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Research article

Characterization and expression analysis of *AGAMOUS*-like, *SEEDSTICK*-like, and *SEPALLATA*-like MADS-box genes in peach (*Prunus persica*) fruit

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ABSTRACT

MADS-box genes encode transcriptional regulators that are critical for flowering, flower organogenesis and plant development. Although there are extensive reports on genes involved in flower organogenesis in model and economically important plant species, there are few reports on MADS-box genes in woody plants. In this study, we have cloned and characterized *AGAMOUS* (*AG*), *SEEDSTICK* (*STK*) and *SEPALLATA* (*SEP*) homologs from peach tree (*Prunus persica* L. Batsch) and studied their expression patterns in different tissues as well as in fruit pericarp during pit hardening. *AG*- *STK*- and *SEP*-like homologs, representative of the C-, D-, E-like MADS-box gene lineages, respectively, play key roles in stamen, carpel, ovule and fruit development in *Arabidopsis thaliana*. Sequence similarities, phylogenetic analysis and structural characteristics were used to provide classification of the isolated genes in type C (*PPERAG*), type D (*PPERSTK*) and type E (*PPERSEP1, PPERSEP3, PPERFB9*) organ identity genes. Expression patterns were determined and in combination with phylogenetic data provided useful indications on the function of these genes. These data suggest the involvement of MADS-box genes in peach flower and fruit development and provide further evidence for the role of these genes in woody perennial trees that is compatible with their function in model plant species.

1. Introduction

MADS-box genes comprise a multigene family of transcription factors regulating different aspects of development in higher plants. The MADS-box family is divided into several subfamilies regarding the primary sequences and expression patterns [17]. Due to the fact that a general co-evolution occurs between the MADS-box and the rest of the genes, the MADS-box genes are considered as a model for the study of evolution [37]. The application of genetic and molecular analysis of floral homeotic mutants of arabidopsis (*Arabidopsis thaliana* L. Heynh) and snapdragon (*Antirrhinum majus* L.) has helped in the discovery of the first MADS-box genes [34]. These initial genetic studies led to the proposal of the ABC model of flower development [6]. In arabidopsis, the action of A-class MADS-box gene, named *AP1* together with a non-MADS-box gene, the *AP2*, form sepals. The aforementioned genes in cooperation with the B-class MADS-box genes *AP3* and *PI* determine the identity of petals. Stamens are determined by the B function genes together with the C function gene *AG*, and the identity of the carpel is determined by *AG*. In snapdragon the functional homologs of *AP3*, *PI*, and *AG* are *DEF*, *GLO*, and *PLE* respectively [23].

Studies on arabidopsis and petunia (*Petunia hybrida* Vilm.) indicated that the ABC model should include the ovule identity genes (*STK* from arabidopsis and *FBP7*, *FBP11* from petunia) and be extended to an ABCD model, where D function specifies ovule development [7]. The C- and D-gene groups are sister lineages as they evolved from a duplication event during early angiosperm evolution [21]. In arabidopsis ovule development is promoted by a complex collaboration of the C-type (*AG* and *SHP*), along with the D-type (*STK*) genes [27].

The ABCD model has been further extended with an additional class indicated as class E genes or *SEP* genes. In arabidopsis this class consists of four members, *SEP1*, *SEP2*, *SEP3* and *SEP4*, encoding MADS-box factors that show partial redundant functions in floral organ identity determination. The homologous genes from petunia are *FBP5*, *PHMADS12*, *FBP2* and *PHFBP4* respectively. Results from Ditta et al. [8], Honma and Goto [14] and Pelaz et al. [26], show that

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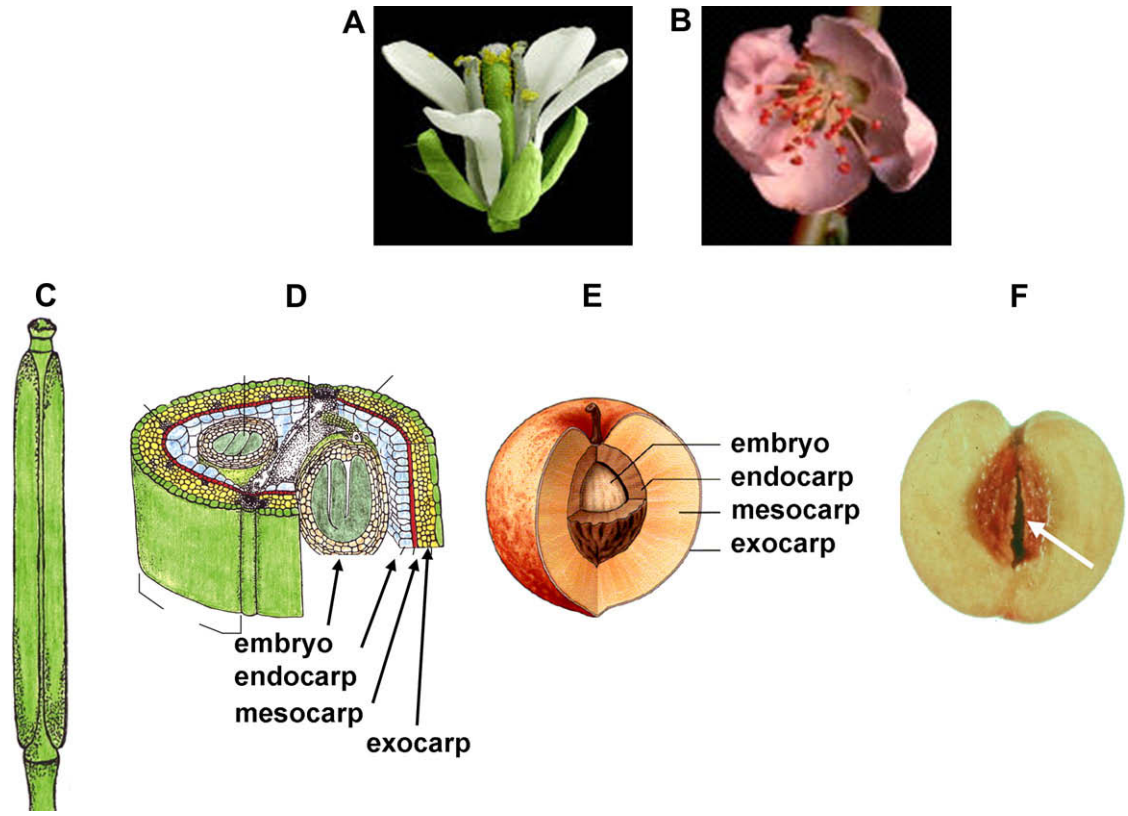


Fig. 1. The arabidopsis flower (A) in comparison with the peach flower (B). The arabidopsis fruit (C) and a transverse section of the wild-type arabidopsis fruit (D) in comparison with the transverse section of a peach fruit (E). The different parts of the fruits are indicated. (F) Split-pit formation in peach endocarp at the dehiscence zone is indicated with an arrow.

the *SEP* genes are necessary for the function of class A, B and C genes and discuss the molecular basis for the genetic relation among floral organ identity. According to their experiments, the single *sep3* mutants displayed a partial transformation of the petals into sepals, therefore the requirement of SEP3 for the development of petals is larger than that of SEP1, SEP2, or SEP4. Interaction studies in yeast showed that SEP3 proteins interact with A, B and C MADS-box factors forming multimeric complexes. The formation of these complexes would allow, at the same time, the transcriptional activation of the target genes [14]. Similar genes have now also been studied in other dicotyledonous plants [33], and very similar structures and expression patterns have been found in more distantly related species.

