Running header: Perianth evolution in *Persea*

Genetic footprints of stamen ancestors guide perianth evolution in *Persea* (Lauraceae)

Andre S. Chanderbali[1]*, Sangtae Kim[1], Matyas Buzgo[1], Patrick Zheng[1], David Oppenheimer[1], Douglas E. Soltis[1], and Pamela S. Soltis[2].

[1]Department of Botany, University of Florida, Gainesville, FL 32611, USA

[2]Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

* For correspondence (fax+1 352-846-2154); e-mail achander@botany.ufl.edu

Abstract

The perianth of *Persea americana*, like that of most Lauraceae, consists of two whorls of morphologically similar petaloid organs, termed tepals. In closely related *Persea borbonia* however, a sepaloid outer whorl of tepals contributes to a dimorphic perianth. To determine whether *Persea* homologues of the A-, B-, C-, and E-class MADS-box genes that determine organ identity in eudicot flowers have played a role in shaping this dimorphic perianth, their expression levels were assessed across the floral whorls of *P. americana* and *P. borbonia*. We find that homologues of A- and B-function genes from *Persea* are expressed at comparable levels in the perianth whorls of the two species, but homologues of *AG* (C-function) and *SEP3* (E-function) have shifted away from sepaloid tepals of *Persea borbonia*. Genetic studies suggest a conserved role for *AG* homologues in specifying stamen and carpel identity in *Persea*, but the coincidence of phenotypic and gene expression shifts argues for a hitherto unprecedented role in perianth development. Phylogenetic conservation of *SEP3-AG* interaction in floral organ identity transcription factor quartets may underlie the coincident shift of *Persea AG* and *SEP3* gene expression. Based on our results, we hypothesize: 1) A role for Persea *AG* and *SEP3* homologues in shaping perianth morphology is indicated by the coincident shift to a dimorphic perianth with loss of their expression in the outer tepals of *Persea borbonia*. 2) Reproductive organ identity in *Persea* is specified through the action of three *AG* homologues. 3) The expression of floral organ identity homologues in the tepals of *Persea*, and perhaps other Lauraceae*,* is suggestive of staminal origin, i.e., andropetals.

Keywords: ABC model, andropetals, tepals, perianth evolution, *Persea*.

Introduction

The ABC model of flower development (Bowman et al., 1991; Coen and Meyerowitz 1991) states that the identities of the four types of floral organs are determined through the action of three classes of homeotic genes. Classified according to function, the A-, B-, and C-function genes act in successive domains in the developing flower, such that sepals form under the influence of A-function genes alone, petals form where A- and B-function gene activities overlap, stamens are produced where B- and C-function genes are active, and carpels are produced under the influence of C- function genes alone. In the model plant, *Arabidopsis*, A-function genes are *APETALA1* (*API*) and *APETALA2* (*AP2*) (Mandel et al., 1992; Bowman et al., 1993), *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) provide B-function (Goto and Meyerowitz, 1994; Jack et al., 1994), and *AGAMOUS* (*AG*) provides C-function (Yanofsky et al., 1990). Genes that specify ovule identity, (*AGAMOUS-LIKE11* (*AGL11*) (Colombo et al., 1995), and contribute to the specification of floral organ identity (*SEPALLATA1* (*SEP1*), *-2*, *-3*, and *-4*, in *Arabidopsis* (Flanagan and Ma, 1994; Pelaz et al., 2000; Theissen, 2001; Ditta et al., 2004), respectively add D-function and E-function to the original ABC model. With the exception of *AP2,* all of these organ identity genes are members of the MADS-box family (reviewed in Ma and dePamphilis, 2000).

The ABCDE model was elucidated primarily through demonstration that loss of genes in these functional classes results in homeotic conversion of floral organs. For example, sepals replace petals and carpels replace stamens in B-function mutants, while C-

function mutants exhibit homeotic conversion of stamens into petals (Bowman et al., 1991; Coen and Meyerowitz 1991). That such dramatic changes in floral structure can be elicited by disrupting the expression of floral genes has led to the hypothesis that evolutionary shifts in floral architecture might involve shifts in the expression domain of these genes, or the "shifting boundary" hypothesis (van Tunen et al., 1993; Bowman, 1997; Albert et al., 1998; Kramer et al., 2003). Conceivably, a perianth comprised of sepals and petals can become entirely sepaloid or petaloid depending on the direction of shift in B-function gene activity. Conversely, an entirely sepaloid or petaloid perianth might differentiate into sepals and petals through outward or inward shifts in B-function gene activity, respectively. Further, stamens can contribute petals to the perianth through an inward shift of the A-C boundary. The E-function genes that interact redundantly with A-, B- and C-function genes to specify sepals, petals, stamens, and carpels (Goto et al., 2001; Theissen, 2001), would conceivable shift their expression domain in concert with their B- and C-function partners.

Although A-function has yet to be demonstrated outside of *Arabidopsis*, evidence for conservation of B-function in angiosperms (e.g., Coen and Meyerowitz, 1991; Sommer et al., 1991; Tröbner, et al., 1992; Schmidt and Ambrose, 1998; Tsuchimoto, et al., 2000; Whipple et al., 2004), and C-function in angiosperms (Bradley et al., 1993; Kempin et al., 1993; Pnueli et al., 1994; Kang et al., 1998; Kater et al., 1998; Yu et al., 1999; Kyozuka and Shimamoto, 2002) and gymnosperms (Rutledge, 1998; Tandre et al., 1998; Winter et al., 1999; Zhang et al., 2004), suggests that core aspects of the ABCDE model have been available for manipulation throughout floral evolution. Few studies, however, have demonstrated that changes in gene expression have led to evolutionary shifts in floral architecture. An outward expansion of B-function gene activity is consistent with the entirely petaloid perianth of *Tulipa* (van Tunen et al., 1993; Kanno et al., 2003), and

many Ranunculaceae (Kramer et al., 2003). However, the broad expression of B-function homologues observed in *Amborella*, *Nuphar*, *Illicium*, and *Magnolia*, suggests that the pattern in *Tulipa* and *Ranunculaceae* might be ancestral, rather than derived (Kim et al., 2005). Accordingly, as articulated also by Albert et al. (1998), a dimorphic perianth of distinct sepal and petal whorls might have evolved through an inward shift of B-function gene activity in core eudicots.

The genus *Persea* (Lauraceae) provides an opportunity to assess whether shifts in gene expression patterns have played a role in shaping a dimorphic perianth outside of the eudicots. The Lauraceae are members of the magnoliids, the largest of the basal angiosperm lineages (Soltis et al., 1999; 2000), and among the oldest known flowering plants (Drinnan et al., 1990; Eklund and Kvacek, 1998). Lauraceae flowers are trimerous with two perianth whorls, three whorls of stamens, a fourth inner whorl of staminodes often present, surrounding a single carpel (Rohwer, 1993). As in *Tulipa*, many Ranunculaceae, and many basal angiosperms, the perianth typically consists of almost identical petaloid organs, termed tepals (Endress, 2001). Many members of the genus *Persea*, however, produce a dimorphic perianth with smaller sepaloid tepals outside petaloid inner tepals. A molecular phylogeny for the Lauraceae (Chanderbali et al., 2001), suggests that perianth dimorphism is derived in *Persea*, and may have evolved independently in several genera in the Lauraceae. To determine whether expression shifts of A-, B-, C-, and E-function genes accompany this morphological transition, we have conducted a comparative investigation of their expression patterns in flowers of two species of *Persea*; *P. americana*, with an undifferentiated perianth, and *P. borbonia*, with a dimorphic perianth. Our results provide the basis for hypotheses about gene function that can be tested through genetic transformation of *Persea americana* (Cruz-Hernandez et al., 1998; Litz et al., 2005).

