

## Identification of transcription factors potentially involved in the juvenile to adult phase transition in *Citrus*

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- **Background and Aims** The juvenile to adult transition (JAT) in higher plants is required for them to reach reproductive competence. However, it is a poorly understood process in woody plants, where only a few genes have been definitely identified as being involved in this transition. This work aims at increasing our understanding of the mechanisms regulating the JAT in citrus.
- **Methods** Juvenile and adult plants from Pineapple sweet orange (*Citrus sinensis*) and Rough lemon (*C. jambhiri*) were used to screen for differentially expressed transcription factors (TFs) using a 1·15K microarray developed on the basis of the CitrusTF database. Murcott tangor (*C. reticulata* × *C. sinensis*) and Duncan grapefruit (*C. paradisi*) were incorporated into the quantitative real-time reverse transcription–PCR validation in order to select those genes whose phase-specific regulation was common to the four species.
- **Key Results** A browsable web database has been created with information about the structural and functional annotation related to 1152 unigenes of putative citrus TFs (CTFs). This database constitutes a valuable resource for research on transcriptional regulation and comparative genomics. Moreover, a microarray has been developed and used that contains these putative CTFs, in order to identify eight genes that showed differential expression in juvenile and adult meristems of four different species of citrus. Those genes have been characterized, and their expression pattern in vegetative and reproductive tissues has been analysed. Four of them are MADS-box genes, a family of TFs involved in developmental processes, whereas another one resembles MADS-box genes but lacks the MADS box itself. The other three showed high partial sequence similarity restricted to specific Arabidopsis protein domains but negligible outside those domains.
- **Conclusions** The work presented here indicates that the JAT in citrus could be controlled by mechanisms that are in part common to those of Arabidopsis, but also somehow different, since specific factors without Arabidopsis orthologues have also been characterized. The potential involvement of the genes in the JAT is discussed.

**Key words:** *Citrus sinensis*, *C. jambhiri*, *C. reticulata*, *C. paradisi*, database, differential gene expression, juvenile to adult transition, juvenility, transcription factor, JAT.

### INTRODUCTION

Development of higher plants has two distinct phases, juvenile and adult. In general, the juvenile phase is characterized by the inability to initiate floral development in response to floral-inductive cues (Zimmerman *et al.*, 1985).

Phase change in Arabidopsis and other herbaceous species involves the activation of new gene expression programmes. Genetic analyses have identified many genes that allow us to better understand how reproductive competence is regulated. Four genetic pathways exist that mediate the effect of hormones, photoperiod, light quality, temperature and other environmental factors in a complex way that involves an intricate network of signalling pathways (Amasino and Michaels, 2010).

One well-known class of regulators involved in floral transition is the MADS-box gene family (Becker and Theissen, 2003). Among them, *FLOWERING LOCUS C* (*FLC*), one of the central flowering regulators, and a small clade of closely

related genes called *MADS AFFECTING FLOWERING* (*MAF* genes), encode repressors of flowering and regulate the autonomous and vernalization pathways (Michaels and Amasino, 1999; Ratcliffe *et al.*, 2003). *FLC* expression is repressed by vernalization treatments, thereby promoting flowering after winter has passed (Michaels and Amasino, 1999). On the other hand, the second central regulator of the transition to reproductive phase, *CONSTANS* (*CO*), mediates the response to long-day photoperiod (Suarez-Lopez *et al.*, 2001). *FLC* and *CO* regulate the expression of genes responsible for the integration of the signals from the multiple flowering pathways (Simpson and Dean, 2002). One of these genes is *FLOWERING LOCUS T* (*FT*), a mobile flowering signal that acts as a floral promoter. *FT* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), a MADS-box floral activator (Simpson and Dean, 2002), leading to induction of *LEAFY* (*LFY*), another MADS-box gene. *FT* is also responsible for the activation of the MADS-box gene *APETALA 1* (*API*), which is

necessary to establish and maintain flower meristem identity. Once established, *LFY* and *API* lead to flower formation according to the ABC model (Ditta *et al.*, 2004; for a review, see Krizek and Fletcher, 2005). *SOC1* is also activated by an age-dependent mechanism in which *SQUAMOSA BINDING FACTOR-LIKE 9* (*SPL9*) and microRNA156 (*miR156*) are involved (J. W. Wang *et al.*, 2009). *MIR172* and other *SPL* and *APETALA2* (*AP2*)-like genes have been described as regulators of shoot maturation and the developmental timing (Schwartz *et al.*, 2009; Wu *et al.*, 2009).

In woody species, the relevant molecular mechanisms do not seem to match exactly those of Arabidopsis (Rottman *et al.*, 2000; Tan and Swain, 2006), although they seem to share some similarities. Only a few genes have been clearly linked to the juvenile to adult transition (JAT). In spruce, the transcription factor *DAL1* (a MADS-box gene) is involved in the transition to adulthood (Carlsbecker *et al.*, 2004). Ectopic expression of *DAL1* in Arabidopsis shortens the juvenile phase and induces flowering (Carlsbecker *et al.*, 2004). In olive tree, the *JAT* gene, which is highly expressed in juvenile phases but repressed in adult phases, seems to be involved in the control of phase transition (Fernandez-Ocaña *et al.*, 2010). Van der Linden *et al.* (2002) suggested that the MADS-box gene *MdMADS12* plays a role in phase transition. *MdMADS12* was isolated from vegetative apple tissues and falls into the *API* clade of genes involved in floral transition and meristem development (Van der Linden *et al.*, 2002). In kiwifruit, nine MADS-box genes have been characterized and the phenotypes of the corresponding transgenic Arabidopsis lines as well as their expression patterns suggest their involvement in phase change, flowering time and flower development (Varkonyi-Gasic *et al.*, 2011). Likewise, overexpression of the gene *CsTFL* of *Citrus sinensis*, which encodes a regulatory protein of the Raf-1/MEK transduction pathway, is able to rescue the *TFL* mutation of Arabidopsis and appears to be involved in the process of juvenility in citrus (Pillitteri *et al.*, 2004). Conversely, overexpression of the Arabidopsis genes *LFY* and *API* in citrus causes a drastic reduction in the juvenile phase (Peña *et al.*, 2001). In poplar and *Poncirus* (a close relative of *Citrus*) trees, the ectopic expression of *FT* or its homologues accelerated the transition from vegetative shoot apical meristems into inflorescence meristems (Endo *et al.*, 2005; Böhlenius *et al.*, 2006), and in apple, constitutive expression of the *FUL*-like *BpMADS4* gene from *Betula pendula* resulted in flowering during *in vitro* culture of some shoots after transformation (Flachowsky *et al.*, 2007). Recently, Wang *et al.* (2011) overexpressed the Arabidopsis *miR156* in transgenic *Populus × canadensis*, resulting in a drastic prolongation of its juvenile phase, suggesting a conserved regulatory role for *miR156* in woody species. However, there is still much work ahead to determine the gene expression programmes that are taking place in the JAT in woody plants.