Despite the progress made studying the MADS-box genes involved in flowering and flower organ formation in annual plants less is known for their role in fruit trees. There have been extensive reports on ABC model of MADS-box genes in annual flowering plants [20] but not in woody plants apart from studies on characterization of A-, B-, C-type MADS-box genes in apple flowers (*Malus domestica* Borkh.) [40], on five MADS-box genes in citrus [10] and on two C-type MADS-box genes in poplar [4]. Peach has become a model tree species for genetic studies within the Rosaceae [1]. The diploid genome ($2n = 16$), the small genome size of 300 Mb and the relatively short reproductive time for a fruit tree (2–3 years until flowering) facilitate genetic studies. Since peach and arabidopsis belong to the same clade of Rosids, and they have anatomical and physiological similarities between their flowers and fruits (Fig. 1), it is applicable to transfer the knowledge obtained from a model plant such as arabidopsis to a crop tree cultivated for its fruit.

Our group has cloned and characterized the peach MADS-box genes *PPERFFRUITFUL* (*PPERFUL*) (A-clade) and *PPERSHATTERPOOF*

Table 1
Primer sequences used in the experiments. Regarding the primers used for in situ hybridization experiments, consensus T3 sequence is shown in bold letters, preceded by a 9 base pair leader sequence in italic and followed after the slash (/) by the gene-specific sequence.

Primer name	Sequence
PPERACTIN-2-F	5′-GTGGGGATGGGACAGAAAGATG-3′
PPERACTIN-2-R	5′-GAGGTCAAGCCGGAGGATGG-3′
PPERAG-F	5′-AGGTTGCTCTCATAGTCTTCTC-3′
PPERAG-R	5′-TGGCTCCTCTCATTCTCAG-3′
PPERAG-F5end	5′-ATGGCCTATGAAAACAAATCC-3
PPERSEEDSTICK-F3	5′-CATTGTCTTCTCCAGTCGAG-3′
PPERSEEDSTICK-R3	5′-TCTGGAGATTGAACCAAGTC-3′
PPERSEP1-F	5′-GTCAAGTGGAAGTCAACAACCTG-3′
PPERSEP1-R	5′-CATCTGCTCTGACCCCACATTA-3′
PPERSEP3-F	5′-TATGAAGCCCTACAACGAAAC-3′
PPERSEP3-R	5′CCGTACTCGTCAGCATTTGG-3′
PPERFBP9-F	5′-GATTTGCCCCACTGAACAC-3′
PPERFBP9-R	5′-TGAGTGAGGATGATGAGGA-3′
PPERSEP1-F-INSITU	5′-TGACATTAGTTCAAGAAACCA-3′
PPERSEP1-R-INSITU	5′-CCAAGCTTC**ATTAACCCTCACTAAAGGG AGA**CATTAGAGTAC-3′
PPERFBP9-F-INSITU	5′-GGCAACAACAATGTTCAGCA-3′
PPERFBP9-R-INSITU	5′-CCAAGCTTC**ATTAACCCTCACTAAAGGG AGACA**CATCTTGTC-3′
PPERSEP3-F-INSITU	5′-CAAACTCAAGCTCATGGCGA-3′
PPERSEP3-R-INSITU	5′-CCAAGCTTC**ATTAACCCTCACTAAAGGGAG A**CCTGCCATGTA-3′
PPERSTK-F-INSITU	5′-GAATGCAATCCAGGCATTAGC-3′
PPERSTK-R-INSITU	5′-CCAAGCTTC**ATTAACCCTCACTAAAGGGAG A**CAGTAGGCAG-3′
PPERAG-F-INSITU	5′-GGGGAAGTTATGAGATCATG-3′
PPERAG-R-INSITU	5′-CCAAGCTTC**ATTAACCCTCACTAAAGGGAG A**ACATACCCACCAT-3′

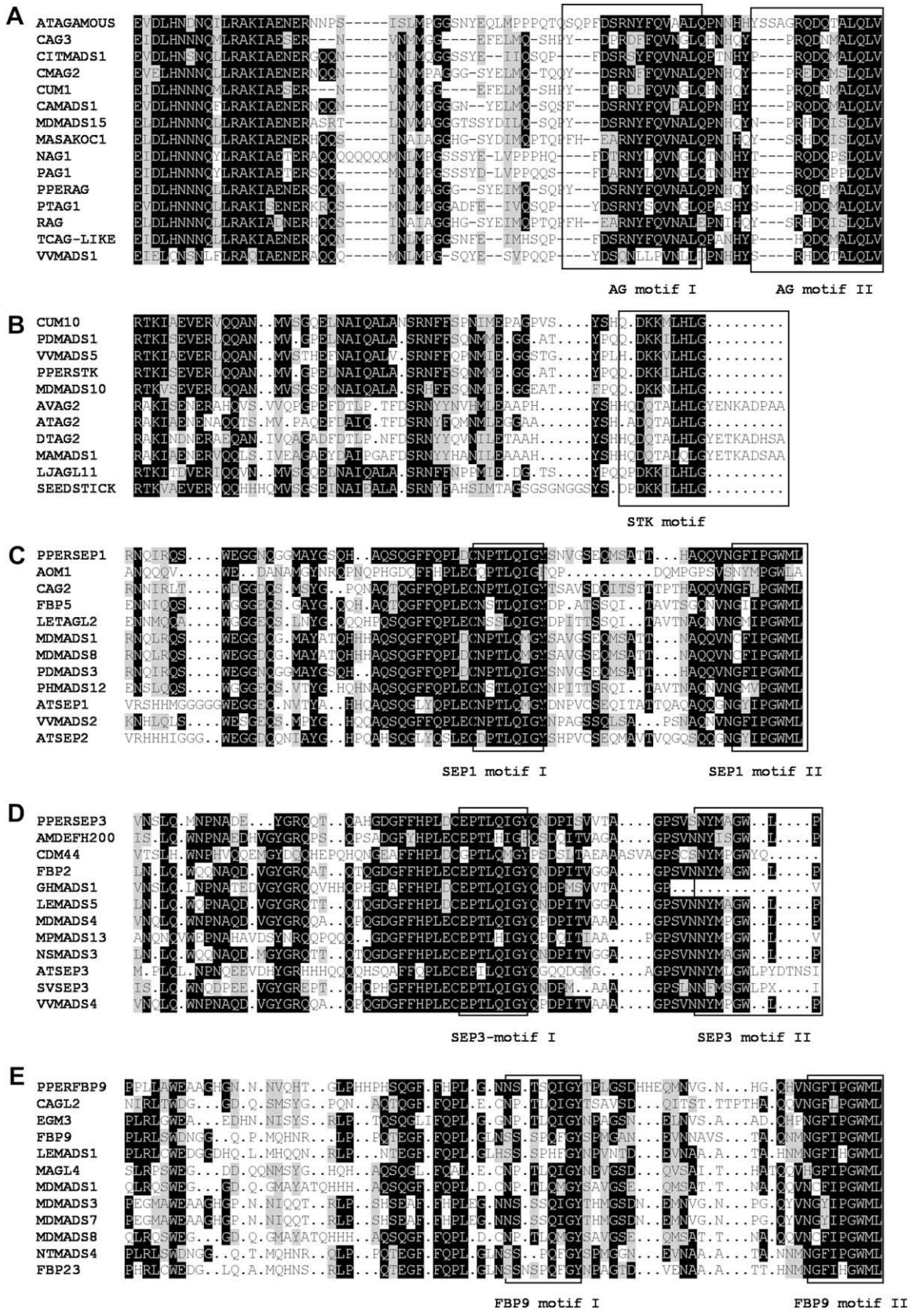
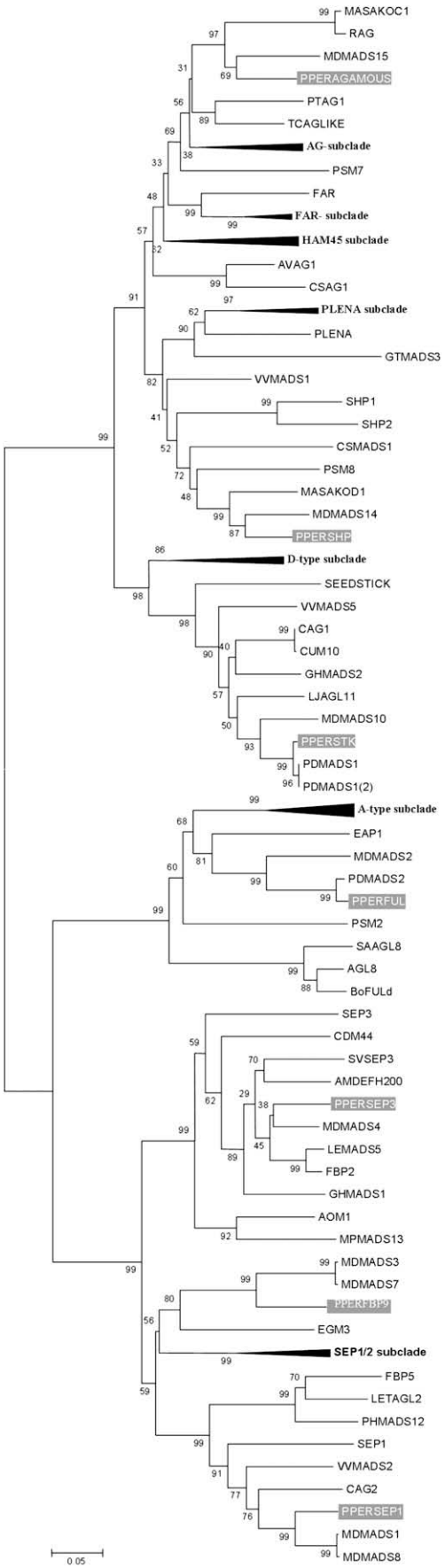


