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ORIGINAL PAPER

Citrus sinensis L. Osbeck orthologs of *FRUITFULL* and *SHATTERPROOF* are differentially expressed during fruit development

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Abstract Several regulatory steps and genes involved in fruit development were identified and characterized in *Arabidopsis thaliana*. *FRUITFULL* (*FUL*) and *SHATTERPROOF* (*SHP*), which belong to the MADS-box family of transcription factors, act together to promote the differentiation of the dehiscence zone and thus control the process of pod shattering in Arabidopsis. Homologs to these genes have been described in fleshy fruit species, but the specific nature of the regulatory hierarchy and interactions among these key regulators remains elusive in most plant species. Here, *Citrus sinensis* putative orthologs to *FUL* and *SHP*, named *CsFUL* and *CsSHP* respectively, were characterized. Phylogenetic comparisons indicated that CsFUL belongs to FRUITFUL sub-clade within the AP1/SQUA major clade while CsSHP falls into PLENA sub-clade from the AG/PLE clade. CsFUL and CsSHP protein sequences possess all of the characteristic conserved domains commonly found in A- and C-lineages of MIKC MADS-box proteins, respectively. Semi-quantitative RT-PCR showed preferential expression of both genes in developing fruits. In situ hybridization and a detailed analysis of *Citrus* fruit development using scanning electron microscopy allowed further characterization of these genes during *C. sinensis* fruit development. *CsFUL* and *CsSHP* are differentially expressed in exocarp, mesocarp and endocarp tissues in early stages of fruit development but their expression diminishes with fruit maturation. Moreover, the co-localization of *CsFUL* and *CsSHP* mRNA during oil glands and juice vesicle formation suggests a potential role in the development of such structures. Altogether, there results might contribute to a better understanding of the molecular mechanisms involved in *Citrus* fruit development.

Keywords *Citrus sinensis* · Fruit development · MADS-box · FRUITFULL · SHATTERPROOF

Introduction

Fruits are specialized organs that develop generally after fertilization as a continuation of carpel (or, in some specific fruit types, additional floral tissues) development (Roeder and Yanofsky 2006). The advent of fruits was a key innovation in the evolution of Angiosperms, conferring not only protection to the developing seeds but also generating a number of mechanisms for seed dispersal (Dinneny and Yanofsky 2005). Fruits exist in a variety of shapes and sizes and are basically separated into dehiscent/dry and non-dehiscent/fleshy fruits, according to their mechanism for seed dispersal (Giovannoni 2001). *Arabidopsis thaliana* has been extensively used as a model for research on dehiscent fruit formation and development, while tomato has emerged as the primary model for fleshy fruit expansion, development and ripening investigations (Giovannoni 2004, 2007). The maturation of dehiscent fruits is characterized by senescence of the mature carpel tissue followed by separation of the valves in the dehiscence zone, which allows the seeds to be released from the dry fruits (Giovannoni 2004; Lozano et al. 2009). In contrast, the development of fleshy fruits involves dramatic biochemical, physiological and

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structural changes to alter texture, color and flavor of the organ and consequently attract seed dispersers (Giovannoni 2004, 2007). Nevertheless, it seems likely that some of the genetic regulatory elements involved in dry fruits development and ripening were conserved during the evolution of fruit fleshiness (Seymour et al. 2008).

Many genes involved in fruit development belong to the MADS-box gene family, a group of transcription factors that regulate several developmental processes in plants ranging from root to fruit development (Parenicova et al. 2003). Changes in gene structure, expression and function of MADS-box transcription factors have been considered a major cause of morphological innovations during land plant evolution. For example, differential expression of MADS-box genes from the classic ABC model is related to the determination of floral organ identity, and loss-of-function of some of these genes causes homeotic modifications during the ontogeny of flowers (Becker and Theissen 2003). Furthermore, MADS-box phylogeny and wide distribution suggest a strong correlation with the origin and evolution of reproductive structures in plants (Theissen et al. 2000). *FRUITFULL* (*FUL*) and *SHATTERPROOF* (*SHP*) genes, which belong to the "A" and "C" clades of the MIKC subfamily of MADS-box genes respectively (Pan et al. 2010), play a critical role during fruit patterning in *Arabidopsis thaliana* (Dinneny et al. 2005). *FUL* expression is first detected during flower development, in a central part of the floral meristem that will later develop as the carpels. *FUL* expression becomes confined to the valves and is maintained until late development of the fruit (Roeder and Yanofsky 2006). The *FUL* gene is responsible for controlling cell division and expansion during carpel development, and for regulating a network of genes that specify cell types in the region of the valve and replum during fruit development (Robles and Pelaz 2005). The *ful* mutant presents very small siliques, defects in the establishment of fruit tissue identities and disrupted or incomplete dehiscence of the valves (Mandel and Yanofsky 1995; Gu et al. 1998; Ferrandiz et al. 1999; Dinneny and Yanofsky 2005).

The redundant genes *SHP1* and *SHP2* are expressed in valve margins of the fruit, among other tissues, and are essential for the specification and differentiation of the dehiscence zone and for lignification of adjacent cells (Robles and Pelaz 2005). Disruption of both *SHP* genes leads to an indehiscent fruit, because the separation and lignified margin layers fail to differentiate and the valves remain firmly attached. Moreover, overexpression of *SHP* promotes ectopic lignification of the valves and premature opening of the fruit, releasing the seeds before they reach maturity (Liljegren et al. 2000; Robles and Pelaz 2005). *SHP1* and *SHP2* positively regulate the expression of *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*), bHLH transcription factors responsible for the specification of both the separation layer and lignified layer of the valve margin and for the formation of the separation layer, respectively (Ostergaard 2009). In this context, *FUL* inhibits the expression of *SHP1*/*SHP2* and, therefore, also represses the expression of *IND* and *ALC* (Ferrandiz et al. 2000). Together with other transcription factors, *FUL*, *SHP1*, *SHP2*, *IND* and *ALC* were proposed to compose a hierarchical network of transcriptional regulation that establishes the pattern of fruit development in *Arabidopsis thaliana* (Dinneny et al. 2005).