Materials and Methods

Plant materials

Samples of *Persea americana* and *Persea borbonia* were collected from individuals cultivated on the campus of the University of Florida (Chanderbali *xxx* and *xxx* respectively, FLAS).

RNA extraction, RT-PCR, sequence determination

To isolate and characterize the *Persea* homologoues of A-, B-, C-, and E-function genes, we BLAST searched an EST dataset generated from a *Persea americana* cDNA library by the Floral Genome Project (Soltis et al., 2002; Albert et al., 2005), and completed the sequences from the library clones. For the genes thus identified in *P. americana*, gene specific primers were designed to obtain the homologs from *P. borbonia*. Additional genes were sought using the MADS specific degenerate primer and a polyT primer on floral cDNA as outlined by Kramer et al. (1998), and Kim et al. (2004).

RNA extractions were performed by a modified method of the RNeasy Plant mini Kit (Qiagen, Stanford, CA), as suggested for basal angiosperms (Kim et al., 2004). The modification includes a part of CTAB DNA extraction protocol (Doyle and Doyle, 1987) and subsequent use of the RNeasy Plant Mini Kit. This Reverse transcription was performed following the manufacturer's directions using SuperScript II RNaseH-reverse transcriptase (Invitrogen, Carlsbad, CA) and polyT primer (5'-CCG GAT CCT CTA G*AG* CGGCCG C(T)17-3'). PCR reactions were performed using a MADS gene-specific degenerate primer (5'-GGGGTA CCA AYM GIC ARG TIA CIT AYT CIA *AG*M GIM G-3')

and the polyT primer used in reverse transcription (Kramer et al., 1998). PCR conditions were those employed by Kramer et al. (1998). PCR bands over 800 bp in size were excised from the agarose gel and purified using the Geneclean II Kit (Q·Bio Gene, Carlsbad, CA). Purified DNAs were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNAs were purified from cloned cells using the FastPlasmid Mini Kit (Eppendorf, Westbury, NY). Cycle sequencing reactions were performed using the CEQ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA), and cDNA sequences were determined using a CEQ 8000 sequencing system (Beckman Coulter, Fullerton, CA).

Characterization and identification of genes

Once gene sequences were obtained, we determined gene identity using a BLAST approach followed by a phylogenetic analysis. To verify the subfamily identities of newly isolated genes from the taxa under investigation, and to address their orthology to previously reported genes, we added our MADS-box sequences to a large published data set representing all subfamilies of MIKC^{C}-type MADS genes (Becker and Theissen, 2003), and complemented with pivotal basal angiosperm sequences (Kim et al., 2005). Amino acid alignment was conducted using CLUSTAL X (ver. 1.83; Thompson et al., 1997). The maximum parsimony (MP) analysis was performed for the amino acid data set using PAUP* 4.0b10 (Swofford, 2001). The search strategy involved 100 random addition replicates with TBR branch swapping, saving all optimal trees. To assess support for each node, a bootstrap analysis (Felsenstein, 1995) was performed using 100 replicate heuristic searches each with 10 random taxon addition sequences and TBR branch swapping, saving all optimal trees.

R-Q-RT PCR studies

Expression of homologs of A-, B-, C-, and E-function genes in floral organs of *P. americana* and *P. borbonia* were assessed through R-Q-RT PCR using total RNA from dissected floral organs as described by Kim et al. (2004). Four sets of floral organs, outer tepal, inner tepal, stamen and carpel, were dissected from mature flower buds. Young leaves were also sampled to represent non-floral tissue.

Total RNAs were extracted from each sample using the RNeasy Plant Mini Kit (Qiagen, Stanford, CA). After the RNA extraction, we treated samples with DNase to avoid potential contamination of genomic DNA (DNase-free kit from Ambion, Austin, TX). Reverse transcription using RNA from each floral part was performed following the manufacturer's directions using SuperScript TM II RnaseH reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was primed with a random-hexamer instead of polyT primer because the 18S ribosomal RNA gene was used as an internal control. R-Q-RT PCR methods used followed those of Kim et al. (2005). We performed multiplex PCR using a gene-specific primer pair (Table 1), 18S rDNA primer pair (internal control), and competitive prime pair of 18S rDNA primers (competimers) following the protocol of QuantumRNA (Ambion, Austin, TX). The 18S rRNA gene was used for the internal control in each reaction. Because the PCR signal of the 18S rRNA gene is higher than that of the specific genes we are studying, the "competimers" of the 18S primer pair included in the Quantum RNA kit were used to reduce the 18S PCR signal. The optimal ratio of the 18S primer pair to competimers was tested for each gene to obtain a similar level of PCR signal between the 18S gene and that of the specific gene. Optimal ratio ranged from 3:7 to 6:4 in all genes that we examined. PCR reactions

for all genes were performed with 27 cycles at 95C (30s), 55C (30s), and 72C (30s) using an Eppendorf Mastercycler (Brinkmann, Westbury, NY). A range of 1-256ng of total RNA (reversely calculated from the amount of cDNA used in the PCR reaction after the RT PCR) was tested, and 16-64ng of total RNA was found to generate unsaturated PCR product accumulation for each gene through 27 cycles of PCR. We used 25ng of total RNA for the R-Q-RT PCR. Twenty microliters from each PCR reaction were fractionated in a 2% (w/v) agarose gel containing 10-4 (w/v) ethidium bromide in tris-acetate EDTA buffer. Gel images were analyzed using KODARK 1D Image Analysis Software (Kodak, Rochester, NY). The gene specificity of each PCR product was confirmed by sequencing all PCR products.

In situ *hybridization*

Based on the results of our R-Q RT PCR experiments, the expression patterns of the three *Persea AG* homologues were studied further through in situ hybridization. Floral buds of various developmental stages were vacuum infiltrated in ice cold 4% PFA (90 ml ddH_2O, 4 g paraformaldehyde, 5 drops 10M NaOH, heated to 65˚C with occasional stirring until paraformaldehyde is dissolved, cooled to room temperature and pH adjusted to 7.0 with H_2SO_4, 10 ml 10 X PBS), for 10 mins, after which the fixative was replaced and tissues left to fix at 4^{o}C overnight. Fixed material was dehydrated, cleared, and embedded in wax (Paraplas Plus) as described by Jackson et al (1991). Sections of 8-10 m were cut, mounted on ProbeOn-Plus slides (Fischer Scientific) and dried overnight at 42^{o}C.

Templates for reverse transcribed gene specific cRNA probes of *Persea americana* and *P. borbonia AG* homologues genes were generated by PCR amplification of the C terminus with the primers used for R-Q RT PCR (Table 1) except for the addition of an SP6 promotor (5' GATTT*AG*GTGACACTATA) to the 5' end of the forward primer and a T7 promoter (5' TAATACGACTCACTAT*AG*GG) to the 5' end of the reverse primer. Sense and antisense riboprobes were generated using the DIG RNA labeling Kit (SP6/T7) from Roche. Probe hybridization, post-hybridization washing and immuno-localization were performed as described previously (Jackson, 1991). Colormetric detection was done with NBT/BCIP staining for 1-2 days and slides photographed using a xxxxx camera with bright field illumination.

Floral secretions

To characterize the role of sepaloid and petaloid tepals in the pollination biology of *Persea borbonia* we followed the method of Vogel (1990) and Dafni (1992) to localize osmophores, or secretory tissue. Freshly picked flowers from morning (9:00 am) and afternoon (3:00 pm) hours were immersed in 1% Neutral Red for 30 minutes and rinsed with distilled water to remove excess dye. Tissues with secretory activity accumulate Neutral Red. Photographs were taken with a XXX camera.

Results

Persea homologues of A-, B-, C- and E-function genes.

