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Characterization and expression analysis of *FRUITFULL*- and *SHATTERPROOF*-like genes from peach (*Prunus persica*) and their role in split-pit formation

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Summary The fruit canning industry processes large quantities of the clingstone varieties of peach (*Prunus persica* L. Batch). The occurrence of split-pit formation—the opening of the pit and sometimes splitting of the fruit—causes deterioration of canned fruit quality. The frequency of split-pit formation is influenced by genetic and environmental factors. To increase understanding of the molecular mechanisms underlying split-pit formation in peach, we cloned and characterized the *PPERFUL* and *PPERSHP* genes that are homologues to the genes *FRUITFULL* and *SHATTERPROOF*, respectively, which are involved in fruit splitting (pod shattering) in *Arabidopsis thaliana*. The deduced amino acid sequences of the two genes had high homology with members of the MADS-box family of transcription factors, and particularly with other members of the FUL-like family of A-type MADS-box proteins and PLENA-like family of C-type MADS-box proteins, respectively. *PPERFUL* and *PPERSHP* were expressed throughout fruit development from full anthesis until fruit harvest. Differences in the mRNA abundance of each gene were compared in a split-pit sensitive and a split-pit resistant variety. Results suggested that temporal regulation of *PPERFUL* and *PPERSHP* expression may have an effect on the split-pit process.

Keywords: MADS-box genes, peach fruit development, RCA-RACE.

Introduction

Deterioration of canned peach fruit quality can occur because of the presence of small pit fragments originating from split-pits during processing. Split-pit formation appears mainly along the suture during stage 2 of fruit growth, which starts 40 days after full anthesis, and may be followed by heavy seed exposure, seed abortion and fruit drop when it happens early and severely (Davis 1941). Any treatment that promotes fruit growth at the start of pit hardening, such as N_2 fertilization and excessive thinning, tends to increase the number of split-pits (Claypool et al. 1972, Nakano and Nakamura 2002, O'Malley and Proctor 2002). Early maturing varieties are particularly prone to split pits, because pit hardening occurs relatively late in these varieties. Understanding the genetic factors underlying peach split-pit sensitivity may provide the means for breeding resistant varieties and for identifying molecular markers that could help improve agronomic practices that minimize the occurrence of split-pit formation.

The fruit is a complex structure unique to flowering plants that is responsible for maturation and dispersal of the seed. Fruit dehiscence is a common mechanism achieving seed dispersal, the control of which is an important trait in many crop plants, such as *Brassica napus* L., where premature seed dispersal leads to significant yield losses (Østergaard et al. 2006). In the last few years, great progress has been made in identifying the molecular mechanisms underlying fruit dehiscence in the model plant arabidopsis (*Arabidopsis thaliana* L. Heynh) (Robles and Pelaz 2005). The process of pod-shatter (fruit splitting) depends on the patterning of the dehiscence zone in the ovary. Correct spatial and temporal regulation in the formation of this zone and in the timing of dehiscence is crucial for successful seed dispersal (Roberts et al. 2002). Although all of the tissue layers present in the mature fruit are already formed in the gynoecium before fertilization (Ferrandiz et al. 1999, Pinyopich et al. 2003), tissues of the valve (peripheral walls of the carpels) and valve margin region require as yet unknown signals produced by post-fertilization processes to reach their final differentiated state.

Genes controlling the post-fertilization differentiation of tissues during fruit development are divided into those that repress valve-margin development and those that promote it (Dinneny and Yanofsky 2005). The first gene identified in the category of repressors was the MADS-box transcription factor *FRUITFULL* (*FUL*) (Gu et al. 1998). In arabidopsis, loss of *FUL* expression results in a severe reduction in fruit size. In contrast to wild-type fruit, the seeds of *ful* mutant fruit are highly compressed because of the failure of the fruit to elon-

gate. The expression of *FUL* is first observed during early flower development in the carpel primordia. Later, *FUL* expression is limited to the valve regions. Loss of *FUL* activity has a strong effect on the distribution of cell types in the valve regions. In a *ful* mutant, cells in the mesophyll tissue layers become lignified late in fruit development and are much smaller than in the wild type (Gu et al. 1998, Ferrandiz et al. 2000). On the contrary, constitutive expression of *FUL* converts cells of the valve margin and outer replum (outer margin of the septum, which is the tissue between the carpels) into valve cells, prevents valve margin lignification and produces indehiscent fruits that fail to disperse their seeds normally. These observations indicated that *FUL* negatively regulates valve-margin identity genes to ensure that valve margin differentiation occurs (Liljegren et al. 2004).

The first transcription factors shown to participate in dehiscence zone specification in arabidopsis were the MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF*2 (*SHP2*). The *SHP* MADS-box genes are necessary for differentiation of the dehiscence zone and for lignification of adjacent cells. Inactivation of the *SHP* genes leads to an indehiscent phenotype in which the fruits fail to open at maturity, thus inhibiting the normal seed dispersal process. *SHP1* and *SHP2* probably represent the top of the hierarchy regulating dehiscence zone formation (Lilgegren et al. 2000). *FUL* controls valve cell fate and formation of the dehiscence zone via *SHP* repression. For example, ectopic expression of *FUL* prevents dehiscence zone formation, suggesting that gain and loss of *SHP* function may provide useful strategies to control seed dispersal in plants of agronomic importance (Ferrandiz et al. 2000, Dinneny and Yanofsky 2005, Østergaard et al. 2006).

Anatomical and physiological comparisons indicate that peach pericarp is an organ analogous to arabidopsis valves, both having originated from the carpel of the ovary, and split-pit occurs exactly at the separation layer of the endocarp that is analogous to the dehiscence zone of the valve's endocarp (Roth 1977). These similarities prompted us to examine the possible roles of the *PPERFUL* and *PPERSHP* genes in split-pit formation in peach. We isolated, characterized and studied the expression of *PPERFUL* and *PPERSHP* genes from *Prunus persica* L. at different developmental stages of fruit development by reverse transcriptase polymerase chain reaction (RT-PCR). Real time PCR was employed for semi quantitative expression analysis in split-pit sensitive (Andross) and resistant (Katherine) varieties. We also carried out histochemical studies of fruits to examine if there were any differences in lignin formation in the endocarp: (1) between the 'Andross' and 'Katherine' varieties; and (2) between control Andross trees and trees subjected to treatments that induce split-pit (i.e., water stress and N_2 fertilization).

Materials and methods

Plant material

During the spring of 2004, flowers and developing fruits were collected from peach trees growing in Veria, northern Greece, which is the main area of peach cultivation, every week after anthesis until fruit maturity. Samples were taken from the varieties 'Andross' and 'Katherine', and frozen in liquid nitrogen and stored at – 80 °C until used. In the second year of the study, samples were taken from 'Andross' trees subjected to water stress (Andross-no irrigation) or excessive N_2 fertilization (Andross-N_2 fertilization) and from untreated trees (Andross-control). Peach trees were subjected to water stress by watering every 30 days, instead of every 15 days, at the stage of pit hardening (mid-May) followed by normal watering after the completion of pit hardening. Extra N_2 fertilization was supplied as 3.5 kg NH_4NO_3 tree^{-1} in mid-May. Samples were collected from initiation of pit hardening (mid-May) until fruit maturation (mid-July).