In recent years, with the availability of additional genomic data from whole genomes and expressed sequence tags (EST) projects, several homologs of *FUL* and *SHP* have been identified in different plant species, including species with fleshy fruits such as tomato and peach. Expression analyzes in peach pericarp and endocarp, which are analogous to Arabidopsis valves and dehiscence zone respectively, suggest that both species present a similar fruit developmental program that include the regulatory transcription factors *SHP* and *STK* (*SEEDSTICK*), which promote endocarp differentiation, *FUL*, which represses the expression of *SHP*, *IND* and *ALC*, and *NST1/3* that regulate lignin deposition (Dardick et al. 2010; Tani et al. 2007). However, functional characterization of *TAGL1*, tomato ortholog of Arabidopsis *SHP1/2*, suggest that these genes are not functionally equivalent at the molecular level (i.e. regulate different arrays of target genes), while retain surprisingly similar roles in plant development regarding seed dispersal (Vrebalov et al. 2009). Therefore, one can speculate that specific functions of MADS-box genes in the development of different fleshy fruit types may exist, which highlights the importance of additional studies in fruit-bearing species.

Citrus, including the commercial sweet orange (*Citrus sinensis* L. Osbeck), is a perennial woody crop with agronomical and economical importance for the production of fresh fruit and juice, amongst other products (Tan and Swain 2007). The majority of the studies regarding the reproductive biology of *Citrus* species have been focused on the processes regulating the transition from vegetative to reproductive growth, since woody perennial trees have a long juvenile period, typically lasting for several years, during which no reproduction occurs (Endo et al. 2006). In addition, the number and distribution of flowers on an individual tree negatively correlates with fruit final size, an economically relevant characteristic of the fruit (Dornelas et al. 2007b; Tan and Swain 2007). Consequently, the biennial bearing pattern of *Citrus*, in which a large number of flowers (thus smaller fruits) are produced in "on" years and relatively few flowers (with bigger fruits) occurs in "off" years, significantly affects the fruit size at harvest (Dornelas et al. 2007b). On the other hand, limited

information on *Citrus* fruit morphology and anatomy is available, and the molecular mechanisms involved in fruit development remains poorly understood. Here we describe the characterization of *Citrus* homologs of *SHP* and *FUL* and provide detailed analysis of their expression patterns during *C. sinensis* fruit development. Moreover, *C. sinensis* fruit development was investigated by means of light and scanning electron microscopy techniques.

Materials and methods

Plant material

Plant material was harvested from adult plants of *Citrus sinensis* Osbeck var. Valencia cultivated in field conditions at Sylvio Moreira *Citrus* Center (IAC), Cordeirópolis, São Paulo, Brazil, from September 2008 to August 2009. Flowers were labeled at anthesis to allow the analysis and collection of material at known developmental stages. Samples were collected at different developmental stage points and prepared for microscopy, in situ hybridization and total RNA extraction as further described.

Analysis of fruit development by light microscopy and scanning electron microscopy

Pieces of ovary/fruits at different developmental stages were fixed in 4 % paraformaldehyde in phosphate buffer (w/v, pH 8.0) under vacuum for 24 h at 4 °C. Fixed material was dehydrated in ethanolic series and embedded in plastic resin—Historesin (Leica, Wetzlar, Germany) following the manufacturer's instructions. Histological sections (5 µm-thick) were obtained with a rotary microtome, mounted in microscope slides and stained with toluidine blue 0.3 % (w/v) in phosphate buffer.

The material for scanning electron microscopy (SEM) was fixed and dehydrated as described, critical-point dried in CO_2 (Balzers Critical Point Drier) and metalized with a colloidal gold layer (40 nm). The prepared samples were observed and documented using a LEO 435 VP at 20 kV equipped with LEOUIF software.

Identification of putative *Citrus* homologs of MADS-box genes and sequence analysis

The *Citrus* EST project (CitEST) database (Targon et al. 2007) was searched for sequences showing significant similarity to MADS-box genes using a strategy similar to that reported by Dias et al. (2005). The obtained EST sequences were assembled in consensus sequences using CAP3 algorithm available in the BioEdit software package (Huang 1992). Multiple amino acid sequence alignments of the deduced sequences from *Citrus* contigs and available homologs from *Arabidopsis thaliana* MIKC subfamily of MADS-box transcription factors were performed using CLUSTALW (Higgins 1994). Protein alignment was manually adjusted and evolutionary relationships were inferred using maximum parsimony method and bootstrap test for 1000 replicates with MEGA 4 software (Tamura et al. 2007). The presence of protein motifs characteristic to MADS-box proteins and FRUITFUL and/or SHATTERPROOF homologs was manually investigated based on previously published data (Becker and Theissen 2003; Kaufmann et al. 2005; Parenicova et al. 2003). Figures highlighting the alignment of the identified motifs among FUL and SHP homologs from several plant species were generated in BOXSHADE 3.21.

Total RNA isolation, cDNA synthesis and semi-quantitative RT-PCR

Total RNA was isolated from roots, shoots, leaves, flowers, fruits and seeds of *Citrus sinensis* using TRIZOL (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. Genomic DNA was removed with DNAse treatment (Ambion, Foster City, USA) and first-strand cDNA was synthesized with SuperScript II (Invitrogen, Carlsbad, USA). Specific primers were designed as following: *Citrus FUL* homolog 5′-CTTCAAAGTGTAGAGCAGCAGA-3′ (forward) and 5′-AAGGTGACGAAGCATCCAAG-3′ (reverse); *Citrus SHP* homolog 5′-TGTGCCGATTCTTCTAACCC-3′ (forward) and 5′-GCTTTCTGATTGCCGTTCTT-3′ (reverse). The expression of the constitutive gene *EF1-α* was used for normalization, using the specific primers 5′-AAGGCTGAGCGT GAACGTGG-3′ (forward) and 5′-ACGGCAATGTGGGAGGT GTG-3′ (reverse). The *EF1-α* primers were designed as reported by Endo et al. (2006). The parameters for semi-quantitative RT-PCR were: first denaturation step at 94 °C for 3 min; 28 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and final extension step at 72 °C for 5 min. 20 µL of the PCR reaction were analysed by electrophoresis in 1 % agarose gel for semi-quantitative analysis and documented with photo documenter using QuantityOne (BioRad, Hercules, USA).

In situ hybridization

The CitEST clones CS00-C3-705-020-D07-CT.F and CS00-C3-703-067-E03-CT.F containing the cDNA sequences of *CsFUL* and *CsSHP*, respectively, were ordered from the Brazilian Clone Collection Center (BCCCenter, FCAV-UNESP, Campus Jaboticabal). After confirmation of sequence identity by plasmid sequencing, these clones were used as templates for the synthesis of in situ hybridization probes for *FUL* with 357 bp and *SHP* with 339 bp length,

respectively. These clones were chosen because they do not contain the conserved MADS-box domain, avoiding cross-hybridization. Plasmid linearization was carried out with NcoI (Fermentas) and SalI (Fermentas) and probes were synthesized by in vitro transcription with SP6 and T7 RNA polymerases using DIG RNA labeling kit (SP6/T7) (Roche Applied Science), according to manufacturer's protocol. Sense probe was used as negative control.

