the plant journal

VOLUME 43 | NUMBER 5 | SEPTEMBER 2005
http:// www.blackwellpublishing.com/journals/tpj | ISSN 0960-7412

Blackwell Publishing

the plant journal

CONTENTS OF VOL. 43, NO. 5, SEPTEMBER 2005

Front cover: The ABC model of floral organ identity is based on studies of eudicots. Although the eudicots constitute 75% of all angiosperms, most of the diversity in arrangement and number of floral parts is found among basal angiosperm lineages. Little is known, however, about the genes that control their floral development. In this issue (pp. 724–744), Kim *et al.* investigate the conservation and divergence of expression patterns of floral MADS-box genes in basal angiosperms compared to eudicot model systems. They isolated several floral MADS-box genes and examined their expression patterns in representative species. Their results show that the expression patterns of floral MADS-box genes in basal angiosperms are broader than those of their counterparts in eudicots and monocots. The broader range of strong expression of *AP3/PI* homologs is inferred to be the ancestral pattern for all angiosperms and is also consistent with the gradual morphological intergradations often observed between adjacent floral organs in basal angiosperms. From top-left to bottom-right: *Amborella trichopoda, Nuphar advena* (photo courtesy of V. Remay), *Illicium floridanum, Asimina longifolia, Magnolia grandiflora* (photo courtesy of D. Callaway), and *Eupomatia bennettii* (photo courtesy of H. Teppner).

The Plant Journal (2005) **43**, 724–744 doi: 10.1111/j.1365-313X.2005.02487.x

Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators

Sangtae Kim[1,*], Jin Koh[1], Mi-Jeong Yoo[1], Hongzhi Kong[2,3], Yi Hu[2], Hong Ma[2], Pamela S. Soltis[4] and Douglas E. Soltis[1,*]
[1]*Department of Botany, University of Florida, Gainesville, FL 32611, USA,*
[2]*Department of Biology, the Institute of Molecular Evolutionary Genetics, and the Huck Institutes of the Life Sciences, the Pennsylvania State University, University Park, PA 16802, USA,*
[3]*Laboratory of Systematic and Evolutionary Botany, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China, and*
[4]*Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA*

Received 16 February 2005; revised 5 June 2005; accepted 10 June 2005.
[*]For correspondence (fax +1 352 846 2154; e-mail sangtae@botany.ufl.edu; dsoltis@botany.ufl.edu).

Summary

The ABC model of floral organ identity is based on studies of Arabidopsis and *Antirrhinum*, both of which are highly derived eudicots. Most of the genes required for the ABC functions in Arabidopsis and *Antirrhinum* are members of the MADS-box gene family, and their orthologs are present in all major angiosperm lineages. Although the eudicots comprise 75% of all angiosperms, most of the diversity in arrangement and number of floral parts is actually found among basal angiosperm lineages, for which little is known about the genes that control floral development. To investigate the conservation and divergence of expression patterns of floral MADS-box genes in basal angiosperms relative to eudicot model systems, we isolated several floral MADS-box genes and examined their expression patterns in representative species, including *Amborella* (Amborellaceae), *Nuphar* (Nymphaeaceae) and *Illicium* (Austrobaileyales), the successive sister groups to all other extant angiosperms, plus *Magnolia* and *Asimina*, members of the large magnoliid clade. Our results from multiple methods (relative-quantitative RT-PCR, real-time PCR and RNA *in situ* hybridization) revealed that expression patterns of floral MADS-box genes in basal angiosperms are broader than those of their counterparts in eudicots and monocots. In particular, (i) *AP1* homologs are generally expressed in all floral organs and leaves, (ii) *AP3*/*PI* homologs are generally expressed in all floral organs and (iii) *AG* homologs are expressed in stamens and carpels of most basal angiosperms, in agreement with the expectations of the ABC model; however, an *AG* homolog is also expressed in the tepals of *Illicium*. The broader range of strong expression of *AP3/PI* homologs is inferred to be the ancestral pattern for all angiosperms and is also consistent with the gradual morphological intergradations often observed between adjacent floral organs in basal angiosperms.

Keywords: *Amborella*, basal angiosperms, *Illicium*, MADS-box, Magnoliales, *Nuphar*, plant evolution, relative-quantitative RT-PCR, *in situ* hybridization.

Introduction

One of the most important developments in our understanding of floral development was the formulation of the ABC model for controlling floral organ identity (Coen and Meyerowitz, 1991). This model is based on genetic studies in Arabidopsis (Brassicaceae) and *Antirrhinum* (Plantaginaceae, formerly placed in Scrophulariaceae; see APGII, 2003) and posits that the specification of floral organ identity is controlled by three genetically separate functions (Figure 1a). The A function specifies sepal identity (whorl 1). The A and B functions together direct petal identity (whorl 2). The combination of both B and C functions determines stamen identity (whorl 3). Finally, the C function alone controls the identity of carpels (whorl 4). A-function genes include the Arabidopsis *APETALA1* (*AP1*) and *APETALA2* (*AP2*) genes (Bowman *et al*., 1993; Mandel *et al*., 1992). The B function requires the *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) genes in *Antirrhinum*, and their respective orthologs in Arabidopsis, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Goto and Meyerowitz,

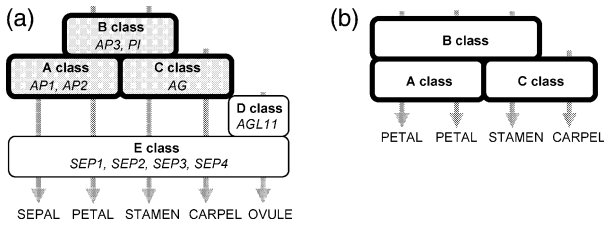

Figure 1. (a) The classic ABC model (Coen and Meyerowitz, 1991) for floral organ identity in Arabidopsis is shown as gray boxes. Based on recent additions to the ABC model (Colombo *et al.*, 1995; Ditta *et al.*, 2004; Pelaz *et al.*, 2000; Theissen, 2001), D- and E-class genes are shown as white boxes. *SEP4* (*AGL3*), a recently recognized E-function gene, is included in the box of E-class genes (Ditta *et al.*, 2004).
(b) Modified ABC model (van Tunen *et al.*, 1993): the boundary of B-class gene function is extended to the first whorl to explain the petaloid perianth ('shifting boundary' of Bowman, 1997, and 'sliding boundary' of Kramer *et al.*, 2003).

1994; Jack *et al.*, 1992, 1994; Schwarz-Sommer *et al.*, 1992; Sommer *et al.*, 1990; Tröbner *et al.*, 1992). The Arabidopsis *AGAMOUS* (*AG*) and *Antirrhinum PLENA* (*PLE*) genes are required for the C function (Bradley *et al.*, 1993; Yanofsky *et al.*, 1990). With the exception of *AP2*, all of these organ identity genes are members of the MADS-box family (reviewed in Ma and dePamphilis, 2000) and are collectively referred to as floral MADS-box genes.

The identification of floral MADS-box genes as essential regulators of early flower development has led to a huge effort at isolation and molecular analysis of additional members of the MADS-box gene family, including the Arabidopsis *AGL* genes (Ma *et al.*, 1991; Mandel and Yanofsky, 1998; Rounsley *et al.*, 1995). Since the original ABC model was presented, MADS-box genes that specify ovule identity were proposed to define the D function (Colombo *et al.*, 1995) (Figure 1a). More recently, the Arabidopsis MADS-box genes *AGL2*, *AGL4*, *AGL9* and *AGL3* were found to have redundant function in specifying the identity of petals, stamens and carpels and were renamed *SEPALLATA1*, *SEPALLATA2*, *SEPALLATA3* and *SEPALLATA4* (*SEP1*, *–2*, *–3* and *–4*); the *SEP* genes were proposed to define the E function (Ditta *et al.*, 2004; Pelaz *et al.*, 2000; Theissen, 2001) (Figure 1a). In addition to model plants such as Arabidopsis and *Antirrhinum*, MADS-box genes have also been isolated from other core eudicots (*sensu* APGII, 2003), including *Petunia* (Solanaceae), tobacco (*Nicotiana*, Solanaceae) and *Gerbera* (Asteraceae) (Angenent *et al.*, 1993; Davies *et al.*, 1996; Kater *et al.*, 1998; Kempin *et al.*, 1993; van der Krol *et al.*, 1993; Yu *et al.*, 1999). Floral MADS-box genes have also been characterized from several basal eudicot families, including Papaveraceae and Ranunculaceae (Kramer and Irish, 2000; Kramer *et al.*, 1998, 2003, 2004), as well as from a diverse array of monocots (Ambrose *et al.*, 2000; Kang *et al.*, 1995; Kyozuka *et al.*, 2000; Mena *et al.*, 1995; Nagasawa *et al.*, 2003; Schmidt *et al.*, 1993) and basal angiosperms (Kim *et al.*, 2004, 2005; Kramer and Irish, 1999, 2000; Kramer *et al.*, 2003, 2004; Litt and Irish, 2003; Zahn *et al.*, 2005a).

Extensive molecular phylogenetic analyses indicate that floral MADS-box genes from model organisms and their homologs form several well-supported major clades, which can be recognized as separate subfamilies (Becker and Theissen, 2003; Nam *et al.*, 2003; Parenicova *et al.*, 2003; Theissen *et al.*, 1996). In fact, functionally similar genes in Arabidopsis and *Antirrhinum* are generally homologs that belong to the same subfamily. For example, the C-function genes *AG* and *PLE* are functional homologs (although not orthologs) in the *AG* subfamily (named after the first described member of the subfamily, as proposed by Becker and Theissen, 2003). Similarly, the B-function genes, *DEF* and *GLO* from *Antirrhinum* and *AP3* and *PI* from Arabidopsis, are members of the *DEF*/*GLO* (or *AP3*/*PI*) subfamily, which can be further divided into the *DEF* (or *AP3*) and *GLO* (or *PI*) lineages. The Arabidopsis A-function gene *AP1* is a putative ortholog of the *Antirrhinum SQUA* gene; *SQUA* plays a less prominent role in the A function than *AP1* (Huijser *et al.*, 1992). Phylogenetic analyses of the *SQUA* subfamily (A-class) identified two gene clades within the core eudicots, the eu*AP1* clade (which includes Arabidopsis *AP1* and *Antirrhinum SQUA*) and the eu*FUL* clade [which includes Arabidopsis *FRUITFULL* (*FUL*)] (Litt and Irish, 2003). The *FUL* gene is important for normal fruit development (Gu *et al.*, 1998) and has a redundant role with *AP1* and *CAULIFLOWER* in regulating meristem identity (Ferrandiz *et al.*, 2000). In angiosperms outside the core eudicots, *SQUA* subfamily members are more similar to *FUL* than to *AP1* and are often referred to as *FUL*-like (Litt and Irish, 2003). The *SEP* genes and their close relatives also form a separate subfamily, whereas the MADS-box genes required for the D function are members of the *AG* subfamily (Becker and Theissen, 2003; Kramer *et al.*, 2004; Zahn *et al.*, 2005a). Other MADS-box genes, such as the Arabidopsis *AGL6* gene (Ma and dePamphilis, 2000; Ma *et al.*, 1991), which defines another subfamily closely related to the *AGL2 (SEP)* subfamily (Becker and Theissen, 2003; Zahn *et al.*, 2005a), are expressed in the flower and may play a role in flower development.

Functional studies in several eudicots and grasses indicate that homologs of *AP3*/*PI* and *AG* often exhibit conserved B and C functions, respectively (Ma and dePamphilis, 2000). In addition, relatively recent gene duplications in some groups of species have resulted in sets of paralogs that carry out a subset of the functions performed by their homologs in Arabidopsis and *Antirrhinum* (reviewed in Baum, 1998; Ma and dePamphilis, 2000; Soltis *et al.*, 2002). For example, two *AG* homologs in maize have undergone subfunctionalization (Mena *et al.*, 1995, 1996). Similarly, multiple paralogs of *AP3* and *PI* are present in petunia, and they seem to fulfill collectively the functions of *AP3* and *PI* (van der Krol and Chun, 1993; van der Krol *et al.*, 1993;

Tsuchimoto *et al.*, 2000; Vandenbussche *et al.*, 2004; Zahn *et al.*, 2005b). These gene duplication and putative subfunctionalization events suggest that, while the functions of individual members of the *DEF*/*GLO* and *AG* subfamilies may vary from species to species, members of each subfamily collectively have a conserved function in regulating floral organ identity as proposed in the ABC model (Zahn *et al.*, 2005b).

Further evidence for conservation and diversification of MADS-box gene expression (and by inference, function) has been found in non-grass monocots. In the monocot *Asparagus* (Asparagaceae), which exhibits only slight morphological differentiation between the outer and inner perianth whorls, *AP3*/*PI* homolog expression follows the classic ABC model (Park *et al.*, 2003, 2004). On the other hand, in the monocot *Tulipa* (Liliaceae), Kanno *et al.* (2003) demonstrated that the organs of both floral whorls 1 and 2, which are morphologically similar, express both A- and B-class genes. These two whorls therefore have the same 'petaloid' identity. To explain the morphology of the lily flower, van Tunen *et al.* (1993) proposed a modified ABC model (Figure 1b) in which the expression of B-class genes was extended to the first floral whorl. The expression of *AP3*/*PI* homologs in both the first and second whorls in *Tulipa* (Kanno *et al.*, 2003) supported this modified ABC model and is consistent with B-class gene expression in the petaloid perianth of *Ranunculus* (Ranunculaceae; Kramer *et al.*, 2003). The 'shifting boundary' (Bowman, 1997) and 'sliding boundary' (Kramer *et al.*, 2003) models allow the boundary of the B function to 'slide' from that observed for Arabidopsis and *Antirrhinum* to include the outer perianth whorl (outer tepals) of *Ranunculus*, *Tulipa* and other species with an entirely petaloid perianth.