From *Persea* we report the following homologues of Arabidopsis A-, B-, C- and E-function genes: *Pe.am.API*, *Pe.bo.API*, *Pe.am.AP3*, *Pe.bo.AP3*, *Pe.am.PI.1*, *Pe.am.PI.2*, *Pe.bo.PI.1*, *Pe.bo.PI.2*, *Pe.am.AG.1*, *Pe.bo.AG.1*, *Pe.am.AG.2*, *Pe.bo.AG.2*, *Pe.am.AG.3*, *Pe.bo.AG.3*, *Pe.bo.SEP3.1*, *Pe.bo.SEP3.2*. Two genes, *Pe.am.SEP3.1*, *Pe.am.SEP3.2*, were previously published (Zhan et al., 2005). Gene annotation is based on our phylogenetic analysis with a representative sampling of MADS-box genes showing their placement in respective sub-familial clades with >70% bootstrap support (Fig. 1). Accordingly, in these two *Persea* species, we have identified one homologue of *API* (A-function), two of *PI* (B-function), one of *AP3* (B-function), three of *AG* (C-function), and two of *SEP3* (E-function).

R-Q RT PCR gene expression

To determine organ-level expression patterns of *Persea* homologoues of A-, B-, C-, and E-function genes we used relative-quantitative RT PCR (R-Q RT PCR) with gene specific primers on outer tepal, inner tepal, stamen (including fourth whorl staminodes), carpel, and leaf cDNA from the two *Persea* species. Our results, as illustrated by representative gel photographs (Fig. 2), indicate that these genes show differential expression among floral organs and between flowers of the two species of *Persea*. In both species of *Persea*, homologues of the A- function gene (A*PI*) are expressed in both perianth whorls and stamens but not detected in carpels. Homologues of the B-function genes (*AP3* and *PI*) are strongly expressed in both perianth whorls and stamens, and one of two paralogous *PI* genes was also weakly detected in carpels. *Persea* homologues of C- and E-function genes show differential expression across the perianth of the two species. In *Persea americana*, with an undifferentiated perianth, two of the

three *AG* homologues (*Pe.am.AG.1* and *Pe.am.AG.2*) are expressed in both perianth whorls as well as stamens and carpels, while the third (*Pe.am.AG.3*) is restricted to stamens and carpels. In *Persea borbonia*, with a dimorphic perianth, *Pe.bo.AG.3*, the orthologue of *Pe.am.AG.3*, is also expressed in stamen and carpels only, but *Pe.bo.AG.1* and *Pe.bo.AG.2* (orthologues of *Pe.am.AG.1* and *Pe.am.AG.2*) show differential expression across the perianth whorls. Both are expressed in the inner petaloid tepals but lacking in the outer sepaloid tepals. Duplicate *Persea SEP3* homologues are similarly absent in the sepaloid tepals of *P. borbonia*, but strongly expressed in petaloid tepals as well as stamens and carpels of the two species.

In situ *Hybridization*

Cellular level expression of *Persea AG* homologues was explored with *in situ* detection in floral buds to better characterize the apparent involvement in tepal development. In both *P. americana* and *P. borbonia*, all three *AG* homologues were detected throughout the floral meristem as well as inner and outer tepal primordia (Fig. 3). *In situ* hybridization was further used to trace spatio-temporal aspects of expression in *P. borbonia* flowers at stages leading to anthesis (Fig. 4). Our findings suggest that the early expression of the three *AG* homologues is maintained in tepals, stamens, and carpels, but by the completion of stamen and carpel differentiation (Stage 9; Buzgo et al., 2004; Buzgo et al., in prep.), neither could be detected in the outer tepals of *P. borbonia*.

Floral secretions

Lauraceae flowers are heterodichogamous (Stout, 1927; Skutch, 1945; Kubitzki and Kurz, 1984; see also Renner 2001) with temporally separate male and female phases. The neutral red staining reveals temporal changes in a protogynous morph. In flowers that were picked the morning, stigma, staminodes, and the margins of petaloid tepals are secretory (Fig. 5 A), while afternoon flowers in the male phase show secretory activity in all anthers, staminal glands of third whorl stamens, and basal glandular fields in sepaloid and petaloid tepals (Fig. 5 B and C).

Discussion

Shifting boundaries and the ABCEs of floral evolution in Persea

Studies have shown that organ identity genes are often expressed in limited domains and/or only during certain stages of flower development, indicating that the spatial and temporal control of gene expression is crucial for the development of floral organs (Yanofsky et al., 1990; Jack et al., 1992; Mizukami and Ma, 1992; Weigel and Meyerowitz, 1993; Goto and Meyerowitz, 1994; Gustafson-Brown et al., 1994; Kempin et al., 1995; Mandel and Yanofsky, 1995; Flanagan et al., 1996; Krizek and Meyerowitz, 1996; Ma and dePamphilis, 2000). Our comparative study of floral developmental genes in *Persea* uncovers spatial and temporal shifts in gene expression that coincide with an evolutionary change in perianth morphology.

The broad expression of *Persea* homologues of *API*, *Pe.am.API* and *Pe.bo.API*, differs from that of *API* in *Arabidopsis* where expression is limited to perianth organs at later stages of development (Mandel et al., 1992a, Bowman et al., 1993) and supports the

widely held view that *API* function is not phylogenetically conserved (Theissen et al., 2000; Irish, 2003; Litt and Irish 2003; Kim et al., 2005). A duplication event at the base of the core eudicots producing paralogous euAPI and euFUL lineages (Litt and Irish, 2003), followed by motif changes in the C terminus of euAPI homologues might underlie the A-function of *API* (Yalofsky et al., 2000). The C-terminus of the *Persea API* homologues, like those of other non-core eudicots, resembles that of *FUL* (Litt and Irish 2003), which plays a role in fruit and leaf development in *Arabidopsis* (Gu et al., 1998). The absence of *Persea API* in carpels departs, however, from the *FUL*-like expression pattern reported for homologues in *Nuphar* and two other magnoliids, *Eupomatia* and *Magnolia* (Kim et al., 2005). It remains possible that a paralogous copy of *Persea API*, not detected in this study, fulfills this role in carpels.

Persea homologues of B-function genes are strongly expressed in perianth organs and stamens, demonstrating phylogenetic conservation of expression, and presumably function, between *Persea* and eudicot models. Low level expression of *Persea PI* homologues, *Pe.am.PI.1* and *Pe.bo.PI.1*, in developing carpels is also not unlike *PI* in *Arabidopsis* (Goto and Meyerowitz, 1994). Their presence in both sepaloid and petaloid tepals however, is a surprising outcome of our study, but closer inspection reveals that morphological traits that might be attributable to B-function gene regulation are present in both tepal types. Neutral red staining of fresh *P. borbonia* flowers localized secretory tissue along tepal margins and, more conspicuously, in paired fields flanking the basal part of the tepals (Fig. 5). It is not known what these glands secret, but numerous volatiles are produced by *Laurus nobilis* flowers (Flamini et al., 2003), another Lauracae. Since pollinator attraction and/or reward are important roles for petals (Endress, 1994), the expression of B-function homologues in *Persea* tepals with secretory glands is consistent with morphology.

The C-function of *AG* homologues is considered to be evolutionarily conserved in seed plants, specifying reproductive organ identity in diverse angiosperms (Mandel et al., 1992b; Kempin et al., 1993; Kang et al., 1998; Kyozuka and Shimamoto, 2001) and gymnosperms (Rutledge, 1998; Tandre et al., 1998; Winter et al, 1999; Zhang et al., 2004). Duplications have occurred in numerous angiosperm lineages, sometimes leading to subfunctionalization and neofunctionalization, but obvious selective advantages have ensured that the role of specifying reproductive organ identity be preserved in at least one descendant gene lineage (Kramer et al., 2004; Zahn et al., in press). The expression of *Persea AG* homologues in developing floral organs is reconcilable with functional conservation when details of the spatio-temporal pattern of gene expression are taken into consideration. In situ hybridization detection of mRNA levels at early floral stages indicate that all three *Persea AG* homologues are expressed throughout the floral meristem and remain present in all developing organs until anther differentiation in the stamens. *Persea AG.3* expression in developing tepals clearly declines with floral maturity since R-Q RT PCR methods on mature floral organs detected expression only in stamens and carpels. The differing perianth morphologies of *P. americana* and *P. borbonia* are also attributable to spatio-temporal restriction of gene expression during later stages of floral development, but now of *Persea AG.1* and *AG.2* genes. Maintained expression of both the genes is evident in the undifferentiated perianth of *P. americana*, while interruption of expression in the outer tepals of *P. borbonia*, by the onset of anther differentiation in stamens, results in sepaloid morphology and a dimorphic perianth.