Fig. 2. Alignment of the C-terminal domains of the predicted amino acid sequence of *PPERAG* (A), *PPERSTK* (B), *PPERSEP1* (C), *PPERSEP3* (D), *PPERFBP9* (E), and all *PPERSEP*-like (F) and with the most similar sequences identified in BLAST. C-motifs characteristic of AG, STK, SEP1, SEP3, FBP9 sequences are boxed.



(*PPERSHP*) (C-type) [36]. In the same study we showed that differential temporal regulation of *PPERFUL* and *PPERSHP* genes in the split-pit sensitive (Andross) and resistant (Katherine) varieties, might affect split pit formation—the opening of the pit and sometimes splitting of the fruit—which causes deterioration of canned fruit quality and great economic losses. Recent studies in peach have characterized A- and B- type MADS-box genes [42] as well as C-type MADS-box genes [35]. However there are no reports for the total group of C-, D-, E-type of MADS-box genes and their interaction in controlling fruit development in woody plants. We report here the cloning of C-, D-, and E-type *PPERAG*, *PPERSTK* and *PPERSEP* genes from peach and the study of their structure, phylogenetic relationship and expression during peach flower, ovule and fruit development.

2. Materials and methods

2.1. Plant material

Developing fruits were collected from peach trees growing in Veria, northern Greece, which is the main area of peach cultivation, in spring 2004, every week after anthesis until fruit maturity. Samples were taken from the varieties 'Andross' and 'Katherine', frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Cloning of PPERAG, PPERSTK, and PPERSEP genes

A peach mesocarp library (cultival name *Loring*) curated at Clemson University [19] was searched for MADS-box gene ESTs, and clones BU043610, AJ825116, BU040997, BU047980, BU043415, and BU046256, with high similarity to arabidopsis *AG*, *STK*, *SEP3*, petunia *FBP9*, and arabidopsis *SEP1/2*, respectively were obtained. In addition, a clone (BU044765) with high similarity to arabidopsis *ACTIN-2* was found (http://www.genome.clemson.edu/gdr/projects/prunus/abbott/PP_LEa/index.shtml). Clones were fully sequenced and primers were designed based on their sequence. Based on the information from the partial *PPERSTK* cDNA, we performed an improved inverse-RACE method, called Rolling Circle Amplification RACE (RCA-RACE), which allows the simultaneous isolation of the unknown 3′ and 5′ ends [28,36]. In order to obtain the internal site between the two inverse primers, we designed two additional primers, namely *PPERSTK-F3*, *PPERSTK-R3* (Table 1).

2.3. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from mesocarp, endocarp and embryo (week 5 after full anthesis), leaves, petals sepals, carpels (including ovules) and stamens of fully developed flower from both varieties using the RNeasy plant RNA isolation kit (Qiagen, Crawley, U.K.). RNA was also isolated from the pericarp of the varieties 'Andross' and 'Katherine' from 8 to 12 week after full anthesis. For the RT-PCR, 1 μg of total RNA was used for first strand cDNA synthesis. The cDNA was synthesized using 1 μg of 3′ RACE adapter Primer 5′-GCCACGCGTCGACTAGTAC(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

Fig. 3. Phylogenetic relationships of the *PPERMADS* proteins shown with white letters in grey background with other MADS-box proteins. 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. Scale indicates number of amino acid substitutions. The sequences homologous to *Prunus persica* MADS-box genes are given in Table 3. The sequence names of the proteins belonging to the PLE clade and FUL clade have been given in our previous study [35].

Table 2
Closest homologous proteins to the peach MADS-box proteins from other plant species.

MADS-box proteins (*Prunus persica*)	Proteins with high similarity from other species	Percent similarity
PPERAG	MDMADS15 (*Malus domestica*)	85%
	CMAG2 (*Castanea mollissima* Blume)	82%
	TCAG-like (*Theobroma cacao*)	81%
PPERSTK	PDMADS1 (*Prunus dulcis*)	98%
	LJAGL11 (*Lotus japonicus*)	87%
	GHMADS2 (*Gossypium hirsutum*)	85%
PPERSEP1	MDMADS8 (*Malus domestica*)	89%
	MDMADS1(*Malus domestica*)	89%
	PTMADS4 (*Populus tomentosa* Carr.)	83%
	CAGL2 (*Cucumis sativus*)	83%
PPERSEP3	VVMADS4 (*Vitis vinifera*)	89%
	NSMADS3 (*Nicotiana sylvestris*)	88%
	LEMADS5 (*Lycopersicum esculentum*)	87%
PPERFBP9	MDMADS3 (*Malus domestica*)	83%
	MDMADS7 (*Malus domestica*)	83%
	EGM3 (*Eucalyptus grandis*)	72%
	FBP9 (*Petunia hybrida*)	71%

DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). PCR primers for *AG*, *STK* and *SEP* homologous genes in peach were designed from the aforementioned ESTs found at the Clemson University's EST library, and their sequence is shown in Table 1. *PPERACTIN-2* was used as the RT-PCR control. The thermocycler program was 30 cycles of: 30 s at 94 °C; 1 min at 53 °C (or 60 °C for *PPERSEP1*); and 1 min at 72 °C, which were preceded by 2 min at 94 °C and followed by 10 min at 72 °C. The thermocycler program for *PPERACTIN-2* was 22 cycles. Fragments were of predicted lengths. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis.