Molecular genetic studies in Arabidopsis and *Antirrhinum*, as well as several other species, indicate that the function of floral MADS-box genes is very well correlated with the expression patterns of these genes, particularly when expression levels are high (Ma and dePamphilis, 2000). In Arabidopsis, *AP1* and *AG* are expressed in the perianth and reproductive regions of the floral meristem, respectively, corresponding to the A and C functions. Although the *DEF*/*GLO* and *AP3*/*PI* genes are initially expressed somewhat broadly, they become restricted to the second and third whorls, as predicted by the B function (Goto and Meyerowitz, 1994; Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992). Also, the petunia *DEF*/*GLO* homologs show differential expression between petals and stamens in a manner consistent with their functions (Angenent *et al.*, 1992; Immink *et al.*, 2003). Conversely, members of the same subfamily that have diverged in function also show distinct expression patterns. For example, the *SHP1* and *SHP2* genes are members of the *AG* subfamily, but have more specialized functions in carpel and ovule development (Liljegren *et al.*, 1999; Pinyopich *et al.*, 2003). They are expressed in developing carpels and ovules, but not stamens (Flanagan *et al.*, 1996; Savidge *et al.*, 1995). Similarly, the *PLE* paralog *FAR* in *Antirrhinum* is strongly expressed in the anther and is required for male reproductive development (Davies *et al.*, 1999). Therefore, within a given subfamily of floral MADS-box genes, expression patterns can be very good predictors of gene function.

Although eudicots comprise approximately 75% of all angiosperm species, the organization of the flower is fairly constant throughout this clade, with floral organs typically arranged in distinct whorls and floral parts in fours, fives or multiples thereof. In contrast, non-monocot basal angiosperms represent only 3% of angiosperm species diversity (Drinnan *et al.*, 1994) but display enormous floral diversity, with some taxa exhibiting an undifferentiated perianth of spirally arranged tepals [e.g. *Amborella* (Amborellaceae); Figure 2a], others having a well-differentiated perianth of distinct sepals and petals [e.g. *Asimina* (Annonaceae) and *Saruma* (Aristolochiaceae); Figure 2d], and still others that apparently lack a perianth altogether [e.g. *Eupomatia* (Eupomatiaceae); Figure 2f]. Reconstructions of perianth evolution indicate, in fact, that a differentiated perianth of sepals and petals evolved independently in several basal angiosperm lineages, as well as in eudicots (Albert *et al.*, 1998; Ronse De Craene *et al.*, 2003; Zanis *et al.*, 2003).

Despite the diversity of floral form and structure in basal angiosperms, information on the expression of floral MADS-box genes in these plants is limited. For example, *AP3*/*PI* homologs are expressed throughout the perianth in a species of *Magnolia* (Magnoliaceae) and in *Calycanthus* (Calycanthaceae) (Kramer and Irish, 2000), both members of the large magnoliid clade of basal angiosperms (Figure 3d; APGII, 2003; D. Soltis *et al.*, 2000). Likewise, *AP1* and *AP3*/*PI* homologs are expressed more broadly than expected from studies of Arabidopsis in both *Magnolia* and *Eupomatia* (Eupomatiaceae), a close relative of *Magnolia* that lacks a perianth (Figure 2; Kim *et al.*, 2005). An *AGL2* (*SEP1*) homolog from *Nuphar* (Nymphaeaceae, water lilies) is expressed in all floral organs (Zahn *et al.*, 2005a).

The basal-most lineages of extant flowering plants, and successive sisters to all other angiosperms, are Amborellaceae, Nymphaeaceae (the water lilies in the broad sense, including Cabombaceae) and Austrobaileyales (e.g. Mathews and Donoghue, 1999; Qiu *et al.*, 1999; Soltis *et al.*, 1999; reviewed in Soltis and Soltis, 2004; see APGII, 2003) (Figure 3). This basal grade is followed by (i) Chloranthaceae, (ii) monocots, (iii) a large magnoliid clade, which comprises four orders (Magnoliales, Laurales, Canellales and Piperales) and includes a number of well-known basal angiosperms, such as *Magnolia*, *Persea* (avocado), *Piper* (black pepper) and *Asimina* (paw-paw) and (iv) eudicots (Figure 3). Although each of these four clades is well supported, relationships among them are unclear.

Figure 2. Photographs of flowers of basal angiosperms investigated in this study.
(a) *Amborella trichopoda*.
(b) *Nuphar advena* (photo credit: V. Remay).
(c) *Illicium floridanum*.
(d) *Asimina longifolia*.
(e) *Magnolia grandiflora* (photo credit: D. Callaway).
(f) *Eupomatia bennettii* (photo credit: H. Teppner).
Arrows indicate sepals of *A. longifolia*.

To investigate the conservation and divergence of expression patterns of floral MADS-box genes, we isolated genes from several basal angiosperms and analyzed their expression. The taxa included in this study are *Amborella* (Amborellaceae), *Nuphar* (Nymphaeaceae), *Illicium* (Illiciaceae; Austrobaileyales), *Magnolia* (Magnoliaceae; Magnoliales) (Figure 3) and *Asimina* (Annonaceae; Magnoliales). The latter is unusual among basal angiosperms in that species of this genus possess a well-differentiated perianth of sepals and petals (Figure 2d). We compare our results here with expression data we reported earlier for several MADS-box genes from *Magnolia* and *Eupomatia* (Eupomatiaceae; Magnoliales) (Kim *et al.*, 2005) and *Nuphar* (Zahn *et al.*, 2005a). We also compare all of the data now available for basal angiosperms, eudicots and monocots. Expression data from basal angiosperms, when coupled with data for model organisms, serve as important reference points for understanding the evolution of floral regulatory genes throughout angiosperms. Our gene expression data can stimulate additional analyses of floral gene expression and function in basal angiosperms, ultimately providing important information regarding the origin of the flower.

Results

Orthology of MADS-box genes from basal angiosperms

We report here the following homologs of MADS-box genes: *Am.tr.AG* (AY936231), *Am.tr.AGL2* (AY936232), *Am.tr.AGL6* (AY936234), *Nu.ad.AP1* (AY936223), *Nu.ad.AP3.1* (DQ004465), *Nu.ad.AP3.2* (DQ004464), *Nu.ad.AG* (AY936230), *Il.fl.PI* (AY936224), *Il.fl.AP3.1* (AY936225), *Il.fl.AP3.2* (AY936226), *Il.fl.AP3.3* (AY936227), *Il.fl.AG* (AY936229), *Ma.gr.AG* (AY936228) and *Ma.gr.AGL6* (AY936233) (Table 1). We isolated two *AP3* homologs from *Nuphar* and three from *Illicium*. In these instances the abbreviation for the taxon and gene is followed by a full stop and then a number to distinguish these multiple homologs.

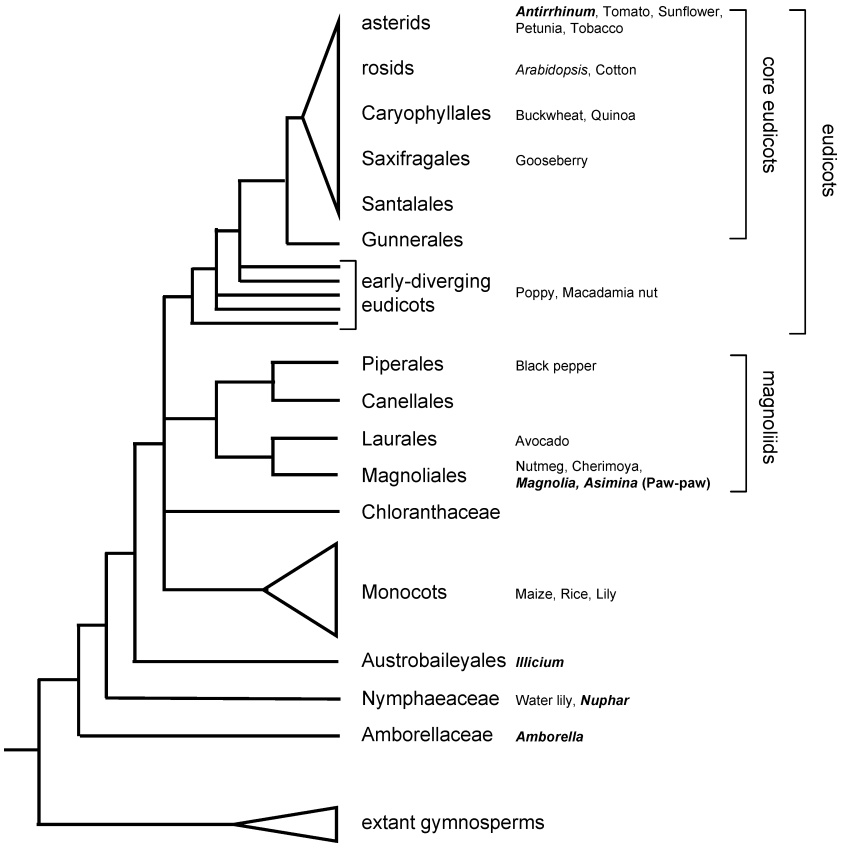

Figure 3. Summary of angiosperm phylogeny. Examples of some clades are indicated after clade names. Names in bold indicate genera included in this study. Nearly all analyses of basal angiosperms have identified *Amborella* as the sister to all other extant angiosperms (e.g. Borsch *et al.*, 2003; Graham and Olmstead, 2000; Graham *et al.*, 2000; Hilu *et al.*, 2003; Magallón and Sanderson, 2001; Mathews and Donoghue, 1999, 2000; Nickerson and Drouin, 2004; Parkinson *et al.*, 1999; Qiu *et al.*, 1999; P. Soltis *et al.*, 1999; D. Soltis *et al.*, 2000; Zanis *et al.*, 2002). In all of these studies Nymphaeaceae and Austrobaileyales followed *Amborella* as successive sisters to the remaining extant angiosperms, as shown here. An alternative topology in which *Amborella* and Nymphaeaceae are sister to each other, with this clade sister to all other extant angiosperms, has been found in some analyses (e.g. Barkman *et al.*, 2000; Kim *et al.*, 2004; Mathews and Donoghue, 2000; Parkinson *et al.*, 1999; Qiu *et al.*, 2000; P. Soltis *et al.*, 2000).

Blast searches in GenBank identified all of these new genes as putative members of the MADS-box gene family. Our phylogenetic analysis of these genes and other MADS-box genes from basal angiosperms showed bootstrap support greater than 70% for clades of A-, B- (*DEF* and *GLO*), C- (and D-) and E-class genes (Figure 4). Considering the new genes reported here, one *Nuphar* gene was identified as an ortholog of *AP1* (A class), two *Nuphar* genes and three *Illicium* genes were identified as orthologs of *AP3* (B class), one *Illicium* gene was identified as an ortholog of *PI* (B class), genes from *Amborella*, *Nuphar*, *Illicium* and *Magnolia* were identified as orthologs of *AG* (C class), one *Amborella* gene was identified as an ortholog of *AGL2* (E class) and genes from *Amborella* and *Magnolia* were identified as orthologs of *AGL6*.

Analysis of gene expression using relative-quantitative RT-PCR

Representative gel photographs (Figure 5) illustrate our relative-quantitative RT-PCR (RQ RT-PCR) results, and a summary of the data is provided in Table 1. No signal was detected in any of the negative controls (i.e. samples that did not contain a cDNA template). Results for the *Antirrhinum DEF* and *GLO* genes, which served as reference samples (Figure 5f), were almost identical to the patterns previously observed using RNA *in situ* and Northern blot hybridizations (e.g. Sommer *et al.*, 1991; Tröbner *et al.*, 1992): both genes exhibited strong expression in petals and stamens, and *GLO* showed very weak expression in carpels in our experiments. Although very weak expression of *DEF* in sepals and carpels was reported in *Antirrhinum* (Sommer *et al.*, 1991), no signal was detected in these organs in our study.

In *Amborella*, *Am.tr.AP3* and *Am.tr.PI* were expressed in the tepals and stamens, consistent with the expression of *AP3* and *PI* in Arabidopsis and *DEF* and *GLO* in *Antirrhinum*. Expression of both *Am.tr.AP3* and *Am.tr.PI* was also detected in carpels, and weak expression of *Am.tr.PI* was found in leaves. *Am.tr.AG* was expressed in reproductive organs, and *Am.tr.AGL2* in tepals, stamens and carpels, both similar to the expression reported for their orthologs in eudicots. In addition, *Am.tr.AGL6* was strongly expressed in tepals, with intermediate levels of expression detected in stamens and carpels (Figure 5a).