Cloning of PPERFUL and PPERSHP genes

A peach mesocarp library curated at Clemson University (Jung et al. 2004) was searched for MADS-box gene ESTs, and clones BU039475 and BU046256 with high similarity to arabidopsis *FUL* and *SHP*, respectively, were obtained. In addition, a clone (BU044765) with high similarity to arabidopsis *ACTIN-2* was found along with a clone (BU043610) with high similarity to arabidopsis *AGAMOUS* (*AG*) (http://www.genome.clemson.edu/gdr/projects/prunus/abbott /PP_LEa/index.shtml).

Clone BU039475 was sequenced and primers PPERFUL-F and PPERFUL-R were designed based on its sequence. The 5′ end of the cDNA was obtained with a GeneRacer Kit (Invitrogen, Paisley, U.K.). First strand cDNA was synthesized from 1 μg of total RNA extracted from Andross fruits collected 1–4 weeks after full anthesis, using the gene-specific primer GSP1 and Superscript™ II. Amplification by PCR was accomplished with *Taq* DNA polymerase, a nested specific primer, GSP2, and the Abridged Anchor Primer (AAP). A second PCR was performed using the nested specific primer GSP3 and the Abridged Universal Anchor Primer (AUAP). The sequences of all the primers used are presented in Table 1. Amplified fragments were then cloned into pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen).

Similar to the cloning of the *PPERFUL* gene, clone BU046256 was sequenced and primers PPERSHP-F and PPERSHP-R were designed according to its sequence. After sequencing the *PPERSHP* clone, we performed an improved inverse-RACE method, called Rolling Circle Amplification RACE (RCA-RACE), which employs CircLigaseTM (Epicentre, Madison, WI) for cDNA circularization followed by rolling circle amplification of the circular cDNA with Phi29 DNA polymerase and random primers (Polidoros et al. 2006). The main advantage of this method is the production of a large amount of PCR template, which allows the simultaneous isolation of unknown 3′ and 5′ ends of a virtually unlimited number of transcripts after a single reverse transcription reaction. It also facilitates cloning of rare transcripts, as well as high throughput cloning of cDNA ends for large numbers of genes from scarce tissue, which cannot be effectively performed

Table 1. Primer sequences used in the experiments.

Primer	Sequence
AAP[1]	5′-GGCCACGCGTCGACTAGTACGGGIIG GGIIGGGIIG-3′
AUAP[2]	5′-GGCCACGCGTCGACTAGTAC-3′
GSP1SHP	5′-AGATGATTCCTGCTGATAAAACTG-3′
GSP2SHP	5′-AGGCGGCCACGGGTAGAGAAG-3′
GSP3SHP	5′-CGCTTAATCTCAATCTTGCCTCTT-3′
PPERACTIN-2-F	5′-GTGGGGATGGGACAGAAAGATG-3′
PPERACTIN-2-R	5′-GAGGTCAAGCCGGAGGATGG-3′
PPERAG-F	5′-AGGTTGCTCTCATAGTCTTCTC-3′
PPERAG-R	5′-TGGCTCCTCTCATTCTCAG-3′
PPERFUL-F	5′-TCAAGGCTAGGGTGGAGGTTTTAC-3′
PPERFUL-R	5′-ATTTGCTGTCGGGGTCTCATTTTC-3′
PPERSHP-F	5′-AGGCATATACTGGGTGAAGC-3′
PPERSHP-R	5′-GGGGAGAACATTGAGAAGC-3′
PPERSHP-2-F	5′-TTGCTCTTATTGTCTTCTCTACCC-3′
PPERSHP-2-R	5′-CCTCTCATTTTCAGCTATCTTTG-3′

[1] Abridged anchor primer.
[2] Abridged universal anchor primer.

with standard RACE methodologies.

A circular cDNA pool was constructed using total RNA from flowers and developing fruits of the 'Andross' variety, followed by rolling circle amplification using the specific primers GSP1SHP, GSP2SHP and GSP3SHP. The PCR fragments, which varied in size from 1400 to 1000 bp, were purified by agarose gel electrophoresis and cloned into pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen), according to the manufacturer's protocol. Analysis of the sequencing results for both genes was made with the SeqMan software package (DNASTAR, Madison, WI).

Reverse transcriptase polymerase chain reaction (RT-PCR)

For the RT-PCR, 1 µg of total RNA, isolated either from flowers (Week 1 of sampling) or from developing fruits without the embryo using the RNeasy plant RNA isolation kit (Qiagen, Crawley, U.K.), was used for first strand cDNA synthesis. The cDNA was synthesized using 1 µg of 3′ RACE adapter Primer 5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′ (Invitrogen), 1 mM dNTPs and 200 U M-MuLV reverse transcriptase (NEB, Beverly, USA) in 50 µl total volume. This cDNA served as a template in the PCR reaction, which used 0.2 pmol gene-specific primers, 0.2 mM dNTPs and 1 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). The sequences of the primers PPERAG-F, PPERAG-R, PPERSHP-F, PPERSHP-R, PPERSHP-2-F, PPERSHP-2-R, PPERFUL-F, PPERFUL-R, PPERACTIN-2-F and PPERACTIN-2-R, designed from the ESTs found at the Clemson University's EST library, are represented in Table 1. *PPERACTIN*-2 was used as the RT-PCR control. PPERSHP-2-F and PPERSHP-2-R were used to compare the expression levels of *PPERAG* and *PPERSHP*. The thermocycler program was 30 cycles of: 30 s at 94 °C; 30 s at 54 °C (or 58 °C for *PPERSHP*); and 30 s at 72 °C, which were preceded by 2 min at 94 °C and followed by 10 min at 72 °C. Fragments were of predicted lengths. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis.