The fruit tissue samples used for in situ hybridization were fixed and dehydrated as described for microscopy, except that fixation was performed for a maximum of 16 h. Ethanol was then replaced by xylene in a graded series (until 100 % xylene), with samples maintained in each solution for 12 h. Incubation with 100 % xylene was repeated 3-times. Paraffin was added to samples in 100 % xylene (3:1, w/v) and incubated at 58 °C to melt paraffin and evaporate xylene. After changing paraffin 3-times (every 12 h), samples were placed on moulds for solidification. Serial sections of 10 µm-thick were cut on a rotary microtome (Leica®) and fixed on slides pre-treated with 2 % 3-aminopropyltriethoxysilane in acetone (Pierce, Rockford, USA).

In situ hybridization protocol was performed according to a modified version of Kidner and Timmermans (2006). Prior to hybridization, paraffin was removed from the sections by immersion in xylene, followed by a proteinase K treatment (1 µg mL^{-1} in 50 mM Tris–HCl pH 7.5) for 10 min at 37 °C. Hybridization was conducted in hybridization buffer (10 mM Tris–HCl pH7.5; 300 mM NaCl; 50 % deionized formamide; 1 mM EDTA; 1 × Denharts; 5 % Dextran Sulfate) containing 300–600 ng of probe, at 42 °C for 16 h. Excess and/or non-bound probe was removed by four stringent washes, twice in 4 × SSC SSC (600 mM NaCl, 60 mM tri-sodium citrate dehydrate pH 7.0) at 42 °C for 30 min, followed by two washes in 2 × SSC (300 mM NaCl, 30 mM tri-sodium citrate dehydrate pH 7.0) under the same conditions. Subsequently, the slides were treated with 1 % Blocking Agent solution (Roche, Basel Schweiz, Switzerland) and the hybridization signal was detected using anti-DIG antibodies conjugated with alkaline phosphatase (1:1000) in buffer (100 mM Tris–HCl pH 7.5; 150 mM NaCl). Visualization of hybridization signal was obtained with Fastblue (Pierce, Rockford, USA) plus suppressor (1 mM levamissole) as substrate. The hybridized material was observed and documented in an AXIOCAM HRc (ZEISS) microscope.

Results

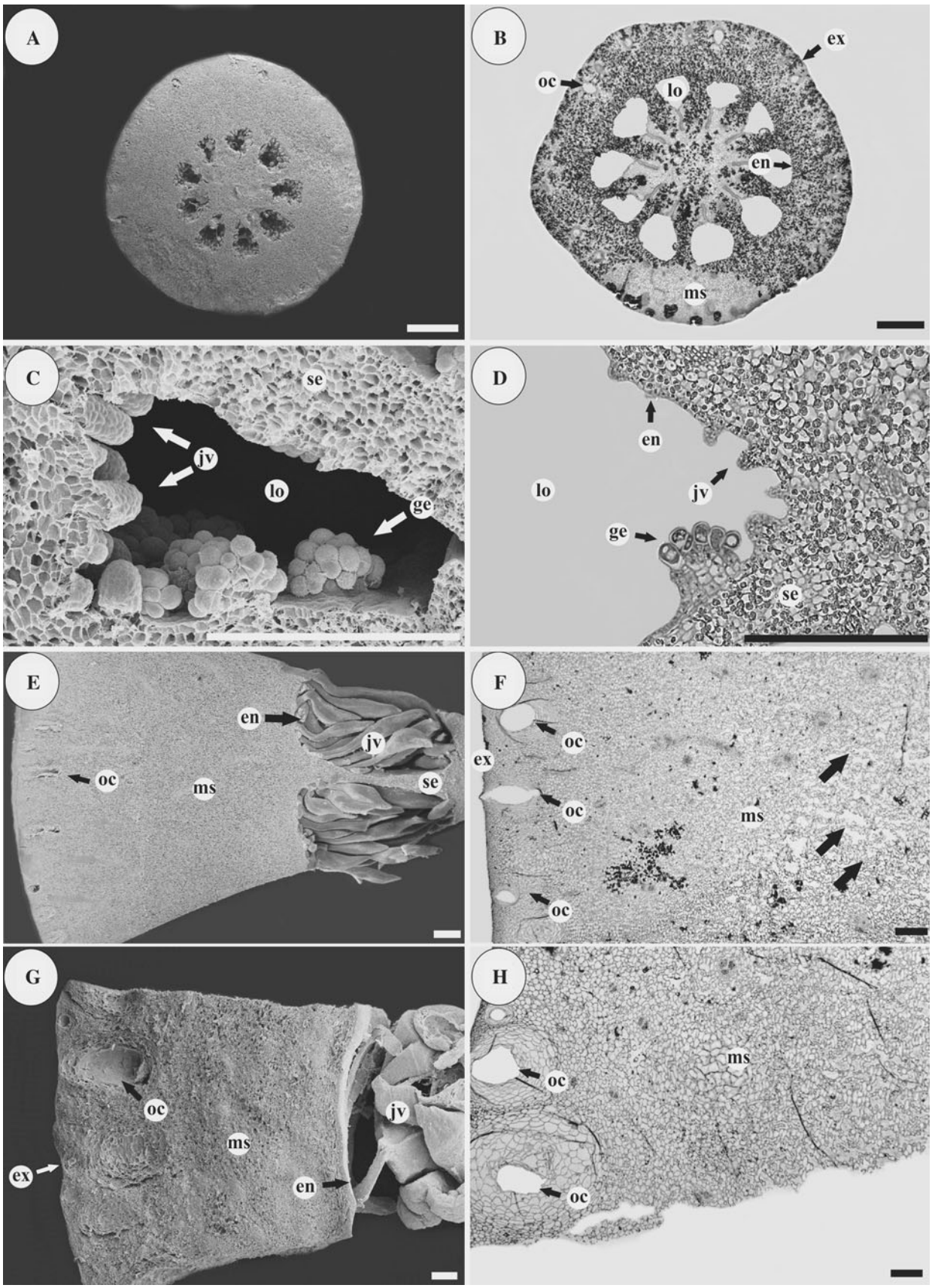
Description of *Citrus* fruit growth and development