2.4. Characterization of peach AGAMOUS-like gene (PPERAGAMOUS)

To extend the characterization of peach *AGAMOUS*-like genes and in order to reveal any other *AG*-like genes apart from *PPERAG*, a PCR amplification was performed with template the RCA-RACE product used in cloning of *PPERSTK* and the primer pair *PPERAG-F5end* and AUAP. The primer *PPERAG-F5end* starts with the starting codon of *PPERAG*. A ~1050 bp PCR fragment was isolated from the agarose gel and was then cloned into pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen). All clones were hybridized with the *PPERAG* probe at the same hybridization temperature like the Southern.

2.5. Protein sequence comparisons and phylogenetic analysis

The nucleotide and deduced amino acid sequences of *PPERAG-AMOUS, PPERSTK* and *PPERSEP*-like 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 deduced amino acid sequences of each set of genes were aligned using the multiple sequence alignment program ClustalW [38]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA3.1 software [22] by the Neighbor-Joining Method with p-distance correction [29].

2.6. Southern hybridization

10 μg of genomic DNA (derived from developing fruits from both varieties 4 weeks after full anthesis) was digested with EcoRI, EcoRV, DraI, XhoI, HindIII and BamHI, (TaKaRa, Otsu, Japan) and transferred to a positively charged Nylon membrane. The digoxigenin labeled gene-specific probes (the same primers were used for RT-PCR analysis) were prepared by PCR amplification using 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

Table 3
Names and accession numbers of protein sequences homologous to *P. persica* MADS-box genes.

Proteins	Organisms	Accession numbers
CUM1	*Cucumis sativus*	AAC08528
CAG3/CSAG1	*Cucumis sativus*	AAD01744
PMADS3	*Petunia hybrida*	BAB79434
NAG1	*Nicotiana tabacum* L.	AAA17033
CAMADS1	*Corylus avellana* L.	AAD03486
PTAG1	*Populus trichocarpa* Torr. & Fray ex Hook.	AAC06237
ATAGAMOUS	*Arabidopsis thaliana*	AAD41556
RAG	*Rosa rugoza*	AAD00025
MASAKOC1	*Rosa rugoza*	BAA90744
MASAKOC2	*Rosa rugoza*	BAA90745
BVMADS6	*Betula verrulosa* Ehrh.	CAB95649
MDMADS15	*Malus domestica*	CAC80858
CITMADS1	*Citrus unshiu Marcow.*	BAF34911
TCAG-like	*Theobroma cacao* L.	ABA39727
PSM7	*Pisum sativum* L.	AAX69069
NAG1	*Nicotiana tabacum*	AAA17033
TAG1	*Solanum lycopersicum* L.	AAA34197
FAR	*Antirrhinum majus*	CAB42988
SLM1	*Silene latifolia* (P. Mill.) Greuter & Burdet	CAA56655
GAG2	*Panax ginseng* L.	CAA86585
HAM45	*Helianthus annus* L.	AAO18228
CDM37	*Chrysanthemum morifolium*	AAO22984
GAGA1	*Gerbera hybrida* Bol. L.	CAA08800
GAGA2	*Gerbera hybrida* Bol.	CAA08801
HAM28	*Helianthus annuus* L.	AAO18228
AVAG1	*Asparagus virgatus*	BAD18011
SEEDSTICK	*Arabidopsis thaliana*	AAC49080
CAG1	*Cucumis sativus*	AAD01742
GHMADS2	*Gossypium hirsutum* L.	AAN15183
LJAGL11	*Lotus japonicus*	AAX13306
PDMADS1	*Prunus dulcis*	AAY30856
CUM10	*Cucumis sativus*	AAC08529
VVMADS5	*Vitis vinifera*	AAM21345
MDMADS10	*Malus domestica*	CAA04324
AVAG2	*Asparagus virgatus*	BAD83772
ATAG2	*Akebia trifoliata* (Thunb.) Koidz.	AAT46096
DTAG2	*Dendrobium thyrsiflorum* Rchb. f. ex André	AAY86365
MAMADS1	*Musa acumate*Colla	AAY53908
NTMADS4	*Nicotiana tabacum*	AAF76381
AMDEFH200	*Antirrhinum majus*	CAA64743
MDMADS1	*Malus domestica*	AAC25922
MDMADS8	*Malus domestica*	CAA04919
MAGL4	*Populus tremuloides* Michx.	AAL08423
CAGL2	*Cucumis sativus*	AAF23363
PHMADS12	*Petunia hybrida*	AAQ72498
FBP5	*Petunia hybrida*	AAK21249
LETAGL2	*Lycopersicum esculentum* Mill.	AAM33104
LEMADS1	*Lycopersicum esculentum*	AAP57412
EGM3	*Eucalyptus grandis* W. Hill ex Maid	AAC78284
MDMADS3	*Malus domestica*	AAC83170
MDMADS7	*Malus domestica*	CAA04323
MDMADS13	*Malus domestica*	AJ25116
AOM1	*Asparagus officinalis* L.	AAQ83834
SEP3	*Arabidopsis thaliana*	AAX15918
SEP2	*Arabidopsis thaliana*	AAA32733
SEP1	*Arabidopsis thaliana*	AAA32732
VVMADS2	*Vitis vinifera*	AAM21342
CDM44	*Chrysanthemum morifolium*	AAO22982
GHMADS1	*Gossypium hirsutum*	AAN15182
MDMADS4	*Malus domestica*	AAM21344
LEMADS5	*Lycopersicum esculentum*	AAP57413
FBP2	*Petunia hybrida*	AAA86854
SVSEP3	*Syringa vulgaris* L.	AAP83410
FBP23	*Petunia hybrida*	AAK21254
PHMADS3	*Petunia hybrida*	CAA51417

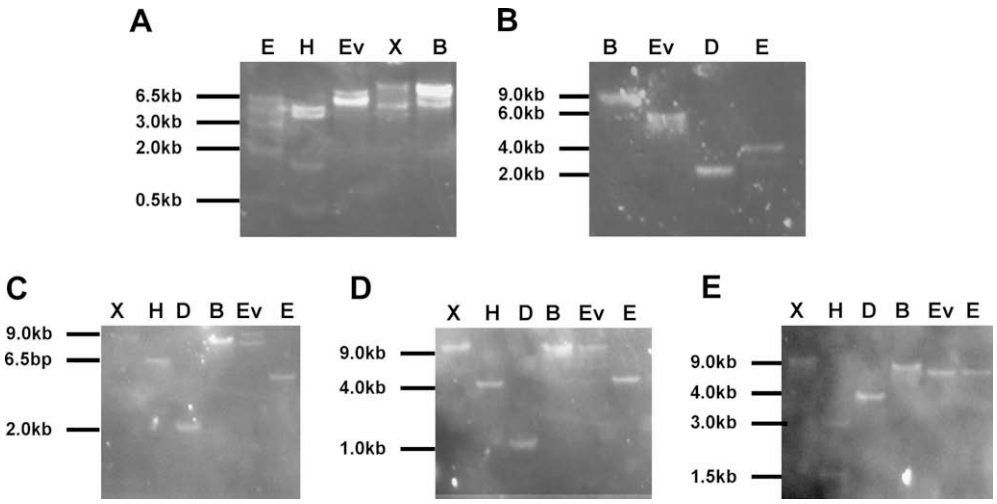


Fig. 4. Southern blot of genomic DNA from peach digested with XhoI (X), Hind III (H) DraI (D), BamHI, (B), EcoRV (Ev), EcoRI (E), probed by a *PPERAG*-specific probe (A), *PPERSTK* (B), *PPERSEP1* (C), *PPERSEP3* (D), *PPERFBP9* (E).

