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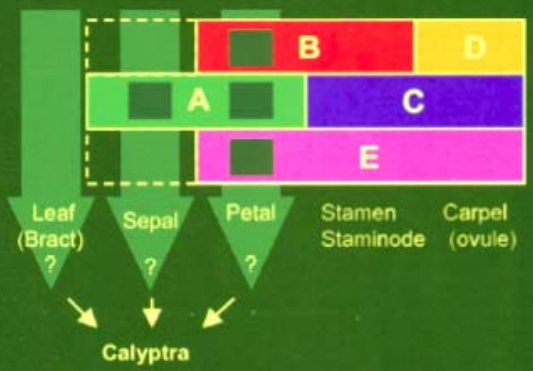

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On the Cover Flower in bud (left) and opened flower (right) of *Eupomatia laurina* R. Br. Flowers of *Eupomatia* do not possess sepals and petals; instead, numerous spirally arranged stamens and showy staminodes are the outermost floral parts. Unlike the flowers of virtually all other angiosperms, the flower of *Eupomatia* includes a special structure termed a "calyptra" (left). The calyptra encloses all other floral parts when the flower is in bud and abscises during anthesis. Kim et al. (in this issue, pp. 185–198) address the origin of calyptra based on the expression patterns of MADS-box floral organ identity genes. Photo credit: Sangtae Kim (left) and Herwig Teppner (right).

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SEQUENCE AND EXPRESSION STUDIES OF A-, B-, AND E-CLASS MADS-BOX HOMOLOGUES IN *EUPOMATIA* (EUPOMATIACEAE): SUPPORT FOR THE BRACTEATE ORIGIN OF THE CALYPTRA

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Eupomatia (Magnoliales, Eupomatiaceae) has flowers that bear a calyptra, an unusual organ that encloses the floral bud. The structural homology and evolutionary derivation of the calyptra are unknown, although some have proposed that it is a bract, while others favor a derivation from the perianth. To address the evolutionary origin of the calyptra, we isolated, sequenced, and characterized the expression of A-, B-, and E-class MADS-box homologues from *Eupomatia bennettii* and a close relative, *Magnolia grandiflora* (Magnoliaceae). The expression patterns of organ identity genes in floral organs of *Eupomatia* and *Magnolia* were very similar. However, the expression patterns of these MADS-box genes indicated that the ABC model is not strictly applicable to either *Eupomatia* or *Magnolia*. For example, A-class homologues were expressed in carpels and leaves of both *Eupomatia* and *Magnolia*. In the calyptra, expression levels of B- and E-class homologues were low and almost identical to those observed in leaf tissue. In contrast, high levels of expression for B- and E-class homologues were observed in the stamens, staminodes, and carpels. These gene expression data agree with recent developmental data and the interpretation of the calyptra as a bract. We also report the presence of various forms of alternatively spliced mRNAs in the cDNA pool from floral organs, and the implications of these mRNAs are discussed.

Keywords: *Eupomatia*, *Magnolia*, relative-quantitative-reverse transcriptase PCR, ABC model, real-time PCR, alternative splicing.

Online enhancements: figures.

Introduction

Basal angiosperms, which include *Amborella*, Nymphaeaceae, and Austrobaileyaceae (the successive sisters to all other extant flowering plants), as well as the large magnoliid clade, exhibit considerable diversity in the type, arrangement, and number of floral parts. Added to this diversity is the appearance of novel structures. Understanding the genetics of floral development in basal angiosperms in general and of novel features in particular should provide key insights into the early evolution of the flower.

One such novel floral structure is the calyptra of *Eupomatia* (Eupomatiaceae). Eupomatiaceae are a monogeneric family of basal angiosperms with three species (*Eupomatia bennettii* F. Muell., *Eupomatia laurina* R. Br., and *Eupomatia barbata* Jessup) of shrubs and trees occurring in tropical to temperate rainforests of Australia and New Guinea (Endress 1993, 2003; Jessup 2002). Recent phylogenetic analyses of molecular and morphological data reveal that Eupomatiaceae belong to a well-supported Magnoliales clade (Savolainen et al. 2000; Soltis et al. 2000; Hilu et al. 2003; Sauquet et al. 2003; Zanis et al. 2003). In Magnoliales, Eupomatiaceae are sister to Annonaceae, and the clade of Degeneriaceae/Himantandraceae is the sister group to Eupomatiaceae/Annonaceae. Magnoliaceae are sister to this clade of four families, and Myristicaceae are sister to all other Magnoliales (Sauquet et al. 2003).

The floral structure of *Eupomatia* is unusual. There are no obvious sepals or petals; instead, numerous spirally arranged stamens and showy staminodes are the outermost parts (fig. 1). Interior to the staminodes, numerous carpels are also spirally arranged. *Eupomatia* also possesses a novel structure for angiosperms, termed a calyptra, which encloses all other floral parts when flowers are in bud (fig. 1); the calyptra abscises during anthesis. A calyptra has been reported elsewhere only in the basal angiosperm family Himantandraceae, also a member of Magnoliales (Cronquist 1981; Endress 2003). The calyptra has been variously interpreted, on the basis of morphological observations, as (1) united sepals and petals (Brown 1814; Bailey 1899; Uphof 1959; Hutchinson 1964; Hiepko 1965); (2) united sepals (von Mueller 1862); and (3) a single, amplexicaul, tubular bract (Baillon 1868*a*, 1868*b*; Diels 1916; Ozenda 1949; Eames 1961; Endress 1977, 2003). Genetic approaches may help to clarify the evolutionary origin of the calyptra.