To aid the interpretation of our in situ hybridization results, we decided to first perform a morphological and anatomical characterization of *C. sinensis* fruit development. The classical description of *Citrus* fruit development by Bain (1958) has only recently been complemented by Laskowski et al. (2006). However, in the present work, scanning electron microscopy was also employed to study the ultrastructure of *C. sinensis* fruit in addition to histochemical analysis using light microscopy. For the sake of clarity, we established three developmental fruit stages. Stage I fruits (Fig. 1a, b, c and d) have unistratified exocarp (Fig. 1b) with radially elongated cells with evident nuclei in a basal position. The mesocarp was composed of parenchymatic cells and could be divided into two regions: one outer region, near the exocarp, where numerous oil cavities were differentiating, which we called external mesocarp. A procambium and vascular bundles were on the inner region, which we called internal mesocarp (Fig. 1b). The unistratified endocarp was composed of radially elongated cells with central voluminous nuclei. At this stage, the initiation of the differentiation of the juice vesicles could be observed in the endocarp, on the opposite side of the placenta (Fig. 1c and d). The juice vesicles were formed by epidermis and sub-epidermal layers of the inner epidermis of the ovary. Near the placenta, a different type of epidermis-derived structures were observed (Fig. 1c and d) whose apical cells probably secreted polysaccharides, as evidenced by the deep staining by toluidine blue. We named these structures glandular emergences.

At the Stage II of fruit development (Fig. 1e and f), significant changes in the mesocarp were observed: intense cell divisions, formation of intercellular spaces, vesicle juice and the end of glandular emergences secretion. Polysaccharides were also detected throughout the locule and during the early lysis of these glandular emergences.

At the Stage III (Fig. 1g and h) the main changes in the mesocarp could be observed, especially in the size and maturation of oil cavities (Fig. 1g). The parenchyma presented a loose arrangement of cells. At this stage, the flavedo, the region corresponding to the exocarp and the external mesocarp, could be perfectly distinguished from the albedo, which corresponded to the internal mesocarp and the endocarp.

Fig. 1 Scanning electron microscopy (*left side*) and light microscopy (*right side*) of corresponding transversal sections of *Citrus sinensis* var. Valencia fruits in different developmental stages. **a–d** Stage fruit I, *right* after the shedding of floral organs, 0.15 mm diameter. **c–b** detail of the locule with the developing juice vesicles and glandular emergences. Stage II fruit, 4 cm diameter, 87 days after anthesis. **e** the oil cavities have developed further and the cells of the mesocarp appear uniform. **f** *arrows* indicate intercellular spaces in the mesocarp. Stage III fruit, ripe, 6.8 cm diameter. **g** the oil cavities in the flavedo are fully developed, **h** Detail of the mesocarp with intercellular spaces. *en* endocarp, *ex* exocarp, *ge* glandular emergence, *jv* juice vesicles, *lo* locule, *ms* mesocarp, *oc* oil cavity, *se* septum. *Bars*: 250 µm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Sequence analysis of putative homologs to MADS-box genes in *Citrus*

A total of 182 reads were obtained from the CitEST database showed sequence similarity to MADS-box genes. The assembly of these reads resulted in two consensus sequences with significant similarity to Arabidopsis *FUL*, which consisted of 3 reads from *C. sinensis* and 3 reads from *C. reticulata*, and 4 contigs with significant similarity to Arabidopsis *SHP*, probably corresponding to *SHP* homologs of *C. sinensis* (7 reads), *C. reticulata* (2 reads), *Poncirus trifoliata* (6 reads) and *Lima tahiti* (1 read). The deduced protein sequences of CsFUL and CrFUL were identical in size and primary amino acid sequence to the reported *Citrus unshiu* FUL homolog (Endo et al. 2006), which was encoded by 244 amino acids, and also presented the characteristic FUL domains: LMQTLTNSSYQMGGGSGE and LLPAWMLR (Fig. 2a). The strictly conserved MADS-box domain of *Citrus* sequences was identical to their Arabidopsis counterparts, with the exception of position 50, where a substitution of S to T was observed (Fig. 2a). PtSHP, CsSHP and CrSHP deduced proteins all showed 258 amino acids with few differences, in contrast to Arabidopsis, in which SHP1 presents 249 amino acids and SHP2 is encoded by 247 amino acids. The characteristic SHP domain SSNPGSITEA was strictly conserved among PtSHP, CsSHP and CrSHP proteins, while a L to F substitution in the second SHP signature domain QPPLQLV was observed in the case of CrSHP, which therefore presented a QPPFQLV domain (Fig. 2b). *Citrus* SHP homologs presented a slightly different MADS-box domain, with I and V in positions 46 and 47, respectively, while Arabidopsis sequences presented V and I, respectively (Fig. 2b). Interestingly, all SHP homologs in species included in Rutaceae family (i.e. *Citrus spp*, *Poncirus spp* and *Lima spp*) presented an N-terminal extension preceding the MADS domain, which is found in some members of the C-lineage of MADS-box genes (Jager et al. 2003). This sequence is not found in any of the other homologs included in the alignment.

To further investigate the evolutionary relationships among *Citrus* FUL and SHP and other corresponding orthologs, a phylogenetic analysis was performed using the maximum parsimony method. The MIKC sequences belonging to the "A-Class" are generally subdivided into two sister clades, corresponding to FRUITFUL and APETALA1 putative orthologs (Becker and Theissen