In citrus, flowering and fruit production are the main characters related to the adult phase (Navarro, 1990). However, although the loss of juvenility and the capacity to flower are associated, juvenility presents some other features apart from flowering, such as thorniness and vigorous growth (Cameron and Frost, 1968). Some species such as limes and lemons have juvenile phases shorter than 4 years when growing in tropical climates, whereas mandarins, grapefruits and sweet oranges may spend up to 10 years in the juvenile phase when grown from seeds.

The long juvenile phase, in citrus in particular but in woody plants in general, constitutes a drawback for studies of reproductive biology and genetics, as well as a serious constraint for molecular and conventional breeding. Due to that, research on flowering in woody plants has historically been done for the purposes of accelerating progeny testing and speeding up the production of genetically improved seeds.

Citrus is an ideal model to approach studies of phase change due to the phenomenon of apomixis that allows asexual reproduction from seeds. This enables the production of juvenile plants by seed that have exactly the same genotype as the seed source plant, which may be hundreds of years old. Clonal propagation of juvenile and adult plants allows plants with the same genotype and similar size to be produced that still maintain their different juvenile or adult characteristics, and that can be grown under the same conditions. In addition, a recently propagated citrus adult plant initially produces only vegetative growth, and only starts flowering when it reaches a certain size. Thus, these recently propagated adult plants are competent to flower (but do not flower) and allow differentiation of the stages of competence and floral induction.

In this work, with the aim of exploring further and understanding the mechanisms regulating the JAT in citrus, we have created a web-browsable database with information about the structural and functional annotation concerning 1152 unigenes of putative citrus transcription factors (CTFs). We have developed a CTF microarray containing these putative unigenes and we have used it to screen for TFs differentially expressed between meristems from juvenile and adult plants from four citrus species, Pineapple sweet orange, Murcott tangor, Duncan grapefruit and Rough lemon, according to the experimental design described in Fig. 1.

## MATERIALS AND METHODS

### Plant material

Plant material used for this study came from the Citrus Germplasm Bank of pathogen-free plants of the Instituto Valenciano de Investigaciones Agrarias (IVIA) (Navarro *et al.*, 2002). Juvenile and adult buds of Pineapple sweet orange [*Citrus sinensis* (L.) Osb.], Murcott tangor (*C. reticulata* × *C. sinensis*), Duncan grapefruit (*C. paradisi* Macf.) and Rough lemon (*C. jambhiri* Lush) were grafted on sour orange (*C. aurantium* L.) and grown for 5 months under the same greenhouse conditions. The juvenile buds came from 3-month-old seedlings. Adult buds came directly from adult trees. Meristems were dissected under a binocular microscope and immediately frozen in liquid nitrogen. Some plants were used for the microarray experiments and the remaining plants were left as a control to examine their flowering time.

Leaves, bark and flowers were collected during the full flowering season, and different floral organs were dissected by hand using tweezers and a scalpel, and immediately frozen in liquid nitrogen.

### Putative transcription factor identification and annotation

All GenBank citrus mRNA sequences ( $n = 148\,813$ ), including expressed sequence tags (ESTs), were downloaded from

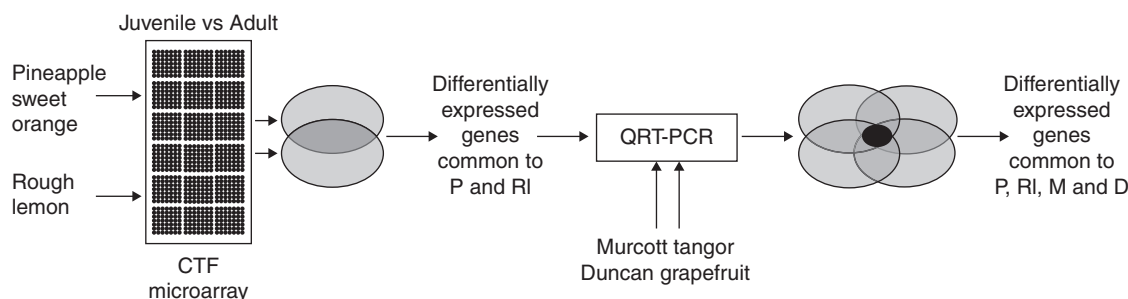


FIG. 1. RNAs from meristems from juvenile and adult plants from Pineapple sweet orange and Rough lemon were labelled for microarray hybridization. A number of transcription factors were identified as differentially expressed. Those common to both lists of genes were selected and further validated by quantitative real-time RT-PCR. At this point, Murcott tangor and Duncan meristem RNAs were incorporated into the quantitative real-time RT-PCR validation in order to select those genes whose phase-specific regulation was common to the four varieties.

NCBI (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/entrez>) and assembled with 85 965 ESTs generated by the Citrus Functional Genomics Project (<http://www.ibmcp.upv.es/genomics/cfgpDB>), using CAP3 (Huang and Madan, 1999). A BLASTX (Altschul *et al.*, 1997) search was then performed against the UniRef90 non-redundant protein database (<http://www.ebi.ac.uk/uniref>) to identify putative TFs in the citrus unigene set. Citrus unigenes were tagged as putative TFs if a BLAST hit annotated the sequence as a TF with an E-value lower than 1E-05.

#### Microarray design and printing

Unigenes with a sequence overlap >300 bp, with an identity >90 % and covering >75 % of the length of one of them, were assumed to represent the same gene, and only one of them was selected for oligo design. In these cases, the longest unigene with a clementine EST was selected. Searching for a specific oligonucleotide probe for each of the citrus unigenes selected was carried out using 'YODA' (<http://pathport.vbi.vt.edu/YODA>). For each unigene, 70-mer oligonucleotides were designed.