Protein sequence comparisons and phylogenetic analysis

The nucleotide and deduced amino acid sequences of PPERFUL and PPERSHP were used for BLAST analysis on the EBI database. Among the best BLAST hits, genes for which there were published reports were selected for comparison. Sequence names were changed to include initials where needed and capitalized. The sequences homologous to PPERFUL were: *Prunus persica* PPMADS6 (AY705973); *Betula verrucosa* Ehrh. BVMADS5 (X99655); *Nicotiana sylvestris* L. NSMADS1 (AF068725); *Petunia hybrida* Vilm. PFG (AF17-7682); *Petunia hybrida* FBP26 (AF176783); *Prunus dulcis* (Mill.) D.A. Webb PDMADS2 (AY947463); *Malus domestica* Borkh. MDMADS2 (U78948); *Nicotiana tabacum* L. NTMADS11 (AF385746); *Nicotiana tabacum* NTNAP1-1 (AF009126); *Eucalyptus globulus* Labill. EAP1 (AF305076); *Solanum tuberosum* L. POTM1-1 (U23758); *Capsicum annuum* L. CAMADS6 (AF130118); *Lycopersicon esculentum* Mill. TDR4 (AY098732); *Solanum commersonii* Dunal ex Poir. SCM1 (AF002666); *Pisum sativum* L. PSM2 (AY88-4287); *Arabidopsis thaliana* AGL8/AtFUL (U33473); *Brassica oleracea* L. BOFULd (AJ505844); *Sinapis alba* L. SAAGL8 (U25695); *Eucalyptus globulus* EAP2L (AAG30923); *Malus domestica* MDAP1-like (AAL61543); *Betula pendula* Roth. BVMADS3 (CAA67967); and *Arabidopsis thaliana* AP1 (CAA78909). The sequences homologous to PPERSHP were: *Antirrhinum majus* L. PLE (AAB25101.1); *Gentiana triflora* Pall. GTMADS3 (AB189431); *Rosa rugoza* L. MASAKO D1 (AB025643); *Petunia hybrida* FBP6 (X68-675); *Malus domestica* MDMADS14 (AJ251117); *Ipomoea nil* (L.) Roth. PEONY (AB006183); *Petunia integrifolia* Lindl. PAGL1 (L33973); *Vitis vinifera* L. VVMADS1 (AF26-5562); *Arabidopsis thaliana* AGL1/SHP1 (AY727623); *Arabidopsis thaliana* AGL5/SHP2 (AY727669); *Cucumis sativus* L. CSMADS1 (AJ312773); *Pisum sativum* PSM8 (AY88-4292); *Malus domestica* MDAGAMOUS-like (AF401637); and *Gerbera hybrida* Bol. L. GAGA2 (Q9ZS29). The deduced amino acid sequences of each set of genes were aligned using the multiple sequence alignment program Clustal W (Thompson et al. 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 3.1 software (Kumar et al. 2004) by the Neighbor-Joining Method with p-distance correction (Saitou and Nei 1987).

Southern hybridization

Five µg of genomic DNA was digested with *Eco*RI, *Eco*RV, *Dra*I, *Xho*I, *Hind*III or *Bam*HI, (TaKaRa, Otsu, Japan) and transferred to a positively charged Nylon membrane. The digoxigenin labeled *PPERFUL* gene-specific probe was prepared by PCR with the primers PPERFUL-F and PPERFUL-R and the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). Hybridization was performed with DIG Easy Hyb buffer at 42 °C according to the manufacturer and stringent

washes at 68 °C in 0.5 × SSC containing 0.1% SDS (twice). Detection was performed with the DIG Luminescent Detection Kit and chemiluminescense was detected with the GeneGenome Bio Imaging System (Syngene, Cambridge, U.K.). The membrane was washed and re-probed with the digoxigenin labeled *PPERSHP* gene-specific probe that was prepared by PCR with the primers PPERSHP-F and PPERSHP-R.

Real-Time PCR

Quantitative expression analysis of *PPERFUL* during fruit development was performed with real-time RT-PCR using an Opticon 2 (MJ Research, Waltham, MA) real-time PCR system. Real-time PCR was also used to provide a relative quantitative estimation of the *PPERSHP* expression ratio (see below) during the crucial period of pit hardening (almost 5 weeks after anthesis until completion of pit hardening). The PCR was performed in 1 × PCR buffer containing 0.2 mM dNTPs, 0.4 pmol of each primer, 0.2 × Sybr-Green (Sigma-Aldrich, Dorset, U.K.) and 1 U of the DyNAzyme II DNA polymerase (Finnzymes) and the template was 1/10 of the cDNA synthesized from 0.5 µg of RNA extracted from developing fruits. The cycling parameters for *PPERFUL* were: incubation at 95 °C for 1 min, followed by 33 cycles of incubation at 95 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s, plate read at 80 °C and a final extension step of 5 min at 72 °C. The cycling parameters for *PPERSHP* were: incubation at 95 °C for 1 min, followed by 33 cycles of incubation at 95 °C for 15 s, 58 °C for 1min, 72 °C for 1 min, plate read at 80 °C and a final extension step of 5 min at 72 °C. To identify the PCR products, a melting curve was performed from 65 to 95 °C with observation every 0.2 °C and a 10-s hold between observations. The reactions were performed in triplicate. SYBR Green I fluorescence dye, which binds to double-stranded DNA, was used to monitor the newly synthesized PCR products (Morrison et al. 1998). Relative quantification and statistical analysis were performed using the REST software for estimation of the normalized gene expression ratio (Pfaffl et al. 2002). *PPERFUL* and *PPERSHP* expression was normalized against the non-regulated reference gene (same copy number per cell and stable expression in every cell) *PPERACTIN-2* (An et al. 1996, Ushijima et al. 2003). The relative expression ratio of *PPERFUL* during fruit development was calculated based on real-time PCR efficiencies (E) and the crossing point (CT) differences of each sample versus a control sample. Crossing point is defined as the cycle number at which the fluorescence rises above the background fluorescence and the efficiency of the reaction reveals the increase in amplicon at each cycle (e.g., 100% efficiency indicates a twofold increase in amplicon per cycle). The sample of the 'Andross' variety at the initiation of pit hardening (5 weeks after anthesis) was chosen as the control sample and the CT of each sample was compared with the control sample CT.

Statistical analysis

Statistical differences in expression between the mean values of control and experimental samples were measured by randomization tests (Pfaffl et al. 2002). In real-time PCR, the quantities of interest are derived from ratios and variances can be high, thus normal distributions would not be expected, and it is unclear how a parametric test could best be constructed. A randomization test avoids making assumptions about distributions, and is instead based on the random allocation of treatments. The randomization test repeatedly and randomly reallocates the observed values to the two groups and notes the apparent effect (expression ratio in our case) each time. The proportion of these apparent effects, which are directly related to the actual experimental observations, gives the *P* value of the test. An average expression was calculated by adding the expression ratio of each gene for all the weeks of sampling and comparisons were evaluated by the *t* test.

Lignin staining

Lignin formation in the peach endocarp was detected by staining with phloroglucinol-HCI reagent (Abeles and Biles 1991, Alba et al. 2000). 'Andross' and 'Katherine' fruits were sampled at 7 weeks after full anthesis. Fruits were also taken from Andross-no irrigation, Andross-control and Andross-N_2 fertilization 8 weeks after full anthesis. Phloroglucinol (2.0 g) was dissolved in 80 ml of 20% ethanol and then 20 ml of 12 *N* HCl was added.

Results

Cloning of PPERFUL and PPERSHP genes

To obtain a full-length peach *FUL* cDNA, we designed *PPERFUL* gene-specific primers based on a partial *PPERFUL* EST sequence and used 5′ RACE to clone the entire *PPERFUL* coding sequence. The gene-specific primers GSP1, GSP2 and GSP3 were used in the 5′ RACE experiments and a fragment of 500 bp was purified from the gel. Analysis of the sequencing results revealed that the 5′ RACE clone and the existing *PPERFUL* sequence belong to the same contig. The complete *PPERFUL* that was assembled had a 1094 bp sequence that contained a 768 bp ORF encoding a 255 amino acid polypeptide, and is almost identical with a putative MADS box sequence that has been deposited in GenBank (AM076976).