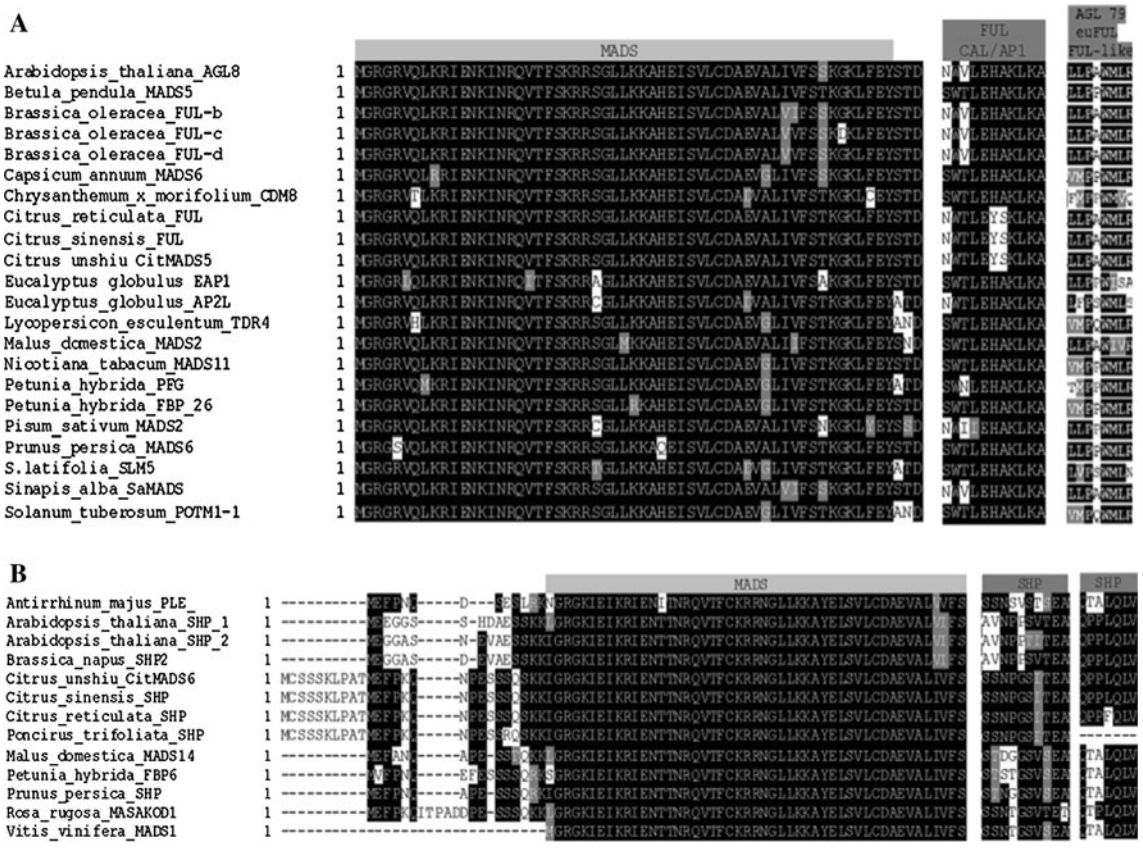


Fig. 2 Partial sequence alignments of FRUITFULL (**a**) and SHATTERPROOF (**b**) homologs. Alignments were performed using TCOFFEE: REGULAR and figure generated with BOXSHADE 3:21. The *gray bars* indicate the different conserved domains used in the alignments, while sequence similarities are highlighted in *gray-scale box shading*, with *black* indicating identical residues

2003; Kaufmann et al. 2005). *Citrus* sequences were clearly positioned into the FUL sub-clade with a significant bootstrap support (Fig. 3a). Similarly, MADS-box sequences belonging to the "C-Class" are also grouped into two sister clades, named PLENA and AGAMOUS (Causier et al. 2005; Pan et al. 2010). All *Citrus* putative SHP homologs were grouped together in a consistently supported clade within PLENA sub-clade (Fig. 3b). In the case SHP, *Citrus* homologs clustered in a separated sister clade to their counterparts from Arabidopsis, which indicates that this gene duplication event (i.e. the production of SHP1/2 in Arabidopsis) occurred after the separation of Brassicaceae and Rutaceae.

Expression analysis of *CsFUL* and *CsSHP* genes by semi-quantitative RT-PCR

The tissue-specificity of *CsFUL* and *CsSHP* expression was investigated in several vegetative and reproductive tissue types of *C. sinensis* using semi-quantitative RT-PCR. Both genes showed a preferential expression in developing fruits (Fig. 4). While transcripts of *CsFUL* could be detected almost exclusively in fruits, weak expression of *CsSHP* were also detected in stems, leaves and flowers, indicating a broader expression pattern. These expression patterns are in agreement with a proposed role for both *CsFUL* and *CsSHP* in *Citrus* fruit development, what prompted us to further analyze the expression of *CsFUL* and *CsSHP* at the cell-type level by in situ hybridization during *C. sinensis* fruit development.

mRNA localization of *CsFUL* and *CsSHP* by in situ hybridization

The mRNA localization of *CsFUL* and *CsSHP* during *Citrus* fruit development was investigated using the same three developmental stages described previously. *CsFUL* mRNA could be detected in the cell layer corresponding to the exocarp in the ovaries of flowers at anthesis (Fig. 5a).

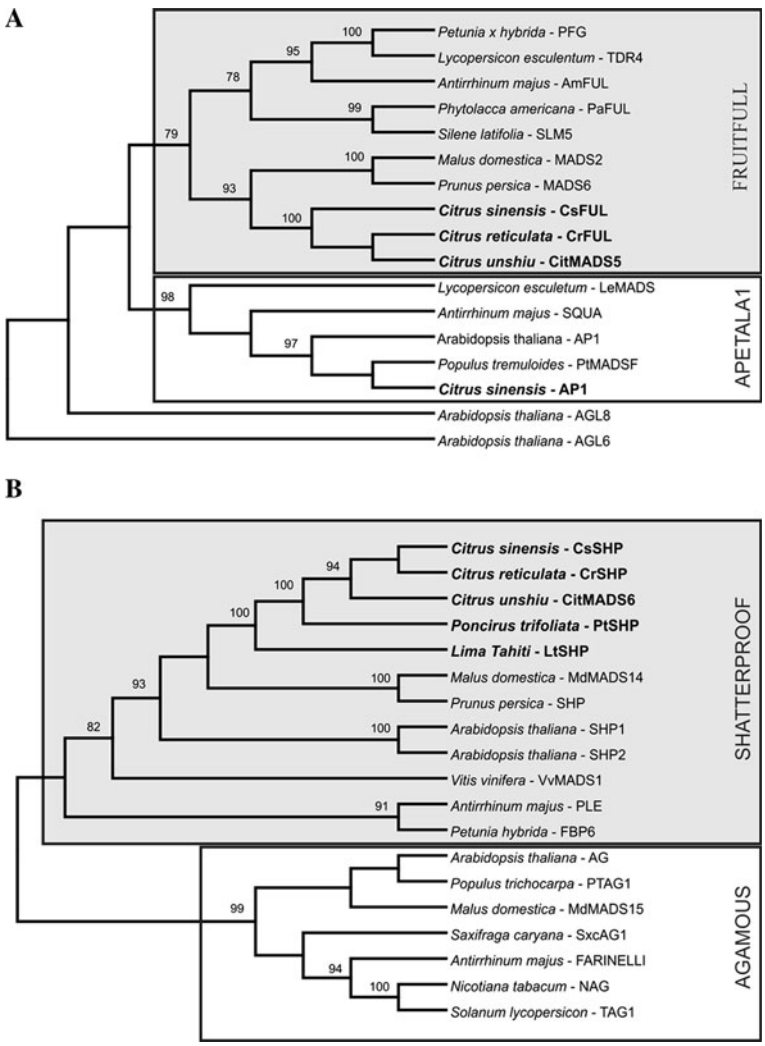


Fig. 3 Maximum parsimony trees showing the comparative analysis of FRUITFULL/APETALA1 (**a**) and PLENA/AGAMOUS (**b**) homologs of different plant species. Only bootstraps greater than 75 % (1000 replicates) are shown