Every citrus clone was spotted twice. All samples were printed onto UltraGAPS aminosilane Corning slides, using a MicroGrid II arrayer (Genomic Solutions) in a 16-block format and 12 × 12 spots per block. Relative humidity was kept to 45 %. After printing, the slides were UV cross-linked at 150 mJ.

#### RNA extraction and labelling

Total RNA was extracted from meristems and individual floral organs using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and from leaf and bark tissues according to Ancillo *et al.* (2007). RNA was then used in an amplification reaction with the AminoAllyl Message Amp<sup>TM</sup> aRNA Amplification kit (Ambion, Inc., Austin, TX, USA) following the manufacturer's instructions. UTP-aminoallyl-amplified RNA (7.5 mg) was labelled using Cy3 or Cy5 dye (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden).

#### Microarray hybridization

Microarray hybridization and washing were performed as previously described by Bassene *et al.* (2010). Four biological

replicates were made, with two of them reverse-labelled (dye switch) with respect to the other two, to correct for dye bias. Afterwards, arrays were scanned at 532 nm for the Cy3 dye and at 635 nm for the Cy5 dye, with a GenePix 4000B scanner (Axon Molecular Devices, Sunnyvale, CA, USA), at a 10 nm resolution and 100 % laser power. Spot intensities were quantified using GenePix Pro 6.0 (Axon Molecular Devices). Spots with a net intensity in both channels <2-fold the mean background intensity were considered low-signal spots and removed.

#### Microarray data analysis and sequence analysis

Data were global median normalized using GenePix Pro 6.0 (Axon Molecular Devices), and LOWESS correction was applied using the Acuity 4.0 software (Axon Molecular Devices). To detect differentially expressed genes, data were analysed with the Significance Analysis of Microarray (SAM) package (Tusher *et al.*, 2001) using two-class unpaired comparison.

For fine homology studies of the selected genes, BLASTX against the NCBI and TAIR9 ([www.arabidopsis.org](http://www.arabidopsis.org)) databases was carried out using default parameters and an arbitrary non-stringent threshold of 1E-05 for the E-value.

#### Quantitative real-time reverse transcription-PCR (RT-PCR)

Prior to cDNA synthesis, total RNA was treated with DNase I according to Bassene *et al.* (2009). Then, 2 µg of DNase-treated RNA was reverse transcribed with oligo(dT)<sub>23</sub> primer in a 20 µL reaction mixture using RevertAid M-MuLV reverse transcriptase (Fermentas). After heat inactivation, 1 µL of the 3-fold diluted cDNA was used as a template to perform real-time quantitative PCR in a total volume of 20 µL. All quantitative PCR analyses were performed with the LightCycler 480system (Roche Applied Science, Indianapolis, IN, USA) and PerfeCTa<sup>®</sup> SYBR<sup>®</sup> Green FastMix<sup>®</sup> (Quanta Bioscience, Gaithersburg, MD, USA). The PCR conditions were as follows: 95 °C for 3 min and 40 cycles of PCR (95 °C for 15 s and 60 °C for 1 min). Primer pairs for each gene are shown in Table 1. Two pooled biological replicates with three technical replicates were performed. Data were normalized against the citrus *ACT11* gene by using the primers 5'-CAGTGTGTTGGATTGG AGGATCA-3' and 5'-TCGCCCTTTGAGATCCACAT-3'.



TABLE 1. Changes in gene expression estimated by microarray hybridization and by quantitative real-time RT-PCR

Unigene ID	Primers	Fold change average in microarray*		Fold change average in qRT-PCR†			
		Rough lemon	Pineapple seet orange	Duncan grapefruit	Rough lemon	Murcott tangor	Pineapple sweet orange
CTF0607	5'-AAGCCATGGCAGCAATCG-3' 5'-GATGTCTGAACTCTGGCCACTT-3'	4.70	3.98	6.41	7.40	6.94	6.29
CTF0899	5'-AACTGCTATGACATGGAAAACATCA-3' 5'-CCTGAACCCAGTTGTTGAAGTCT-3'	2.17	1.85	1.58	1.62	1.68	1.30
CTF0931	5'-GGACATCTGAATGAAATTGATGCT-3' 5'-CATGCTGTTTCAATCTCAGGTTCT-3'	7.00	1.48	2.90	2.80	6.12	1.93
CTF0516	5'-AAGTTCTCTCATCGACTAGCA-3' 5'-TGATTGAACTGGGTAGCGATTG-3'	2.47	3.14	1.93	1.89	2.70	2.03
CTF0558	5'-CGAATGAGGTCTCGAGAAGCA-3' 5'-AATCTTGATCCCTTCAAAATCGAA-3'	1.57	2.05	5.39	4.83	6.67	1.94
CTF0173	5'-AAAGAAGGTAAAGAAAAGGAGAAGCT-3' 5'-GGATGAGTTCCAGTCATGATTTAGC-3'	6.33	13.05	10.02	7.00	3.79	14.25
CTF0316	5'-CGCTACCAGAGTCGCATTACAG-3' 5'-AGTTGCAGGCTCGAATATCCA-3'	2.87	3.17	7.22	5.27	3.10	1.63
CTF0397	5'-CGATCCCTGCGGTATCAAGT-3' 5'-GCTCATCTGAACCAGAGTTTGAGTT-3'	2.37	1.41	3.90	2.55	3.58	3.32

\*Average fold change of the mean of expression values of four replicates in the microarray in adult samples with respect to juvenile samples.

†Average fold change of three independent RT-PCRs for three independent samples for each variety.

## RESULTS

### Database construction and development of an oligonucleotide microarray of CTFs

In order to obtain a comprehensive non-redundant set of citrus unique sequences, all available citrus mRNA sequences, including ESTs, were downloaded and assembled, resulting in a total of 49 155 non-redundant unigenes (see the Materials and Methods). Of these, 1834 were identified as putative TFs, based on their similarity to a UniRef90 protein annotated as a TF. To reduce further the sequence redundancy in the putative CTFs found, very similar unigenes were assumed to represent the same gene, and only the longest unigene with clementine ESTs was selected, resulting in 1306 non-redundant putative CTFs. When searching for a specific oligonucleotide probe for each of these selected citrus unigenes, a total of 154 unigenes did not yield an oligonucleotide specific enough to be used as a probe, resulting in a specific oligonucleotide set for putative CTFs of 1152 sequences. An oligo-based microarray was constructed using 70-mer specific oligonucleotides representing these 1152 putative CTFs.

