flower development, so the expression levels of these genes was further investigated by real-time PCR using the primers designed within the 5′ untranslated region (Table 1). Of the four selected genes, C1-C16 and C3-L11 showed high identity with two proteins related to flower development in Arabidopsis (Accession nos. BX819153 and BX827054). C5-B16 and C5-E3 shared close identity with the Arabidopsis F-box gene (Accession no. NM_123904) and a gene encoding receptor-like kinase (Accession no. ABT18100.1), respectively.

During the transition from the vegetative to the reproductive condition in the adult precocious mutant, flower induction and flowering are regulated by temperature in subtropical regions. Flower induction occurs as temperature decreases in autumn and winter and growth rate decreases substantially or growth ceases. This period of low or no growth is important for flower induction and the transition from vegetative to flowering shoots. Flowering occurs the following spring as temperatures increase. There is generally one major flowering period, a second much less intense bloom may also occur a few months later (termed the June or off-bloom). During these periods, the mRNA levels of C1-C16, C3-L11, C5-B16 and C5-E3 began to increase after November, peaked in January, decreased after flower induction, and were maintained at low levels during the vegetative growth stage, and then increased in September. The results suggested that expression of these genes was closely correlated with floral induction, inflorescence development and flowering in the adult stage (Figure 4).

To examine the expression patterns of C1-C16, C3-L11, C5-B16 and C5-E3 in more detail, their mRNA levels were analyzed by real-time PCR in roots, spring shoot flushes, leaves, flowers at anthesis and whole fruits at 30 days after flowering.

Figure 2. Differential screening of clones from the flower-related suppression subtractive hybridization library. Two colony dot blots were prepared, and the membranes were hybridized with labeled probes. Arrows indicate positives clones: (a) reverse subtracted cDNA probes; (b) forward subtracted cDNA probes.
Precocious trifoliate orange has three main flushes of growth, and the main and most important one occurs in late winter or early spring (spring flush), when vegetative sprouts and inflorescences are formed, so the mRNA level of flower-related genes were also investigated in spring flushes. These genes showed broad expression patterns, with transcripts detected in all plant organs (Figure 5). Expression levels of the genes were particularly high in flowers (Figure 5), suggesting that the expression may be associated with floral development.

Table 1. Primer sequences for the quantification of transcripts by real-time PCR. Abbreviation: EST = expressed sequence tag.

<table>
<thead>
<tr>
<th>EST</th>
<th>Size (bp)</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-C16</td>
<td>178</td>
<td>Sense CGTTCATCACAGACGACCTT Anti TGCTTTCCAATGAGGCTACT</td>
</tr>
<tr>
<td>C3-L11</td>
<td>182</td>
<td>Sense TTGACTGCTGTTCTATGA Anti GACAGGTCCTTGGCTAT</td>
</tr>
<tr>
<td>C5-B16</td>
<td>184</td>
<td>Sense GTTATCCGACGAGAACAG Anti AGCTAAAGCGACGCCACAAG</td>
</tr>
<tr>
<td>C5-E3</td>
<td>132</td>
<td>Sense GTTATCCGACGAGAACAG Anti AGCTAAAGCGACGCCACAAG</td>
</tr>
</tbody>
</table>

Figure 3. Distribution of the 190 isolated expressed sequence tags (ESTs) by functional category. The putative functions of identified ESTs were determined by sequence comparison with the GenBank database using BLASTx homology search program. All ESTs were functionally classified based on the MIPS website (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp).

Figure 4. Relative quantities of C1-C16, C3-L11, C5-B16 and C5-E3 transcripts in adult precocious trifoliate orange. Values are the means ± SE of at least four replications for the relative expression, which was calibrated against the amount of the β-actin control expression. The primers used for the analyses are shown in Table 1.

Figure 5. Relative quantities of C1-C16, C3-L11, C5-B16 and C5-E3 transcripts in various tissues of adult precocious trifoliate orange: roots, spring flushes, leaves, flowers at anthesis and whole fruits at 30 days after flowering. Values are the means ± SE of at least four replications for the relative expression, which was calibrated against the amount of the β-actin control expression. The primers used for the analyses are shown in Table 1.
rescence development and flowering stages. The profile data for these genes showed flower-specific expression of two unknown proteins. Thus, the expression patterns of C1-C16 and C3-L11 and their sequences similarity to unknown proteins in flower tissue of Arabidopsis suggested that they are required for flower development in precocious trifoliate orange.