The *PPERSHP* gene was cloned by the newly developed RCA-RACE method using *GSP1SHP*, *GSP2SHP* and *GSP3-SHP* specific primers. Analysis of the sequencing results using the SeqMan software package (DNA Star, Madison, WI) revealed that the RCA-RACE clones and the existing *PPERSHP* sequence belong to the same contig. The *PPERSHP* transcript was 1040 bp and contained a 735 bp ORF encoding for 244 amino acids. The sequence was deposited in the GenBank under Accession Number DQ777635.

Amino acid sequence comparisons and phylogenetic analysis

FUL belongs to the AP1/FUL lineage of the MADS-box family of transcription factors that can be divided into two major clades: the AP1 clade and the FUL clade (Shchennikova et al.

2004). The PPERFUL sequence was aligned with the closest matching homologous proteins from other plant species. The MADS domain of the aligned proteins was highly conserved and conserved regions were also observed in the I and K domains, whereas the C terminal domain was more divergent. However, at the C terminus, all the FUL-like sequences, including that of PPERFUL (Figure 1A), harbored a highly conserved paleoAP1 motif:L/MPPWMLRHLNE (Litt and Irish 2003, Vandenbussche et al. 2003, Skipper et al. 2005). This motif was almost identical to those of closely related species *Prunus persica*, *Prunus dulcis* and *Malus domestica*. The AP1-like sequences showed a different C-terminus motif (euAP1 motif), because of a frame shift mutation as described in Vandenbussche et al. (2003). These changes in amino acid sequence are believed to play crucial roles in gene-specific functions (Lamb and Irish 2003). A phylogenetic tree was calculated for the amino acid sequence of the PPERFUL protein and the selected FUL-like proteins using the Neighbor-Joining Method (Figure 1B). A MADS-box protein identified from a Chinese peach variety 'YouTao', which was named PPMADS6 and was integrated into UniProtKB/TrEMBL on October 2004 (Accession Number AY705973), showed 99% similarity with our PPERFUL. Data indicated that the closest homologues to the peach protein from other species were a *Prunus dulcis* protein (PDMADS2), which showed 98% homology, a *Malus domestica* protein (MDMADS2), which showed 82% similarity with PPERFUL protein, and a protein from *Betula verrucosa* (BVMADS5) which showed 76% homology.

The PPERSHP sequence was aligned with the closest matching homologous proteins from other plant species (Figure 2A). One characteristic commonly found in C-lineage members of MADS-box genes is the presence of an N-terminal extension preceding the MADS domain (Jager et al. 2003). The MADS domain of the aligned proteins was highly conserved and conserved regions were also observed in the I and K domains, whereas the C terminal domain was more divergent. However, at the very C-terminal end of the proteins, there were two short, highly conserved regions, the AG motif I and the AG motif II, which are boxed (Figure 2A). PPERSHP fell into the PLE lineage of C-type, MADS-box proteins along with other AG-like proteins from *Petunia hybrida* (FBP6), *Arabidopsis thaliana* (SHP1), *Malus domestica* (MDMADS14), *Rosa rugoza* (MASAKO D1) and *Vitis vinifera* (VVMADS1) (Kramer et al. 2004).

A phylogenetic tree was calculated for the amino acid sequence of the PPERSHP protein and the selected SHP-like proteins using the Neighbor-Joining Method (Figure 2B). The closest homologues to the peach gene were a gene from *Malus domestica* (MDMADS14), which showed 87% similarity with PPERSHP protein, a protein from *Rosa rugosa* (MASAKO D1), which showed 83% homology, and a *Pisum sativum* (M8) protein, which showed 79% homology.

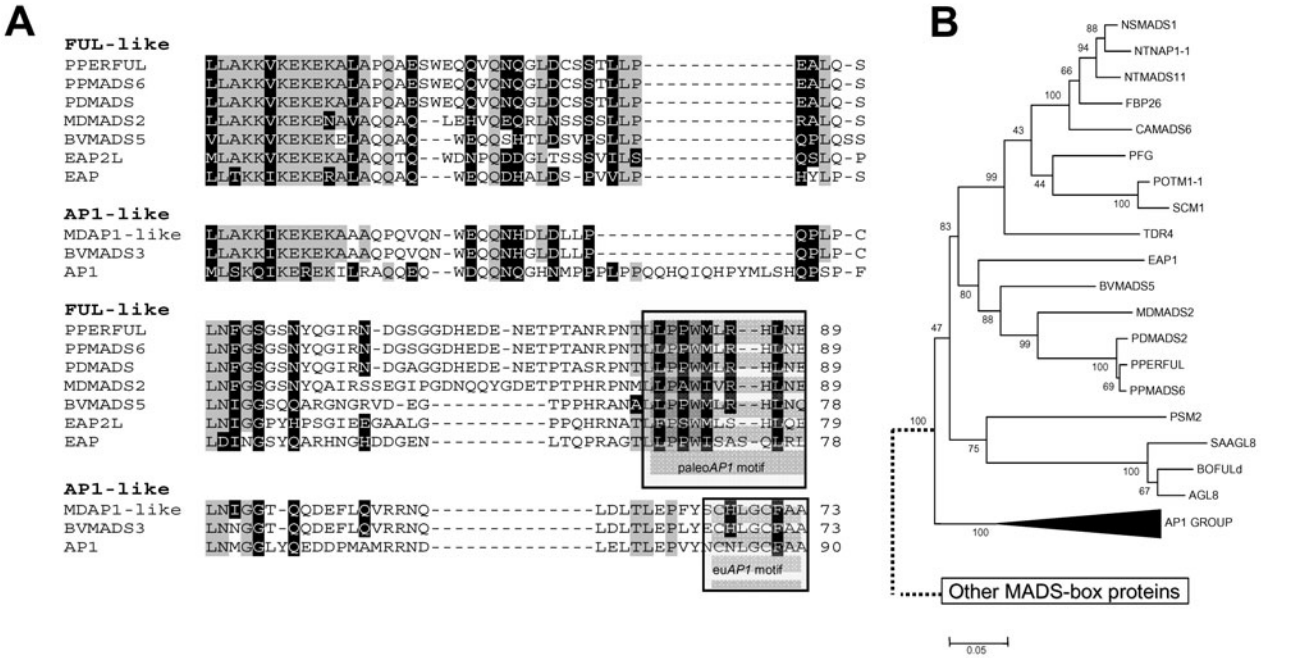

Figure 1. A: Alignment of the C-terminal domain of the predicted amino acid sequence of PPERFUL with the most similar FUL-like sequences identified in BLAST along with characteristic AP1-like sequences in other plants. A paleoAP1 motif characteristic of FUL-like sequences and a euAP1-motif characteristic of AP1-like sequences are boxed. The C-terminal domain of PDMADS2 is almost identical with that of PPERFUL and differences in two amino acid sites are indicated by open boxes. B: Phylogenetic relationships of PPERFUL with other FUL-like MADS-box proteins. The tree showing the FUL clade with FUL-like sequences and a condensed AP1 branch was generated by the Neighbor-Joining method using the p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications. The dashed line connects the tree with other MADS-box proteins that are beyond the *x*-axis scale. Scale indicates number of amino acid substitutions.