A web-browsable CTF database was created containing information about the 1152 identified putative CTFs. The database is available at <http://www.ibmcp.upv.es/genomics/citrusTF>. The web interface to the CitrusTF database is not just a simple query to search by using sequence identifiers or keywords, or a collection of mere data tables. It can also be used with any combination of almost every different functional and structural annotation criterion in the queries. In addition, bulk queries using a file with a list of unigene names are implemented. The unigenes obtained as query results can be inspected individually, but also bulk downloads of the sequences, names or orthologues are allowed. The individual unigene web page view contains graphical and textual summaries of the assembly and annotation processes, and hyperlinks to the first hits of the external databases

searched with BLAST are provided, as well as their descriptions and E-values. The full BLAST results can also be retrieved. Gene Ontology annotation results are also shown in a table with links to the GO term description pages, using the AmiGO tool (<http://amigo.geneontology.org>).

### Identification of TFs differentially expressed in meristems from juvenile and adult plants

In order to identify TFs differentially expressed in meristems from juvenile and adult plants, four citrus species showing significant phenotypic differences in both developmental phases were chosen. Thus, juvenile and adult plants from Pineapple sweet orange, Murcott tangor, Duncan grapefruit and Rough lemon were grown as described in the Materials and Methods. Some of the plants were collected 5 months after grafting and used to dissect their meristems. Some others were used as control plants to examine their flowering time in order to ensure that, in our experimental conditions, adult plants were competent to flower but did not undergo floral induction. Control plants did not flower during the first spring following harvest of the experimental samples and only flowered during the second spring, thus ensuring that plants did not undergo floral induction at the moment of harvesting the material. RNA was extracted from meristems and, according to the experimental design shown in Fig. 1, that of Pineapple sweet orange and Rough lemon was hybridized to the oligonucleotide microarray. Four replicates were analysed for each species, comparing juvenile vs. adult expression using a dye switch experimental design (GEO accession numbers GSE39798 and GSE39799). After data normalization, expression levels were analysed with the SAM package (Tusher *et al.*, 2001) using two-class unpaired comparison. Several TFs were identified as differentially expressed between adult and juvenile plants for both species. The SAM output for Rough lemon identified 53 genes as

differentially expressed between adult and juvenile plants, with a false discovery rate of 4.9 %. Forty of them were overexpressed in juvenile plants and 13 in adult plants (Supplementary Data Table S1). In the case of Pineapple sweet orange, 46 genes were identified as differentially expressed. Twelve of them were overexpressed in juvenile plants and 34 in adult plants (see Supplementary Data Table S1). The two SAM outputs were then compared and 12 genes were common to both lists of differentially expressed genes (Table 2). These genes were selected and considered for further analysis. In order to identify the genes whose phase-specific regulation was recurrent not only in the two species analysed in the microarray approach but also in two other *Citrus* species, i.e. Duncan grapefruit and Murcott tangor, quantitative real-time RT-PCR was accomplished and RNA from the four species was included in the analysis. Eight out of 12 genes showed significant expression level differences between the two phases in the four species studied (Table 1). All eight genes common to the four species showed higher expression in adult samples than in juvenile samples.

#### *Expression pattern of the identified genes in young and adult vegetative tissues*

Expression analysis of the eight selected genes in meristems, leaf and bark tissues from young and adult plants was accomplished by quantitative real-time RT-PCR. According to the experimental design and quantitative validation, all CTFs analysed showed a marked difference in expression between meristems from juvenile and adult plants, being significantly more expressed in adult plant meristems than in juvenile meristems (Fig. 2A; Supplementary Data Table S2A). When the differences in CTF expression between leaves and bark from young and adult plants were analysed, most of the genes (*CTF0607*, *CTF0931*, *CTF0173*, *CTF0899*, *CTF0516*, *CTF0397* and *CTF0316*) were in the same range as that present in meristems (Fig. 2A; Supplementary Data Table S2A), showing no expression in juvenile tissues or a much higher expression in adult than in juvenile tissues. *CTF0588* showed a broader expression pattern.

Differences between juvenile and adult leaf could be observed in Rough lemon and sweet orange, but not in grapefruit or tangor, where transcripts were detected in both juvenile and adult leaf and bark tissues (Fig. 2A; Supplementary Data Table S2A). In bark, differences could be observed in Rough lemon, sweet orange and tangor, but not in grapefruit.

#### *Expression pattern of the identified genes in reproductive organs*

When gene expression was analysed by quantitative real-time RT-PCR in floral organs, two groups of genes could be distinguished according to their different pattern of expression (Fig. 2B; Supplementary Data Table S2B). *CTF0607*, *CTF0931* and *CTF0316* showed no expression or very low levels of expression in all reproductive organs (Fig. 2B; Supplementary Data Table S2B). The second, more heterogeneous group, consisted of *CTF0516*, *CTF0588*, *CTF0899*, *CTF0397* and *CTF0173*, which showed considerable levels of expression in specific reproductive organs (Fig. 2B; Supplementary Data Table S2B). *CTF0397*, *CTF0588* and *CTF0173* were mainly expressed in sepals, carpels and fruit primordia (Fig. 2B; Supplementary Data Table S2B), whereas *CTF0899* and *CTF0516* appeared to be overexpressed in sepals and to a lesser extent in carpels and fruit (Fig. 2B; Supplementary Data Table S2B).