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 striped and re-probed twice (for all *PPERSEP*-like genes). For detection of *PPERAG* and *PPERSTK* genes a fresh membrane was used.

2.7. Lignin staining

Lignin formation in the peach endocarp was detected by staining with phloroglucinol–HCl reagent [2]. Andross and Katherine fruits were sampled at 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.

2.8. In situ hybridization

In situ hybridization experiments were performed as previously described [12]. *Prunus persica* fully developed buds were fixed in 4% (w/v) paraformaldehyde supplemented with 0.25% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) for 4 h in a vacuum aspirator. Fixed buds were block-stained in 0.5% (w/v) safranin, dehydrated through ethanol series, embedded in paraffin and cut into 8 lm-thin sections. Antisense RNA probes labeled with digoxigenin-11-rUTP (Boehringer Mannheim, Mannheim, Germany) were originated from PCR-generated templates incorporating T3 polymerase sites [13]. The primers used are presented in Table 1. Each probe was designed close to the 3′end of the genes and their length was as follows: *PPERAG* probe-214 bp, *PPERSTK* probe-206 bp, *PPERSEP3* probe-155 bp, *PPERSEP1* probe-177 bp, *PPERFBP9* probe-254 bp. Sections were prepared for hybridization according to Scheres et al. [31] and hybridized overnight at 42 °C in 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.025% (w/v) bovine serum albumin (BSA), 10% (v/v) dextran sulfate and 60 mM DTT. After hybridization the sections were treated with a solution containing 500 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl and 50 lg/ml RNase A. Finally, sections were washed several times

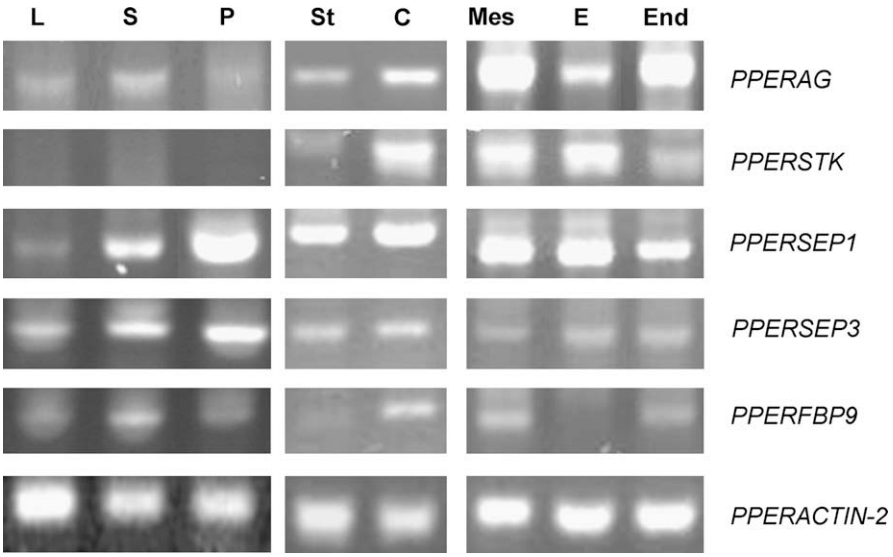


Fig. 5. Expression analysis of *PPERAG, PPERSEP1, PPERSEP3,* and *PPERFBP9* and *PPERSTK* in leaves (L), sepals (S) petals (P), pericarp (Per.), embryo (E), endocarp (End.), stamens (St.) and carpels (C) by semiquantitative RT-PCR with ACTIN-2 as the control. Agarose gels, stained with ethidium bromide, showing a 409 bp *PPERAG* fragment (A), a 603 bp *PPERSTK* fragment, (B), a 452 bp *PPERSEP1* fragment (C), a 269 bp *PPERSEP3* fragment (D), and a 306 bp *PPERFBP9* fragment (E). The 402 bp *PPERACTIN-2* fragment is shown at the bottom part of the figure.

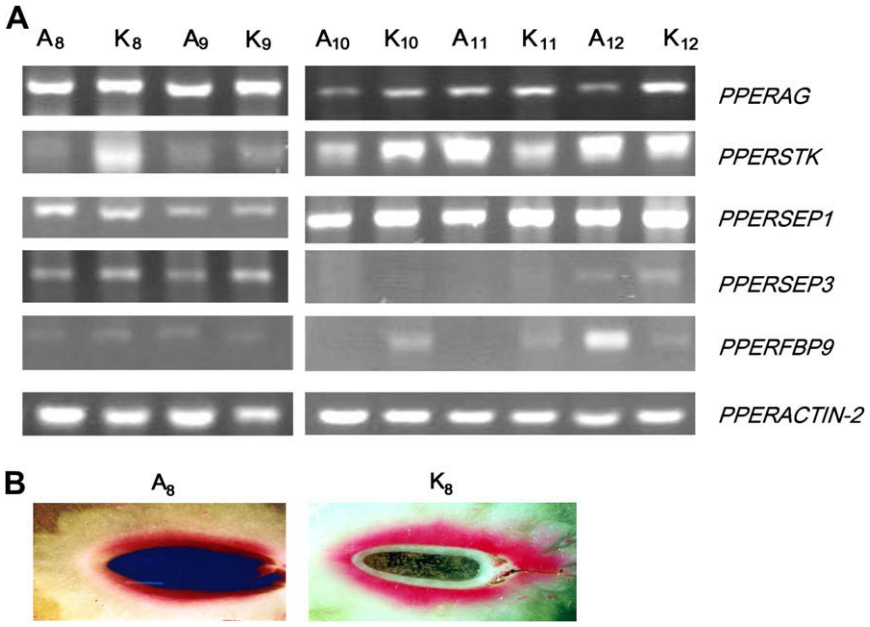


Fig. 6. A. Expression analysis in Andross (A) and Katherine (K) varieties 8–12 weeks after full anthesis. The numbers under the letters indicate the weeks after full anthesis (e.g. 10 = week 10 after full anthesis). Agarose gels, stained with ethidium bromide, showing a 409 bp *PPERAG* fragment (A), a 603 bp *PPERSTK* fragment (B), a 452 bp *PPERSEP1* fragment (C), a 269 bp *PPERSEP3* fragment (D), and a 306 bp *PPERFBP9* fragment (E). The 402 bp *PPERACTIN-2* fragment is shown at the bottom part of the figure. B. Lignin staining with phloroglucinol-HCl.

in a 2xSSC solution. Hybridization signals were visualized with anti-digoxigenin antibodies conjugated with alkaline phosphatase. Images were processed using Photoshop 6 software (Adobe Systems Inc., San Jose, CA, USA).