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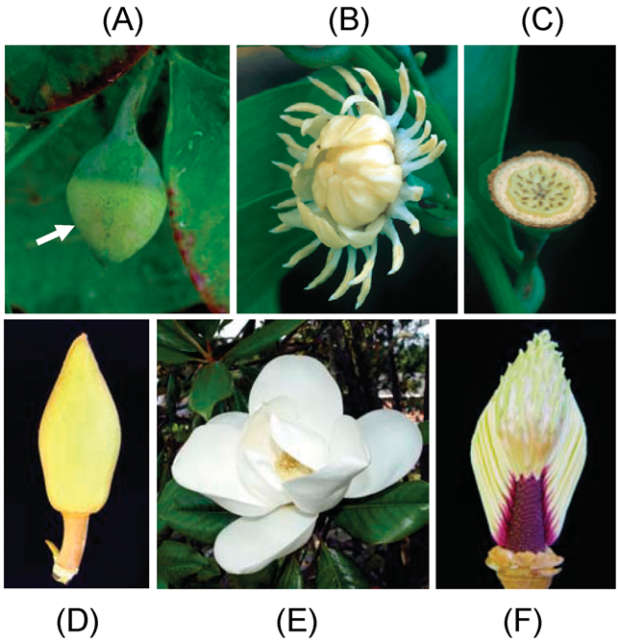

Fig. 1 *A–C*, *Eupomatia laurina*. *A*, Flower buds with intact calyptra (arrow). *B*, Opening flower. *C*, Stamens and staminodes abscised. *D–F*, *Magnolia grandiflora*. *D*, Flower buds with spathaceous bract. *E*, Opening flower including prominent tepals. *F*, Tepals and some stamens are removed. Photographs *B* and *C* by H. Teppner, Botanic Garden of the Institute of Botany, University of Graz, Austria.

Based on studies of *Arabidopsis* and *Antirrhinum*, the ABC model was proposed to explain functional interactions of floral genes that determine floral organ identity (Coen and Meyerowitz 1991; Coen et al. 1991; Meyerowitz et al. 1991). In this model, three homeotic activities, designated A, B, and C, specify four different organ identities in a combinatorial manner: A-function genes determine sepal identity, A- and B-function genes together determine petal identity, B- and C-function genes together determine stamen identity, and C-function genes alone determine carpel identity. In *Arabidopsis*, *APETALA1* (*AP1*) and *APETALA2* (*AP2*) are A-function genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are B-function genes, and *AGAMOUS* (*AG*) is the known C-function gene. With the exception of *AP2*, all of these genes encode members of the MADS-box family of transcription factors (Shore and Sharrocks 1995; Theissen et al. 2000). After the ABC model was published, a D-function was proposed, on the basis of analysis of *Petunia* floral genes (*FBP7* and *FBP11*), as responsible for the establishment of ovule identity (Colombo et al. 1995). Three other MADS-box genes, *AGL2*, *AGL4*, and *AGL9*, were shown to be expressed during early flower development (Ma et al. 1991; Flanagan and Ma 1994; Savidge et al. 1995; Mandel and Yanofsky 1998). Recent studies have shown that these genes (renamed *SEPALLATA1*, *2*, and *3* and collectively referred to as E-function genes) are also involved in specifying the identity of petals, stamens, and carpels in *Arabidopsis* (Pelaz et al. 2000; Honma and Goto 2001; Theissen 2001; Theissen and Saedler 2001). The B and C functions of this model also extend to maize and rice, although conservation of the A function remains to be demonstrated (Ambrose et al. 2000; Nagasawa et al. 2003).

The strong genetic and molecular evidence for the conservation of at least portions of the ABC model indicates an ancient regulatory network perhaps applicable to most angiosperms (Ma and de Pamphilis 2000). However, various modifications of the specific components of the ABC model may have occurred in different lineages of angiosperms (van Tunen et al. 1993; Kanno et al. 2003; Kramer et al. 2003). For example, van Tunen et al. (1993) proposed a modified ABC model in which the expression of B-class genes was extended to the first floral whorl to explain the morphology of the lily flower, in which the first and second whorls of floral parts (tepals) are identical. This modified model has subsequently been supported by developmental genetic studies: B-class genes are expressed in both the first and second whorls in the tulip, which also exhibits morphologically identical organs in the first and second whorls (Kanno et al. 2003; but see Park et al. 2004 for contrasting results for *Asparagus*).