#### *Sequence and bioinformatic analysis of CTF genes*

Selected genes were re-checked by BLASTX searches against the TAIR9 ([www.arabidopsis.org](http://www.arabidopsis.org)) database. Results (shown in Table 3) revealed that five of them showed high similarity to Arabidopsis MADS-box factors, although one lacks the MADS domain and could not strictly be considered a bona fide MADS-box TF. *CTF0173* showed high similarity to the Arabidopsis gene *FRUITFULL* (*AtFUL*), showing 99, 81 and 34 % similarity at the amino acid level in the MADS domain, K-box domain and in the rest of the protein, respectively (Fig. 3). The best blast hit for *CTF0516* was *SEPALLATA1* (*AtSEPI*), for *CTF0607* it was *AGAMOUS-LIKE 42* (*AGL42*)

TABLE 2. List of differentially expressed genes common to Rough lemon and Pineapple sweet orange

Unigene ID	Annotation	Rough lemon		Pineapple sweet orange	
		M-value*	FDR (%) <sup>†</sup>	M-value*	FDR (%) <sup>†</sup>
<i>CTF0607</i>	MADS-box protein PTM5	-2.232	0	-1.993	0
<i>CTF0899</i>	MYB transcription factor MYB84	-1.120	0	-0.891	0
<i>CTF0931</i>	SVP-like floral repressor	-2.807	0	-0.568	0
<i>CTF0516</i>	MADS-box protein 2	-1.305	4.9	-1.650	0
<i>CTF0558</i>	APETALA1	-0.648	5	-1.037	0
<i>CTF0173</i>	MADS5 protein	-2.663	0	-3.707	0
<i>CTF0316</i>	MADS-box protein 15	-1.523	4.9	-1.663	0
<i>CTF0397</i>	At3g49760	-1.244	4.9	-0.498	1.9
<i>CTF0515</i>	AtMYB2	-0.377	4.9	-1.038	0
<i>CTF0588</i>	Putative homeodomain protein	-0.348	4.9	-0.278	2.87
<b><i>CTF0170</i></b>	Ethylene response factor 5	0.655	4.1	14.405	5.1
<b><i>CTF0723</i></b>	Pathogenesis-related transcription factor and ERF	2.337	0	0.368	5.1

Genes highlighted in bold were upregulated in the juvenile phase; the other genes were overexpressed in the adult phase.

\*The M-value is the base two logarithm of the ratio between the background-subtracted foreground intensity measured in the red and the green channels.

<sup>†</sup>Negative values mean higher expression in adult samples and positive ones higher expression in juvenile samples. FDR is the false discovery rate estimated for each gene by SAM (Significance Analysis of Microarrays) as the percentage of genes falsely identified as differentially expressed.

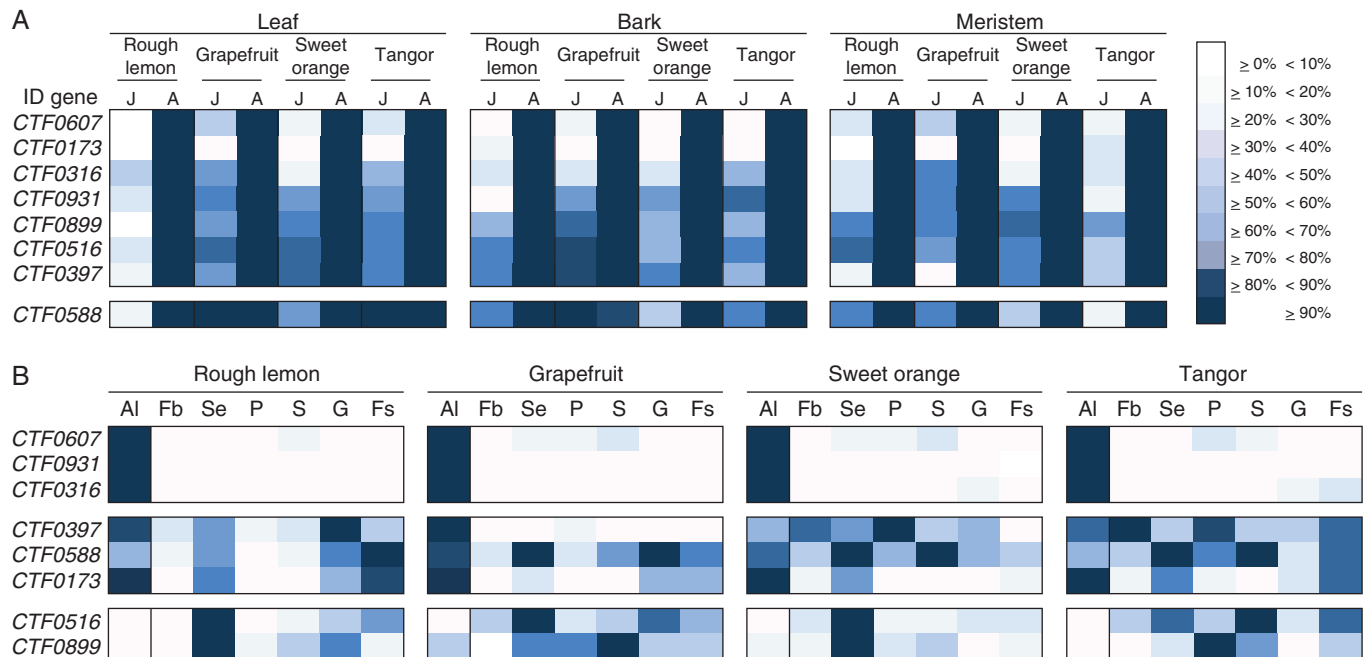


FIG. 2. Expression patterns of selected CTF genes in juvenile and adult plant tissues (A) and in different floral organs (B) in citrus plants. The analysis was performed in four *Citrus* species (Rough lemon, Duncan grapefruit, Pineapple sweet orange and Murcott tangor). The figure illustrates relative expression levels of each gene measured by quantitative real-time RT-PCR given as heat maps in blue-scale, where darker colours represent stronger expression. For each gene, all gene level profiles were normalized as follows. The highest signal intensity, for each comparison, is given a value of 100 % (shown in darkest blue), whereas absence of signal is given a value of 0 % (shown in white). Normalization was assessed by analysis of the citrus *ACTIN 11* transcript in the same samples. J, juvenile; A, adult; Al, adult leaf; Fb, flower bud; Se, sepals; P, petals; S, stamens; G, gynoecium; and Fs, fruit set.