3. Results

3.1. Amino acid sequence comparisons and phylogenetic analysis

Complete sequencing from both ends of the EST clones selected for further analysis revealed that the sequence of *PPERAG*, *PPERSEP1*, *PPERSEP3* and *PPERFBP9* have already been deposited in GenBank (GenBank accession nos. AAU29513, ABO27622, ABO27621 and AAZ16241 respectively). *PPERSTK* was not found in Genbank and the EST was incomplete. *PPERSTK* gene was cloned by the newly developed RCA-RACE method [28]. The *PPERSTK* transcript was 1040 bp and contained a 735 bp ORF encoding for 244 amino acids. The sequence was deposited in the GenBank (GenBank accession nos. EF602037).

Similarity of the predicted amino acid sequence of PPERAG, PPERSTK, PPERSEP1, PPERSEP3, PPERFBP9, with other MADS-box proteins was examined by sequence alignment and phylogenetic analysis was performed to identify phylogenetic relationships. PPERAG fell into the euAG lineage of C-type, MADS-box proteins along with other AG-like proteins from *P. hybrida* (PMADS3), *M. domestica* (MDMADS15), and *Rosa rugoza* L. (MASAKO C1) [21]. One characteristic commonly found in C-lineage members of MADS-box genes is the presence of an N-terminal extension preceding the MADS domain [18]. 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 (Fig. 2A).

PPERSTK fell into the D-lineage/AGL11-lineage along with other D-type MADS-box proteins like *Prunus dulcis* (Mill.) D.A. Webb (PDMADS1), *M. domestica* (MDMADS10), and *Lotus japonicus* (Regel) K. Larsen (LJAGL11) [9]. The conserved C-terminus of the D lineage sequences is characterized by a fairly conserved extension (YET/AKA/DDXX) following the stop codon position of the AG motif II (Fig. 2B).

PPERSEP1 possesses two motifs conserved between members of the SEP1/2 clade, an internal motif NPTLQIGY and a C-terminal motif GFIPGWML [24]. These motifs were almost identical to those of related species such as *M. domestica*, *Cucumis sativus* L. and *Vitis vinifera* L. (Fig. 2C). PPERSEP3 shows an internal motif EPTLQIGY and a C-terminal motif YMAGWLP. These motifs were found in homologous proteins of species such as *Gossypium hirsutum*, *P. hybrida* and *Antirrhinum majus* (Fig. 2D). Finally PPERFBP9 protein harbours the motifs NSTSQIGY (internal motif) and GFIPGWML (C-terminal motif) which are identical in homologous proteins of *M. domestica* and *P. hybrida* (Fig. 2E).

Deduced amino acid sequences from all cloned peach genes were aligned with the closest matching homologous proteins from other plant species. The phylogenetic tree included the amino acid sequence of the PPERAG protein and the selected AG-like proteins and other C-type MADS-box proteins belonging to the PLENA clade using the Neighbor-Joining Method (Fig. 3). The closest homologs to the peach MADS-box proteins and the percentages of similarity with proteins of other species are presented in Table 2.

3.2. Southern hybridization

Results from restriction enzyme digestions for the number of *PPERAG* copies in peach were inconclusive (Fig. 4A) since multiple bands were observed and further experiments were performed (see below). Southern blots provided strong indication for the presence of one putative *STK*-like and *SEP3*-like ortholog in peach since for both genes a single band could be detected after digestion with each enzyme tested (Fig. 4B,D). *PPERSEP1* and *PPERFBP9* gene-specific probes hybridized with one band for all but one enzyme, since the former hybridized with two bands after EcoRV digestion,