The inward shift of *Persea SEP3* genes in the dimorphic perianth is also significant. SEP genes, SEP1, 2, 3 and 4, are not sufficient to specify organ identify but act together with

the A-, B-, and C -function genes in quartet transcription factor complexes to specify sepal, petal, stamen, and carpel identity (Pelaz et al., 2001; Honma and Goto, 2001; Theissen, 2001; Ditta et al., 2004). Of particular significance is that, in *Arabidopsis*, the BC component of these floral quartets is dependent on *SEP3* providing a scaffold between *AG* and *AP3-PI* (Honma and Goto, 2001; Goto et al., 2001). Thus, *AG* requires interaction with *SEP3* in order to contribute to the floral quartets. Our observation that *Persea SEP3* and *AG* genes are co-expressed and shift in concert with changes in floral morphology, suggests that *AG-SEP3* interaction is phylogenetically conserved, and ancient, originating prior to the separation of magnoliids and eudicots early in angiosperm evolution.

Discussions of perianth evolution have focused on shifts in B-function gene expression producing variant perianth morphologies (van Tunen et al., 1993; Bowman, 1997; Albert et al., 1998; Kanno et al., 2003; Kramer et al., 2003). Our results suggest that, in *Persea*, a dimorphic perianth evolved through an inward shift in the expression domain of C- and E-function homologues. The coincidence of phenotypic change with gene expression argues for a hitherto unprecedented role for *AG* homologues in perianth development, and provides the basis for hypotheses of how floral developmental regulators might be interacting to specify floral organ identity in *Persea* flowers (Fig. 6). Stamens are expected to be specified through the combined action of three B-function homologues, two E-function homologues, and three C-function homologues. Tepals appear to be initiated under the influence of the same set of genes that act to specify stamen identity, but subsequent spatio-temporal restriction of *Persea AG* homologues decides their tepal-fate. In both species, restriction of *Persea AG.3* genes to reproductive organs results in petaloid tepals, whereas, as observed in *P. borbonia*, repression of all three *AG* homologues early in outer tepal development results in sepaloid morphology. It is

conceivable, therefore, that the genetic basis of A-function in *Persea* would include a repressive influence on *Persea AG.3* expression. Carpel identity in *Persea* appears to be specified by the full complement of *AG* and *SEP3* homologues.

On the origin of perianth in Persea

The classical view of angiosperm flower evolution holds that stamens and carpels (the reproductive structures) evolved just once, whereas the sterile perianth organs have had multiple origins (e.g., Eames, 1961; Tahktajan, 1991). This line of thinking is based on the idea that angiosperms are derived from apetalous ancestors and the perianth is an evolutionary novelty. The resemblance of sepals to foliar bracts and petals with stamens has encouraged the view that sepals are evolutionarily derived from foliar bracts and petals from stamens. Such stamen-derived petals, called andropetals (Takhtajan 1991), are primarily associated with eudicots and monocots. The perianth of basal angiosperms typically consists of morphologically similar organs, termed tepals (Endress 2001), which could be assigned bracteal (bracteopetal) or staminal (andropetal) origins depending on whether sepal-like or petal-like features prevail. By these criteria, the tepals of Lauraceae have been considered bracteopetalous (Albert et al., 1998; de Craene et al., 2003). The “sliding boundary” corollary of the ABC model offers a molecular genetic mechanism for the evolution of the angiosperm perianth. Conceivably, the acquisition of a perianth around reproductive organs could be accomplished through expansion of the floral genes to pre-existing sterile appendages, effectively recruiting these organs into the floral plan. This genetic scenario would be in line with the bacteopetal hypothesis. Alternatively, reduction of C-function gene activity in outer stamen whorls would herald the origin of andropetals (Bowman, 1997; Albert et al., 1998; Baum and Whitlock, 1999).

The expression of *AG* homologues we observe in *Persea* tepals might be genetic footprints of a stamen ancestor. Alternatively, we might consider whether C-function resides only with *Persea AG.3* genes, with *AG.1* and *AG.2* adopting B-function. However, this scenario is inconsistent with strong *AG.1* and *AG.2* expression in stamens and carpels, as well as early *AG.3* expression in tepal primordia. The spatio-temporal patterns of gene expression are more readily reconciled with subfunctionalization. The influence of all three *AG* paralogues appears to be required to elicit reproductive organ identity, and in the absence of one of the trio, namely the *AG.3* paralogue, organ development shifts to produce petaloid tepals. Thus, *Persea AG.1* and *AG.2* genes are not sufficient to confer reproductive organ identity but the development of sepaloid tepals in their absence argues for a role in the development of petaloid tepals. The later role could be considered a form of neofunctionalization, but it should be noted that *AG* mRNA is absent from sporogenous tissues but accumulates in sterile tissue types late in *Arabidopsis* stamen and carpel development (Bowman et al., 1991). If *Persea* tepals are sterile stamens, or staminodes, the role of *Persea AG.1* and *AG.2* in petaloid tepals might be equivalent to the late role of *AG* in *Arabidopsis*.

Mutant phenotypes encountered during the course of this study potentially provide additional insight into the origin of *Persea* tepals. Occasionally, in *P. americana*, members of the outermost stamen whorl were replaced by organs resembling petaloid tepals but with basal patches of glandular tissue (Fig. 7, A and B). Basal glands are not usually associated with tepals, but neutral red staining reveals basal glandular fields as a standard feature of *Persea* tepals, and basal glands were occasionally also found on the inner tepals of *P. americana* (Fig. 7, C). Basal glands are a standard feature of Lauraceae stamens, and are always present on the third whorl stamens of *Persea*

flowers, but can regularly be found on all stamens of other genera, for example *Chlorocardium* (Rohwer et al., 1991), and occasionally also in *Umbellularia* and *Laurus* (Kasapligil, 1951). A second type of mutant stamen observed was a hybrid structure in which one side of the organ developed as a stamen, complete with filament and pollen sacs, while the other side was tepaloid (Fig. 7, D). The developmental genetic anomalies that underlie these mutant stamens are unknown, although it is tempting to speculate that repression of *Persea AG.3* during stamen development is responsible. Nevertheless, in support of the aforementioned genetic indications of staminal origin for *Persea* tepals, these mutants demonstrate that the genetic developmental pathways needed to transformation stamens into tepals are available and at work.

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Table 1. Gene specific primers used to amplify partial sequences of AP1, AP3, PI, and SEP3 homologues in *Persea americana* and *P. borbonia*.