TABLE 3. Sequence analysis of the CTF genes

Unigene ID	Protein length	Homologous locus*	Locus description/function	BLASTX E-value	Query coverage <sup>†</sup>	Domains <sup>‡</sup>
<i>CTF0173</i>	244	At5g60910.1	FRUITFULL, AGAMOUS-LIKE 8	1.00E-63	98 %	MADS and K-box
<i>CTF0516</i>	249	At5g15800.1	SEPALLATA1, AGAMOUS-LIKE 2	2.00E-77	100 %	MADS and K-box
<i>CTF0607</i>	212	At5g62165.3	FOREVER YOUNG FLOWER, AGAMOUS-LIKE 42	1.00E-54	97 %	MADS and K-box
<i>CTF0316</i>	188	At5g10140.2	FLOWERING LOCUS C, AGAMOUS-LIKE 25	1.00E-33	100 %	MADS and K-box
<i>CTF0931</i>	152	At2g22540.2	SHORT VEGETATIVE PHASE, AGAMOUS-LIKE 22	2.00E-23	75 %	K-box
<i>CTF0588</i>	642	At5g02030.1	BEL1-LIKE HOMEODOMAIN 9	1.00E-107	80 %	POX and homeodomain
<i>CTF0397</i>	185	At3g49760.1	BASIC LEUCINE-ZIPPER 5	2.00E-16	87 %	bZIP1
<i>CTF0899</i>	324	At1g68320.1	MYB DOMAIN PROTEIN 62	4.00E-62	44 %	SANT1 and SANT2

The deduced protein sequences of citrus cDNAs were searched against the Arabidopsis Information Resource (TAIR) database.

\*The results refer to the best hit when using the BLASTX algorithm to query the non-redundant protein sequence data set of TAIR at [www.arabidopsis.org](http://www.arabidopsis.org).

<sup>†</sup>Extension of the successful alignment between citrus and Arabidopsis proteins using the NCBI BLAST 2 sequences software (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>).

<sup>‡</sup>The conserved domains in each citrus deduced protein were predicted with the Conserved Domain Database (CDD) from the NCBI (<http://www.ncbi.nlm.nih.gov/cdd>).

and for *CTF0316* it was *FLC*. As for *CTF0173*, the percentage similarity in the case of the amino acid sequence of *CTF0516*, *CTF0607* and *CTF0316* with their respective best blast hits was very high in the MADS domain (ranging from 84 to 96 %), also high but somewhat lower in the K-box domain (ranging from 64 to 81 %), and lower in the rest of the protein

(ranging from 45 to 62 %) (Fig. 3). *CTF0931* lacks the MADS domain but showed 82 % similarity to the K-box domain of *SHORT VEGETATIVE PHASE* (*AtSVP*) and 68 % in the rest of the protein.

The other three CTF genes showed high partial sequence similarity restricted to specific domains but negligible outside those

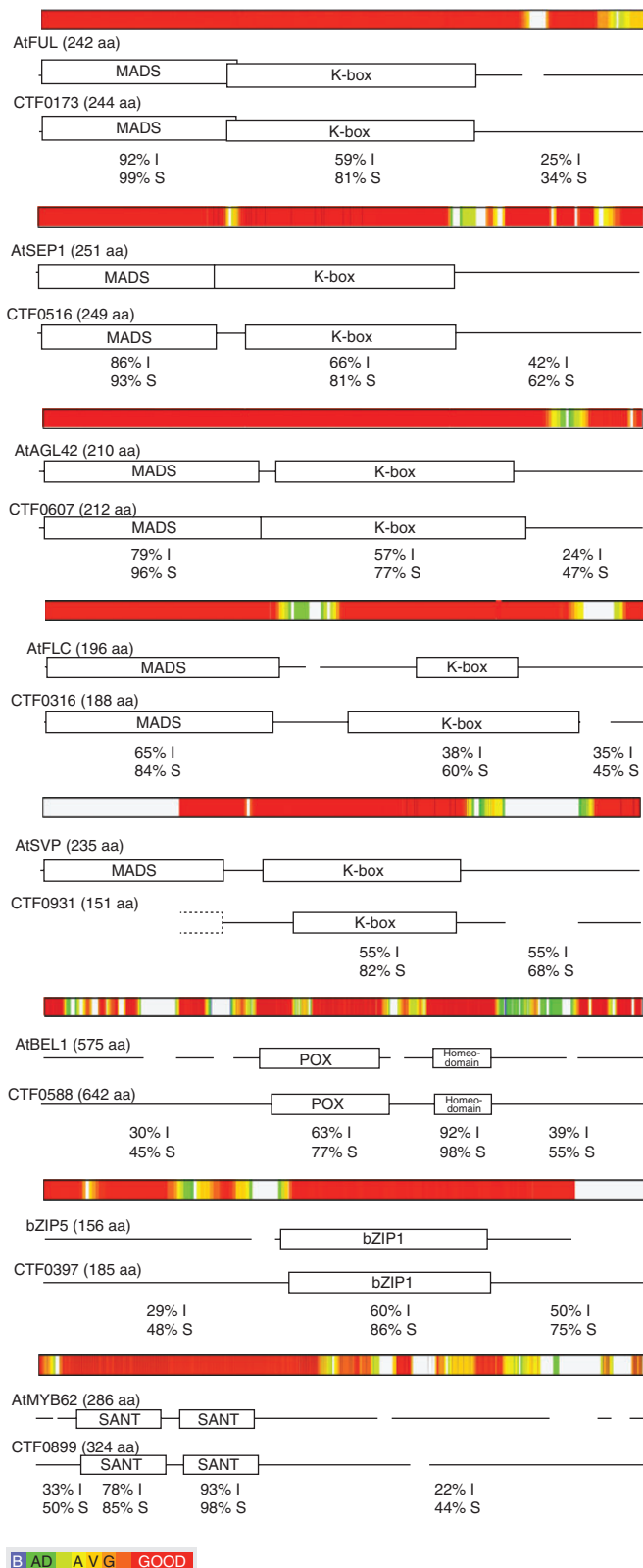


FIG. 3. For each protein, the boxes represent the conserved domains described in the Conserved Domain Database (CDD). The colour-coded alignments between citrus deduced protein and the corresponding Arabidopsis protein showing the best BLAST hit were done by Expresso Alignment of the T-Coffee software (<http://www.tcoffee.org>). The graphic coloured output indicates the level of

domains. CTF0588 contains a POX domain and a homeodomain that shares 77 and 98 % similarity, respectively. CTF0397 bears a bZIP1 domain with a similarity percentage of 86 %. Finally, CTF0899 is a MYB-like protein that contains two SANT domains with 85 and 98 % similarity to those of the MYB62 protein of Arabidopsis.