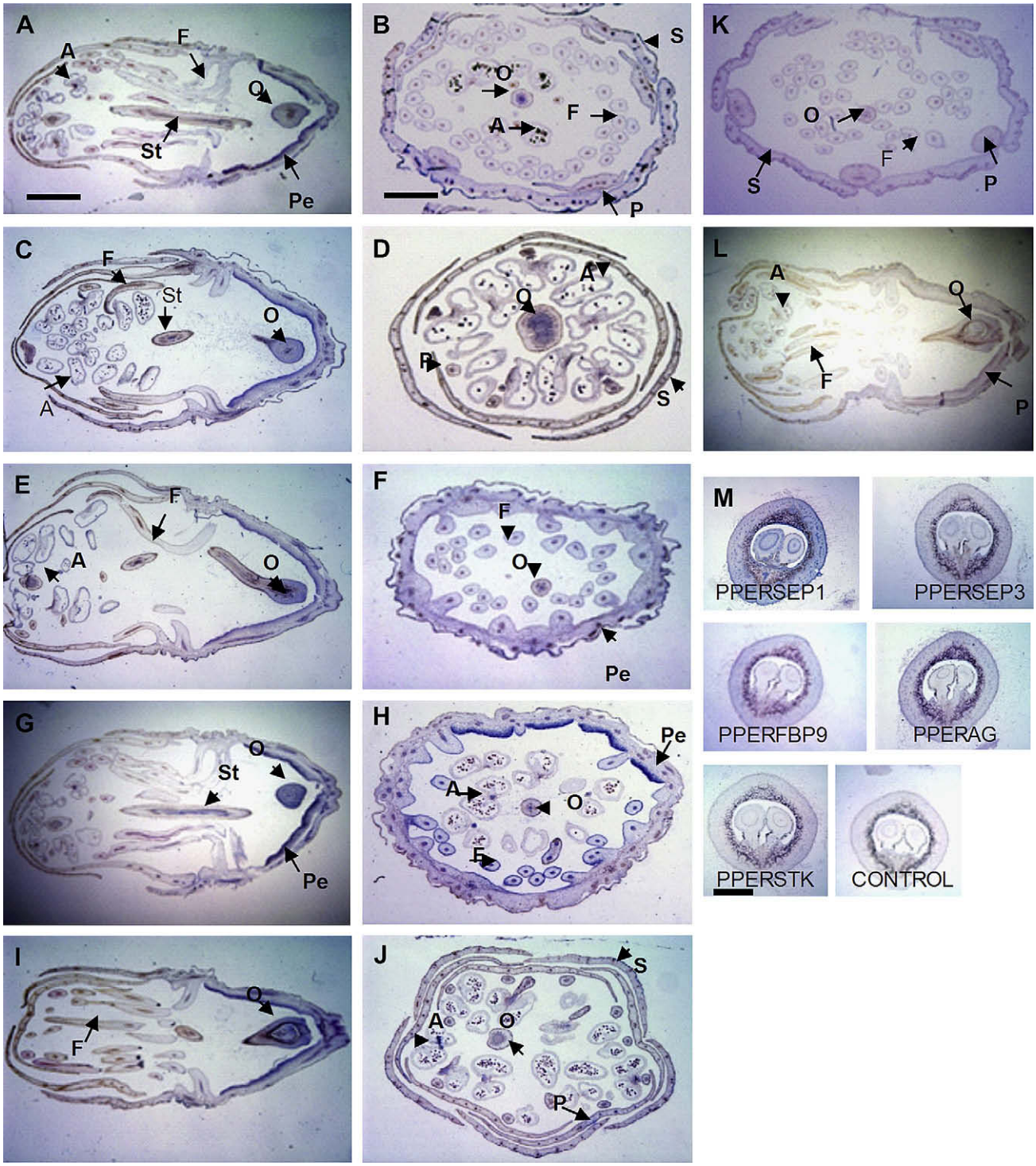


Fig. 7. Detection of the expression of *PPERAG* (A,B) *PPERSTK* (C,D), *PPERFBP9* (E,F) *PPERSEP1* (G,H), and *PPERSEP3* (I,J). Hybridization signal was visible as a blue-purple precipitate. Expression of *PPERAG* is most prominent at the ovary, style and along the filaments of the stamens (the signal is ambiguous in the anthers) (A). *PPERAG* transcription is reduced to a weak signal in the sepals whereas no signal is detected in petals (B). On the other hand, detection of the *PPERSTK* transcript is strong in the ovary (C) and almost no signal is detected in any other tissues apart from a faint signal in the perianth which is not detectable in the transverse section (D). *PPERFBP9* expression is localized at low levels in ovary and perianth (E). On the other hand *PPERSEP1* and *PPERSEP3* transcripts are visible in all flower organs (G,H,I,J) although *PPERSEP3* hybridization signal is low for the sepals and petals in the transverse section (J). Finally, detection of *PPERSEP1* transcript is very strong in all floral organs, especially in the perianth (H). A close-up in the anthers clearly demonstrated that *PPERSEP1* is the only gene strongly expressed (M). As a negative control, transverse and longitudinal sections (K,L) were hybridized to sense RNA probes transcribed from *pGEM-luc* Vector (Promega, Madison, WI, U.S.A.). In this case, no hybridization signal was visible. Size bars 2 mm (A,C,E,G,I,L), 1,6 mm (B,D,F,H,J,K) and 1,2 mm (M). A, Anthers; F, filament; O, ovary; P, petals; Pe, perianth; S, Sepals; St, style.

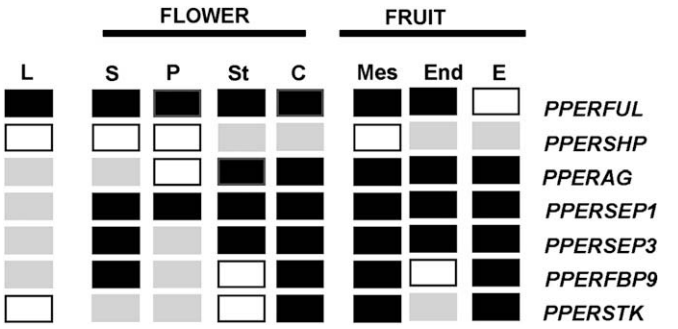


Fig. 8. mRNA expression profiles of MADS-box genes in peach leaf, flower and fruit. The plant tissues examined are: L (leaves), P (petals), S (sepals), St. (stamens), C (carpels), PER (pericarp), END (endocarp), E (embryo). Filled rectangles indicate gene expression, unfilled rectangles indicate no expression and grey rectangles indicate low level of gene expression.

and the latter hybridized with two bands after HindIII digestion (Fig. 4C,E), possibly due to presence of recognition sites of the aforementioned restriction enzymes inside an intron. However, existence of multiple copies for these genes, although unlikely, cannot be excluded.

3.3. Characterization of peach AGAMOUS-like sequences

Four bands were observed in Southern hybridization with the *PPERAG* gene-specific probe after digestion of the genomic DNA with the restriction enzymes EcoRI and HindIII and two bands with EcoRV, XhoI and BamHI (Fig. 4A). It should be mentioned that the probe was designed outside the MADS domain and that there was no recognition site for the restriction enzymes used inside the *PPERAG* probe. Southern blot analysis revealed that the *PPERAG* probe at the high stringency conditions employed did not cross-hybridize with *PPERSHP* and *PPERSTK* which share high similarity with *PPERAG* (data not shown). To obtain information regarding expressed *AG*-like sequences in peach we performed PCR amplification of RCA-RACE products using an *AG*-specific primer. A ~1050 bp band was cloned and 17 clones were sequenced. Sequence information revealed 12 single nucleotide polymorphism (SNP) differences, in addition to a 3 bp insertion within the coding region which was found in two clones. These SNPs produced 8 modifications in amino acid sequence and the 3 bp insertion produced an extra glutamine found in two clones. The clone which was found with the 3 bp insertion also had 4 SNPs, indicating that is a different *PPERAG* allele. Furthermore, five clones were found to have different 3′terminus, indicating that *PPERAG* transcripts have multiple polyadenylation sites. Some of the SNPs resulted in the production of new recognition sites (in particular new HindIIII recognition sites). These data confirm the existence of different *PPERAG* alleles, and strongly support the presence of additional *AG*-like genes in peach. We also tried to find other *AG*-like genes in databases by blast search using *PPERAG* as query. Several similar sequences were obtained but phylogenetic analysis suggested that they belong to the *SHATTERPROOF*-like lineage.

3.4. Expression analysis

Initial RT-PCR experiments were carried out on cDNAs derived from peach mesocarp, endocarp and embryo 30 days after full anthesis as well as on cDNAs from leaves, sepals, petals, carpels and stamens of a fully developed flower. All 5 genes were expressed in mesocarp, endocarp and embryo except from *PPERFBP9* which was not expressed in embryo. Results from RT-PCR experiments have shown that *PPERAG* and *PPERSTK* transcripts were mainly accumulated in carpels. *PPERSTK* mRNA was not detectable in any other tissue whereas *PPERAG* transcripts were detected in all other tissues apart from the petals. *PPERFBP9* expression was very low in leaves, sepals and petals, whereas no transcripts were detected in stamens. On the other hand *PPERSEP3* and *PPERSEP1* mRNA was detectable in all tissues examined (Fig. 5). The localization of the expression of the aforementioned genes in fully developed buds was also studied with in situ hybridization (Fig. 7A–I). Expression of *PPERAG* is most prominent at the ovary and along the stamens (Fig. 7A). *PPERAG* transcripts are detected along the sepals (Fig. 7B), whereas *PPERAG* transcription is reduced to a weak signal in the petals (Fig. 7B). On the other hand, detection of the *PPERSTK* transcript is strong in the ovary (Fig. 7D) and almost no signal is detected in any other tissues apart from a weak signal visible in the perianth (Fig. 7C). *PPERFBP9* expression resembles *PPERSTK* (a weak signal is detected in the ovary and the perianth) (Fig. 7E). On the other hand both *PPERSEP1* and *PPERSEP3* are expressed in all flower organs (Fig. 7G–J) although *PPERSEP1* expression is stronger in all organs, especially in petals, sepals and anthers (Fig. 7H,M).

Expression of *PPERSEP*-like genes, *PPERAG* and *PPERSTK* was compared in Andross and Katherine varieties in pericarp during the crucial developmental stages of pit hardening (8–12 weeks after full anthesis) (Fig. 6A). *PPERAG* and *PPERSEP1* were stably expressed in both varieties. However, *PPERFBP9* transcript was not detected in Andross while *PPERSEP3* transcript was detected neither in Andross nor in Katherine, 10 and 11 weeks after full anthesis. Moreover *PPERSTK* expression was not detected in Andross 8 weeks and in both Andross and Katherine, 9 weeks after full anthesis. Together with *PPERFUL* and *PPERSHP* mRNA expression profiles (data not shown), Fig. 8 gives a sum of mRNA expression patterns of all MADS-box genes examined in our lab so far.