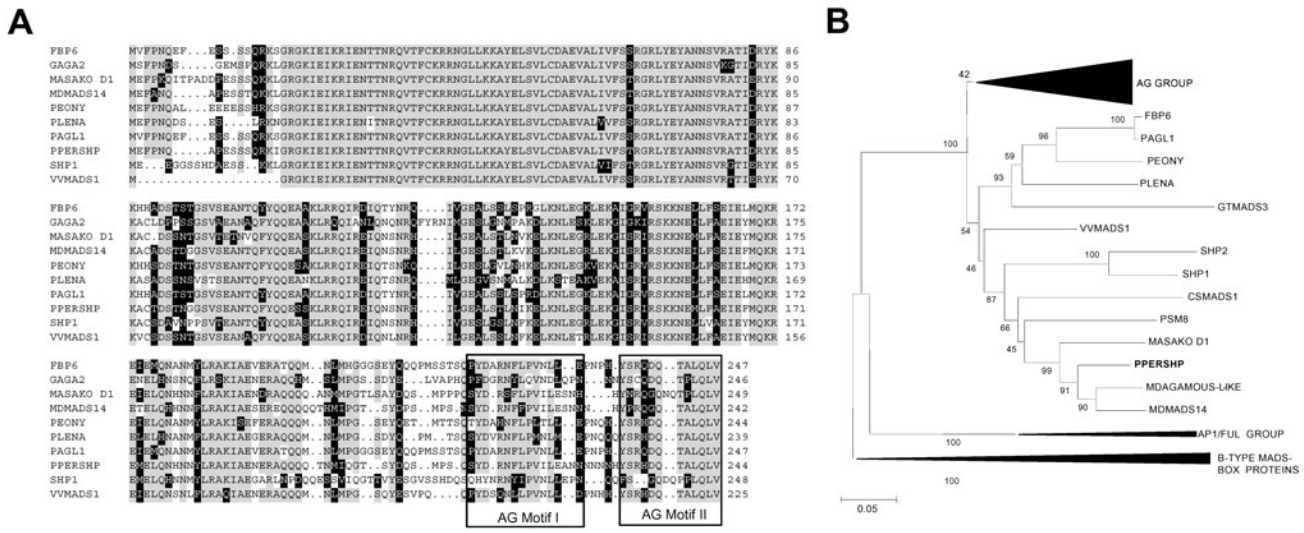

Figure 2. A: Alignment of the predicted amino acid sequence of the PPERSHP protein and members of the SHP family in other plants. The AG-I and AG-II motifs characteristic of C-type MADS-box proteins are boxed. The alignments were generated using ClustalW. B: Phylogenetic relationship of PPERSHP with members of other PLE-like sequences and the AG, AP1/FUL and B-type MADS-box protein clades. The tree was generated by the Neighbor-Joining method using the p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications.

Comparison of expression levels of PPERSHP and PPERAG

AGAMOUS is a C-type MADS-box gene that determines reproductive organ development in arabidopsis (Causier et al. 2005). *SHP1* and *2* can also direct male and female organ identity when ectopically expressed in transgenic arabidopsis plants. Nevertheless, whereas *AG* is expressed in both male and female reproductive organs, *SHP* genes are expressed only in the fourth whorl. To test whether this is the case in *Prunus persica*, RT-PCR experiments on cDNAs derived from carpels and stamens were performed to determine expression levels of *PPERSHP* and *PPERAG* in both tissues. Expression of *PPERSHP* was detected primarily in carpels and a low level of expression was found in stamens. *PPERAG* expression was high in both stamens and carpels (Figure 3). These findings are in agreement with the expression profiles of *MdMADS14* and *MdMADS15*, the orthologues of *SHP* and *AG*, respectively, in apple (Van der Linden et al. 2002).

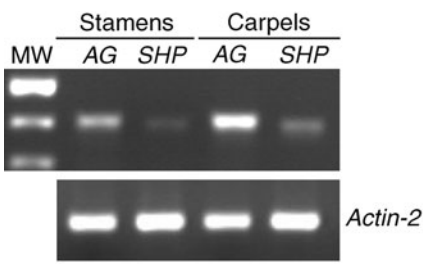

Figure 3. Expression analysis of *PPERSHP* and *PPERAG* in stamens and carpels by semiquantitative RT-PCR with *ACTIN-2* as the control. Agarose gels, stained with ethidium bromide, showing a 363 bp *PPERSHP* fragment and a 409 bp *PPERAG* fragment on top. The 402 bp *ACTIN-2* fragment is shown in the bottom part of the figure.

Southern hybridization

A Southern blot revealed the presence of one putative *FUL*-like ortholog in peach (Figure 4A). One band was observed with the *PPERFUL* gene-specific probe except in Lane 6, where the *PPERFUL* gene-specific probe hybridized with two bands after *Bam*HI digestion. This is explained by the presence of a recognition site for the particular restriction enzyme inside the intron fragment amplified by the *PPERFUL* primers, which was used as the *PPERFUL* probe (data not shown).

Results from restriction enzyme digestions for the number of *PPERSHP* copies in peach were inconclusive. *Hind*III and *Bam*HI digestions revealed two hybridizing bands consistent with the presence of two copies, whereas the rest of the restriction enzymes produced a single band, which was more intense in the *Eco*RI and *Xho*I digestions (Figure 4B). To resolve the number of copies of *PPERSHP* in peach, we amplified the genomic fragment corresponding to the *PPERSHP* probe with the primers used for probe preparation. Data in the inset picture indicate that the primers could amplify a ~600 bp fragment from genomic DNA indicating the presence of an intron in the genomic sequence of *PPERSHP*. No additional bands were detected) (data not shown) when the genomic DNA was digested with all the restriction enzymes used for the Southern analysis, supporting the hypothesis that two copies of *PPERSHP* are present in peach.