Sequence and bioinformatics analysis of C5-B16 and C5-E3

To further characterize early-flower-regulated genes in precocious trifoliate orange, the full-length cDNAs of C1-C16, C3-L11, C5-B16 and C5-E3 were cloned by 5' and 3' RACE. Unfortunately, only the full-length C5-B16 and the 3' region of C5-E3 were obtained.

The C5-B16 sequence, which has been deposited in GenBank under Accession no. EU325896, contained 1368 bp with 990 nucleotides of an ORF. The deduced protein sequence of C5-B16 contained 330 amino acids with a predicted molecular mass of 37.4 kDa. Among these amino acids, 45 are basic (K: 24, 7.3%; R: 15, 4.5%; H: 6, 1.8%) and 55 are acidic (D: 26, 7.9%; E: 29, 8.8%), giving it a predicted pI of 4.92. This sequence shared high homology to an Arabidopsis F-box family protein, which has not yet been functionally characterized. F-box proteins are considered to bind substrates for ubiquitin-mediated proteolysis (Kipreos and Pagano 2000). In contrast to the many studies on F-box proteins in Arabidopsis, the function of F-box proteins in woody plants is poorly understood. We aligned the amino acid sequences of C5-B16 with six reported F-box protein homologs, and all seven proteins shared high homology along their entire length.

The sequence obtained for C5-E3 by RACE was 1628 bp encoding a partial ORF with 421 amino acids. The C5-E3 sequence has been deposited in GenBank under Accession no. EU325897. BLASTx analysis of C5-E3 showed that it was closely related to the Arabidopsis FERONIA receptor-like kinase gene. A comparison with the Prosite database using MotifScan identified a protein kinase domain in the partial amino acid sequence of C5-E3.

Expression of F-box in shoot apical meristems of precocious trifoliate orange

To understand the possible role of F-box, its expression in various tissues of precocious trifoliate orange was examined to provide clues to its physiological function during floral induction, inflorescence development and the flowering process. Shoot apical meristems at different development phases were fixed, sectioned and hybridized with probes complementary to the F-box probe (Figure 6). F-box was detected in axillary bud meristem of the shoot apical meristem during the juvenile vegetative growth phase (Figure 6a). At the floral determination phase, expression of F-box was gradually increased in the shoot apical meristem (Figure 6c). At the inflorescence forming phase, the expression of F-box was upregulated to high levels in floral organ primordia, whereas the expression of F-box was high in anther (Figure 6d). These results indicated that F-box mRNA expression was low in meristem during the vegetative growth phase and in axillary buds before floral determination, and its expression was upregulated to high levels in meristem during the floral determination phase. Thus, F-box expression was correlated with floral induction, inflorescence development and flowering.

Discussion

Over the last two decades, genes related to flower development have been identified, such as LEAFY (LFY), APETELA1 (AP1), TERMINAL FLOWER (TFL1), FLOWERING LOCUS T (FT), APETELA3 (AP3), SUPPRESSOR OF OVER-EXPRESSION OF CO1 (SOC1) and WUSCHEL (WUS) (Kobayashi et al. 1999, Pillitteri et al. 2004a, 2004b, Tan and Swain 2007). These genes have been isolated from different types of citrus and their cDNA sequences have been successfully isolated from precocious trifoliate orange (Accession nos. EU400600, EU400601, EU400602, AY970823.1 and AY970824.1). Although the mRNA transcripts of these genes showed no significant differences between the juvenile and adult stage, their expression patterns were closely correlated with floral induction, inflorescence development and flowering in adult mutant plants (Zhang et al., unpublished data). There are two possible explanations why these previously reported flowering-related genes were not discovered in our SSH library. First, suppression PCR prevented amplification.
of these genes during enrichment of target molecules because their mRNA transcript levels did not differ significantly between the juvenile and adult stage. Second, only 467 differentially expressed clones were sequenced. In this study, a total of 190 significantly upregulated genes were discovered during early flower process.