## DISCUSSION

Genomics and microarray technology constitute a powerful tool to accomplish analysis of large numbers of genes at the same time, enabling a comprehensive knowledge of the processes under study to be gained. Genome-wide identification of CTFs and the subsequent construction of a database of these TFs are of major importance to explore in greater depth the biological function of TFs and the mechanisms of transcriptional regulation. The CitrusTF database constitutes a valuable resource for research on transcriptional regulation and comparative genomics. Together with DPTF from poplar (<http://dptf.cbi.pku.edu.cn/>), CitrusTF is the only existing database of TFs from woody plants. The citrus genome has been predicted to have 29 445 protein-coding genes (Xu *et al.*, 2013) and Arabidopsis 27 416 (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)). The Arabidopsis database RARTF (<http://rarge.psc.riken.jp/rartf/>), developed by Iida *et al.* (2005), contains 1978 TFs. On the other hand, Zhu *et al.* (2007) have been able to identify 1922 TFs in poplar, and the DPTF database currently has 2576 putative TFs. We have been able to identify 1152 CTFs, a relatively high number of TFs that may reflect a significantly high percentage of the total CTFs.

By using the CitrusTF database resource, a microarray of 1·152K was generated, that has been used as a tool for the identification of TFs involved in the JAT in citrus. We have identified a number of TFs differentially expressed in juvenile and adult phases in four citrus species. Since we are interested in general regulatory mechanisms in citrus, we selected those common to the four analysed species and characterized eight TFs putatively involved in this process. These genes exhibited a strong increase at the mRNA level in adult plant meristem samples with respect to juvenile samples and, in addition, some of them are expressed in reproductive tissues, suggesting that they may be involved in the JAT in citrus.

In citrus it is well known that meristems from juvenile plants only produce vegetative growth for several years after seed germination (Cameron and Frost, 1968). In contrast, meristems from adult or mature plants produce both vegetative growth and flowering. A recently propagated citrus adult or mature plant initially produces only vegetative growth, and only starts flowering when it reaches a certain size. Under greenhouse conditions, where the experiments presented here were carried out, 5-month-old plants of the four species studied are still too small to start flowering. Thus, juvenile plants and adult plants competent to flower (but not flowering) were used in this experimental design. Consequently, neither the juvenile nor the adult propagated plants produced flowers during the course of the study. In fact, control plants grown in parallel did not flower until the second

consistency between the final alignment and the library used by T-Coffee (Di Tomaso *et al.*, 2011). The red colour corresponds to alignment portions with a strong consistency, and blue colour corresponds to poorly supported alignments.



spring. In citrus, grafting does not affect the maturity status at all (Navarro, 1990) and this is the basis for commercial propagation by grafting of hundreds of millions of mature plants at the nurseries.

Blast sequence analyses of CTF genes against the TAIR9 protein data set revealed that five of them are highly similar to MADS-box proteins of Arabidopsis (i.e. CTF0607, CTF0931, CTF0516, CTF0173 and CTF0316). *CTF0173* showed high similarity to *AtFUL*, which controls development of the dehiscent valves of the Arabidopsis seed case as well as flower development (Gu et al., 1998). Endo et al. (2006) isolated and characterized a MADS-box gene from *Citrus unshiu* Marc. (*CitMADS5*) which, on the basis of its similarity and its expression pattern, was assumed to represent the citrus *FUL* orthologue. The *CitMADS5* sequence is 100 % identical to *CTF0173*, and in a similar manner as occurs in Arabidopsis, both *CTF0173* and *CitMADS5* were detected in both adult vegetative and reproductive tissues, with only small differences that can be attributed to differences in the *Citrus* species analysed, in the growing conditions or in the harvesting time of the samples. The mRNA of *CTF0516*, which has high similarity to *AtSEP1*, accumulated abundantly in reproductive tissues, especially in sepals, gynoecium and the fruit primordium, and it was also abundant in juvenile tissues of leaves and bark. Similarly, in Arabidopsis, *SEP1* is expressed in early flower development and in developing seed (Ma et al., 1991; Pelaz et al., 2000). *SEP1* encodes a MADS-box TF involved in flower and ovule development, which is functionally redundant with *SEP2*, *SEP3* and *SEP4*. Nishikawa et al. (2009) analysed the expression profile of two *SEP* homologues (*CuSEP1* and *CuSEP3*) from *C. unshiu*. The similarity of *CTF0516* to these genes is 76 and 66 %, respectively. As the *SEP* subfamily in Arabidopsis is formed by four *SEP* genes (Malcomber and Kellogg, 2005), it is quite probable that several *SEP* genes also exist in citrus. Whether these members are functionally redundant or not is a question to be elucidated. The best blast hit for *CTF0607* in Arabidopsis is *AGL42*, which is a *SOC1*-like gene involved in the vegetative to floral transition in Arabidopsis (Dorca-Fornell et al., 2011). The expression of *SOC1* increases according to developmental age (Moon et al., 2003) in the same way as *CTF0607*. Tan and Swain (2007) functionally characterized two *SOC1*-like genes from *C. sinensis* (*CsSL1* and *CsSL2*). Overexpression of these genes in Arabidopsis shortened the time taken to flower, performing the endogenous function of Arabidopsis *SOC1*. The amino acid sequence of *CsSL2* showed 100 % identity to that of *CTF0607*. Thus, *CTF0607* may function as the *SOC1* orthologue in citrus.