Expression analysis

Initial RT-PCR experiments on cDNAs derived from all the developmental stages of fruit development (from full anthesis to fruit harvest) revealed that *PPERFUL* and *PPERSHP* were

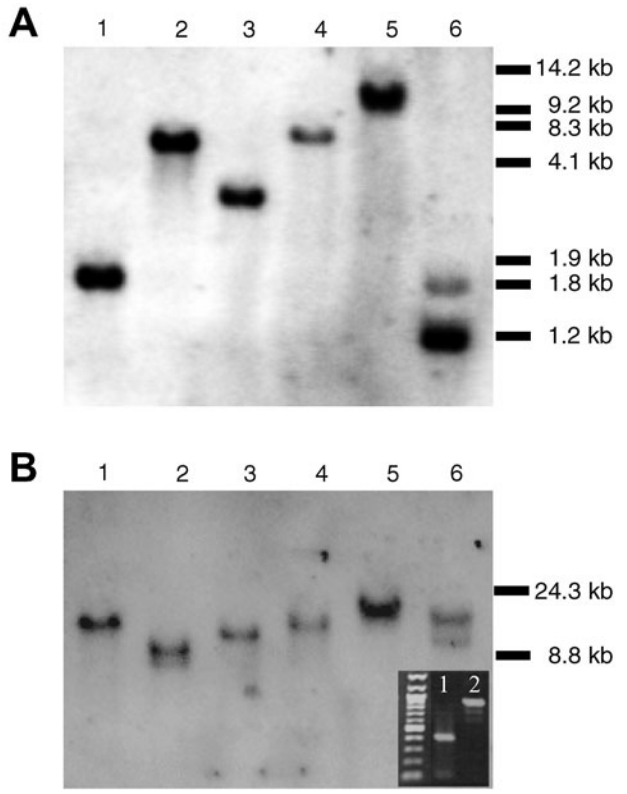

Figure 4. A: Southern blot of genomic DNA from peach digested with *Eco*RI (1), *Hind* III (2) *Dra*I (3), *Eco*RV (4), *Xho*I (5) or *Bam*HI (6) and probed with a *PPERFUL*-specific probe. A single hybridizing band was observed in all digests except the digest with *Bam*HI that displayed two bands. B: Southern blot revealing the presence of *SHP* ortholog(s) in peach. Five µg of peach genomic DNA was digested with *Eco*RI (1), *Hind* III (2) *DraI* (3), *EcoRV* (4), *XhoI* (5) or *BamHI* (6) and hybridized with a *PPERSHP* gene-specific 408 bp fragment. Inset: PCR was performed with the primers used for the *PPERSHP*-specific probe and cDNA (Lane 1) and genomic DNA (Lane 2). Fragments amplified in Lanes 1 and 2 are 408 bp and ~1000 bp, respectively.

present throughout fruit development (Figures 5A and 6A).

Real-time PCR provided a relative quantitative estimation of *PPERFUL* and *PPERSHP* expression compared with *PPER-ACTIN-2* expression in the same samples (Figures 5B and 6B). The growth curves of fruits of the two peach varieties during the sampling period is shown in Figure 5C. The two curves were similar, showing a double sigmoid pattern, in four phases of growth. Mean fruit diameter growth is depicted in Figure 5B for comparison with *PPERFUL* expression during fruit development.

The *PPERFUL* expression ratio during the first 1–4 weeks after anthesis was high in both the susceptible variety ('Andross') and the resistant variety ('Katherine') compared with the later stages of fruit development (Table 2). During the phase of rapid increase in fruit diameter, 4–7 weeks after anthesis, there was no variation in *PPERFUL* expression between the two varieties although it was higher than the control.

Table 2. Statistical analysis of differences of *PPERFUL* expression during each week of sampling and *PPERSHP* expression during pit hardening (Weeks 5–11) between 'Andross' and 'Katherine' using REST software. Factor refers to up- or down-regulation of expression in 'Katherine' compared with 'Andross' in each pair. The *P* values were estimated based on a pair-wise fixed reallocation randomization test statistical model. Abbreviation: n.s. = not significant.

Week	*PPERFUL*		*PPERSHP*	
	Factor	*P* value	Factor	*P* value
1	1.038	n.s.		
3	–1.027	n.s.		
4	1.419	0.0275		
5	–1.068	n.s.	–1.953	n.s.
6	1.462	n.s.	–1.933	n.s.
7	–2.373	0.0455	–1.227	n.s.
8	2.139	0.0455	1.007	n.s.
9	2.394	n.s.	1.01	n.s.
10	1.059	n.s.	–1.589	0.0325
11	2.387	0.001	–1.38	0.001
12	–3.87	0.022		
13	1.434	n.s.		
14	–6.844	0.032		
15	3.736	n.s.		

In the crucial period of pit hardening, 7–11 weeks after anthesis, *PPERFUL* expression declined in both varieties, with a dramatic reduction in *PPERFUL* expression in 'Andross' compared with 'Katherine' (Table 2). Later, in the third stage of fruit growth (12–15 weeks after anthesis), an increase in *PPERFUL* expression occurred that was more evident in 'Andross' than in 'Katherine'. Mean *PPERFUL* expression during the sampling period was not significantly different between 'Andross' and 'Katherine' ($t = 0.246$, $P > 0.05$); however, there were significant differences when each sample was compared with its pair (e.g., pair A4–K4) using the randomization test from the REST software (Pfaffl et al. 2002). During the period of pit hardening, the differences observed between the pairs became highly significant (pair A11–K11) (Table 2).

The mean *PPERSHP* expression during pit hardening was not significantly different between the two varieties ($t = 1.393$, $P > 0.05$). Although the *PPERSHP* relative expression ratio was lower in 'Katherine' than in 'Andross' during the initial period of pit hardening (6–7 weeks after anthesis), it was not statistically different. During the intermediate phase of pit hardening (8–9 weeks after full anthesis) there was no difference in the *PPERSHP* relative expression ratio between the two varieties; however, significant differences appeared during the last period of pit hardening (10–11 weeks after full anthesis), when the expression ratio of *PPERSHP* was significantly lower in Katherine than in Andross (Table 2).

Lignin staining

As an additional parameter that may affect split pit in peach we examined lignin formation during the period of initiation of pit