The evolutionary origin of enigmatic organs and their homologues in closely related taxa may be addressed through the comparison of expression patterns of organ identity genes (e.g., Albert et al. 1998). Over large phylogenetic distances, such comparisons may be difficult, but among close relatives within a well-defined clade, these comparisons may be fruitful (Baum 2002). Although the applicability of the ABC model in *Eupomatia* and other basal angiosperms has not been established, expression patterns of homologues of floral genes in leaves, calyptra, and other floral organs can provide clues about the molecular identity of the calyptra. If the ABC model is strictly applicable in *Eupomatia* flowers, expected expression patterns of floral genes in the calyptra vary according to three evolutionary scenarios (fig. 2): (1) if the calyptra originated from united sepals and petals (Hiepko 1965 and references therein), expression of A-, B-, and E-class genes is expected; (2) if it originated from sepals (von Mueller 1862),

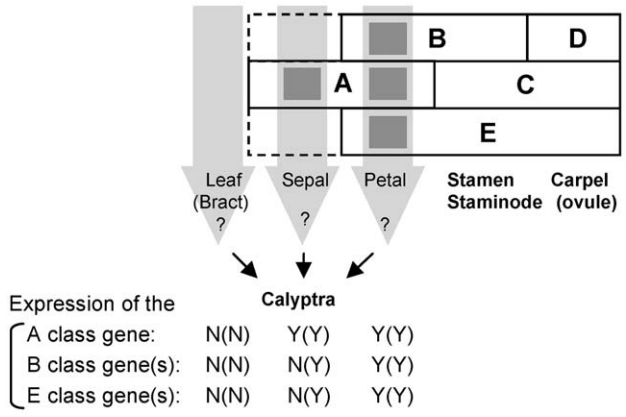

Fig. 2 Extended ABC model (including D- and E-class genes) for floral organ identity and the possible expression pattern of floral genes in the calyptra when we strictly apply the ABC model to *Eupomatia*. Dashed boxes indicate the modified ABC model from lily and tulip, in which the borders of B- and E-class genes are extended to the first whorl. When we apply this model to *Eupomatia*, the expected expression pattern is indicated in parentheses.

expression of A- and perhaps E-class genes is expected; and (3) if it originated from bracts (Endress 2003 and references therein), no expression of any of the A-, B-, or E-class genes is expected. Alternatively, if the modified ABC model from lily and tulip is applied to *Eupomatia* and the calyptra originated from either sepals or petals (or tepals), expression of A-, B-, and E-class genes would be expected in the calyptra (fig. 2). However, expression patterns of floral organ identity genes in *Eupomatia* may differ from expectations on the basis of either the classic ABC model or the modified ABC model of lily and tulip. If this is the case, comparison of expression patterns in *Eupomatia* with those of a close relative of Eupomatiaceae may be useful in inferring the evolutionary origin of the calyptra. Finally, these models assume that A-, B-, and E-class genes are not expressed in extrafloral tissues. However, the *PI* homologue in tulip is also expressed in leaves (Kanno et al. 2003). Distinguishing among hypotheses on the origin of the calyptra can best be accomplished by comparing gene expression patterns in the calyptra with (1) those in vegetative organs, such as leaves of *Eupomatia* or bracts of closely related taxa, and (2) those predicted for perianth organs and/or observed for perianth organs in a close relative.

To address the evolutionary origin of the calyptra, we (1) isolated homologues of A-, B-, and E-class genes of *Eupomatia* and determined their sequences, (2) examined the expression patterns of these floral genes in each floral organ, and (3) tested the applicability of the ABC model in *Eupomatia*. Because comparison of the expression patterns of floral genes in *Eupomatia* and closely related taxa is necessary to identify instances of conservation and shifts in gene expression in homologous organs, we also examined the expression of A-, B-, and E-function genes of *Magnolia grandiflora* (Magnoliaceae; Magnoliales). In contrast to the putatively perianthless *Eupomatia* (Endress 2003), *Magnolia* has prominent tepals (fig. 1*E*), which are not differentiated into well-defined sepals and petals. *Magnolia* also has a spathaceous bract (fig. 1*D*), which encloses the floral organs in bud.

Material and Methods

We collected samples from the following sources: *Eupomatia bennettii* and *Eupomatia laurina* (used for the *in situ* hybridization; see below) plants cultivated at the greenhouse of the Botanical Garden of the University of Zurich (Endress 5197 and Endress 4019) and a *Magnolia grandiflora* plant cultivated on the campus of the University of Florida, Gainesville, FL (S. Kim 1138). Entire flowers at varying stages of early development up to anthesis were removed and dropped immediately into liquid nitrogen and stored at −80°C.

Determination of Gene Sequences

Five sequences of B-class homologues of *Eupomatia* and *Magnolia* were reported in a previous study of B-class gene phylogeny (Kim et al. 2004): homologues of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) from *E. bennettii* (*Eu.be.AP3-1*: AY337750, *Eu.be.AP3-2*: AY337751, and *Eu.be.PI*: AY337740); a *PI* homologue from *E. laurina* (*Eu.la.PI*: AY337741); and an *AP3* homologue from *M. grandiflora* (*Ma.gr.AP3*: AY337752). Sequences of two *AP3* homologues of *E. bennettii*, *Eu.be.AP3-1* and *Eu.be.AP3-2*, differ from each other by only one nucleotide in the middle of exon 4 and may represent alleles of the same locus. It was impossible and unnecessary to address their expression patterns separately. We therefore treated both *Eu.be.AP3-1* and *Eu.be.AP3-2* as *Eu.be.AP3*. In addition to the previously reported B-class genes of *Eupomatia* and *Magnolia*, A- and E-class genes were determined using the method described by Kim et al. (2004).