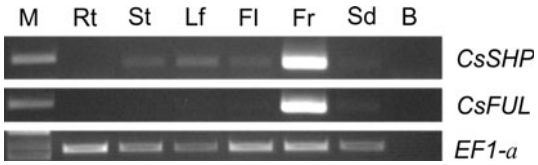


Fig. 4 Tissue-specific expression of *CsSHP* and *CsFUL* by semi-quantitative RT-PCR. The expression of *EF1-α* gene was used as a control. The *marker* (M) band for *CsSHP* and *CsFUL* is 400 bp and *EF1-α* is between 750 and 1000 bp. *B* negative control reaction lacking DNA template, *Fl* Flower, *Fr* Fruit, *Lf* Leaf, *M* fragment size marker; *Rt* Root, *Sd* Seed, *St* Shoot

Additionally, hybridization signal was also observed in the secretory cells of developing oil cavities (Fig. 5a), as well as in the endocarp cells and in the primordia of juice vesicles (Fig. 5c). The inner integument of the seed also showed strong hybridization signal (Fig. 5d). When immature fruits reached around 3 cm in diameter, *CsFUL* transcripts were present in the secretory cells of oil cavities, as previously observed, and in cells at the outermost layer of the developing juice vesicles (Fig. 5g). In mature *C. sinensis* fruits, the expression of *CsFUL* was drastically reduced. *CsFUL* transcripts could only be detected in the mesocarp cells, close to the exocarp, in the endocarp (Fig. 5k), and in the outer layers of the juice vesicles (not showed). The expression in cells surrounding the oil cavities, observed in more immature stages, could no longer be detected (Fig. 5k). Sense probes were used as negative control in different stages of development and only very low background was observed (Fig. S1).

In general terms, the hybridizations performed with the antisense probe of *CsSHP* showed less extensive expression when compared to the results obtained with *CsFUL*. In the early stages of fruit development, hybridization signal for *CsSHP* was concentrated primarily in the exocarp and the endocarp of the ovary in flowers at anthesis (Fig. 5b, e and f). Transcripts could be detected mainly in the epidermal (exocarp) cell layer (Fig. 5e) and in very few epidermal cells in the endocarp that will generate the primordia of the juice vesicles (Fig. 5f). Later on, when the fruits reached 3 cm in diameter, the hybridization signal became more diffuse in all tissues (Fig. 5i and j). Finally, when *C. sinensis* fruits reached maturity, the *CsSHP* expression became very low and its expression pattern resembles that observed for Cs*FUL*: transcripts could only be detected in the mesocarp cells, close to the exocarp, in the endocarp and in the outer layers of the juice vesicles (Fig. 5l).

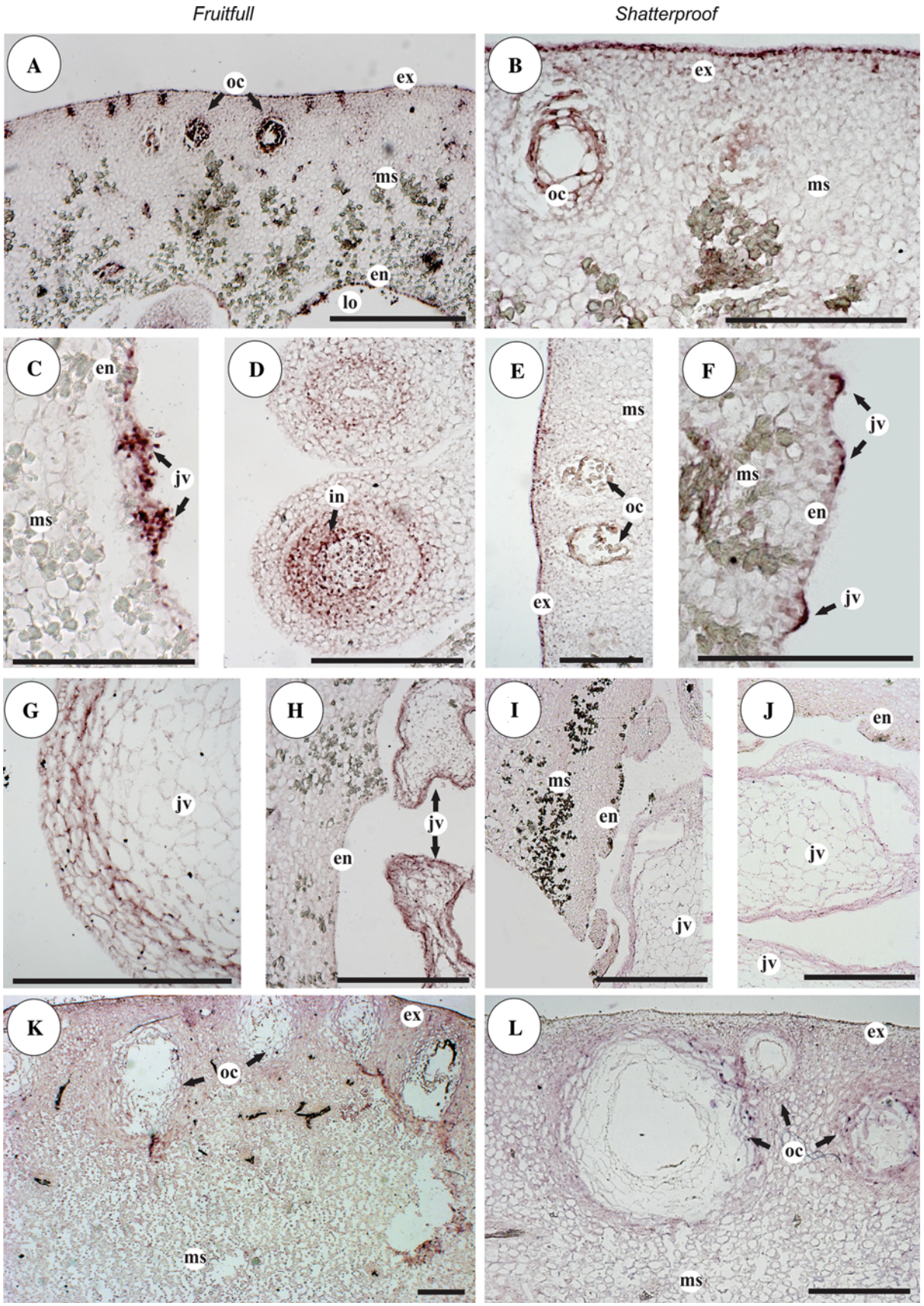
Discussion

Citrus fruit is a specific type of berry called hesperidium whose development consists in the formation of the