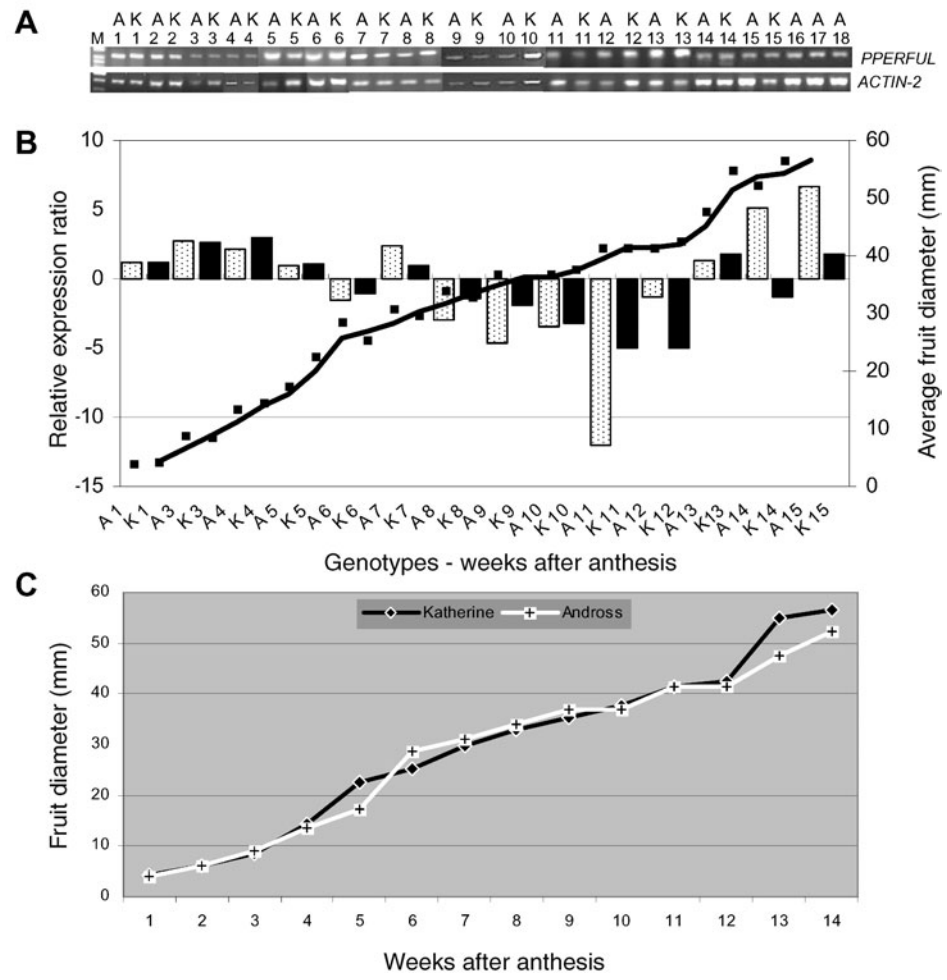

Figure 5. A: Qualitative analysis of the *PPERFUL* transcript accumulation in peach. The RT-PCR of *PPERFUL* was performed during all stages of peach fruit development in the varieties 'Andross' (A) and 'Katherine' (K). The numbers under the letters indicate weeks after full anthesis (e.g., 1 = Week 1 after full anthesis). Amplicons of the expected size were observed. Control RT-PCR was performed with *PPERACTIN-2* primers. B: Quantitative analysis of *PPERFUL* expression and fruit growth in peach. Sampling was from full anthesis until the end of fruit growth (1–15 weeks after full anthesis). The relative expression ratio of each sample in comparison with the control sample, which was 'Andross' at 5 weeks after anthesis, is represented by a factor of up- or down-regulation and is shown with bars for the varieties 'Andross' (A, dotted bar) and 'Katherine' (K, black bar). The increase in fruit diameter is shown as an average trend-line (calculated from the mean of two consecutive measurements) showing a characteristic double sigmoid form. C: The increase in fruit diameter in 'Andross' (white line) and 'Katherine' (black line).

hardening. Seven weeks after full anthesis (1 week after initiation of pit hardening), the endocarp of 'Andross' fruits was not stained, whereas the endocarp of 'Katherine' was clearly stained in the inner part (Figures 7A and 7B). Eight weeks after full anthesis, fruits from trees subjected to water stress were stained only in the inner part of the endocarp, whereas the entire

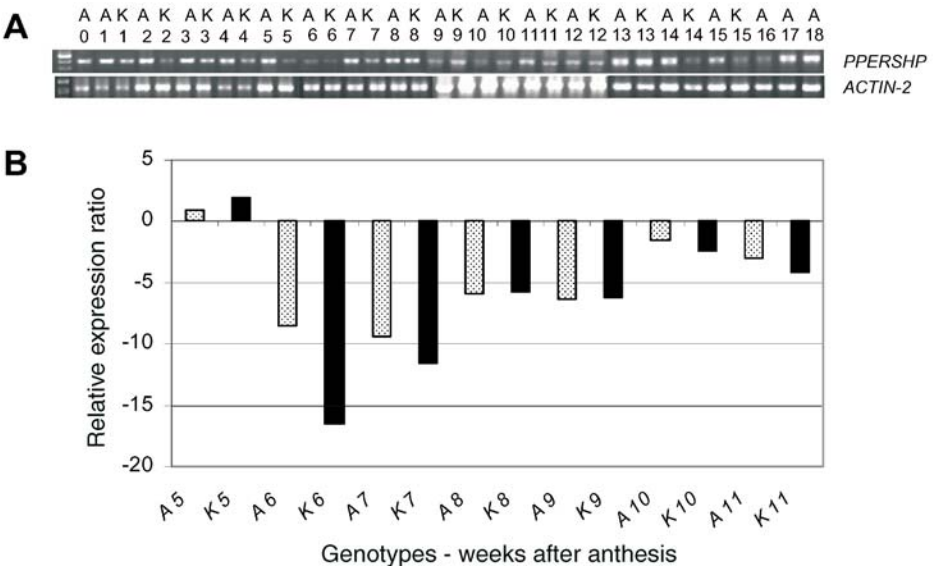

Figure 6. A: An RT-PCR analysis of the *PPERSHP* gene expression during the stages of peach fruit development in the varieties 'Andross' (A) and 'Katherine' (K). B: Quantitative analysis of *PPERSHP* in the critical stages of fruit development (50 days from full anthesis until pit hardening). Relative expression ratio of each sample in comparison to the control sample ('Andross' at 5 weeks after anthesis) is represented by a factor of up- or down- regulation and is shown with bars for 'Andross' (A, dotted bar) and 'Katherine' (K, black bar). The numbers under the letters indicate the weeks after full anthesis (e.g., 6 = Week 6 after full anthesis).

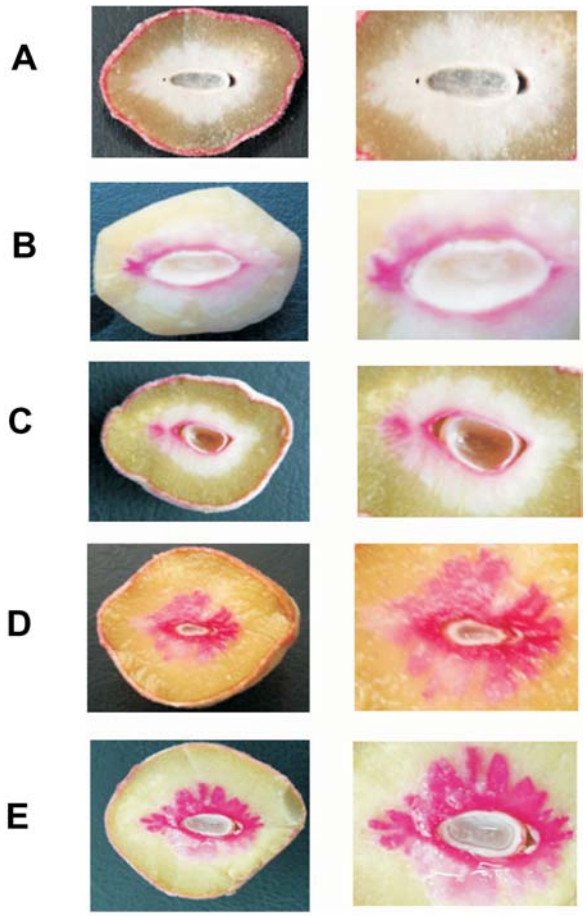

Figure 7. Lignin stained with phloroglucinol-HCl. Samples are: A, 'Andross' (7 weeks after anthesis); B, 'Katherine' (7 weeks after full anthesis); C, Andross-no irrigation (8 weeks after anthesis); D, Andross-control (8 weeks after anthesis); and E, Andross-N_2 fertilization (8 weeks after anthesis).

endocarp was stained in control 'Andross' samples as well as in 'Andross' fruits from trees subjected to excessive N_2 fertilization (Figures 7C–E).