The 5′ end of the translated region was not available in the previously reported sequences of B-class genes of *Eupomatia*. To obtain additional cDNA sequence of *Eu.be.AP3*, the 5′ end of the cDNA was obtained using the SMART RACE cDNA Amplification Kit (Clontech, Alameda, CA). To determine the genomic DNA sequence of *Eu.be.AP3*, several different primers were designed in exon regions (EF6A, EF11A, and EF13A-EF20A; the relative positions of these primers and their sequences are shown in fig. A1). We compared the genomic sequence with the cDNA sequence to determine the sizes of the exons and introns. Alternatively spliced mRNAs were detected by PCR using primers designed at the 5′ and 3′ ends of the translated region (EF11A and EF6A; fig. A1) and templates of cDNAs generated using polyT primer. PCR conditions for analyses of genomic DNA and alternatively spliced mRNAs followed Kim et al. (2001). PCR bands were purified using the Geneclean II kit (Qbiogene, Carlsbad, CA) and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). After plasmid extraction, cycle sequencing reactions were performed using the CEQ DTCS-Quick Start kit (Beckman Coulter, Fullerton, CA), and sequences were determined using a CEQ 8000 sequencing system (Beckman Coulter, Fullerton, CA).

Phylogenetic Analyses

Recent phylogenetic studies have revealed major clades in the MADS family (Theissen et al. 1996, 2000; Alvarez-Buylla et al. 2000; Henschel et al. 2002; Becker and Theissen 2003; Nam et al. 2004). To verify the subfamily identities of newly isolated genes from *Eupomatia* and *Magnolia* and to address their orthology to previously reported genes, we added our sequences to a large data set of 88 sequences representing all subfamilies of MIKC^{C}-type MADS genes (Becker and Theissen 2003). Amino acid alignment was conducted using CLUSTAL X (ver. 1.83; Thompson et al. 1997) and then adjusted manually. The maximum parsimony (MP) analysis was performed for the amino acid data set using PAUP*, version 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 replicate heuristic searches each with 10 random taxon addition sequences and TBR branch swapping, saving all optimal trees. Although we also conducted maximum likelihood and neighbor-joining analyses in addition to the MP analysis, we include only the results of the MP analysis in this article because all three analyses showed the same subfamilial groupings for new genes.

Southern Blot Analysis

To obtain an independent estimate of the number of copies of *AP3*-like genes, we performed Southern blot analyses. We chose *AP3* for these studies because our cloning and sequencing investigations suggested that at least two *AP3* genes are present in *Eupomatia*. Total DNA was isolated from leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Ca. 2.5 μg of total DNA was digested with each of five restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, and *Xho*I), electrophoresed on 0.5% agarose gel, and blotted onto positively charged nylon membranes (Roche, Indianapolis, IN). Gene-specific hybridization probes were obtained from PCR with specific primers designed for exon 7 (EF21A and EF6A; located in the C-domain region) of *Eu.be.AP3*. This probe sequence does not have restriction sites for any of the enzymes that were used to digest the genomic DNA. The probe was labeled with the DIG-High Prime kit (Roche). Hybridization was performed at 65°C with the DIG Luminescent Detection Kit, following the recommendations of the supplier (Roche). Membranes were washed two times in 2X SSPE (with 1% SDS) for 5 min and two times in 0.5X SSPE (with 1% SDS) for 15 min.

Quantification of RNA Expression in Mature Flowers

Reverse transcriptase (RT) PCR is a powerful method for expression analysis of gene families because amplification from mRNAs can be highly specific and quantification of expression signals can be rapidly performed (McDowell et al. 1996; Wang et al. 1999). To quantify levels of gene expression, we used relative-quantitative-reverse transcriptase polymerase chain reaction (R-Q-RT PCR) and real-time PCR. For these analyses, 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: calyptras, stamens, staminodes, and carpels for *Eupomatia*; spathaceous bract, tepals, stamens, and carpels for *Magnolia*. Young leaves of both *Eupomatia* and *Magnolia* were also included. In preliminary experiments, the expressions of outer, middle, and inner tepals of *Magnolia* were tested separately. However, there were no differences among these tissues for any of the genes that we tested (data not shown). Therefore, we combined all three groups of tepals for the comparison with *Eupomatia*.

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

For the R-Q-RT PCR, we performed multiplex PCR, using a gene-specific primer pair (see fig. A1), 18S rDNA primer pair (internal control), and a competitive primer pair of 18S rDNA primers (competimers), following the protocol of QuantumRNA (Ambion, Austin, TX). The 18S rRNA gene was used for the internal control in each reaction. Because the PCR signal of the 18S rRNA gene is higher than that of the specific genes we are studying, the competimers of the 18S primer pair were used to reduce the 18S PCR signal. The optimal ratio of the 18S primer pair to competimers was tested for each gene to obtain a similar level of PCR signal for both the 18S gene and the specific gene. Optimal ratios ranged from 3 : 7 to 6 : 4. PCR reactions for all genes were performed with 25 cycles at 95°C (30 s), 55°C (30 s), and 72°C (30 s) using an Eppendorf Mastercycler (Brinkmann, Westbury, NY). A range of 1–256 ng of total RNA (reversely calculated from the amount of cDNA used in the PCR reaction after the RT PCR) was tested, and 16–64 ng of total RNA was found to generate unsaturated PCR product accumulation for each gene through 25 cycles of PCR. We used 25 ng of total RNA for the R-Q-RT PCR, and three independent PCR reactions were performed for each gene. 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 KODAK 1D Image Analysis Software (Kodak, Rochester, NY). Relative PCR signals of each gene to 18S rDNA from three independent PCR results and their standard deviations were calculated for each floral organ. The gene specificity of each PCR product was confirmed by sequencing all PCR products. For all PCRs, we used a negative control that did not contain cDNA template.