Primer name: Sequence
Pe.am.AP1F: 5’ GAA GGC ATT GCA GGA GCA GAA CAT
Pe.am.AP1R: 5’ ATC CAA GGT GGG ACA AGG CTG TTA
Pe.am.AP3F: 5’ CCG GAA TCA ACT TGT GGA ATT C
Pe.am.AP3R: 5’ TTA TCG CCG AGT GCA CAA TC
Pe.am.PI.1F: 5’ CAG ATG GAG TTC TTA AGG GCA CTC
Pe.am.PI.1R: 5’ GAT ATT TGC TGC TGA TGC AA
Pe.am.PI.2F: 5' CAG GAG GAG CTG TTA TCG ATC CTG
Pe.am.PI.2R: 5’ GCC AAA TGA TGA TGA TGC AA
Pe.am.AG1F: 5’ CAG AAC GCA AAC AGG CAT CTG
Pe.am.AG2R: 5’ GTT TGC TCC TGG TGA GAG TAA TG
Pe.am.AG1F: 5’ AGG AGC TCA AGC AGC TGG AAA C
Pe.am.AG2R: 5’ GTT TGC TCA TGG TTG GAG TAA CC
Pe.am.AG3F: 5’ AGG AGC TCA AAC AAC TGG AGA G
Pe.am.AG3R: 5’ GTT TGA TCT TGG CGA TTG TAA TG
Pe.am.SEP3.1: F 5’ TGG AGA TCT TCA ACG AAG GGA GCA
Pe.am.SEP3.1: R 5’ GGG TGC AGC GAT TGT GAT TTG ACT
Pe.am.SEP3.2: F 5’ TGC AGA TCT CCA ACG AAG GGA ACA
Pe.am.SEP3.2: R 5’ AGG TGC GGC AAT CGT TAT TTG ATC

Figure Legends

Fig 1. One of 795 equally parsimonious trees from parsimony analyses of amino acid sequences of *Persea* and representative seed plant MIKC^{C} MADS-box genes. Consistency index (CI) = 0.409; Retention index (RI) = 0.634. All *Persea* sequences are members of major clades, thickened branches, with bootstrap values (indicated above branches) >70%. Clade names and functional class are indicated on the basis of the *Arabidopsis* members.

Fig. 2. Flowers of *Persea americana* (A) and *P. borbonia* (B), and R-Q RT PCR reactions on RNA prepared from leaves and organs dissected from mature floral buds. The 18S PCR product is a positive control, and product from *Persea* homologues of *API, AP3, PI, AG*, and *SEP3*, were obtained with gene-specific primers. OTE=outer tepals; ITE=inner tepals; STM=stamens; CAR=carpels; LVE=leaves.

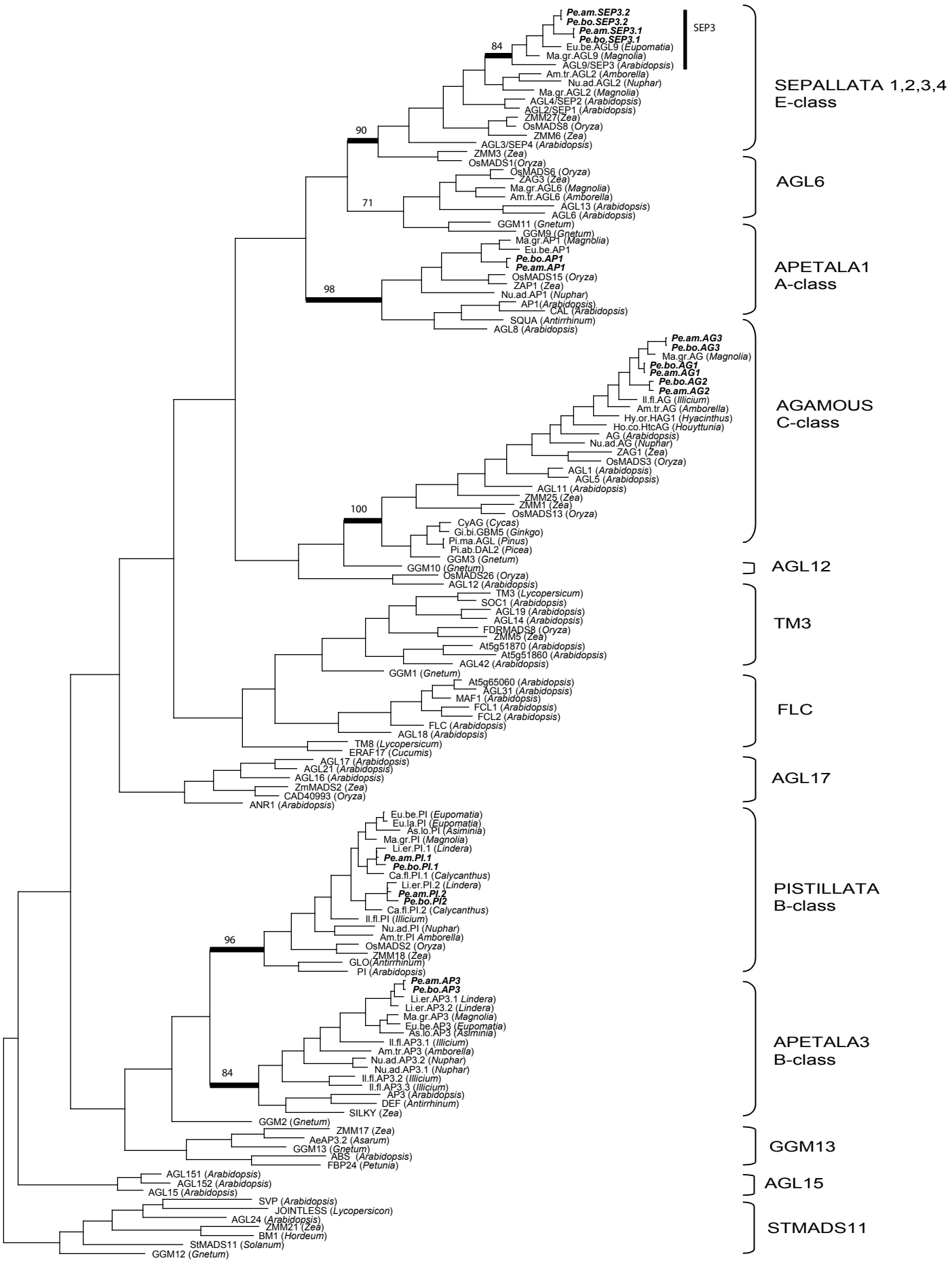
Fig. 3. *In situ* hybridization of *Persea AG* homologues in young floral buds at tepal initiation (stage 2-3 flowers). A-D, *Persea americana* probed with anti-sense (A) *Pe.am.AG.1*, (B) *Pe.am.AG.2*, (C) *Pe.am.AG.3*., and (D) sense *Pe.am.AG.1*. E-F, *Persea borbonia* probed with anti-sense (E) *Pe.bo.AG1*, (F) *Pe.bo.AG.2*, (G) *Pe.bo.AG3*, and (H) sense *Pe.am.AG1*. Anti-sense probes show expression of target genes throughout the floral meristem and in initiating tepals. FM=floral meristem; OTE=outer tepals; ITE=inner tepals.

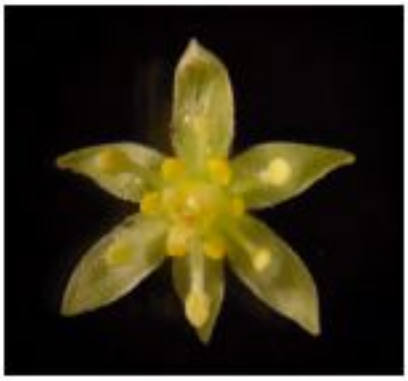
Fig. 4. In situ hybridization of *Persea borbonia* AG homologues in floral buds during (A-D) stamen initiation (stage 4) and (E-H) prior to anthesis (stage 9). Sections in A & E are probed with anti-sense *Pe.bo.AG.1*; in B & F with anti-sense *Pe.bo.AG.2*; in C & G with anti-sense *Pe.bo.AG.3*; in D & H with sense *Pe.am.AG.1*. Anti-sense probes show strong expression of target genes in initiating stamens and somewhat lower expression levels in inner and outer tepals (A-C), but expression is not detected in outer tepals after carpel initiation and anther differentiation (E-G). OTE=outer tepals; ITE=inner tepals; STM=stamens; CAR=carpels.