Discussion

Fruit development has been extensively studied in arabidopsis and key regulatory steps and the genes involved have been identified and their roles characterized (Ferrandiz et al. 1999, Ferrandiz 2002). Anatomical and physiological analogies exist between arabidopsis valves and peach pericarp. In arabidopsis, *FUL* expression is required for the expansion and differentiation of the fruit valve after fertilization (Gu et al. 1998). *FUL* is expressed throughout the valves, except in a narrow strip where the dehiscence zone is formed. The expression of two other genes, *SHP1* and *SHP2*, is required for valve margin development; these genes encode proteins that control dehiscence zone differentiation and promote the lignification of adjacent cells (Liljegren et al. 2000, Dinneny and Yanofsky 2005). Results from Østergaard et al. (2006) showed that ectopic expression of the *FRUITFULL*-like gene in *Brassica juncea* (L.) Czern. leads to a lack of valve margin specification in the fruit and, consequently, to pod-shatter resistance. Because there are no published studies reporting attempts to transfer the knowledge and technology on fruit development derived from the model plant arabidopsis to a tree species, we isolated and examined the expression patterns of *PPERFUL* and *PPERSHP* genes in peach and studied their involvement in split-pit formation.

Based on existing information, we designed PCR primers and isolated full length cDNAs of *PPERFUL* and *PPERSHP* through 5′-RACE and RCA-RACE techniques, respectively. The PPERFUL sequence was aligned with the closest matching homologous proteins from other plant species (Figure 1A). At the very C terminus the sequence showed an L/MPPWMLRHLNE motif that is highly conserved in all FUL-like sequences (Litt and Irish 2003, Skipper et al. 2005). Phylogenetic analysis of PPERFUL revealed that peach sequences form a separate branch that also includes *Prunus dulcis* PDMADS2, *Malus domestica* MDMADS2 and the *Eucalyptus globulus* sequence EAP1 as well as the *Betula verrucosa* sequence BVMADS5 (Figure 1B).

Alignment of the PPERSHP protein sequence with the closest matching homologous proteins from other plant species revealed that it possesses all of the characteristics commonly found in C-type, MADS-box proteins (Figure 2A). A gene duplication in the arabidopsis *PLE* homologue led to the formation of the two arabidopsis *SHP* genes (Causier et al. 2005). Phylogenetic analysis indicated that PPERSHP falls into the PLE lineage of the C-type, possibly originating from a PLE ortholog from peach (Figure 2B). We examined *PPERFUL* and *PPERSHP* expression in *Prunus persica* fruits and found that both were normally expressed during fruit development.

The growth curves of peach fruits from the varieties examined showed the classical double sigmoid pattern, with four phases of growth (Figure 5C) (Bregoli et al. 2002). Fruit diameter growth is characterized by an initial phase of rapid increase up to 7 weeks after full anthesis, a second phase of slow growth between 7 and 11 weeks after anthesis, a third phase of exponential growth from 12 to 15 weeks after anthesis and a final phase of growth (15–18 weeks after anthesis) characterized by a slight increase in diameter. Pit hardening as a result of lignification occurs during the second phase of fruit growth (7–11 weeks after full anthesis). When the expression patterns of *PPERFUL* and *PPERSHP* were compared in split-pit susceptible and resistant varieties, a characteristic regulation that might be relevant to split-pit formation was observed. Temporal differences in the level of *PPERFUL* expression were recorded. Initially, *PPERFUL* in both varieties was expressed at high levels, followed by a decrease during pit hardening, and then an increase in expression at the final fruit growth phases. The differences between the split-pit sensitive variety and the resistant variety were initially small when *PPERFUL* expression was high. During the phase of pit hardening when *PPERSHP* expression decreased, the resistant variety had significantly lower *PPERSHP* expression than the sensitive variety. During the final phases of fruit growth, *PPERFUL* expression

increased and was higher in the sensitive variety than in the resistant variety. Our findings indicate that a change in *PPERFUL/PPERSHP* expression equilibrium during the second phase of fruit growth could be an important factor affecting split-pit formation sensitivity.

Split-pit formation occurs at the third phase of fruit growth, after pit hardening. During hardening and lignification, the pit gradually loses flexibility and becomes very rigid while the flesh is still tightly attached to it. In the next and final stage of fruit growth, the flesh creates forces pulling out on the pit. If great enough, these forces will cause the pit to break in the weakest spot, which is along the suture. This resembles the arabidopsis model of fruit dehiscence, in which the valves shatter because of internal tension created when the fruit dries. We suggest that early formation and lignification of the dehiscence zone may contribute to suture weakening. Consistent with this hypothesis we found evidence that the genes *PPERFUL* and *PPERSHP* are involved in the formation of the separation layer, having a temporal expression pattern that allowed earlier formation of the dehiscence zone in the split-pit sensitive variety than in the split-pit resistant variety. The susceptible variety showed a larger decrease in *FUL* expression and a lower suppression in *SHP* expression when compared with the resistant variety. This pattern of gene expression could lead to fast formation and lignification of the dehiscence zone, allowing separation of the endocarp halves under the pulling forces of the growing fruit.

In arabidopsis, in addition to *SHP* and *FUL*, several other genes appear to be involved in fruit dehiscence. *ALCATRAZ* (*ALC*), a basic-helix-loop-helix (b-HLH) transcription factor, is involved in the differentiation of the separation layer (Rajani and Sundaresan 2001), and *INDEHISCENT* (*IND*), another b-HLH transcription factor, is involved in the differentiation of all three layers (Lijegren et al. 2004). Furthermore, *REPLUMLESS* (*REP*), a member of the bell subfamily of homeodomain transcription factors, negatively regulates *SHP1* and *2* expression in the replum (Roeder et al. 2003). Together, *IND*, *ALC* and *SHP* form a regulatory network that orchestrates the differentiation of the valve margins, allowing seed dispersal to take place. *FUL* negatively regulates *IND*, *ALC* and *SHP1* and *2* to ensure that valve margin differentiation occurs at the edge of the valve (Gu et al. 1998, Liljegren et al. 2004). It is not known whether a similar model could control the opening of the endocarp margins in peach; however, we found that two components, the *FUL* and *SHP* homologues in peach, are present and their expression patterns fit the model.

Further experiments and expression analysis of other genes involved in formation of the fruit dehiscence zone, as well as pit hardening, will give more information about split-pit formation and possibly indicate an approach to minimize its occurrence. The detection of differences in the expression of each gene involved in the formation of the fruit dehiscence zone and in pit hardening between varieties resistant and susceptible to split-pit would enable the design of molecular markers to distinguish varieties prone or resistant to pit opening not only for molecular breeding but also for monitoring split-pit formation during fruit development.

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