As an alternative method to R-Q-RT PCR for the B-class homologues of *Eupomatia*, we also used real-time PCR (Chiang et al. 1996; Leutenegger et al. 1999). Because this method includes a third primer as a probe and a fluorescent-dye-labeled system, the quantification of real-time PCR is more precise than R-Q-RT PCR. The probe and primers for real-time PCR were designed using the Primer Express 2.0 program (Applied Biosystems, Foster City, CA). The designations are as follows: EF5A (primer), EuAP3RT1 (probe), and EF12A (primer) for *Eu.be.AP3*, and EF3P (primer), EuPIRT2 (probe), and EF6P (primer) for *Eu.be.PI* (fig. A1). 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. 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 ratio of threshold cycle (Ct) values between the 18S rRNA gene and the specific gene was calculated for each sample.

In Situ *Hybridization of B-Class Genes of* Eupomatia

For the *in situ* hybridization study, we used material of *E. laurina* instead of *E. bennettii*. Because *Eupomatia* plants are rarely cultivated and flower only once per year, it was impossible to collect samples of the same species for all experiments. However, floral structure does not differ between *E. bennettii* and *E. laurina*, so patterns of gene expression for floral regulators are expected to be similar in the two species. Gene-specific primers for *Eu.la.AP3* (EF9A and EF6A) and *Eu.la.PI* (EF5P and EF4P) were designed in the K- and