The outer and inner tepals of *Nuphar* are morphologically similar, but the outer tepals are green, whereas the inner tepals are yellow (Figure 2b). Because of this color difference, the outer and inner tepals are often considered to be sepals and petals, respectively (e.g. Cronquist, 1988; Judd *et al.*, 2002). However, outer and inner tepals of *Nuphar* exhibited very similar expression levels for all of the genes we investigated (Figure 5b; Table 1). *Nu.ad.AP1* was expressed in all floral organs and leaves, with the strongest expression

Table 1 Summary of expression patterns of floral genes in basal angiosperm flowers just prior to anthesis

Taxa	Gene name	Outer perianth[e] 1st	Inner perianth 2nd	Inner stamens 3rd	Carpels 4th	Leaves
SQUA subfamily						
Nuphar	***Nu.ad.AP1***	+	+	++	+++	+++
Eupomatia	*Eu.be.AP1*[a]	NA	NA	++	+++	+++
Magnolia	*Ma.gr.AP1*[a]	++	++	++	++	+++
Persea	*Pe.am.AP1*[b]	+++	+++	+++	–	+++
GLO subfamily						
Amborella	*Am.tr.PI*	?[d]	+++	+++	+++	+
Nuphar	*Nu.ad.PI*	+++	+++	+++	++	–
Illicium	***Il.fl.PI***	+++	+++	+++	–	–
Asimina	*As.lo.PI*	–	+++	+++	–	–
Eupomatia	*Eu.be.PI*[a]	NA	NA	+++	++	–
Magnolia	*Ma.gr.PI*[a]	++	+++	+++	+	–
Persea	*Pe.am.PI.1*[b]	+++	+++	+++	+	–
Persea	*Pe.am.PI.2*[b]	+++	+++	+++	–	–
DEF subfamily						
Amborella	*Am.tr.AP3*	?[d]	+++	+++	+++	–
Nuphar	***Nu.ad.AP3.1***	+++	+++	+++	+++	–
Nuphar	***Nu.ad.AP3.2***	+++	+++	+++	+++	–
Illicium	***Il.fl.AP3.1***	–	+++	+++	–	–
Illicium	***Il.fl.AP3.2***	++	+++	+++	–	–
Illicium	***Il.fl.AP3.3***	–	+++	–	–	–
Asimina	*As.lo.AP3*	+	+++	+++	–	–
Eupomatia	*Eu.be.AP3*[a]	NA	NA	+++	+++	++
Magnolia	*Ma.gr.AP3*[a]	+++	+++	+++	–	–
Persea	*Pe.am.AP3*[b]	+++	+++	++	–	–
AG subfamily						
Amborella	***Am.tr.AG***	?[d]	+	+++	+++	–
Nuphar	***Nu.ad.AG***	–	–	+++	+++	–
Illicium	***Il.fl.AG***	–	+++	+++	+	–
Magnolia	***Ma.gr.AG***	–	–	+++	+++	–
Persea	*Pe.am.AG*[b]	+++	+++	+++	+++	++
AGL2 subfamily						
AGL2/3/4 lineage						
Amborella	***Am.tr.AGL2***	?[d]	+++	+++	+++	–
Nuphar	*Nu.ad.AGL2*[c]	+++	+++	+++	+++	–
Magnolia	*Ma.gr.AGL2*[b]	+++	+++	+++	+++	++
AGL9 lineage						
Eupomatia	*Eu.be.AGL9*[b]	NA	NA	+++	+++	+
Magnolia	*Ma.gr.AGL9*[b]	+++	+++	+++	+++	+
AGL6 subfamily						
Amborella	***Am.tr.AGL6***	?[d]	+++	++	++	–
Magnolia	***Ma.gr.AGL6***	+++	+++	–	–	–

Newly identified genes in this study are indicated in bold. NA indicates not applicable. *Eupomatia* lacks a perianth.
[a]Data from Kim *et al.* (2005).
[b]Data from Chanderbali *et al.* (in prep.).
[c]Data from Zahn *et al.* (2005a).
[d]We did not obtain information from the outermost perianth organs of *Amborella* because of the small size of the outer tepals.
[e]Some taxa investigated do not have a whorled arrangement of floral parts (e.g. *Amborella* has all parts spirally arranged; *Magnolia* has whorled perianth parts, but spirally arranged stamens and carpels).

observed in carpels and leaves (Figure 5b; Table 1). Expression of *Nu.ad.PI*, *Nu.ad.AP3.1* and *Nu.ad.AP3.2* (Figure 5b) was detected in both outer and inner tepals, as well as in stamens and staminodes. Furthermore, the level of expression of *Nu.ad.PI* in the carpels was dependent on the stage of the floral bud (data not shown). In the carpels from floral buds 10–13 mm in diameter (near the time of male meiosis) relatively high expression was observed. However, expression of *Nu.ad.PI* was lower in carpels from flowers that were 30 mm in diameter (just before anthesis) and was not detected in carpels from open flowers (40 mm in diameter) (see also the results for real-time PCR). Hence, the data indicate a gradual decrease in expression of this *PI* homolog in carpels as the flower matures.

Although the two *AP3* homologs of *Nuphar* had very similar expression patterns, the expression of *Nu.ad.AP3.2*

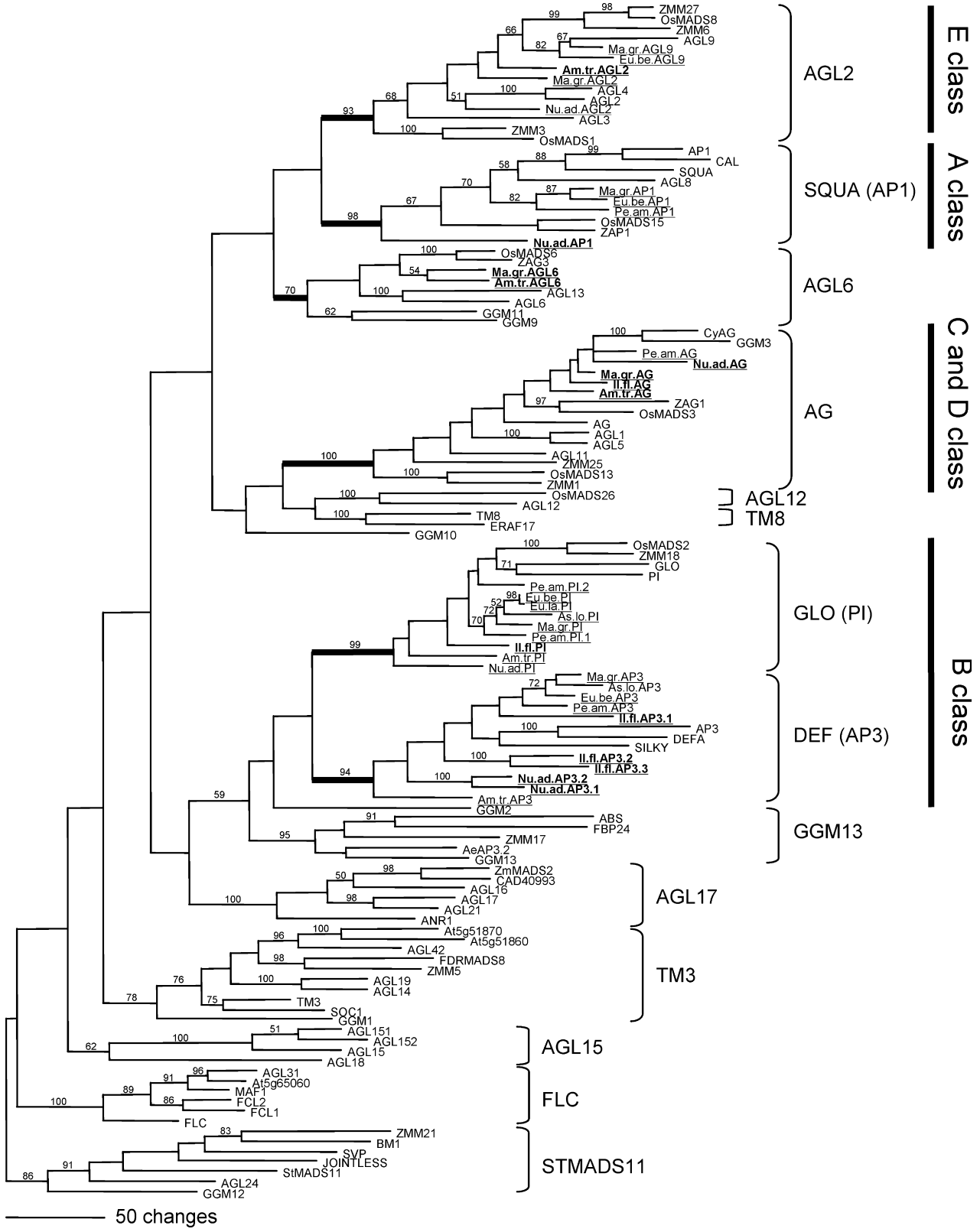

Figure 4. Strict consensus of two shortest trees from a maximum parsimony analysis of MADS genes (7608 steps, Consistency Index (CI) = 0.40 and Retention Index (RI) = 0.58).

Selected representatives of each major clade of MIKC^{C}-type MADS-box genes were analyzed together with genes newly identified in this study (bold) and genes used in this study (underlined). Each new gene is a member of a well-supported (>70% bootstrap value) major clade of the MADS-box family (thickened nodes).

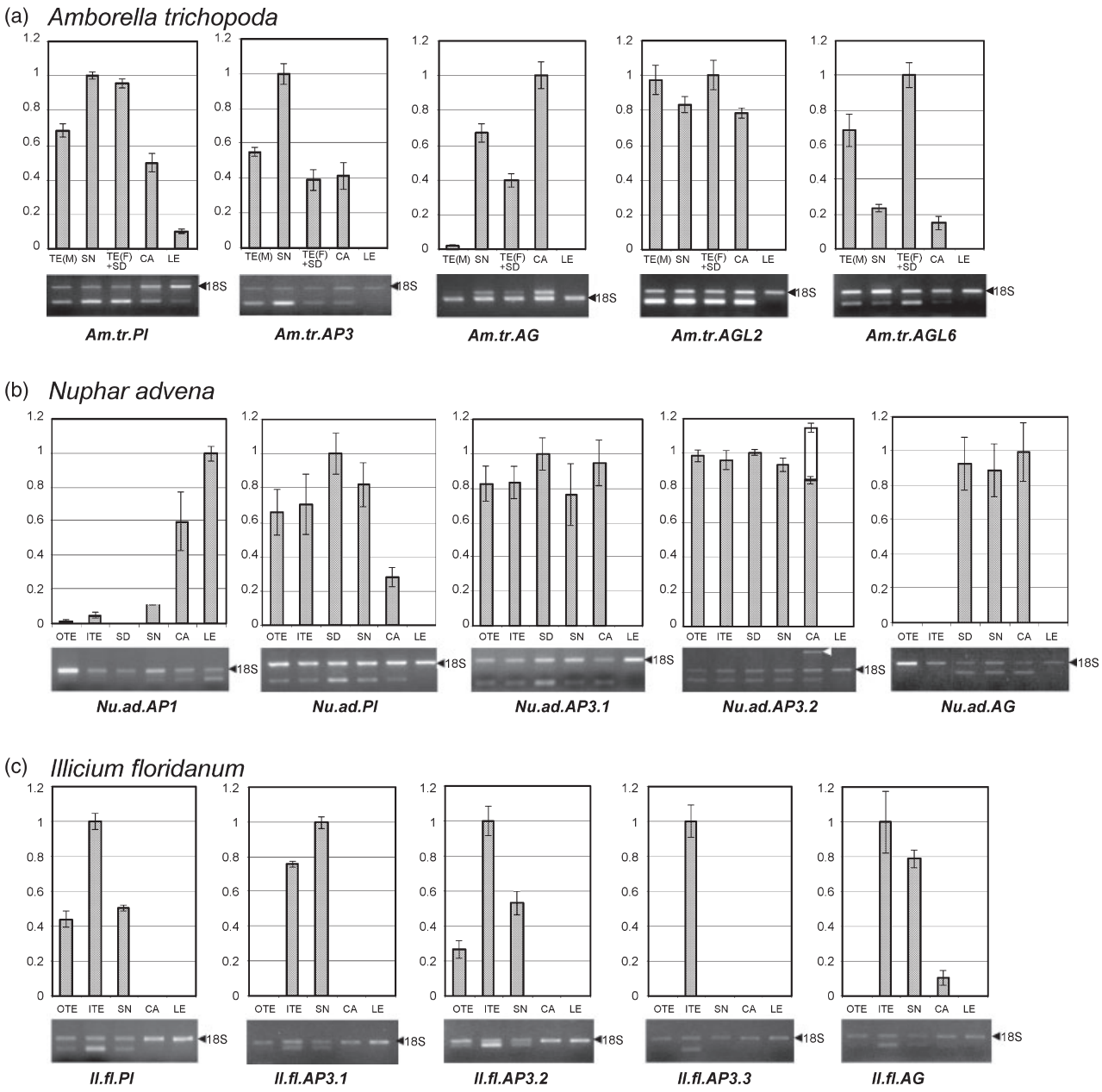

Figure 5. Relative quantitative RT-PCR results of floral MADS-box genes in basal angiosperms. Standard deviations are indicated for each value.
Flower buds collected just before anthesis were used. In the case of *Magnolia*, floral buds of 15 and 30 mm in diameter (just before anthesis) were examined. The open arrowhead indicates a longer band than expected (see text). For *Nu.ad.AP3.2*, the expression signal of expected bands and the sum of signals of longer and expected bands were calculated separately in carpels. TE, tepals; TE(M), tepals in male flowers; TE(F) tepals in female flowers; OTE, outer tepals; ITE, inner tepals; SE, sepals; OPE, outer petals; IPE inner petals; SN, stamens; SD, staminodes; CA, carpels; LE, leaves; BR, spathaceous bracts; TE1, three outer tepals; TE2, three middle tepals; TE3, three inner tepals; SE1, immature seeds of 1 mm in diameter; SE2, immature seeds of 2 mm in diameter.

was relatively weak compared with *Nu.ad.AP3.1*: the signal of *Nu.ad.AP3.2* was only detectable after 29 cycles (all other RQ RT-PCR experiments were performed with 26 or 27 cycles; see Experimental procedures). For *Nu.ad.AP3.2* an additional band was detected only in carpels (open arrowhead in Figure 5b, fourth panel). The genomic sequence of the corresponding region of this additional band (DQ070749) indicates that this band represents an unspliced precursor RNA. The primer pairs used for RQ RT-PCR of *Nu.ad.AP3.2* correspond to putative exon3 and exon7 of *Nu.ad.AP3.2*. The sequence of the additional band contained intron3, intron4, intron5 and intron6. Alternatively spliced or partially spliced RNA fragments, which are restricted to certain floral organs, have been reported for several MADS genes (Kim *et al.*, 2005; Stellari *et al.*, 2004). *Nu.ad.AG* was expressed in stamens (and staminodes) and carpels.