Fig. 5. Neutral red staining of a protogynous *Persea borbonia* flower. A. Morning flower in female phase showing deeply stained stigma and fourth whorl staminodes. Margins of inner tepals are also stained. B. Afternoon flower in male phase showing deeply stained anthers, third whorl staminal glands, and basal patches of inner tepals. C. Afternoon flower in male phase from below to reveal stained basal patches of outer tepals.

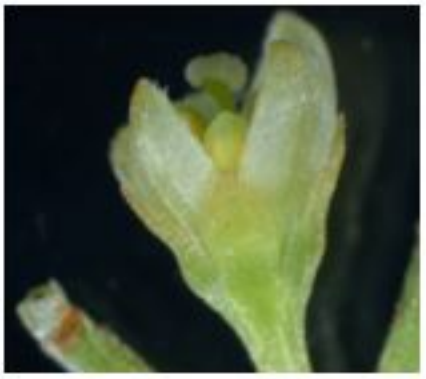
Fig. 6. Hypothesized activity domains of Persea A-, B-, C-, and E-function homologues in flowers of *P. americana* (left) and *P. borbonia* (right),

Fig. 7. Mutant phenotypes in *Persea americana* flowers. *O*ne (A) and two (B) first whorl stamens transformed into tepaloid organs with basal patches of glandular tissue. C. Petaloid inner tepal with basal patches of glandular tissue. D. Mutant first whorl stamen showing partial stamen and tepal features.


Pe.am.SEP3.2
Pe.bo.SEP3.2
Pe.am.SEP3.1
Pe.bo.SEP3.1
SEP3
84
Eu.be.AGL9 (Eupomatia)
Ma.gr.AGL9 (Magnolia)
AGL9/SEP3 (Arabidopsis)
Am.tr.AGL2 (Amborella)
Nu.ad.AGL2 (Nuphar)
Ma.gr.AGL2 (Magnolia)
AGL4/SEP2 (Arabidopsis)
AGL2/SEP1 (Arabidopsis)
ZMM27(Zea)
OsMADS8 (Oryza)
90
ZMM6 (Zea)
AGL3/SEP4 (Arabidopsis)
ZMM3 (Zea)
OsMADS1(Oryza)
SEPALLATA 1,2,3,4
E-class
OsMADS6 (Oryza)
ZAG3 (Zea)
Ma.gr.AGL6 (Magnolia)
Am.tr.AGL6 (Amborella)
71
AGL13 (Arabidopsis)
AGL6 (Arabidopsis)
AGL6
GGM11 (Gnetum)
GGM9 (Gnetum)
Ma.gr.AP1 (Magnolia)
Eu.be.AP1
Pe.bo.AP1
Pe.am.AP1
OsMADS15 (Oryza)
ZAP1 (Zea)
98
Nu.ad.AP1 (Nuphar)
AP1(Arabidopsis)
CAL (Arabidopsis)
APETALA1
A-class
SQUA (Antirrhinum)
AGL8 (Arabidopsis)
Pe.am.AG3
Pe.bo.AG3
Ma.gr.AG (Magnolia)
Pe.bo.AG1
Pe.am.AG1
Pe.bo.AG2
Pe.am.AG2
Il.fl.AG (Illicium)
Am.tr.AG (Amborella)
Hy.or.HAG1 (Hyacinthus)
Ho.co.HtcAG (Houyttunia)
AG (Arabidopsis)
Nu.ad.AG (Nuphar)
ZAG1 (Zea)
OsMADS3 (Oryza)
AGL1 (Arabidopsis)
AGL5 (Arabidopsis)
AGL11 (Arabidopsis)
ZMM25 (Zea)
ZMM1 (Zea)
100
OsMADS13 (Oryza)
CyAG (Cycas)
Gi.bi.GBM5 (Ginkgo)
Pi.ma.AGL (Pinus)
Pi.ab.DAL2 (Picea)
GGM3 (Gnetum)
AGAMOUS
C-class
GGM10 (Gnetum)
OsMADS26 (Oryza)
AGL12 (Arabidopsis)
AGL12
TM3 (Lycopersicum)
SOC1 (Arabidopsis)
AGL19 (Arabidopsis)
AGL14 (Arabidopsis)
FDRMADS8 (Oryza)
ZMM5 (Zea)
At5g51870 (Arabidopsis)
At5g51860 (Arabidopsis)
AGL42 (Arabidopsis)
GGM1 (Gnetum)
TM3
At5g65060 (Arabidopsis)
AGL31 (Arabidopsis)
MAF1 (Arabidopsis)
FCL1 (Arabidopsis)
FCL2 (Arabidopsis)
FLC (Arabidopsis)
AGL18 (Arabidopsis)
FLC
TM8 (Lycopersicum)
ERAF17 (Cucumis)
AGL17 (Arabidopsis)
AGL21 (Arabidopsis)
AGL16 (Arabidopsis)
ZmMADS2 (Zea)
CAD40993 (Oryza)
ANR1 (Arabidopsis)
AGL17
Eu.be.PI (Eupomatia)
Eu.la.PI (Eupomatia)
As.lo.PI (Asiminia)
Ma.gr.PI (Magnolia)
Li.er.PI.1 (Lindera)
Pe.am.PI.1
Pe.bo.PI.1
Ca.fl.PI.1 (Calycanthus)
Li.er.PI.2 (Lindera)
Pe.am.PI.2
Pe.bo.PI2
Ca.fl.PI.2 (Calycanthus)
Il.fl.PI (Illicium)
Nu.ad.PI (Nuphar)
Am.tr.PI Amborella)
96
OsMADS2 (Oryza)
ZMM18 (Zea)
GLO(Antirrhinum)
PI (Arabidopsis)
PISTILLATA
B-class
Pe.am.AP3
Pe.bo.AP3
Li.er.AP3.1 Lindera)
Li.er.AP3.2 (Lindera)
Ma.gr.AP3 (Magnolia)
Eu.be.AP3 (Eupomatia)
As.lo.AP3 (Asiminia)
Il.fl.AP3.1 (Illicium)
Am.tr.AP3 (Amborella)
Nu.ad.AP3.2 (Nuphar)
Nu.ad.AP3.1 (Nuphar)
84
Il.fl.AP3.2 (Illicium)
Il.fl.AP3.3 (Illicium)
AP3 (Arabidopsis)
DEF (Antirrhinum)
SILKY (Zea)
APETALA3
B-class
GGM2 (Gnetum)
ZMM17 (Zea)
AeAP3.2 (Asarum)
GGM13 (Gnetum)
ABS (Arabidopsis)
FBP24 (Petunia)
GGM13
AGL151 (Arabidopsis)
AGL152 (Arabidopsis)
AGL15 (Arabidopsis)
AGL15
SVP (Arabidopsis)
JOINTLESS (Lycopersicon)
AGL24 (Arabidopsis)
ZMM21 (Zea)
BM1 (Hordeum)
StMADS11 (Solanum)
GGM12 (Gnetum)
STMADS11
10 changes