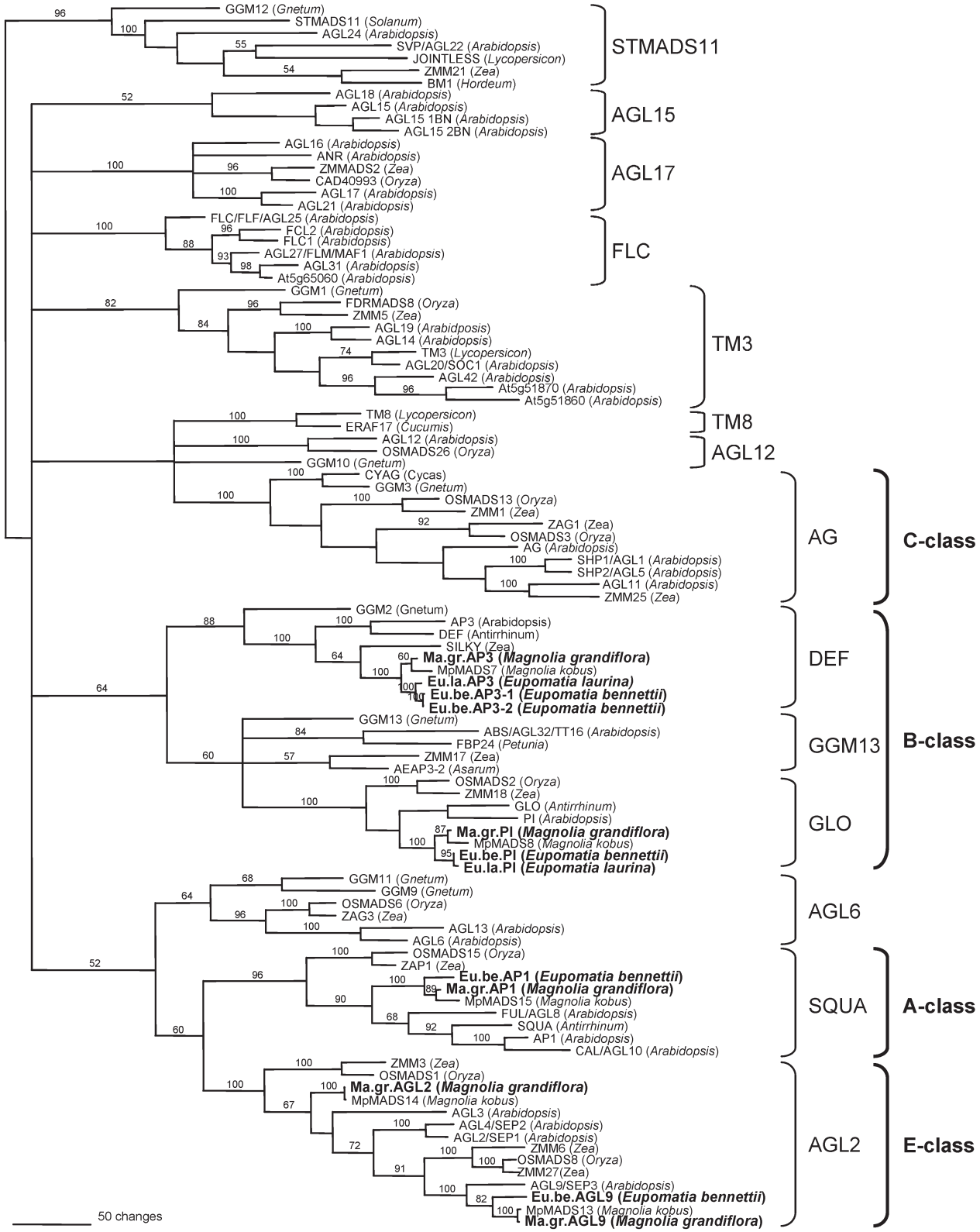

Fig. 3 Strict consensus of two shortest trees from the maximum parsimony analysis (6656 steps, Consistency Index = 0.44, Retention Index = 0.60). Selected representatives of each major clade of MIKC^{C}-type MADS-box genes were analyzed, together with genes used in this study (bold). Numbers above the branches indicate bootstrap values.

C-domain regions to make RNA probes (fig. A1). PCR products of these regions were purified and cloned using the pGEM T-vector system (Promega, Madison, WI). ^{35}S-dATP-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). The transcripts were partially hydrolyzed by incubation at 60°C in 0.1M Na_2CO_3·$NaHCO_3$ buffer, pH 10.2, for 45 min. Sample-embedding procedure, hybridization, washing, and autoradiography followed the protocols of the Meyerowitz lab (http://www.its.caltech.edu/~plantlab).

Results

Sequence Determination and Phylogenetic Analysis

We report seven new A-, B-, and E-class homologues from *Eupomatia bennettii*, *Eupomatia laurina*, and *Magnolia grandiflora*: *Eu.be.AP1* (AY821776), *Ma.gr.AP1* (AY821777), *Eu.la.PI* (AY821778), *Ma.gr.PI* (AY821779), *Eu.be.AGL9* (AY821780), *Ma.gr.AGL9* (AY821782), and *Ma.gr.AGL2* (AY821781). Blast searches in GenBank identified these genes as putative members of the MADS-box gene family. Our phylogenetic analysis confirmed the orthology of the new genes: genes were included in *DEF* (B-class), *GLO* (B-class), *SQUA* (A-class), and *AGL2* (E-class) clades (fig. 3), and these clades were generally well supported (>79%). Genes isolated from *Eupomatia* and *Magnolia* representing the A-, B-, and E-class genes exhibited relationships that tracked organismal relationships. In each clade, genes from *M. grandiflora* grouped with previously reported MADS genes of *Magnolia kobus* (*MpMADS7*, *MpMADS8*, *MpMADS13*, *MpMADS14*, and *MpMADS15*), and genes of *Eupomatia* were sister to the genes of Magnoliaceae, as would be expected from molecular phylogenies inferred from plastid, mitochondrial, and nuclear ribosomal genes, given that no sequences of Annonaceae, Himantandraceae, or Degeneriaceae were available for inclusion in the analyses.

In the *AGL2* clade (E-class), *Ma.gr.AGL9* and *MpMADS13* grouped with *AGL9*, but *Ma.gr.AGL2*/*MpMADS14* and *AGL2*/*AGL4* did not form a clade. Only one *AGL2* homologue of *Eupomatia* was detected in this study, and this sequence grouped with *Ma.gr.AGL9*, *MpMADS13*, and *AGL9*.

We also further characterized the B-class homologue of *AP3* in *Eupomatia*. We determined the complete sequence of the translated region of *Eu.be.AP3* from Kim et al. (2004);