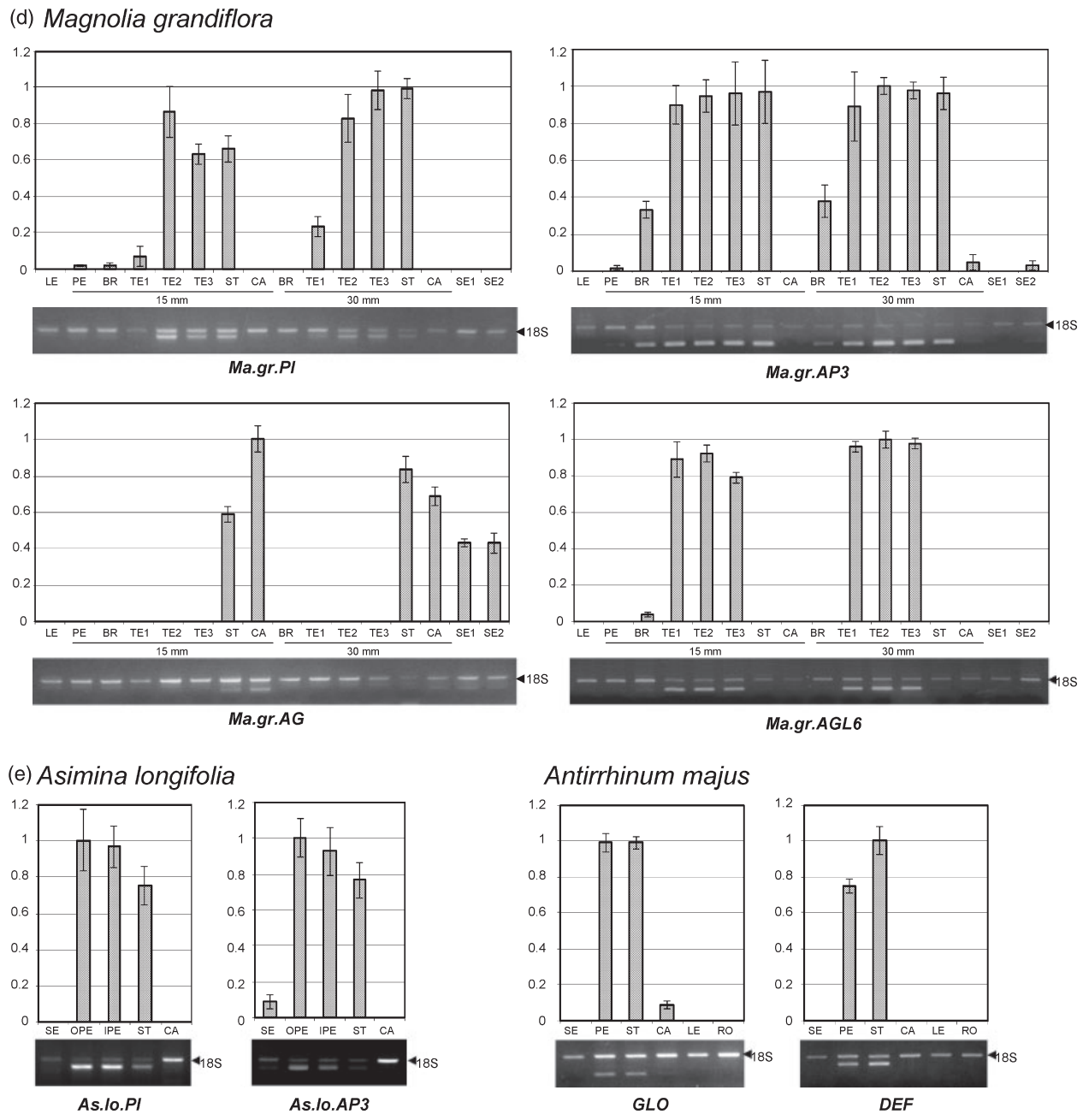

Figure 5. Continued.

The *Illicium PI* homolog (*Il.fl.PI*) was strongly expressed in outer and inner tepals and stamens. The three *Illicium AP3* homologs (*Il.fl.AP3.1*, *Il.fl.AP3.2* and *Il.fl.AP3.3*) exhibited different expression levels among floral organs. *Il.fl.AP3.1* was expressed strongly in stamens and inner tepals. *Il.fl.AP3.2* was expressed at a high level in inner tepals and stamens and a medium level in outer tepals. *Il.fl.AP3.3* was strongly expressed in inner tepals. Strong expression of the *AG* homolog (*Il.fl.AG*) was observed in inner tepals and stamens, and weak expression was observed in carpels.

Expression of the homologs of *AP1*, *AP3*, *PI* and *AGL2/9* from *Magnolia grandiflora* was described previously (Kim *et al.*, 2005) and is summarized in Table 1. Here, we present expression data for an *AG* homolog (*Ma.gr.AG*) and an *AGL6* homolog (*Ma.gr.AGL6*) from *M. grandiflora*. The large size of *Magnolia* flowers also permits a comparison of expression levels between floral organs from floral buds of 15 and 30 mm in diameter. Expression of *Ma.gr.AG* was observed in both stamens and carpels (Figure 5d). For *Ma.gr.AGL6*, strong expression was observed only in tepals, a result

similar to that observed for the *Amborella AGL6* homolog. The levels of expression at two different stages of floral development in *M. grandiflora* (flowers of 15 and 30 mm in diameter) were very similar for all four genes investigated (Figure 5d), but with minor differences. For example, a weak signal of *Ma.gr.AGL6* was detected in the spathaceous bract of the15 mm floral buds but not in the spathaceous bract of older buds (30 mm; just before anthesis). Also, weak expression of *Ma.gr.AP3* was detected in seeds 2 mm in diameter, but not in seeds 1 mm in diameter.

Homologs of *AP3* and *PI* were previously isolated (*As.lo.AP3* and *As.lo.PI*; Kim *et al.*, 2004) from *Asimina longifolia*. Both *As.lo.AP3* and *As.lo.PI* were expressed in petals and stamens (Figure 5e), but were either not expressed or only weakly expressed in sepals; the lack of detected expression in sepals differs from the results reported here for *AP3* and *PI* homologs in the outer perianth of other basal angiosperms.

Determining expression levels using real-time PCR

The real-time PCR results for *Nu.ad.PI* and *Nu.ad.AP3.1* generally agree with those obtained using RQ RT-PCR (Figure 5b). Both genes showed strong expression in all floral parts in relatively young floral buds (10–13 mm in diameter). However, *Nu.ad.PI* expression was not detected in carpels from open flowers (40 mm in diameter) using real-time PCR (Figure 6a) whereas expression was detected using RQ RT-PCR. Importantly, identical results were obtained for *Nu.ad.PI* using both RQ RT-PCR and real-time PCR when the same floral samples were used (Figure 6b). In addition, *Nu.ad.AP3.1*, but not *Nu.ad.PI*, was also expressed in primary roots and the immature seed (1 mm in diameter) (Figure 6a,b).

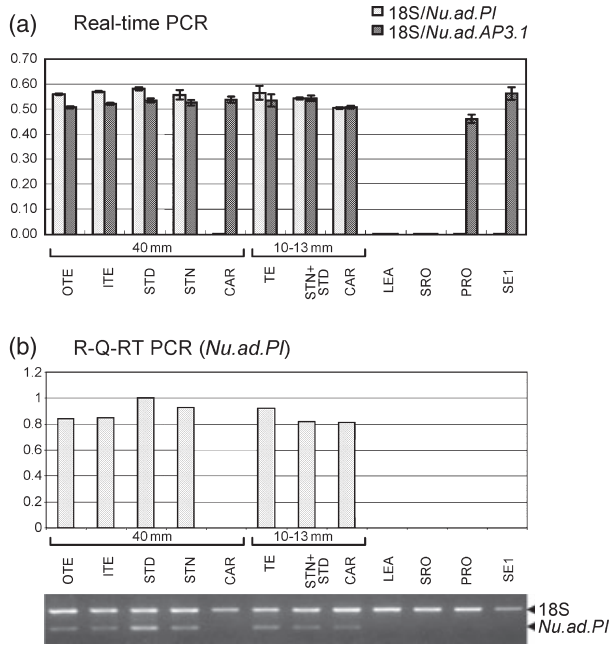

Figure 6. (a) The real-time PCR results for *AP3* and *PI* homologs of *Nuphar* (*Nu.ad.PI* and *Nu.ad.AP3.1*). The Ct value of the *18S rRNA* gene control was divided by those of *Nu.ad.PI* and *Nu.ad.AP3.1*.
(b) The relative quantitative (RQ) RT-PCR result for *Nu.ad.PI* using the same samples that were used in the real-time PCR experiment. Abbreviations are as in Figure 5. PRO, primary roots; SRO, secondary roots.

In situ *hybridization studies of* AP3 *and* PI *homologs in* Amborella *and* Nuphar

Our RQ RT-PCR experiments suggest that *AP3* and *PI* homologs in basal angiosperms tend to have broader expression than that reported for their eudicot counterparts. To obtain information on the spatial expression pattern of *AP3* and *PI* homologs, particularly at relatively early stages of flower development before the stages analyzed by the RT-PCR experiments, we performed RNA *in situ* hybridization experiments using gene-specific probes for *AP3* and *PI* homologs of *Amborella* and *Nuphar*. For *Amborella*, only male flowers were included in this study because of the very limited amount of material available for this taxon, which is restricted in nature to New Caledonia and is cultivated only rarely in botanical gardens.

A recent study of floral development in *Amborella* (Buzgo *et al.*, 2004) suggested the following developmental stages for male flowers: stage 1, flower initiation; stage 2, initiation of the transverse receptacular bracts and outer tepals; stage 3, initiation of inner perianth organs; stage 4, initiation of stamens; stage 6, development of sporophylls and of microsporangia; and stage 8, male meiosis (stages 5 and 7 occur in female flowers). For *Am.tr.PI* a strong signal was detected in the initiating tepals and in the primordia of other floral organs during stages 1–3 (Figure 7a). At stage 4, a strong signal was detected in the outer and inner tepals and also in initiating stamens (Figure 7b). At stages 6 and 8 (which follow the development of anthers), signals detected in tepals and in the connective tissue of the stamens were particularly high. A very strong signal was also detected in the anthers (Figure 7c). No expression was detected in bracteoles or receptacular bracts in any stage investigated (Figure 7a–c). The expression pattern of *Am.tr.AP3* was similar to that of *Am.tr.PI*. In stages 1–3, a strong signal was detected in the initiating tepals and in the primordia of stamens (Figure 7d). In stages 6 and 8, a relatively weak signal was detected in the tepals and filaments of the stamens, and a strong signal was detected in anthers (Figure 7f). Weak expression was also detected in the vascular bundles (Figure 7f).

The floral materials of *Nuphar advena* used in this study were limited; floral buds must be collected from natural populations and are only available seasonally. In addition, *Nuphar* floral buds develop individually in the axils of leaf

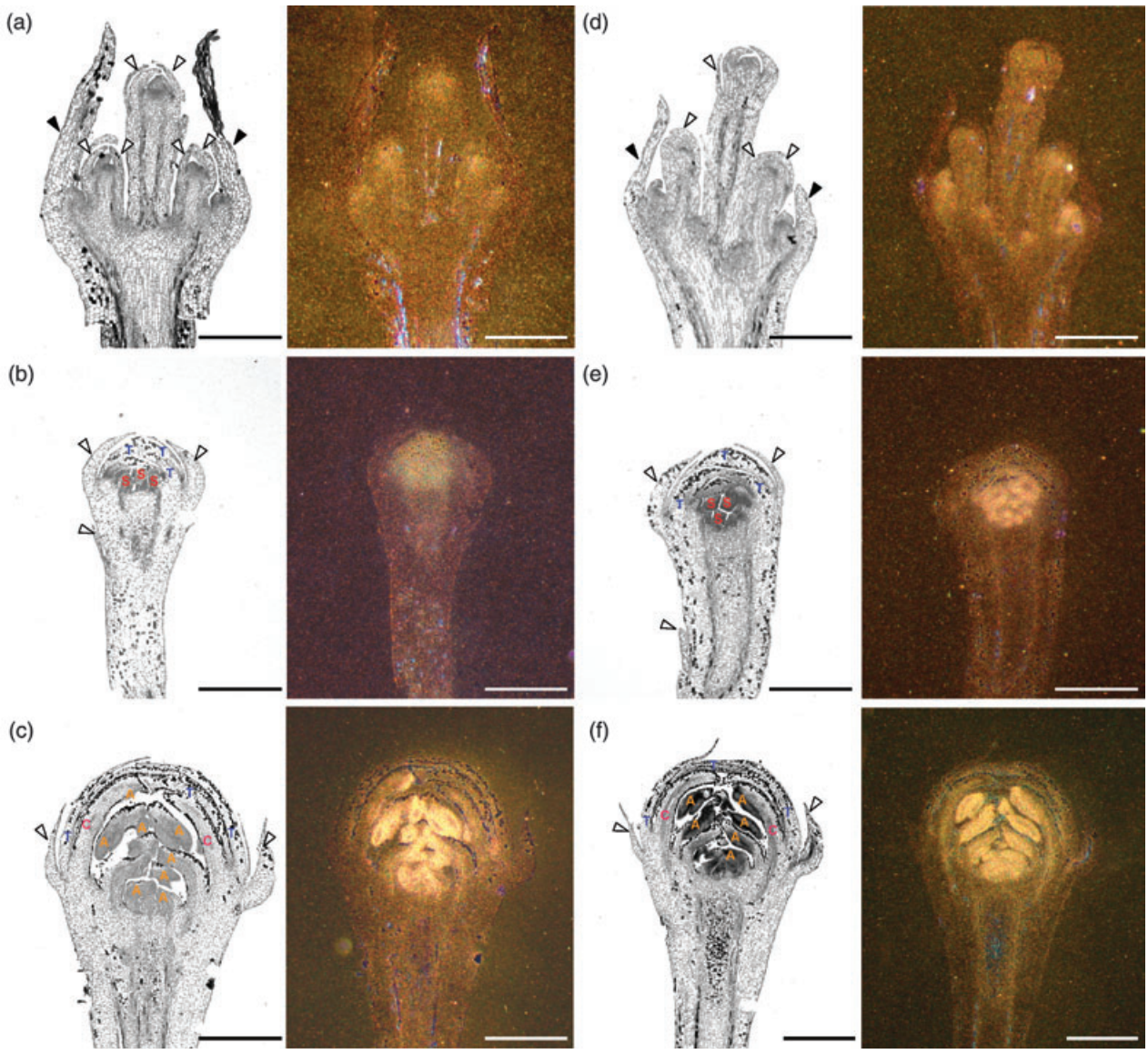

Figure 7. *In situ* hybridization using *Am.tr.PI* and *Am.tr.AP3* gene probes to longitudinal sections of developing *Amborella* flowers. Developmental stages follow Buzgo *et al.* (2004): a–c, *Am.tr.PI*; d–f, *Am.tr.AP3* (a and d stages 1–3, b and e stage 4, c and f stages 6, 8). Solid arrows indicate bracteoles, and open arrows indicate receptacular bracts. T, tepal; S, stamen; C, connective; A, anther. In each pair of images, the left image is bright field and the right image is dark field. All scale bars are 0.5 mm.

primordia. Therefore, the total number of very young floral buds available for analysis was small. Nonetheless, our experiments revealed that both *PI* and *AP3* homologs were expressed strongly in stamen and staminode primordia (Figure 8). In addition, expression of the *Nu.ad.AP3.2* and *Nu.ad.PI* genes was also clearly detectable in the inner tepals, and *Nu.ad.AP3.1* is also possibly expressed in the inner tepals (Figure 8, arrows). Very weak expression of these genes may have been present in the tips of the outer tepals (Figure 8, arrowheads). This pattern of expression was observed for a number of sections, suggesting that it is representative of the floral material at this stage of development. There was no detectable expression in the fused carpels at the center of the flower. The bright spots in the region below the gynoecium are not a true signal because they are not from the silver grains of the photosensitive emulsion, but rather from the tissue. Therefore, these spots represent non-specific background which can also be seen to some extent in the sense control (Figure S1).