The *CTF0316* mRNA is expressed only in vegetative tissues, showing higher accumulation in adult than in juvenile stages. *CTF0316* shares close amino acid identity with Arabidopsis *FLC*. In Arabidopsis, *FLC* acts as a repressor of *SOC1* by sensing seasonal changes. In summer/autumn, *FLC* levels are high to prevent flowering by repressing floral integrators (*CO/FT*). In winter, vernalization leads to repression of *FLC*, and the level of expression decreases until finally it is repressed when the spring season arrives. Therefore, relieved from repression, floral integrators activate flowering. In perennials such as *Arabis alpina*, the regulation of the response to vernalization appears to be similarly preserved. *PEP1*, the *A. alpina* orthologue of *FLC*, is downregulated during prolonged exposure to

cold temperatures (coinciding with the initiation of flowering) and is upregulated after vernalization to repress flowering (R.H. Wang et al., 2009). In *Poncirus trifoliata* (a deciduous relative of *Citrus* which is an evergreen species), five amplicons produced by alternative splicing were characterized for *FLC* (Zhang et al., 2009). Furthermore, in *Citrus clementina* hort. ex Tanaka, Muñoz-Fambuena et al. (2011) reported the effect of fruits on the expression of a citrus *FLC* homologue among other genes involved in alternate bearing. However, whether these genes are indeed *FLC* orthologues has been questioned due to the methodology used for their identification (Samach, 2013). *CTF0316* was 100 % identical in amino acid sequence to the variant 5 characterized by these authors (*PtFLC5*). In a similar way to *CTF0316*, *PtFLC5* expression was only detectable in the adult period; however, their expression patterns in *P. trifoliata* differ from citrus in reproductive organs, since *PtFLC5* was detected in flowers. However, Nishikawa et al. (2009) found that some flowering-related genes that were analysed in parallel in *P. trifoliata* and *C. unshiu* showed different expression patterns, indicating that the contribution of some flowering genes might differ between evergreen and deciduous species.

*CTF0931* shares high similarity with the MADS-box genes *SVP* and *PtSVP* (an *SVP* homologue from *P. trifoliata*; Li et al., 2010) in the I and K domains and to a lesser extent in the C region, but lacks the MADS domain, the most highly conserved region of the MADS proteins (Riechmann et al., 1996; Hartmann et al., 2000). The MADS domain is the major determinant of DNA binding, but it also performs dimerization and accessory factor binding functions (Shore and Sharrocks, 1995). Although several *SVP*-like genes have been characterized in different plant species (Brill and Watson, 2004; Bielenberg et al., 2008; Diaz-Riquelme et al., 2009; Li et al., 2010; Wu et al., 2011) and an alternative splicing has been reported for *SVP* (Severing et al., 2012), to our knowledge no *SVP*-like protein lacking the MADS domain has been reported. Therefore, *CTF0931* may constitute a specific TF of citrus. The lack of the MADS domain would prevent *CTF0931* binding to the CArG-box sequence motif in the DNA, but whether an alternative DNA-binding motif not yet described is present in *CTF0931* is something that still remains to be discovered. Apart from this, *CTF0931* still retains the dimerization and transactivation domains, so it may act by binding to other TFs and modulating their functions. Autoregulatory loops are a common phenomenon in the regulation of MADS-box genes in plants (Honma and Goto, 2001; Gomez-Mena et al., 2005; Zhu and Perry, 2005). Severing et al. (2012) demonstrated that the deletion of a short fragment with a predicted interaction motif in the sequence of *SVP* by alternative splicing resulted in different interaction specificities and therefore different functionalities. In fact, *SVP* has been predicted to have different dimerization partners (Van Dijk et al., 2010), and different *SVP*-like genes have been proven to perform similar but distinct functions (Hartmann et al., 2000; Fornara et al., 2008; Wu et al., 2011).

As in *SVP*, *CTF0931* also contains the EAR repression motif LxLxL. Proteins containing these motifs play key roles in diverse biological functions by negatively regulating genes involved in developmental, hormonal and stress signalling pathways (Kagale et al., 2010). One of these proteins is AGAMOUS-LIKE15 (AGL15), a MADS-box TF that, along with AGL18, is known to act as a repressor of floral transition



in *Arabidopsis* (Adamczyk *et al.*, 2007). Whether CTF0931 functions as a repressor of the phase transition in citrus is something that will require additional experiments.

CTF0588 carries a POX domain and a homeodomain that confers high similarity to the *BELL1* (*BEL1*) *Arabidopsis* gene. *BEL1* participates in ovule development and morphogenesis, and the specification of outer and inner integuments (Reiser *et al.*, 1995) but, besides this, numerous *BEL1*-like homeobox genes have been identified ubiquitously in the plant kingdom that regulate numerous developmental processes as diverse as tuber formation in potato (Chen *et al.*, 2003) or the leaf shape in *Arabidopsis* (Kumar *et al.*, 2007). Overexpression of barley *BEL1* in tobacco plants caused dwarfism and leaf and flower malformation (Müller *et al.*, 2001). On the other hand, *MDH1*, a *BEL1*-like homeodomain gene of apple, is involved in growth, fertility, development of carpels and fruit shape (Dong *et al.*, 2000). Interestingly, two *Arabidopsis* null mutants of the *BEL1*-like genes *PENNYWISE* and *POUND-FOOLISH* disrupt the transition from vegetative to floral development (Smith *et al.*, 2004). Both the expression pattern of CTF0588 and its homology with other genes that are involved in the transition from vegetative to reproductive phase in *Arabidopsis* suggest that CTF0588 also plays a role in this phase transition in citrus, although further analysis will be required to elucidate its specific function.

Sequence analysis showed that CTF0397 and CTF0899, despite their high percentage of similarity in specific domains (bZIP1 and SANT, respectively), are novel genes that have not been functionally characterized in other plants, so it was not possible to predict their function, although some bZIP and MYB proteins involved in flowering development have been identified in *Arabidopsis* (Chuan *et al.*, 1999; Abe *et al.*, 2005; Tominaga *et al.*, 2008). Overexpression in *Arabidopsis* will be an important clue to elucidate their specific roles in phase transition.

The results presented here indicate that the molecular mechanisms operating in the JAT in citrus plants could be controlled by genetic and/or environmental pathways that are in part common to those of *Arabidopsis*, but also somehow different since specific factors without *Arabidopsis* orthologues have been characterized. The approach used in this study has allowed us to identify both TFs with a high similarity to those of *Arabidopsis thaliana* (some of them already identified just because of this high similarity) and also TFs very distant from those of *A. thaliana* in terms of homology that may be involved in those pathways that are not common. How the regulation of the transition from juvenile to adult phase takes place in woody plants and how these specific TFs relate to this regulation are the most interesting questions to be investigated in the future. Additional work is still required to answer these questions, but our preliminary results could help to decipher the molecular mechanisms involved in the JAT in citrus plants as well as to reduce the generation time for genetic studies and breeding programmes and offer the prospect of rapid transfer of information to other woody plants.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: differentially expressed genes identified by SAM. Table S2: expression

analysis of selected CTF genes in juvenile and adult plant tissues and in different floral organs in citrus plants.

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