Fig. 5 In situ hybridization of *CsFUL* and *CsSHP* in developing fruit tissues of *C. sinensis* var. Valencia. The positive hybridization signal (*red/pink color*) for *CsFUL* can be observed in Stage I fruit in **a** exocarp, **c** secreting cells of oils cavities, **d** endocarp and inner integument of the seeds. *CsSHP* signal is concentrated in exocarp cells (**b**–**e**); the secretory cells of the oil cavities (**b**) and in the endocarp cells that will give rise to the juice vesicles (**f**). Stage II fruit, *CsFUL* hybridization signal is detected in the cells surrounding the secretory cells of the oil cavities and the outer cells of the juice vesicles (**g** and **h**), while a very weak signal for *CsSHP* is observed widely in most tissues including the outer cells of the flavedo and the cells around the secretory cells of the oil cavity, the endocarp and the outer cell layers of the juice vesicles (**i**–**j**). Stage III fruit, the signal is very weak for *CsFUL* and can be detected in the flavedo near to the exocarp (**k**), while is excluded from the oil cavity cells, the endocarp and the outer layer of the juice vesicles. A weak broad hybridization signal of *CsSHP* is observed in most tissues (**l**) The sense probe was used as negative control (supplemental Figure S1). Legend: *en* endocarp, *ex* exocarp, *in* inner integument of the seed, *jv* juice vesicles, *ms* mesocarp, *oc* oil cavity. *Bars*: 250 μm

exocarp (flavedo), the mesocarp (albedo) and the endocarp (Laskowski et al. 2006). The outermost layer of the exocarp contains cavities filled of essential volatile oils, while the mesocarp constitutes an intermediary thick spongy layer and the fleshy interior is separated in sections filled with juice vesicles (Bennici and Tani 2004; Laskowski et al. 2006). Our analysis of *C. sinensis* fruit morpho-anatomy is in agreement with previous characterization of *Citrus* fruit development (Bain 1958; Roth 1977). Moreover, the description of the exocarp, mesocarp and endocarp structures and development of *C. sinensis* var. Valencia were very similar to the reported for fruits of *C. sinensis* var. Salustino (Laskowski et al. 2006). The development of oil cavities, which start to differentiate in the subepidermal region of the ovary walls, follows the fruit growth kinetics, with the secretory lumen increasing in size and volume. Such a description is in agreement with the observations made by Knight et al. (2001) in *C. sinensis* var. Washington Navel, who also observed oil cavity initiation only during very early stages of fruit development. At maturity, the secretory cells of the oil glands have collapsed and the gland itself has become an oil-containing sac. Nonetheless, our observations refute the statement of Schneider (1968) who suggested oil gland differentiation throughout the fruit development. Interestingly, the development of additional glandular emergences from the endocarp epidermis, easily distinguishable from developing juice vesicles, has not been mentioned by other authors describing *Citrus* fruit development. Such structures were observed very early during *C. sinensis* fruit development, and they senesce as the juice vesicles grow and take the ovary cavity space. These glandular emergences are apparently absent from the mature fruit and their function remains elusive.

Several regulatory steps and genes involved in dry fruit development were identified and characterized in the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model plant *Arabidopsis thaliana*, from early patterning events of the gynoecium to the control of seed dispersal (Ostergaard 2009). In tomato, the model plant for fleshy fruit species, investigations have been centered in organ expansion, maturity, ripening and nutritional quality rather than initial steps of fruit development (Giovannoni 2007). Nevertheless, the anatomical and physiological analogies between specific structures of dehiscence and indehiscence fruits, as well as the identification of a number of common genetic regulatory elements suggest that different types of fruits might be controlled by very similar molecular mechanisms. In this regard, MADS-box family of transcription factors may represent a potential starting point in a search for common mechanisms controlling the reproductive development (Giovannoni 2001). Research on reproductive biology of *Citrus* species have been focused mainly in the elucidation of the molecular mechanism of flower induction, because of the negative correlation between fruit size and the number of flowers in an individual *Citrus* tree (Dornelas et al. 2007b; Muñoz-Fambuena et al. 2012, 2011; Tan and Swain 2007). However, the molecular basis of fruit patterning and development in *Citrus* remains largely unknown. Here, we isolated and characterized the expression pattern of the putative orthologs of MADS-box genes *FUL* and *SHP* in *Citrus* and discussed their potential roles during *Citrus* fruit development.

Based on annotated genes from other plant species, a total of 182 reads with significant similarity to MADS-box genes were obtained from 242.790 valid reads in the CiEST database, which is derived from several *Citrus* species such as *C. sinensis*, *C. reticulata*, *Poncirus trifoliata* and *Lima Tahiti* (Targon et al. 2007). Interestingly, only a single consensus sequence for both *FUL* and *SHP* orthologs were found in each *Citrus* species, which reinforces the idea that *SHP1* and *SHP2* paralogs observed in Arabidopsis are probably products from a recent duplication event, posterior to the previous duplication event that originated the PLE and euAG lineages, which probably occurred before the divergence of the core eudicots (Pan et al. 2010). The alignment of FUL and SHP protein sequences from *Citrus* species with close homologous proteins from other plant species revealed that they possess all of the characteristic conserved domains commonly found in other A- and C-lineages of MIKC MADS-box proteins, respectively. Another interesting observation was the presence in CsSHP of an N-terminal extension preceding the MADS domain found in *Arabidopsis thaliana* AGAMOUS and *Antirrhinum majus* PLENA but absent from all MADS-box genes belonging to A- and B-functional groups (Jager et al. 2003). This N-extension is variable in length and sequence among different proteins and it might have a role in modulating the DNA-binding activity of MADS-box protein, but not necessarily by affecting specificity (Jager et al. 2003; Riechmann and Meyerowitz 1997). In addition, a wider sequence variation could be observed within the intervening domain, responsible for the specificity in dimer formation, and in the C-terminal domain, which is involved in protein–protein interactions (Kaufmann et al. 2005). Such variation may be extremely important since MADS-box role driving the evolution of diverse plant morphology might be due to their ability to evolve new protein interaction capabilities and thus modulate a new developmental process (Vrebalov et al. 2009).