We tested the specificity of our *in situ* probes using Southern blots. For the various MADS genes we tested, each probe was specifically hybridized to the gene from which it was derived under the same hybridization temperature

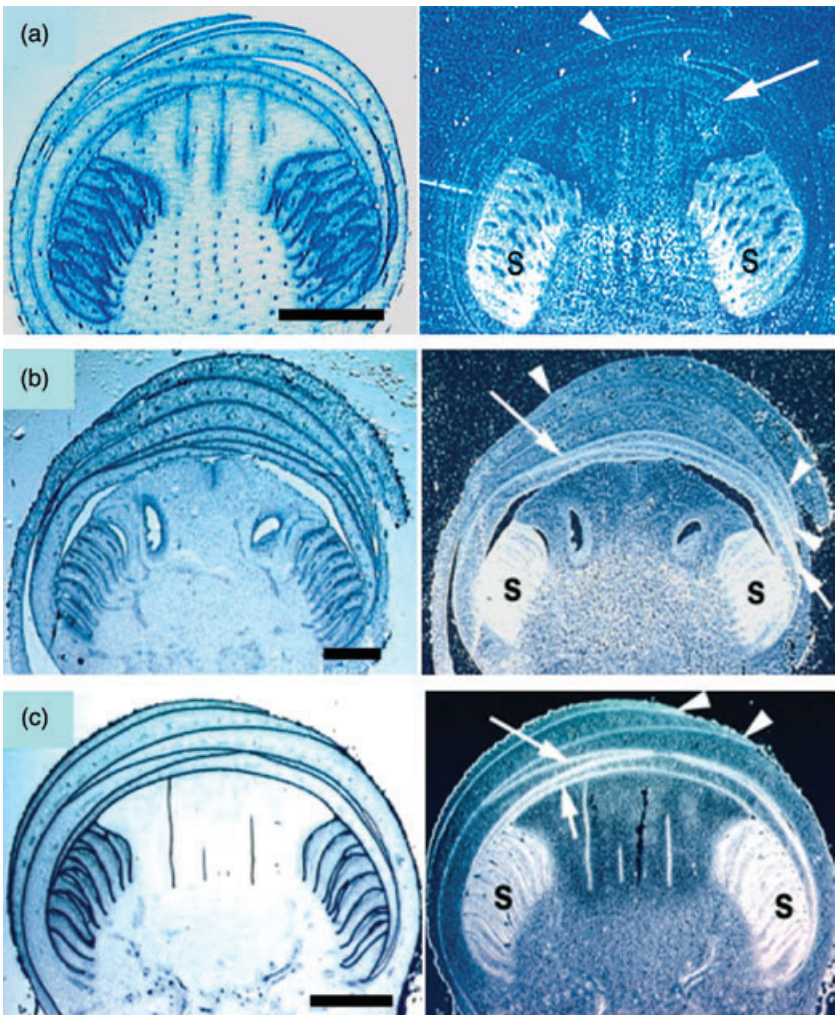

Figure 8. *In situ* hybridization using *Nu.ad.PI*, *Nu.ad.AP3.1* and *Nu.ad.AP3.2* gene probes to longitudinal sections of developing *Nuphar* flowers: (a) *Nu.ad.AP3.1*, (b) *Nu.ad.AP3.2*, (c) *Nu.ad.PI*. In each pair of images, the left image is bright field and the right image is dark field. All scale bars are 1.0 mm.

(42°C) and washing stringency (salt conditions) as those of the *in situ* experiments (Figure S2).

Evolutionary reconstruction of gene expression patterns

Our analyses of evolutionary transformations of floral MADS-box gene expression revealed that the *AP3* and *PI* homologs were 'strongly expressed' in all floral organs in the common ancestor of all extant angiosperms (Figure 9). However, after the first two branches of extant angiosperms (represented here by *Amborella* and *Nuphar*), the ancestral state of both *AP3* and *PI* homologs was 'not expressed/weakly expressed' in the carpels, and *AP3* was 'equivocal' in the outer perianth. The ancestral state for *PI* and *AP3* homologs in the eudicots was reconstructed in each as 'not expressed/weakly expressed' in the outermost floral organs.

The ancestral pattern of expression of *AG* homologs in angiosperms was restricted to the reproductive organs (Figure 9). However, in *Illicium* the *AG* homolog was expressed in the inner tepals and stamens, with 'no/weak' expression in carpels. In *Persea*, *AG* homologs were expressed in all floral organs. These expression patterns in *Illicium* and *Persea* appear to represent derived states for these taxa, based on our reconstructions, but more data are needed. For *AP1* homologs we reconstructed expression patterns for eu*FUL* genes and eu*AP1* genes separately. *AP1* homologs from basal angiosperms were compared with eu*FUL* genes. The ancestral state of the eu*FUL* lineage for all angiosperms was 'strongly expressed' in reproductive organs and 'equivocal' in perianth (Figure 9). Because eu*AP1* genes were found only in core eudicots (Litt and Irish, 2003), we reconstructed the ancestral state of eu*AP1* gene expression in core eudicots separately. Strong expression was restricted to the perianth in the ancestor of core eudicots (Figure 9). When we compare eu*AP1* genes and *FUL*-like genes of basal angiosperms, the perianth-specific expression reported for core eudicots (Hardenack *et al.*, 1994; Mandel *et al.*, 1992; Taylor *et al.*, 2002) is derived in our reconstructions.

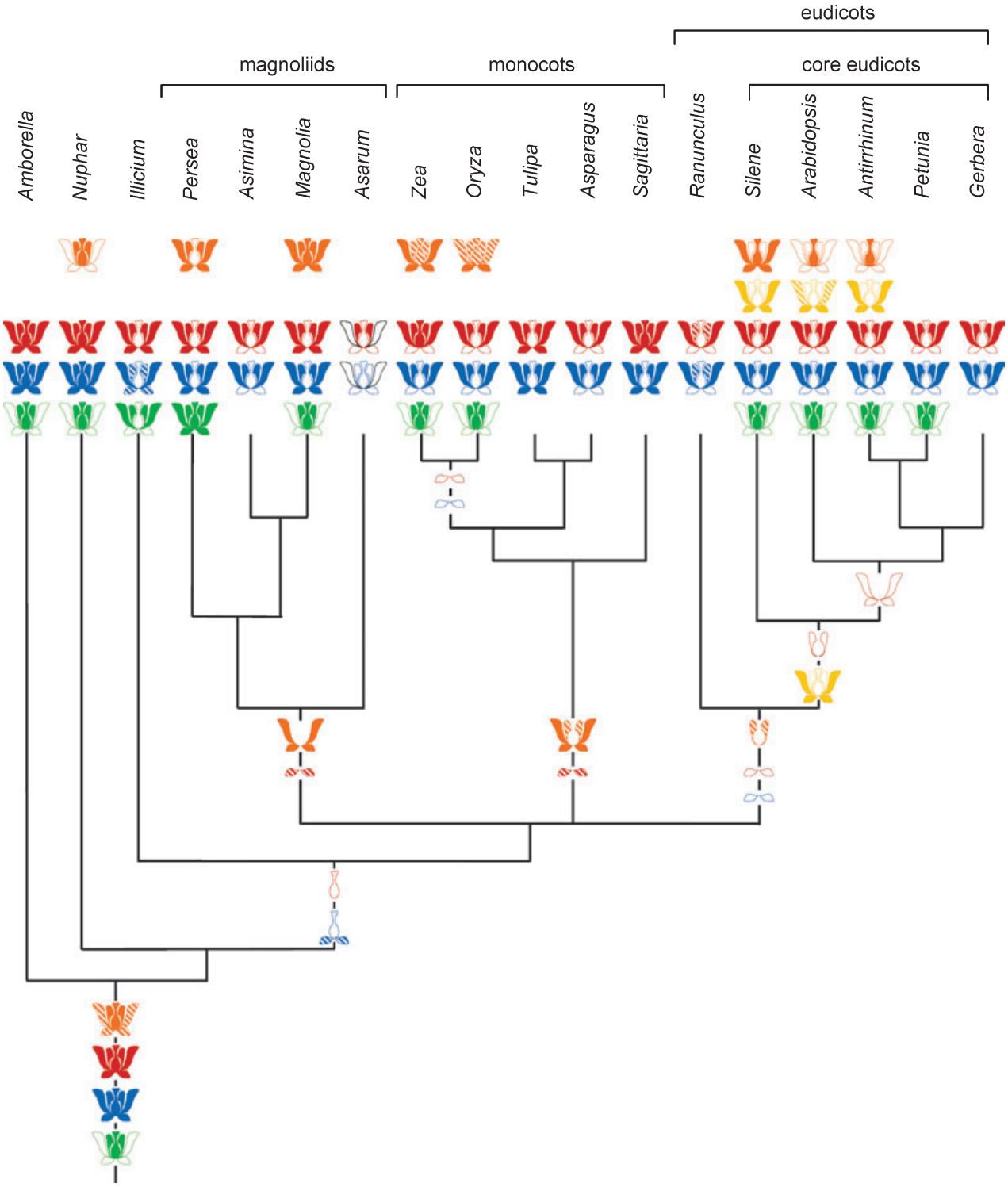

Figure 9. Evolution of gene expression patterns of floral MADS-box genes in angiosperms. Character states are indicated for each floral part: colored floral parts represent 'strongly expressed' genes (+++ and ++ in Table 1); open floral parts indicate that the genes are 'not expressed/weakly expressed' (– and + in Table 1). Dashed organs in the nodes indicate 'equivocal' status, and those in the terminals indicate 'uncertain (contain both character states)' status. Symplesiomorphic character states are indicated at each node. Each color represents a MADS gene subfamily/lineage: brown, *SQUA* (excluding the eu*AP1* lineage); yellow, the eu*AP1* lineage; red, *GLO*; blue, *DEF*; green, *AG*. Where expression data are not available or the homologous organ is not present, we used black.

Discussion

Conservation and divergence of expression patterns of floral MADS-box genes in angiosperms

To gain insights into the evolution of expression and function of floral MADS-box genes we examined the expression of homologs of known floral MADS-box genes from several basal angiosperms. Included among the taxa analyzed here are *Amborella* and *Nuphar*, phylogenetically pivotal taxa that are the successive sister groups to all other extant flowering plants. We obtained expression data using RQ RT-PCR, real-time PCR and RNA *in situ* hybridization experiments. The results obtained from these different approaches agreed closely when the same stage of floral development was assayed, supporting the reliability of the results. The minor differences in expression patterns among methods are likely to be due to sampling and technical differences. For example, the RQ RT-PCR data are generally for later stages of floral development, but *in situ* hybridization data are presented for early stages of floral development. It will be very useful in the future to obtain additional *in situ* hybridization data for earlier stages of floral development for additional genes in more basal angiosperm taxa to complement the RQ RT-PCR results reported here.

The results we provide here, together with expression data reported from other studies (summarized in Table 1), indicate that most of the homologs of known floral genes isolated from basal angiosperms are expressed in those floral organs that are functionally and/or morphologically similar to the organs in genetic model organisms (e.g. Arabidopsis and *Antirrhinum*) that require these genes. For example, in basal angiosperms *AP3* and *PI* homologs are expressed in petal-like perianth organs (e.g. tepals) and stamens, and *AG* homologs are expressed in stamens and carpels. Similarly, *SEP* homologs are expressed in all floral organs. At the same time, many of these genes also exhibit expression in organs other than those expected on the basis of the known function of their homologs in Arabidopsis or *Antirrhinum*. For example, expression of some *AP3*/*PI* homologs was detected in carpel tissue of basal angiosperms, and expression of *AG* homologs was observed in the perianth of some basal taxa. Therefore, homologs of *AP3*, *PI*, *AG* and *SEP* from basal angiosperms generally have expression patterns that include a conserved component, as well as a broader component, when compared with their counterparts in well-characterized genetic systems (Goto and Meyerowitz, 1994; Jack *et al.*, 1992; Ma *et al.*, 1991; Mandel and Yanofsky, 1998; Mandel *et al.*, 1992). In contrast, homologs of the *AP1* gene from basal angiosperms show a pattern of expression that is very different from that reported in model systems: homologs of *AP1* from basal angiosperms show strong expression in leaves, perianth and reproductive organs, unlike *AP1* which is expressed only in the perianth in Arabidopsis and other eudicots (Mandel *et al.*, 1992).

In *Amborella*, *Nuphar* and the other basal angiosperms having an undifferentiated perianth (e.g. *Magnolia*, *Illicium*, *Persea*), homologs of the B-class genes *PI* and *AP3* are generally expressed in both the outer and inner perianth organs. Hence, it may be that expression of *PI* and *AP3* homologs in the outer whorl typifies most basal angiosperms. There is also evidence for the expression of *PI* and *AP3* homologs in the outer perianth in some basal eudicots (e.g. Ranunculaceae). Even in the core eudicots, expression of *PI* and *AP3* has been detected at very early stages in the outer perianth (Jack *et al.*, 1992; van der Krol *et al.*, 1993; Schwarz-Sommer *et al.*, 1992; Tsuchimoto *et al.*, 2000).