The cDNA subtraction strategy identified 190 unique candidate genes (FE896593–FE896782) that were upregulated during the early flowering process, indicating that they may play important roles in the transition from the vegetative phase to floral development in woody plants. Almost 99.5% of these upregulated genes from precocious trifoliate orange have a match with wild-type trifoliate orange within the HarvEST citrus EST database (http://harvest.ucr.edu/). These results reflect the high similarity between the mutant and wild-type genotypes, which is consistent with the single-locus mutation hypothesis and common developmental program between these genotypes.

BLAST searches of the 190 genes showed that many of the isolated genes had significant homology to known sequences in GenBank. About 17% of the ESTs had a high identity with known transcription and post-transcriptional regulatory genes, indicating that these genes may be key regulators controlling flower development by activating or repressing numerous genes. For example, UBQ3 and 26S proteasome were the most frequently isolated genes in this study. The UBQ3 gene belongs to the ubiquitin gene family, an important component of the post-transcriptional regulatory pathway. UBQ3 allows cells to respond rapidly to intracellular signals and change environmental conditions by adjusting the levels of key proteins (Schenk and Snaar-Jagalska 1999, Manning et al. 2002). Protein kinases play critical roles in various aspects of plant growth and development. For example, ASK1, an F-box protein in Arabidopsis (closely related to the Arabidopsis FERONIA gene) is identified as a receptor protein kinase gene. In Arabidopsis, FERONIA expression is closely correlated with floral development. Our examination of the temporal and spatial expression pattern of C5-B16 during vegetative and reproductive development corresponded with changes in morphology (Figures 4–6). When buds were swelling in January, C5-B16 showed a high expression level (Figure 4). The mRNA abundance of C5-B16 progressively increased, reaching a maximum during floral development, and decreased when flushes started to grow out (Figure 4). The highest C5-B16 expression level corresponded to flower initiation; the expression of C5-B16 was high in anthers at the inflorescence formation phase, according to in situ hybridization (Figure 6).

These findings suggest that the gene plays an important role in inducing early flowering and shortening the juvenile phase in mutant plants. Because of a variety of technical limitations associated with woody plants, it is generally not feasible to confirm the function of a gene through molecular genetic analysis of mutant trees lacking the gene in question. Instead, the function of a tree gene is usually predicted based on the extent and nature of the similarity between the tree gene and the related gene from the model plant Arabidopsis whose function has been defined by molecular genetic analysis (Tan and Swain 2006). Unfortunately, sequence analysis showed that C5-B16 is a novel gene that has not been functionally characterized in other plants, so it was not possible to predict its function, although many F-box proteins involved in self-incompatibility and floral development have been identified in plants (Zhao et al. 2001, Gagne et al. 2002, Wang et al. 2003, Somers et al. 2004). Functional analysis of the gene will require over-expression in the Arabidopsis genome and assessing the effect on plant development to elucidate its specific roles in floral induction, inflorescence development and flowering. Such information will greatly enhance our understanding of the flowering process in precocious trifoliate orange.

Many protein kinases were found in our library, with about 4% of ESTs identical to this class (Figure 3). Protein kinases are involved in many cellular processes, including regulation of metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, differentiation and responses to a variety of stimuli (Schenk and Snaar-Jagalska 1999, Manning et al. 2002). Protein kinases play an important role in the transition from the vegetative to reproductive phase; for instance, protein kinase CK2 and never-in-mitosis-A (NIMA)-like kinase are involved in the regulation of flowering (Sugano et al. 1999). Calmodulin-binding protein kinase functions as a negative regulator of flowering in tobacco (Hua et al. 2004). We found that C5-E3 (closely related to the Arabidopsis FERONIA gene) is identical to a receptor protein kinase gene. In Arabidopsis, FERONIA expression is closely correlated with floral development. Our examination of the temporal and spatial expression pattern of C5-E3 showed that it was closely correlated with floral induction, inflorescence development and flowering in precocious trifoliate orange (Figures 4 and 5), consistent with previous reports on the FERONIA gene (Escobar-Restrepo et al. 2007).

Two genes related to flower development (C1-C16 and C3-L11) that were discovered in our SSH library showed high identity with flower-related genes from Arabidopsis, and their
expression levels were closely correlated with flower development (Figures 4 and 5), suggesting that they are involved in flower development in precocious trifoliate orange. Functional analysis is being undertaken to determine if these newly identified genes are involved in flower development in other woody plant species.

Acknowledgments

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