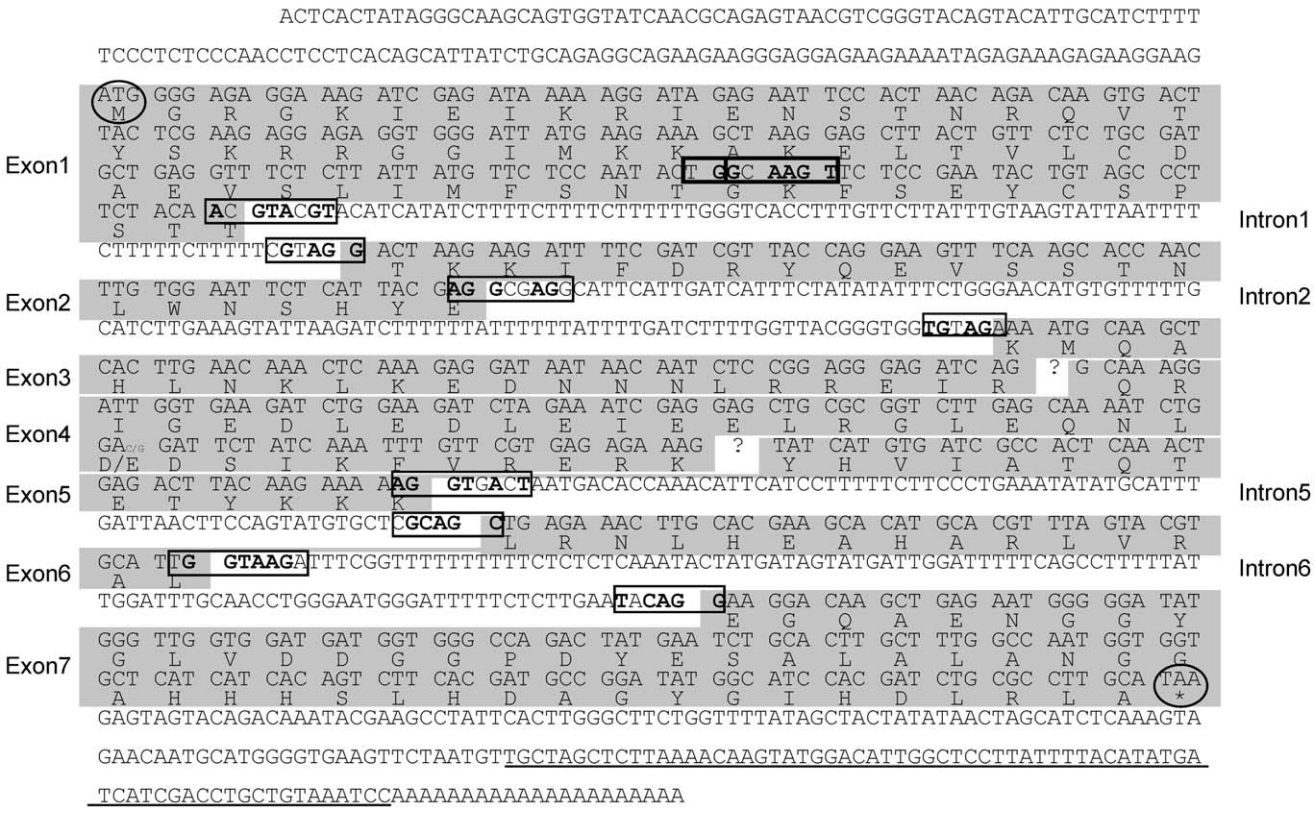

Fig. 4 Gene structure of *Eu.be.AP3*. Introns 3 and 4 were not determined (question marks). Shaded areas indicate exon regions. Circles indicate the initiation and stop codons. Boxes indicate intron donor and acceptor sequences. Matched sequences with intron donor/acceptor sequences of *Arabidopsis* are indicated in bold. The thick box indicates the intron donor site found in exon 1 (see text). The vertical line in the thickened box indicates the splice position.

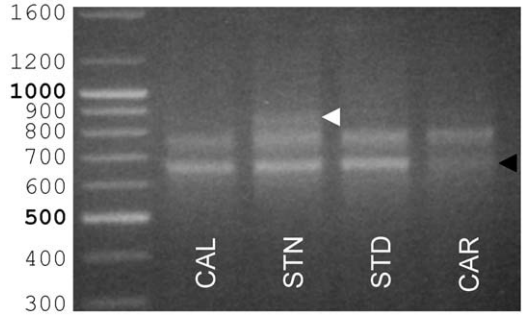

Fig. 5 PCR bands produced by specific primers designed in the 3′ and 5′ ends of *Eu.be.AP3* in each floral organ. The size of the expected band from normal mature mRNA is 650 bp. Stamen-specific bands around 850 bp (white arrowhead) were recognized, and bands around 650 bp (black arrowhead) in carpels were weaker than others. *CAL* = calyptra; *STN* = stamens; *STD* = staminodes; *CAR* = carpels.

some introns were sequenced, and putative splicing sites were determined by comparing cDNA and genomic DNA sequences (fig. 4). We determined genomic DNA sequences of introns 1, 2, 5, and 6 in *Eu.be.AP3*. Although we performed several PCR reactions, using variable combinations of 10 different primers designed in the different exon regions (fig. A1), we did not recover the sequences of introns 3 and 4 (fig. 4). These introns may be very long, or PCR of these regions using genomic DNA was inhibited for unknown reasons.

Alternative Splicing

During the determination of genes and R-Q-RT PCR (see below), we recognized that several alternatively spliced mRNAs of *Eu.be.AP3* were represented in the cDNA pool. Intensive screening for various types of mRNAs was performed using PCR with specific primer pairs designed at the 5′ and 3′ ends of the gene in each floral organ. PCR products for each floral organ showed smeared bands in addition to the expected band (650 bp) (fig. 5). Furthermore, some of the bands were restricted to certain organs: stamens have an additional band of ca. 850 bp, and carpels have a very weak band of ca. 650 bp (fig. 5). When we repeated the PCR several times with slightly different conditions (e.g., annealing temperature), we observed the same band pattern.