Considering the patterns of expression of homologs of *AG*, *AP3*/*PI* and *AP1*, our reconstructions indicate that, within angiosperms, the ancestral expression pattern of *AG* homologs is most similar to that observed in core eudicots (Bradley *et al.*, 1993; Davies *et al.*, 1999; Kater *et al.*, 1998; Kempin *et al.*, 1993; Yanofsky *et al.*, 1990). In contrast, the *AP3* and *PI* homologs have ancestral states suggesting broader expression patterns in early angiosperms than those observed in eudicots (Angenent *et al.*, 1993; Davies *et al.*, 1996; Goto and Meyerowitz, 1994; Hardenack *et al.*, 1994; Jack *et al.*, 1994; van der Krol *et al.*, 1993; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Yu *et al.*, 1999). Finally, the ancestral state for the expression of *AP1* homologs differs dramatically from the expression pattern found in eu*AP1* genes of core eudicots (Hardenack *et al.*, 1994; Mandel *et al.*, 1992; Taylor *et al.*, 2002), suggesting that the perianth-specific expression reported for the core eudicots (Hardenack *et al.*, 1994; Mandel *et al.*, 1992; Taylor *et al.*, 2002) is derived and associated with a gene duplication (see below).

Functional implications for MADS-box genes in basal angiosperms

Genetic and molecular studies in core eudicots and monocots support a strong correlation between the pattern of expression and function of floral MADS-box genes, although sometimes transient and/or weak expression does not correspond to a known genetic function (Goto and Meyerowitz, 1994; Jack *et al.*, 1992; van der Krol *et al.*, 1993; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Tsuchimoto *et al.*, 2000). Therefore, the expression patterns reported here strongly suggest that *AG* homologs in basal angiosperms probably promote the development of reproductive organs, as *AG* and *PLE* do in Arabidopsis and *Antirrhinum*, respectively. The detection of *AG* homologs in the perianth of both *Persea* and *Illicium* suggests that expression of *AG* in all floral organs in these taxa is independently derived. These two genera are not closely related among basal angiosperms, with *Illicium* being a member of

Austrobaileyales and *Persea* a member of Laurales in the magnoliid clade (Figure 1). Our results indicate that patterns of expression of the *AP3*/*PI* homologs in basal angiosperms are generally broader than the patterns of expression of these genes in model core eudicots, such as Arabidopsis. For example, in both *Amborella* and *Nuphar, AP3* and *PI* homologs are expressed in the perianth and carpels, as well as in stamens. Because the expression in the inner perianth and stamens is either stronger or detected at both early and late stages it is likely that these genes function to control the identity of these organs, similar to their respective homologs in core eudicots (Angenent *et al.*, 1993; Davies *et al.*, 1996; Goto and Meyerowitz, 1994; Hardenack *et al.*, 1994; Jack *et al.*, 1994; van der Krol and Chun, 1993; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Yu *et al.*, 1999; Zahn *et al.*, 2005b). The expression of these *AP3*/*PI* homologs in the outer perianth and carpels could represent function, because the outer perianth of many basal angiosperms has petal-like characteristics. Furthermore, a series of morphological transitions from bracts through tepals and stamens to carpels observed in *Amborella* (Buzgo *et al.*, 2004) also supports possible functioning of these genes in outer perianth and carpels. Alternatively, such expression could be a 'molecular fossil' left from an ancestral state that is no longer needed today.

Functional studies of *AP1* homologs are very limited, and members of this subfamily are functionally divergent (reviewed in Litt and Irish, 2003). To date, *AP1* is the only gene in the *SQUA* subfamily that has been shown to confer the A function (Bowman *et al.*, 1993; Litt and Irish, 2003). In *Antirrhinum*, the expression pattern of the *AP1* homolog *SQUA* is the same as that of *AP1*; however, mutant analysis did not demonstrate the A function of *SQUA* (Huijser *et al.*, 1992; Taylor *et al.*, 2002). A gene duplication generated the eu*AP1* and eu*FUL* lineages during the evolution of the *SQUA* subfamily, perhaps in the common ancestor of the eudicots (Litt and Irish, 2003). Expression of *AP1* homologs is broader in basal angiosperms than in core eudicots (i.e. eu*AP1* genes), suggesting that *AP1* homologs may have different functions in basal taxa. In *Magnolia* and *Nuphar* expression of the *AP1* homolog was higher in leaves and carpels than in the perianth and stamens, resembling the expression of members of the eu*FUL* lineage rather than that of members of the eu*AP1* lineage (Irish, 2003; Litt and Irish, 2003).

Phylogenetic analyses support a close relationship among the *SQUA*, *AGL2 (SEP)* and *AGL6* subfamilies of the MADS-box gene family (Becker and Theissen, 2003). *SEP* homologs from basal angiosperms are expressed in all floral organs, similar to some *SEP* genes in Arabidopsis, suggesting that these genes may have similar functions in basal angiosperms to those reported in Arabidopsis.

When the ABC model was proposed to explain the genetic control of the identity of floral organs on the basis of similar homeotic mutants in Arabidopsis and *Antirrhinum*, most of the corresponding floral homeotic genes had not yet been cloned (Coen and Meyerowitz, 1991). The subsequent molecular analysis of these genes revealed that most of them encode highly similar MADS-box genes, raising the question of whether their homologs in other angiosperms also have similarly conserved functions. Our data support the idea that *AG* homologs have a conserved function in angiosperms that is required for the C function of the ABC model. Similarly, *AP3* and *PI* homologs probably also have conserved functions necessary for the B function for petal (inner perianth) and stamen identities. In addition, *AP3* and *PI* homologs in basal angiosperms may have a broader function, extending to the outer perianth, particularly if the outer perianth resembles petals as in the case of *Amborella*, *Nuphar*, *Magnolia* and many other basal angiosperms. Furthermore, this broader pattern of B function may represent the ancestral condition for angiosperms. On the other hand, *AP1* homologs in basal angiosperms do not seem to share conserved functions that specifically control the identity of the outer perianth, as in Arabidopsis (Bowman *et al.*, 1993; Mandel *et al.*, 1992). Recent studies indicate that four *SEP* genes (previously *AGL2*, *–3*, *–4* and *–9*) redundantly control sepal identity and contribute to the specification of other floral organs (Ditta *et al.*, 2004; Pelaz *et al.*, 2000; Theissen, 2001). Because the *SQUA* and *AGL2* subfamilies are phylogenetically closely related it is possible that, in basal angiosperms, both *SQUA* and *AGL2* subfamily members together control the identity of the outer perianth and also contribute to the identity of other floral organs. The expression of *AP1* homologs in basal angiosperms is also similar to that of *AGL3 (SEP4)* in Arabidopsis (Huang *et al.*, 1995). In addition, the *AGL6* homolog of *Magnolia* was strongly expressed only in tepals (Figure 5d). In the case of *Amborella*, although *Am.tr.AGL6* is expressed in all floral organs, the strongest expression was found in tepals (Figure 5a). Therefore, other candidates for A-function genes in basal angiosperms could be *AGL6* homologs.

MADS-box gene expression and perianth differentiation

Most basal angiosperms do not exhibit a well-differentiated perianth of sepals and petals (e.g. Endress, 2001). Rather, the perianth typically consists of tepals. The broad expression of *AP3*/*PI* homologs observed throughout the undifferentiated perianth of many basal angiosperms (e.g. *Amborella*, *Nuphar* and *Magnolia*) complements the lack of a clear morphological distinction between sepals and petals in these taxa. In *Nuphar*, the outer perianth organs are green and more 'sepal-like'; the inner perianth organs are brightly colored and more 'petal-like' (Figure 2b). However, this distinction is not clear-cut, and the perianth is often described as consisting of tepals.

In *Asimina*, a morphologically well-differentiated perianth of sepals and petals (Figure 2d) corresponds very well with

the observation that the expression pattern of both *AP3* and *PI* homologs in *Asimina* is the same as that in Arabidopsis: expression of *AP3* and *PI* homologs was not observed in sepals, but was detected in petals and stamens. *Asimina* clearly represents an independent derivation of a differentiated perianth from that observed in eudicots (e.g. Albert *et al.*, 1998; Ronse De Craene *et al.*, 2003). It is noteworthy, therefore, that the pattern of expression of the *AP3*/*PI* homolog is similar in these phylogenetically well-separated taxa.

Experimental procedures

Plant materials

We collected samples from the following sources: *Amborella trichopoda*, plants cultivated at the National Tropical Botanical Garden, HI, USA (DL8346, TF6481, DHL8350, HAW); *N. advena*, plants collected in Black Moshannon State Park, PN, USA (*S. Kim 1140*, FLAS); *Illicium floridanum*, plants cultivated on the campus of the University of Florida, Gainesville, FL, USA (*S. Kim 1139*, FLAS); *A. longifolia*, a plant collected in Morningside Park, Gainesville, FL, USA (*S. Kim 1129*, FLAS); *M. grandiflora*, a plant cultivated on the campus of the University of Florida, Gainesville, FL, USA (*S. Kim 1138*, FLAS). Entire flowers at varying stages of early development up to anthesis were removed and dropped immediately into liquid nitrogen and stored at −80°C.

We used two general methods to isolate and characterize genes for floral organ identity. The first method used expressed sequence tags (ESTs) obtained as part of the Floral Genome Project (Albert *et al.*, 2005; Soltis *et al.*, 2002; http://fgp.bio.psu.edu/cgi-bin/fgpmine/index.cgi). We subsequently obtained finished sequences of these ESTs using M13 and M13 reverse universal primers. The second method involved isolating RNA from floral buds and developing flowers, making cDNA, and using RT-PCR following the general methods of Kim *et al.* (2004) (described below).

RNA extraction, RT-PCR and cDNA sequence determination

RNA extractions for all taxa were performed following a modified method of the RNeasy Plant Mini Kit (Qiagen, Stanford, CA, USA). The modification includes a portion of the CTAB DNA extraction protocol (Doyle and Doyle, 1987) and subsequent use of the RNeasy Plant Mini Kit. This method was originally developed for the successful extraction of RNA from basal angiosperms such as *Amborella* and *Nuphar* (Kim *et al.*, 2004). Reverse transcription was performed following the manufacturer's directions using SuperScript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and polyT primer (5′-CCG GAT CCT CTA GAG CGG CCG $C(T)_{17}$-3′). PCR reactions were performed using a MADS gene-specific degenerate primer (5′-GGG GTA CCA AYM GIC ARG TIA CIT AYT CIA AGM 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, USA). Purified DNAs were cloned using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNAs were purified from cloned cells using the FastPlasmid Mini Kit (Eppendorf, Westbury, NY, USA). Cycle sequencing reactions were performed using the CEQ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA), and cDNA sequences were determined using a CEQ 8000 sequencing system (Beckman Coulter).

Characterization and identification of genes

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 data set of 82 sequences representing all subfamilies of $MIKC^C$-type MADS genes (Becker and Theissen, 2003).

For each gene identified as a putative member of the MADS-box gene family, we used the following naming system (see also Kim *et al.*, 2004). Each gene was named using the first two letters of the genus name, followed by a full stop, and the first two letters of the specific epithet; this was, in turn, followed by the abbreviated name of its phylogenetically closest homolog in Arabidopsis. For example, a putative *AGAMOUS* homolog isolated and identified from *A. trichopoda* was abbreviated as: *Am.tr.AG*.

Amino acid alignment was conducted using CLUSTAL X (ver. 1.83; Thompson *et al.*, 1997) with manual adjustment. Maximum parsimony (MP) analysis was performed on the amino acid data set using PAUP* 4.0b10 (Swofford, 2001). The search strategy involved 100 random addition replicates with tree-bisection–reconnection (TBR) branch swapping, saving all optimal trees. To assess support for each node, bootstrap analysis (Felsenstein, 1985) was performed using 100 bootstrap replicates, each with 10 random addition replicates and TBR branch swapping, saving all optimal trees.

Expression studies

For the examination and quantification of gene expression, we used RQ RT-PCR. We also present comparable data for *Nuphar* B-class homologs based on real-time PCR. *In situ* hybridization studies were employed for B-class homologs in both *Amborella* and *Nuphar*.

For the RQ RT-PCR and real-time PCR analyses we used samples collected just before anthesis for all taxa used in this study. Flowers were dissected while completely frozen. Separated piles of floral parts from almost-opened flowers were made while carefully working to ensure that all parts remained frozen: tepals, stamens, staminodes (for *Nuphar*) and carpels. Young leaves were also analyzed. For *Amborella*, both male and female flowers were included. Although tepals of *Amborella* showed gradual morphological changes from the outermost to the innermost positions, only inner tepals were sampled separately because the outer tepals were too small to collect. Tepals from female flowers were collected together with staminodes. Tepals of *Illicium* exhibit similar morphological transitions to those of *Amborella*. Samples of tepals from both the outermost and innermost whorls of *Illicium* were prepared and analyzed for expression of floral genes. As a reference, we also examined the expression patterns of the *Antirrhinum DEF* and *GLO* genes using RQ RT-PCR.

Total RNAs were extracted from each sample using the RNeasy Plant Mini Kit (Qiagen). After RNA extraction we treated samples with DNase to avoid potential contamination by genomic DNA (DNase-free kit from Ambion, Austin, TX, USA). Reverse transcription using RNA from each floral part was performed following the manufacturer's directions using SuperScriptTM II RNase H-reverse transcriptase (Invitrogen). We used random-hexamer oligonucleotides for the reverse transcription instead of polyT primer because the *18S rRNA* gene was used as an internal control of quantification in both RQ RT-PCR and real-time PCR.