Persea americana



Persea borbonia

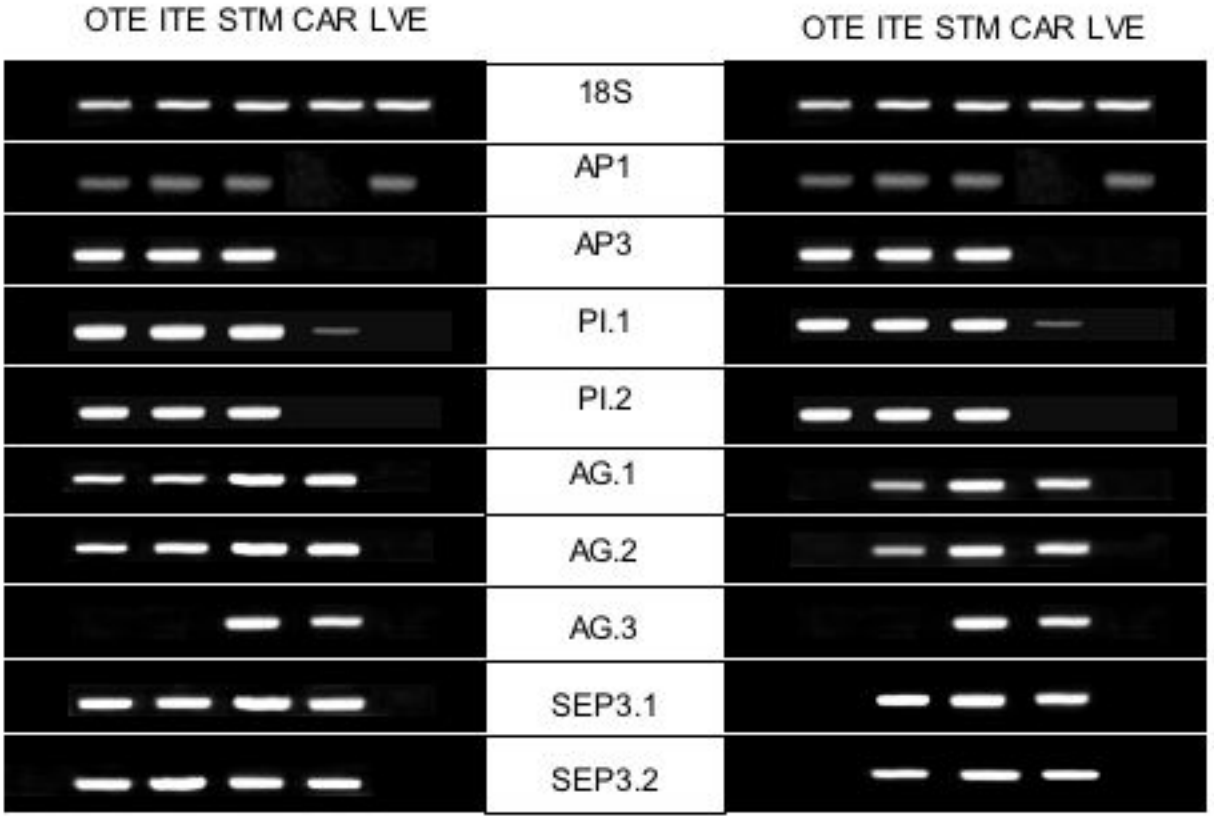


Fig. 2

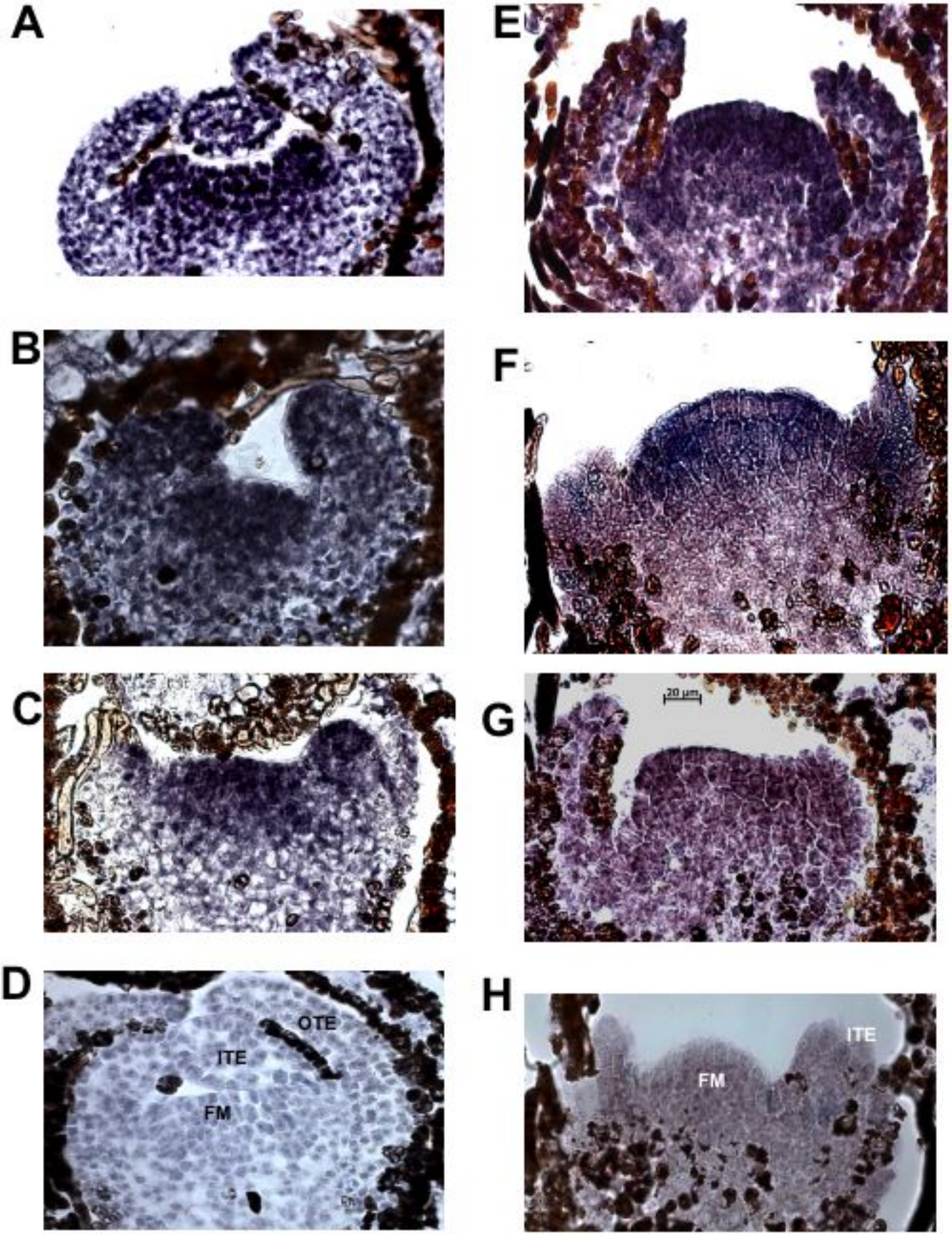


Fig. 3

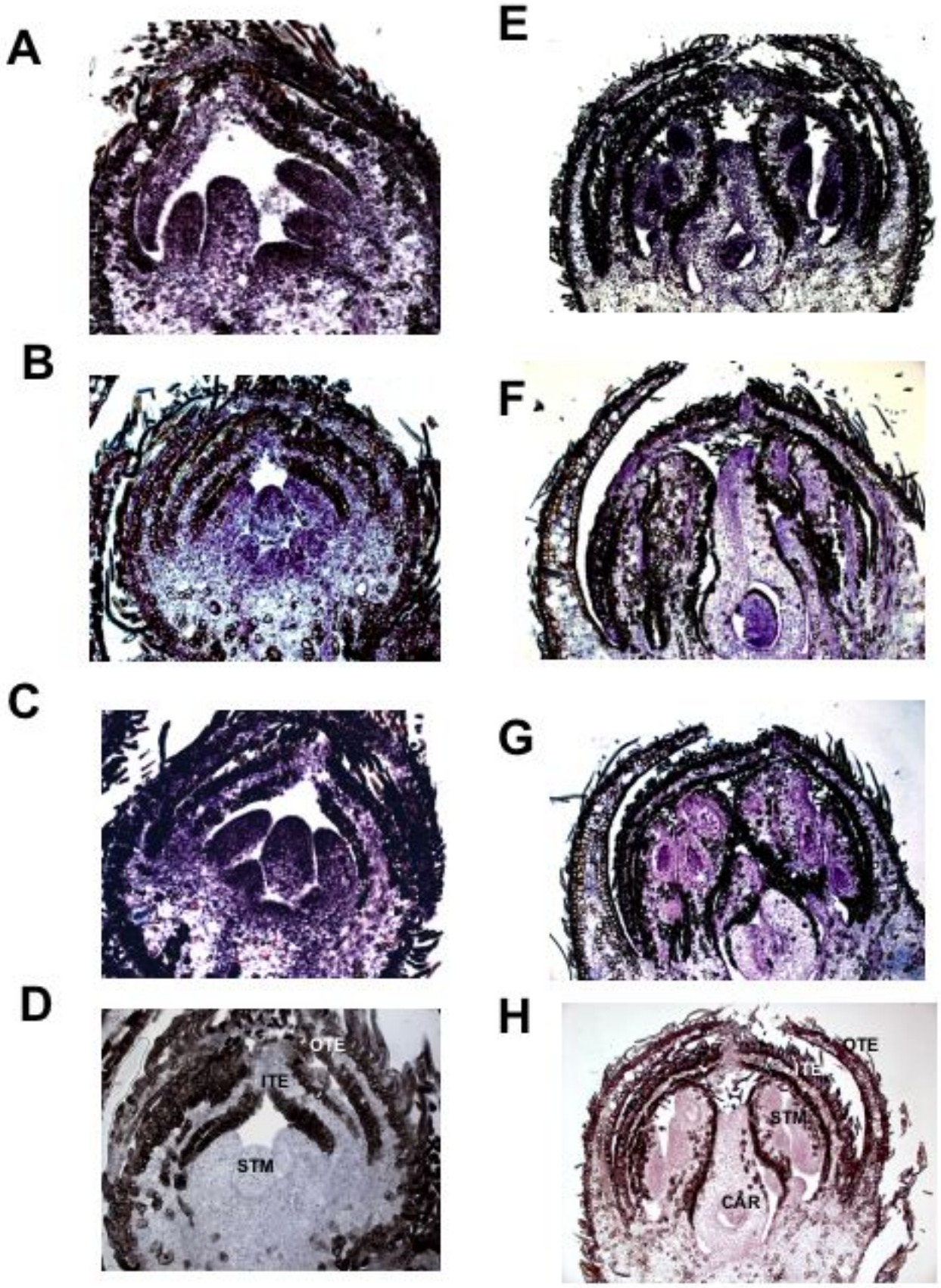


Fig. 4

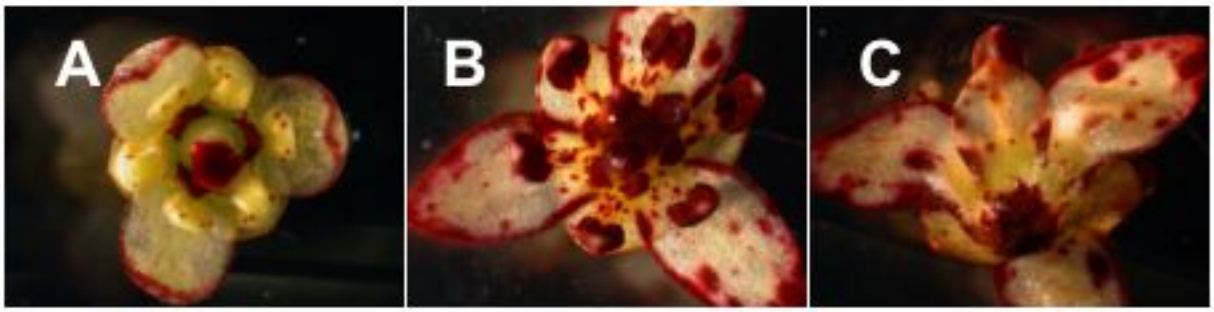


Fig. 5