3.5. Lignin staining

Lignin formation was examined during the period of initiation of pit hardening. Eight weeks after full anthesis (2 weeks after initiation of pit hardening), the endocarp of Andross fruit was not fully stained, whereas the endocarp of Katherine was clearly stained (Fig. 6B).

4. Discussion

The economic value of clingstone peach varieties depends on their resistance to split-pit. In a previous study we demonstrated that temporal regulation of a *FRUITFUL*-like (*PPERFUL*) and a *SHATTERPOOF*-like (*PPERSHP*) gene in a split-pit sensitive (Andross) and a resistant (Katherine) variety, might affect split-pit formation [36]. Here we report the characterization of five additional MADS-box genes, namely *PPERAG*, *PPERSTK*, *PPERSEP1*, *PPERSEP3* and *PPERFBP9* in order to provide a better understanding of flowering and fruit development in peach. Each gene had a unique pattern of expression in the tissues and developmental stages examined. Localization of expression with in situ hybridization was in good agreement with RT-PCR expression patterns in most cases. The minor differences among the two methods in few specific cases were likely due to difficulties in sampling where tissues could not be very well distinguished and/or differences in the stage samples were derived. For example, the RT-PCR data derived from later stages of floral development (open flower) because tissue separation can be easier, whereas in situ hybridization data are presented for earlier stages of floral development (closed buds) where all organs can be stained on the same sample. *PPERAG* and *PPERSEP1* were expressed in almost all organs and stages examined while *PPERSTK* and *PPERFBP9* appeared to have a more restricted expression profile

(Fig. 8). This may reflect the different functions each gene fulfils during floral and fruit development.

Three of the characterized genes, *PPERSEP1*, *PPEPSEP3*, and *PPERFBP9*, are single copy genes (Fig. 4) that encode putative proteins that phylogenetically belong to the SEP1/2, SEP3 and FBP9 subclades of the SEPALLATA family, respectively [24]. *PPERSEP1* was expressed at high levels in all tissues examined which is in agreement with previous data showing that all *SEP1/2* genes are expressed in inflorescences and also that these genes are expressed in the second, third and fourth whorls, and in the fruit [24]. *PPERSEP3* was expressed at low levels in all fruit tissues examined, whereas it was clearly detectable in all flower tissues, especially in the outer parts of the flower (sepals, petals) and also in leaves. *SEP3*-like genes are mostly expressed in inflorescences and the inner three floral whorls of all species examined, but expression in leaves has not been observed [24]. Thus, PPERSEP3 has a distinct expression pattern and could be essential for the development of the outer parts of peach flower and the fruit as well. *PPERFBP9* expression in all the tissues examined resembled the expression of genes belonging to the FBP9/23 subclade. Proteins of the FBP9/23 subclade, which is lost in arabidopsis, appear to function redundantly with other SEP proteins during floral development but might have other roles during transition to flowering [15]. In petunia PhFBP23 interacts with AP1/FUL proteins [16] and a similar interaction of PPERFBP9 with PPERFUL if exists, may be very important in split-pit formation given the possible role of PPERFUL in this phenomenon [36]. *SEP* genes range from developmentally redundant, as in arabidopsis, to nonredundant, with unique roles for several developmental processes in other plants [24]. Expression patterns of the peach *SEP*-like genes where coincide may indicate functional redundancy while when differ may indicate unique and specific roles that remain to be examined.

The *PPERAG* gene characterized in this report is homologous to arabidopsis *AG* and to snapdragon *FARINELLI* (*FAR*). Our expression data is in accordance with expression analysis of Tadiello et al. [35] and demonstrated that *PPERAG* expression is strong in both stamen and carpel tissues similarly to *AG* [3]. Furthermore we have detected it although at low levels in sepals and in leaves. Apple *AG*-like (*MdMADS15*) is also expressed in sepals suggesting that it may be essential for further development of sepal tissue into fruit flesh [40]. Transcripts of poplar *PTAG1* and *PTAG2* were detected in vegetative tissues as well as in stamens and carpels of developing flowers [4]. Accumulation of citrus *CitMADS1* mRNA was also detected in all flower organs [10]. Although there are species containing two AG homologs that in some cases exhibit diversification in function and/or expression [25,39] in most woody plants examined such as apple and citrus, a single copy of *AG*-like gene was found. However, peach may contain more than 1 *AG*-like gene since Southern hybridization revealed multiple bands and cDNA cloning resulted in the isolation of two clones different for an in-frame insertion at the coding region. Whether peach has two *AG* genes as Tadiello et al. [35] mentioned is still under question and could be clarified only after the analysis of syntenic loci conservation between arabidopsis and peach.

In model species D-class genes (*STK* of arabidopsis and *FPB11* of petunia) show specific expression in carpels–ovules whereas the phylogenetically related C-class genes are also expressed in stamens. *STK* is required for ovule development and the normal development of the funiculus, the cord-like structure in arabidopsis that connects the developing seed to the fruit [30]. *PPERSTK* was expressed in carpel-ovary, embryo and the fruit tissues pericarp and endocarp, but not in stamens (although a faint signal was detected in anthers) (Fig. 7). This pattern of expression closely resembled D-class gene expression and might indicate that *PPERSTK* has a role in ovule development and also at later stages in embryo and fruit development of peach. Similar results have been obtained in *D. thyrsiflorum* by in situ localization experiments revealing high expression of the *AG* ortholog *DthyrAG1* at the initial stage of placenta- and ovule development, while the D-type gene *DthyrAG2*, like *PPERSTK* in peach, is expressed in the ovary and throughout ovule development [33], suggesting a role in ovule development as could be the case in peach as well. Interestingly, *PPERSTK* transcription was detected at low levels in the perianth as well, and could be an ancestral pattern of expression that is no longer needed today. *PPERSTK* occurs as a single copy gene, like *AVAG2* a putative D-class gene from an ornamental asparagus (*Asparagus virgatus* Baker) [41], and *EScaAGL11* from California poppy (*Eschscholzia californica* Cham.) [5]. The overlapping functions of C/D-type genes in core eudicots are reviewed in [30].

Finally, the differential mRNA accumulation of the five MADS-box genes during pit hardening (8–12 weeks after full anthesis) suggests they may play roles in other aspects of peach fruit development since several studies in other plants demonstrate their homolog interactions with *FUL/SHP* genes [11,32]. Previous results showed differential temporal regulation of PPERFUL and PPERSHP that might affect split-pit formation [36]. Interestingly, different extend of lignin formation was observed at the same stage of the beginning of pit hardening in the two peach varieties that differ also in split-pit formation (Fig. 6B). A synergistic effect of MADS-box gene regulation of fruit development and lignification could be responsible for split-pit formation in the sensitive variety but this should be further examined.

In summary, we have presented here five new members of the MADS-box gene family from *P. persica*. Sequence similarities, phylogenetic analysis and structural characteristics were used to provide classification in type C (*PPERAG*), type D (*PPERSTK*) and type E (*PPERSEP1, PPERSEP3, PPERFB9*) organ identity genes. Expression patterns were determined and combined with phylogenetic data was used to predict function of these genes. However, because of the presence of paralogues, functional conservation may be difficult to determine and expression does not always correlate with function. Thus, further experiments are planned to determine putative functional roles of the C-, D-, and E-type genes mainly in peach fruit development.

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