Relative-quantitative-RT PCR. Methods followed those used previously (Kim *et al.*, 2005). We performed multiplex PCR using a gene-specific primer pair (Figure S3), the *18S rRNA* gene primer pair (internal control), and a competitive primer pair to the *18S rRNA* gene primers (competimers) following the protocol of QuantumRNA (Ambion). 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 QuantumRNA kit were used to reduce the *18S rRNA* 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 rRNA* and that of the mRNA of each gene. The optimal ratio ranged from 3:7 to 6:4 for the genes that we examined. PCR reactions for all genes were performed with 26 or 27 cycles (except *Nu.ad.AP3.2* which needed 29 cycles) at 95°C (30 sec), 55°C (30 sec) and 72°C (30 sec) using an Eppendorf Mastercycler (Brinkmann, Westbury, NY, USA). A range of 1–256 ng of total RNA (back-calculated from the amount of cDNA used in the PCR reaction after the RT-PCR) was tested, and 16–64 ng of total RNA were found to generate accumulation of unsaturated PCR product for each gene through 26 or 27 cycles of PCR. We used 25 ng of total RNA for the RQ RT-PCR. For all PCRs, we used a negative control that did not contain cDNA template. 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, USA). Three to 15 replicates of RQ RT-PCR were performed for each gene using cDNAs from more than two independent RNA samples extracted from different individuals. The relative PCR signal of the specific gene to the *18S rRNA* gene and its standard deviation were calculated for each floral organ. The gene specificity of each PCR product was confirmed by sequencing all PCR products. To compare relative expression levels among taxa we (i) converted the highest expression value in each gene to 1, (ii) made a histogram showing the expression of each gene in each organ using relative values based on the conversion in (i), and (iii) evaluated the relative amount of expression in each histogram as follows: −, not expressed; +, <0.1; ++, 0.1–0.4; +++, 0.4–1. We analyzed two different developmental stages using floral materials of *M. grandiflora* for *Ma.gr.AP3*, *Ma.gr.PI*, *Ma.gr.AG* and *Ma.gr.AGL6*. Samples of floral organs were obtained from the dissection of floral buds 15 and 30 mm in diameter, respectively.

Real-time PCR. We also used real-time PCR to investigate expression of the *AP3* and *PI* homologs of *N. advena* (*Nu.ad.AP3.1* and *Nu.ad.PI*). This method includes a third primer as a probe and a fluorescent dye-labeled system and can provide a relative quantification of expression (Chiang *et al.*, 1996; Leutenegger *et al.*, 1999). We analyzed samples of floral organs from young floral buds (1.0–1.5 mm in diameter) and open flowers (40 mm in diameter) and samples from vegetative organs. The probe and primers for real-time PCR were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). The designations of primers and probes are indicated in Figure S3. We performed PCR reactions with TaqMan® Universal PCR Master Mix (Applied Biosystems) using a GeneAmp® 5700 Sequence Detection System (Applied Biosystems) following the recommendations of the manufacturer. A TaqMan® Ribosomal RNA Control Reagent (Applied Biosystems) was used for the internal control of each sample. Five independent reactions were performed for each sample. The relative ratio of threshold cycle (Ct) values between the *18S rRNA* gene and the specific gene and their standard deviations were calculated for each sample.

RNA in situ *hybridization.* For the *in situ* hybridization study we employed B-class homologs of *A. trichopoda* and *N. advena*. Fresh *Amborella* and *Nuphar* floral bud samples were immediately fixed in formaldehyde–acetic acid–ethanol (FAA) for 4–6 h. For *Amborella*, gene-specific primers were designed in the C-domain and a part of the K-domain to make RNA probes (Figure S3). PCR products of these regions were purified and cloned using the pGEM® T-vector system (Promega, Madison, WI, USA). ^{35}S-dUTP-labeled RNA probes were synthesized by *in vitro* transcription: antisense and sense (negative control) transcripts were generated by using either T7 or T3 RNA polymerase (Promega). For *Nuphar*, an antisense RNA probe for *Nu.ad.PI* was transcribed from the plasmid 30M-A20 from the Nad03 floral cDNA library linearized with *Bam*HI with T7 RNA polymerase in the presence of ^{35}S-UTP (K and C domains). Antisense RNA probes for *Nu.ad.AP3.1* and *Nu.ad.AP3.2* were synthesized using T7 RNA polymerase with *Eco*RI-linearized plasmid 19M-E24 and *Apo*l-linearized plasmid 37M-E01, respectively, from the Nad03 floral cDNA library. A sense RNA probe was generated using T3 RNA polymerase with *Xho*l-linearized 30MS2-A10. Information on clones used in this experiment is available at http://fgp.bio.psu.edu/cgi-bin/fgpmine/index.cgi. The transcripts were partially hydrolyzed by incubation at 60°C in 0.1 M Na_2CO_3·$NaHCO_3$ buffer, pH 10.2, for 45 min. The sample embedding, hybridization, washing and autoradiography were performed as described previously (Drews *et al.*, 1991; Flanagan and Ma, 1994).

Character-state reconstruction

To investigate the diversification of expression patterns of A-, B-, C- and E-class homologs across angiosperms we conducted a character-state reconstruction using MACCLADE (ver. 3.04; Maddison and Maddison, 1992) and a phylogenetic framework for angiosperms inferred from recent multigene analyses (e.g. Qiu *et al.*, 1999; P. Soltis *et al.*, 1999; D. Soltis *et al.*, 2000; Zanis *et al.*, 2002; reviewed in Soltis and Soltis, 2004). We used the 'all most parsimonious states' optimization in MACCLADE because the accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN) optimizations cannot be applied when a polytomy is present.

In addition to our expression data for A-, B-, C- and E-class homologs in basal angiosperms, we added expression data from major lineages of angiosperms (see Figure S4). We selected *Silene*, Arabidopsis, *Antirrhinum*, *Petunia* and *Gerbera* as well-studied representatives of core eudicots (summarized in De Bodt *et al.*, 2003; Irish, 2003); *Ranunculus* was used as a representative of basal eudicots (Kramer *et al.*, 2003), and *Zea*, *Oryza*, *Tulipa*, *Asparagus* and *Sagittaria* were added as representatives of monocots (summarized in De Bodt *et al.*, 2003; Irish, 2003; Kanno *et al.*, 2003; Park *et al.*, 2003, 2004). For basal angiosperms, *Nuphar* (Zahn *et al.*, 2005a), *Asarum* (Piperales; Kramer and Irish, 2000), *Persea* (Laurales; A. Chanderbali, SK, M. Buzgo, Z. Zheng, PS and DS, unpublished data) and *Magnolia* (Magnoliales; Kim *et al.*, 2005) were added to the data presented here. Because some of the MADS-box gene subfamilies contain major duplications, only genes representing one of the duplicates were included. In our analyses of B-class genes, *TM6* genes (in the *DEF* subfamily) were excluded because they do not show B function (Kim *et al.*, 2004). D-class genes in the *AG* subfamily (Kramer *et al.*, 2004) were excluded. The expression of *AP1* homologs in basal angiosperms was more similar to that reported for eu*FUL* genes than for eu*AP1* genes (Litt and Irish, 2003). Therefore, we compared *AP1* homologs of basal angiosperms with eu*FUL* genes. For eu*AP1*

genes, we only reconstructed character evolution in core eudicots. Because our purpose was to address the evolution of 'strong' expression (which is more likely than 'weak' expression to be associated with function) and because we used binary character coding, weak expression (i.e. '+' in Table 1) was treated as '0'. If patterns of expression of multiple genes within a species differed (e.g. the expression of *II.fl.AP3.1*, *II.fl.AP3.2* and *II.fl.AP3.3* in outer perianth and stamens of *I. floridanum*), we considered the expression for that gene in that species as 'equivocal' (Figure S4). For *Amborella*, with the exception of *PI* and *AP3* (which were examined via *in situ* hybridization), we did not address expression in the outermost tepals because of the small size of the flowers. Therefore, the expression of C-class homologs in the outer perianth of *Amborella* was considered 'uncertain' (Figure 9 and Figure S4).

Acknowledgements

We thank Doug Yul Sung and Ilho Kang for technical advice regarding RQ RT-PCR experiments and Laura Zahn and Donglan Tian for help with *Nuphar in situ* hybridization experiments. We also thank Andre Chanderbali, Matyas Buzgo, Günter Theissen, Samuel Brockington, Laura Zahn and Jim Leebens-Mack for helpful comments and discussion. This study was supported by NSF grant PGR-0115684.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Sense control of *in situ* hybridization.

Figure S2. Specificity test of probes used for *in situ* hybridization experiments.

Figure S3. Primers used for R-Q-RT PCR, real-time PCR, and *in situ* hybridization.

Figure S4. Character states used in the maximum parsimony character reconstruction.

References

Albert, V.A., Gustafsson, M.H.G. and Di Laurenzio, L. (1998) Ontogenetic systematics, molecular developmental genetics, and the angiosperm petal. In *Molecular Systematics of Plants II* (Soltis, P.S., Soltis, D.E. and Doyle, J.J., eds). Boston: Kluwer Academic Publishers, pp. 349–374.

Albert, V.A., Soltis, D.E., Carlson, J.E. *et al.* (2005) Floral gene resources from basal angiosperms for comparative genomics research. *BMC Plant Biol.* **5**, 1–15.

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F. and Schmidt, R.J. (2000) Molecular and genetic analyses of the *Silky1* gene reveal conservation in floral organ specification between eudicots and monocots. *Mol. Cell*, **5**, 569–579.

Angenent, G.C., Busscher, M., Franken, J., Mol, J.N.M. and van Tunen, A.J. (1992) Differential expression of two MADS box genes in wild-type and mutant *Petunia* flowers. *Plant Cell*, **4**, 983–993.

Angenent, G.C., Franken, J., Bussher, M., Colombo, L. and van Tunen, A.J. (1993) Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1*. *Plant J.* **4**, 101–112.

APGII (2003) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Bot. J. Linn. Soc.* **141**, 399–436.

Barkman, T., Chenery, J.G., Mcneal, J.R., Lyons-Weiler, J. and De Pamphilis, C.W. (2000) Independent and combined analyses of sequences from all three genomic compartments converge on the root of flowering plant phylogeny. *Proc. Natl Acad. Sci. USA*, **97**, 13166–13171.

Baum, D.A. (1998) The evolution of plant development. *Curr. Opin. Plant Biol.* **1**, 79–86.

Becker, A. and Theissen, G. (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Mol. Phylogenet. Evol.* **29**, 464–489.

Borsch, T., Hilu, K.W., Quandt, D., Wilde, V., Neinhuis, C. and Barthlott, W. (2003) Non-coding plastid *trnT-trnF* sequences reveal a highly supported phylogeny of basal angiosperms. *J. Evol. Biol.* **15**, 558–567.

Bowman, J.L. (1997) Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. *J. Biosci.* **22**, 515–527.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D.R. (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development*, **119**, 721–743.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N. and Coen, E. (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of *Antirrhinum*. *Cell*, **72**, 83–95.

Buzgo, M., Soltis, D.E. and Soltis, P.S. (2004) Floral developmental morphology of *Amborella trichopoda* (Amborellaceae). *Int. J. Plant Sci.* **165**, 925–947.

Chiang, P.W., Song, W.J., Wu, K.Y., Korenberg, J.R., Fogel, E.J., Van Keuren, M.L., Lashkari, D. and Kurnit, D.M. (1996) Use of a fluorescent-PCR reaction to detect genomic sequence copy number and transcriptional abundance. *Genome Res.* **6**, 1013–1026.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31–37.

Colombo, L., Franken, J., Koetje, E., Vanwent, J., Dons, H.J.M., Angenent, G.C. and Vantunen, A.J. (1995) The petunia MADS box gene *FBP11* determines ovule identity. *Plant Cell*, **7**, 1859–1868.

Cronquist, A. (1988) *The Evolution and Classification of Flowering Plants*, 2nd edn. Bronx: New York Botanical Garden.

Davies, B., DiRosa, A., Eneva, T., Saedler, H. and Sommer, H. (1996) Alteration of tobacco floral organ identity by expression of combinations of *Antirrhinum* MADS-box genes. *Plant J.* **10**, 663–677.

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1999) *PLENA* and *FARINELLI*: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. *EMBO J.* **18**, 4023–4034.

De Bodt, S., Raes, J., de Peer, Y.V. and Theissen, G. (2003) And then there were many: MADS goes genomic. *Trends Plant Sci.* **8**, 475–483.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S. and Yanofsky, M.F. (2004) The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr. Biol.* **14**, 1935–1940.

Doyle, J.J. and Doyle, J.L. (1987) A rapid DNA isolation from small amount of fresh leaf tissue. *Phytochem. Bull.* **19**, 11–15.

Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell*, **65**, 991–1002.

Drinnan, A.N., Crane, P.R. and Hoot, S.B. (1994) Patterns of floral evolution in the early diversification of non-magnoliid dicotyledons (eudicots). *Plant Syst. Evol. Suppl.* **8**, 93–122.

Endress, P.K. (2001) The flowers in extant basal angiosperms and inferences on ancestral flowers. *Int. J. Plant Sci.* **162**, 1111–1140.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Ferrandiz, C., Gu, Q., Martienssen, R. and Yanofsky, M.F. (2000) Redundant regulation of meristem identity and plant architecture by *FRUITFULL*, *APETALA1*, and *CAULIFLOWER*. *Development*, **127**, 725–734.

Flanagan, C.A. and Ma, H. (1994) Spatially and temporally regulated expression of the MADS-box gene *AGL2* in wild-type and mutant *Arabidopsis* flowers. *Plant Mol. Biol.* **26**, 581–595.

Flanagan, C., Hu, Y. and Ma, H. (1996) Specific expression of the *AGL1* MADS-box gene suggests regulatory functions in *Arabidopsis* gynoecium and ovule development. *Plant J.* **10**, 343–353.

Goto, K. and Meyerowitz, E.M. (1994) Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes Dev.* **8**, 1548–1560.

Graham, S.W. and Olmstead, R.G. (2000) Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. *Am. J. Bot.* **87**, 1712–1730.

Graham, S.W., Reeves, P.A., Burns, A.C.E. and Olmstead, R.G. (2000) Microstructural changes in noncoding chloroplast DNA: interpretation, evolution, and utility of indels and inversions in basal angiosperm phylogenetic inference. *Int. J. Plant Sci.* **161** (Suppl.), S83–S96.

Gu, Q., Ferrandiz, C., Yanofsky, M.F. and Martienssen, R. (1998) The *FRUITFULL* MADS-box gene mediates cell differentiation during *Arabidopsis* fruit development. *Development*, **125**, 1509–1517.

Hardenack, S., Ye, D., Saedler, H. and Grant, S. (1994) Comparison of MADS box gene expression in developing male and female flowers of the dioecious plant white campion. *Plant Cell*, **6**, 1775–1787.

Hilu, K.W., Borsch, T., Muller, K. *et al.* (2003) Inference of angiosperm phylogeny based on *matK* sequence information. *Am. J. Bot.* **90**, 1758–1776.