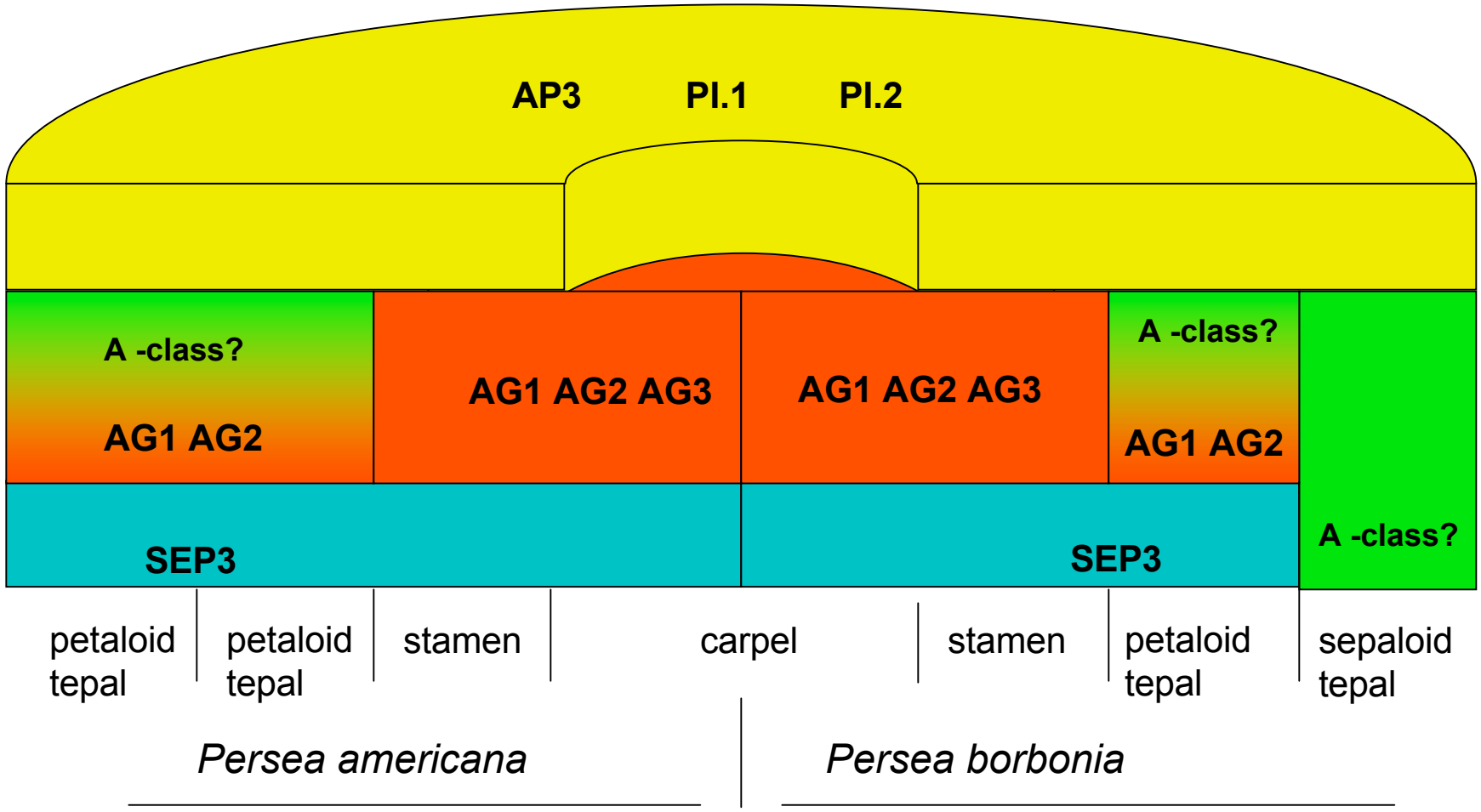

AP3
PI.1
PI.2
A -class?
AG1 AG2
AG1 AG2 AG3
AG1 AG2 AG3
A -class?
AG1 AG2
A -class?
SEP3
SEP3
petaloid tepal
petaloid tepal
stamen
carpel
stamen
petaloid tepal
sepaloid tepal
Persea americana
Persea borbonia

Fig. 6