Phylogenetic comparisons indicated that CsFUL belongs to FRUITFUL sub-clade within the APETALA1/SQUAMOSA major clade while CsSHP falls into PLENA subclade from the AGAMOUS/PLENA clade. In both cases, the similarity among *Citrus* sequences and those of other woody species like peach and apple was greater than when the comparison was between *Citrus* and herbaceous species, even thought *Citrus* and, for example, Arabidopsis, belong to rosid II subclass of eudicotiledons while peach and apple belong to rosid I subclass. The same was observed in the case of *Citrus* flower-derived MADS-box sequences (Dornelas et al. 2007a). This observation could indicate that proteins involved in the regulation of reproductive development of woody perennial species would share, at least to some extent, particular motifs not found in proteins from herbaceous plants (Dornelas et al. 2007a). Noteworthy, our phylogenetic analysis suggest that CsSHP is orthologous to PpSHP from peach (Tani et al. 2007). This gene was recently shown to be actually an ortholog to *PLENA* from *Antirrhinum majus* since its gene structure resembles the conserved structure of *PLE* genes while substantially differs from that of Arabidopsis *SHP1/2* (Tadiello et al. 2009). This observation is remarkable since it is still not clear, based only on protein similarity and gene structure, which gene is the true ortholog to *PpPLE*. One possibility is that *PpPLE* actually correspond to the second *AG* gene that Causier et al. (2005) have hypothesized as lost in Arabidopsis (Tadiello et al. 2009), in a situation similar to that described for poplar, which possesses two *AG* genes and apparently lacks *SHP* (Leseberg et al. 2006). Interestingly, *Citrus* CsSHP and Arabidopsis AG proteins both possess the N-terminal extension while in Arabidopsis SHP1/2 this feature is not present.

The expression analysis of CsFUL by semi-quantitative RT-PCR revealed strong expression in developing fruit and reduced levels in seeds. This expression pattern is relatively similar to the observed for *TDR4*, the putative FUL ortholog in tomato, whose mRNA is also detected in developing seeds, but differs from its counterparts in Arabidopsis and Antirrhinum, whose expression is limited to carpel walls (Busi et al. 2003). A broader expression profile was observed for *CsSHP*, whose transcripts were detected in all analyzed tissues except in roots. Endo et al. (2006)

reported the expression profiles of *C. unshiu FUL* and *SHP* putative orthologs and observed the presence of significant transcript levels in both vegetative and reproductive tissues. Such discrepancy could be explained by the utilization of different analytical methods to quantify gene expression and by the fact that we used a semi-quantitative approach in which the number of PCR cycles was optimized to observe differences in transcript levels between distinct cDNA preparations. We could also observe this broader expression pattern for both genes, but only under saturated RT-PCR conditions (more than 40 cycles; data not shown). Therefore, the relatively high expression levels of *CsFUL* and *CsSHP* in developing fruits compared to other tissues suggest that these genes are mainly involved in developmental processes during fruit formation, but do not exclude the possibility of additional roles in vegetative development.

In Arabidopsis, the expression of *FUL* is detected in the central region of floral meristem, in cells that further constitute the carpels, and becomes restricted to the valves with carpel maturation. On the other hand, *SHP* expression is detected broadly in the developing gynoecium and becomes limited to the valve margins and ovules in latter stages of development (Roeder and Yanofsky 2006). In such scenario, *FUL* controls the formation of dehiscence zone by negatively regulating *SHP1/2* expression (Ferrandiz et al. 2000). In peach fruits, a similar but not identical mechanism was proposed (Dardick et al. 2010). Peach *FUL* ortholog is expressed exclusively in mesocarp and exocarp, while *SHP* expression is endocarp-specific. Moreover, *FUL* expression did not increase in the endocarp as *SHP* levels declined. Therefore, it seems that, at least in peach, the relative ratio of FUL enables SHP to promote endocarp differentiation and stone formation, but SHP is not actively regulated by dynamic FUL levels (Dardick et al. 2010). In tomato, the genetic interaction between *FUL* and *SHP* orthologs remain to be investigated, but the overlapping expression patterns displayed by those genes suggest that the negative interaction observed in Arabidopsis is not applicable (Busi et al. 2003). Similarly, *CsSHP* and *CsFUL* are co-expressed in many common cell types during *Citrus* fruit development, which also suggest that CsSHP might not be negatively regulated by CsFUL. The overall decrease in *CsFUL* and *CsSHP* transcript levels observed during fruit maturation also suggests that these genes might be more important during initial steps of fruit patterning than during the ripening process. Additionally, the co-localization of *CsFUL* and *CsSHP* mRNA in early stages of oil glands and juice vesicle development indicates a new potential role in the formation of such structures. Indeed, it seems likely that changes in the strength and spatiotemporal pattern of gene expression, often caused by sequence variation within promoter regions, may be a major reason for changes in MADS-box gene function during the phylogenetic diversification of flowering plants (Theissen et al. 2000). The assumption of new functions might rely on different protein–protein interactions and by different target recognition in the new expression site (Colombo et al. 2010). As described previously, *SHP1/2* are expressed in the valve margins of Arabidopsis fruits where they interact with *IND* and *ALC* to promote dehiscence zone differentiation. However, *SHP1/2* are also broadly expressed in the developing carpel primordium, as well as in nectaries, ovules, septum and style (Flanagan et al. 1996; Savidge et al. 1995). Recently, Colombo et al. (2010) demonstrated that *SHP1* and *SHP2*, together with *AINTEGUMENTA* (*ANT*) and *CRABS CLAW* (*CRC*), promote the formation of stigma, style and marginal tissues in the medial ridge during gynoecium development. These results suggest that there are still unidentified functions of already characterized MADS-box genes that might be hidden by functional redundancy. Since differential gene expression might had been important for the co-option of MADS-box genes as key regulatory elements of specialized plant structures (Theissen et al. 2000), it seems likely that *CsFUL* and *CsSHP* had developed new regulatory functions due to the co-localization of their gene expression during the initial development of specialized structures such as oil glands and juice vesicles. Nevertheless, additional studies are still necessary for total elucidation of the molecular mechanisms by which *CsSHP* and *CsFUL* promote *Citrus* fleshy fruit development.

The literature seems to be consensual about the potential roles of the orthologs of *FUL* and *SHP* in different developmental processes during fleshy fruit formation (Dardick et al. 2010; Gimenez et al. 2010; Itkin et al. 2009; Lozano et al. 2009; Tadiello et al. 2009; Vrebalov et al. 2009). Due to the great variety of fruit types and the tremendous flexibility of MADS-box transcription factors in assuming new functions during reproductive developmental processes, extensive research remains to be performed in different fruit-bearing species. Therefore, the initial characterization of *FUL* and *SHP* putative orthologs in *Citrus* and the detailed analysis of their expression patterns during fruit development may contribute to an enhanced understanding of the regulatory hierarchy controlling the fruit development in this important woody perennial species.

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