Huang, H., Tudor, M., Weiss, C.A., Hu, Y. and Ma, H. (1995) The *Arabidopsis* MADS-box gene *AGL3* is widely expressed and encodes a sequence-specific DNA-binding protein. *Plant Mol. Biol.* **28**, 549–567.

Huijser, P., Klein, J., Lonnig, W.E., Meijer, H., Saedler, H. and Sommer, H. (1992) Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *SQUAMOSA* in *Antirrhinum majus*. *EMBO J.* **11**, 1239–1249.

Immink, R.G.H., Ferrario, S., Busscher-Lange, J., Kooiker, M., Busscher, M. and Angenent, G.C. (2003) Analysis of the petunia MADS-box transcription factor family. *Mol. Genet. Genomics*, **268**, 598–606.

Irish, V.F. (2003) The evolution of floral homeotic gene function. *BioEssays*, **25**, 637–646.

Jack, T., Brockman, L.L. and Meyerowitz, E.M. (1992) The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683–692.

Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994) *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell*, **76**, 703–716.

Judd, W.S., Champbell, C.S., Kellogg, E.A., Stevens, P.F. and Donoghue, M.J. (2002) *Plant Systematics: A Phylogenetic Approach*. Sunderland, MA: Sinauer Associates.

Kang, H.-G., Noh, Y.-S., Chung, Y.-Y., Costa, M.A., An, K. and An, G. (1995) Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. *Plant Mol. Biol.* **29**, 1–10.

Kanno, A., Saeki, H., Kameya, T., Saedler, H. and Theissen, G. (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*). *Plant Mol. Biol.* **52**, 831–841.

Kater, M.M., Colombo, L., Franken, J., Busscher, M., Masiero, S., Van Lookeren, M.M. and Angenent, G.C. (1998) Multiple *AGAMOUS* homologs from cucumber and petunia differ in their ability to induce reproductive organ fate. *Plant Cell*, **10**, 171–182.

Kempin, S.A., Mandel, M.A. and Yanofsky, M.F. (1993) Conversion of perianth into reproductive organs by ectopic expression of the tobacco floral homeotic gene *NAG1*. *Plant Physiol.* **103**, 705–718.

Kim, S., Yoo, M.-J., Albert, V.A., Farris, J.S., Soltis, P.S. and Soltis, D.E. (2004) Phylogeny and diversification of B-function MADS-box genes in angiosperms: evolution and functional implications of a 260-million-year-old duplication. *Am. J. Bot.* **91**, 2102–2118.

Kim, S., Koh, J., Ma, H., Hu, Y., Endress, P.K., Buzgo, M., Hauser, B.A., Soltis, P.S. and Soltis, D.E. (2005) Sequence and expression studies of A-, B-, and E-class MADS-box genes in *Eupomatia* (Eupomatiaceae): support for the bractate origin of the calyptra. *Int. J. Plant Sci.* **166**, 185–198.

Kramer, E.M. and Irish, V.F. (1999) Evolution of genetic mechanisms controlling petal development. *Nature*, **399**, 144–148.

Kramer, E.M. and Irish, V.F. (2000) Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *Int. J. Plant Sci.* **161**, S29–S40.

Kramer, E.M., Dorit, R.L. and Irish, V.F. (1998) Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics*, **149**, 765–783.

Kramer, E.M., Di Stilio, V.S. and Schluter, P.M. (2003) Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the Ranunculaceae. *Int. J. Plant Sci.* **164**, 1–11.

Kramer, E.M., Jaramillo, M.A. and Di Stilio, V.S. (2004) Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. *Genetics*, **166**, 1011–1023.

van der Krol, A.R. and Chun, N.H. (1993) Flower development in Petunia. *Plant Cell*, **5**, 1195–1203.

van der Krol, A.R., Brunelle, A., Tsuchimoto, S. and Chua, N.H. (1993) Functional analysis of petunia floral homeotic MADS box gene *pMADS1*. *Genes Dev.* **7**, 1214–1228.

Kyozuka, J., Kobayashi, T., Morita, M. and Shimamoto, K. (2000) Spatially and temporally regulated expression of rice MADS box genes with similarity to *Arabidopsis* class A, B, and C genes. *Plant Cell Physiol.* **41**, 710–718.

Leutenegger, C.M., Mislin, C.N., Sigrist, B.E., Markus, U., Hofmann-Lehmann, R. and Lutz, H. (1999) Quantitative real-time PCR for the measurement of feline cytokine mRNA. *Vet. Immunol. Immunopathol.* **71**, 291–305.

Liljegren, S., Gustafson-Brown, C., Pinyopich, A., Ditta, G. and Yanofsky, M. (1999) Interactions among *APETALA1*, *LEAFY*, and *TERMINAL FLOWER1* specify meristem fate. *Plant Cell*, **11**, 1007–1018.

Litt, A. and Irish, V.F. (2003) Duplication and diversification in the *APETALA1/FRUITFULL* floral homeotic gene lineage: implication for the evolution of floral development. *Genetics*, **165**, 821–833.

Ma, H. and dePamphilis, C. (2000) The ABCs of floral evolution. *Cell*, **101**, 5–8.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1991) *AGL1-AGL6*, an *Arabidopsis* gene gamily with similarity to floral homeotic and transcription factor genes. *Genes Dev.* **5**, 484–495.

Maddison, W.P. and Maddison, D.R. (1992) *MacClade: Analysis of Phylogeny and Character Evolution*. Sunderland, MA: Sinauer Associates.

Magallón, S. and Sanderson, M.J. (2001) Absolute diversification rates in angiosperm clades. *Evolution*, **55**, 1762–1780.

Mandel, M.A. and Yanofsky, M.F. (1998) The *Arabidopsis AGL9* MADS box gene is expressed in young flower primordial. *Sex Plant Reprod.* **11**, 22–28.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1992) Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature*, **360**, 273–277.

Mathews, S. and Donoghue, M.J. (1999) The root of angiosperm phylogeny inferred from duplicate phytochrome genes. *Science*, **286**, 947–950.

Mathews, S. and Donoghue, M.J. (2000) Basal angiosperm phylogeny inferred from duplicate phytochromes A and C. *Int. J. Plant Sci.* **161** (Suppl.), S41–S55.

Mena, M., Mandel, M.A., Lerner, D.R., Yanofsky, M.F. and Schmidt, R.J. (1995) A characterization of the MADS-box gene family in maize. *Plant J.* **8**, 845–854.

Mena, M., Ambrose, B., Meeley, R., Briggs, S., Yanofsky, M. and Schmidt, R. (1996) Diversification of C-function activity in maize flower development. *Science*, **274**, 1537–1540.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H. and Nagato Y. (2003) *SUPERWOMAN1* and *DROOPING LEAF* genes control floral organ identity in rice. *Development*, **130**, 705–718.

Nam, J., dePamphilis, C.W., Ma, H. and Nei, M. (2003) Antiquity and evolution of the MADS-box gene family controlling flower development in plants. *Mol. Biol. Evol.* **20**, 1435–1447.

Nickerson, J. and Drouin, G. (2004) The sequence of the largest subunit of RNA polymerase II is a useful marker for inferring seed plant phylogeny. *Mol. Phylogenet. Evol.* **31**, 403–415.

Parenicova, L., de Folter, S., Kieffer, M. *et al.* (2003) Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in *Arabidopsis*: new openings to the MADS world. *Plant Cell*, **15**, 1538–1551.

Park, J.H., Ishikawa, Y., Yoshida, R., Kanno, A. and Kameya, T. (2003) Expression of *AODEF*, a B-functional MADS-box gene, in stamens and inner tepals of the dioceious species *Asparagus officinalis* L. *Plant Mol. Biol.* **51**, 867–875.

Park, J.H., Ishikawa, Y., Ochiai, T., Kanno, A. and Kameya, T. (2004) Two *GLOBOSA*-like genes are expressed in second and third whorls of homochlamydeous flowers in *Asparagus officinalis* L. *Plant Cell Physiol.* **45**, 325–332.

Parkinson, C.L., Adams, K.L. and Palmer, J.D. (1999) Multigene analyses identify the three earliest lineages of extant flowering plants. *Curr. Biol.* **9**, 1485–1488.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E. and Yanofsky, M.F. (2000) B and C floral organ identity functions require *SEPALLATA* MADS-box genes. *Nature*, **405**, 200–203.

Pinyopich, A., Ditta, G., Savidge, B., Liljegren, S., Baumann, E., Wisman, E. and Yanofsky, M. (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature*, **424**, 85–88.

Qiu, Y.-L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V. and Chase, M.W. (1999) The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. *Nature*, **402**, 404–407.

Qiu, Y.-L., Lee, J.-Y., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E., Chen, Z., Savolainen, V. and Chase, M. (2000) Phylogeny of basal angiosperms: analyses of five genes from three genomes. *Int. J. Plant Sci.* **161** (Suppl.), S3–S27.

Ronse De Craene, L.P.R., Soltis, P.S. and Soltis, D.E. (2003) Evolution of floral structures in basal angiosperms. *Int. J. Plant Sci.* **164**, S329–S363.

Rounsley, S.D., Ditta, G.S. and Yanofsky, M.F. (1995) Diverse roles for MADS box genes in *Arabidopsis* development. *Plant Cell*, **8**, 1259–1269.

Savidge, B., Rounsley, S. and Yanofsky, M. (1995) Temporal relationship between the transcription of 2 *Arabidopsis* MADS box genes and the floral organ identity genes. *Plant Cell*, **7**, 721–733.

Schmidt, R.J., Veit, B., Mandel, M.A., Mena, M., Hake, S. and Yanofsky, M.F. (1993) Identification and characterization of *ZAG1*, the maize homolog of the *Arabidopsis* floral homeotic gene *AGAMOUS*. *Plant Cell*, **5**, 729–737.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H. and Sommer, H. (1992) Characterization of the *Antirrhinum* floral homeotic MADS-box gene *deficiens*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11**, 251–263.

Soltis, P.S. and Soltis, D.E (2004) The origin and diversification of the angiosperms. *Am J. Bot.* **91**, 1614–1626.

Soltis, P.S., Soltis, D.E. and Chase, M.W. (1999). Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature*, **402**, 402–404.

Soltis, D.E., Soltis, P.S., Chase, M.W. *et al.* (2000) Angiosperm phylogeny inferred from a combined data set of 18S rDNA, *rbcL* and *atpB* sequences. *Bot. J. Linn. Soc.* **133**, 381–461.

Soltis, P.S., Soltis, D.E., Zanis, M.J. and Kim, S. (2000) Basal lineages of angiosperms: relationships and implications for floral evolution. *Int. J. Plant Sci.* **161** (Suppl.), S97–S107.

Soltis, D.E., Soltis, P.S., Albert, V.A., Oppenheimer, D.G., de Pamphilis, C.W., Ma, H., Frohlich, M.W. and Theissen, G. (2002) Missing links: the genetic architecture of flower and floral diversification. *Trends Plant Sci.* **7**, 22–31.

Sommer H., Beltran J.P., Huijser P., Pape H., Lonnig W.E., Saedler H. and Schwarz-Sommer Z. (1990) *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. *EMBO J.* **9**, 605–613.

Sommer, H., Nacken, W., Beltran, P., Huijser, P., Pape, H., Hansen, R., Flor, P., Saedler, H. and Schwarz-sommer, Z. (1991) Properties of *DEFICIENS*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*. *Development*, **1** (Suppl. 1), 169–175.

Stellari, G.M., Jaramillo, M.A. and Kramer, E.M. (2004) Evloution of the *APETALA3* and *PISTILLATA* lineages of MADS-box-containing genes in the basal angiosperms. *Mol. Biol. Evol.* **21**, 506–519.

Swofford, D.L. (2001) *PAUP* 4.0b10: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sunderland, MA: Sinauer Associates.

Taylor, S.A., Hofer, J.M.I., Murfet, I.C., Sollinger, J.D., Singer, S.R., Knox, M.R. and Noel Ellis, T.H. (2002) PROLIFERATING INFLORESCENCE MERISTEM, a MADS-box gene that regulates floral meristem identity in Pea. *Plant Physiol.* **27**, 69–78.

Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.* **4**, 75–85.

Theissen, G., Kim, J.T. and Saedler, H. (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. *J. Mol. Evol.* **43**, 484–516.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W.E., Saedler, H., Sommer, H. and Schwarz-sommer, Z. (1992)

GLOBOSA: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO J.* **11**, 4693–4704.

Tsuchimoto, S., Mayama, T., van der Krol, A. and Ohtsubo, E. (2000) The whorl-specific action of a petunia class B floral homeotic gene. *Genes Cells*, **5**, 89–99.

van Tunen, A.J., Eikelboom, W. and Angenent, G.C. (1993) Floral organogenesis in *Tulipa*. *Flow. Newsl.* **16**, 33–38.

Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K. and Gerats, T. (2004) The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in *Petunia hybrida* flower development. *Plant Cell*, **16**, 741–754.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature*, **346**, 35–39.

Yu, D.Y., Kotilainen, M., Pollanen, E., Mehto, M., Elomaa, P., Helariutta, Y., Albert, V.A. and Teeri, T.H. (1999) Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). *Plant J.* **17**, 51–62.

Zahn, L.M., Kong, H., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., de Pamphilis, C.W. and Ma, H. (2005a) The evolution of the *SEPALLATA* subfamily of MADS-box genes: a pre-angiosperm origin with multiple duplications throughout angiosperm history. *Genetics*, **169**, 2209–2223.

Zahn, L.M., Leebens-Mack, J., de Pamphilis, Ma, H. and Theissen, G. (2005b) To B or not to B a flower: the role of *DEFICIENS* and *GLOBOSA* orthologs in the evolution of the angiosperms. *J. Hered.* **96**, 225–240.

Zanis, M.J., Soltis, D.E., Soltis, P.E., Mathews, S. and Donoghue, M.J. (2002) The root of the angiosperms revisited. *Proc. Natl Acad. Sci. USA*, **99**, 6848–6853.

Zanis, M.J., Soltis, D.E., Soltis, P.S., Qiu, Y.-L. and Zimmer, E. (2003) Phylogenetic analyses and perianth evolution in basal angiosperms. *Ann. Mo. Bot. Gard.* **90**, 129–150.