In addition to the expected transcript in the *Eu.be.AP3* cDNA pool, we detected 11 different forms of transcripts, which we refer to hereafter using Roman numerals (fig. 6). Type I, II, and III sequences (fig. 6) are partially spliced mRNAs. The sequences inserted between exons exactly

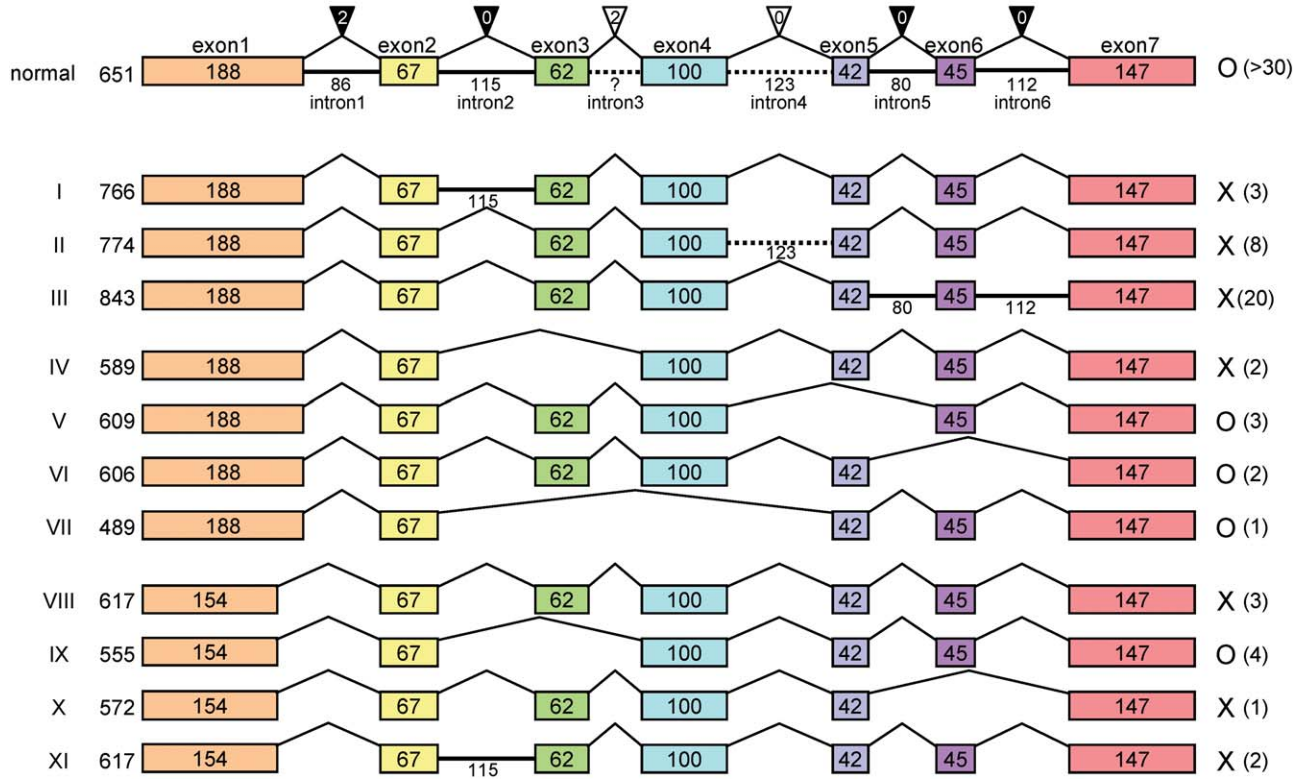

Fig. 6 Various alternatively spliced forms of *Eu.be.AP3*. Each exon is distinguished by a different color. Intervening solid lines between exons indicate confirmed introns from the genomic DNA sequences; dashed lines indicate unconfirmed introns. Numbers inside inverted triangles indicate the "phase" of frame of splicing site in the codon triplet. Filled triangles are for confirmed splicing sites from the alignment of cDNA and genomic DNA and from shared intron donor/acceptor sequences; open triangles are for putative splicing sites from Kim et al. (2004). Size of fragments is indicated at the left side of each fragment. Numbers in parentheses indicate the number of clones found in each fragment. *O* = full-length translation possible; *X* = stop codon found in the middle of the fragment.

matched intron sequences determined from genomic DNA in the type I and III sequences. The sequence inserted between exon 4 and exon 5 in the type II sequences seems to be intron 4 or a part of intron 4 of the gene. However, this region has not yet been confirmed with genomic sequence. If these three types of sequences are translated, they will result in truncated polypeptides, because the inserted sequences in each case contain in-frame stop codons.

In type IV, V, VI, and VII sequences (fig. 6), one or two exons were entirely deleted. In the B-class genes, the first and third splicing sites are placed after the second base position in the codon ("phase two"; Henschel et al. 2002), and the second, fourth, fifth, and sixth splicing sites are placed between codons ("zero phase"; Henschel et al. 2002). When exon(s) contain zero-phase splicing sites at both ends, these sequences can potentially be used to produce polypeptides that have internal deletions and end at the normal terminus if they are translated (fig. 6; *V*, *VI*, and *VII*).

Thirty-four base pairs at the end of exon 1 were deleted in the type VIII, IX, X, and XI sequences. Sequences near the abnormal 3′ end of exon 1 in these sequences nearly matched the *Arabidopsis* intron donor sequence: six out of eight base pairs were identical. This intron donor also has GC instead of GT, as observed at the 5′ end of exon 2. Because the deletion of 34 bp shifted the reading frame, in-frame stop codons were observed after the deletion. In type IX sequences, however, the reading frame was recovered by the deletion of exon 3, which has zero-phase and the phase two splicing sites in the 5′ and 3′ ends, respectively. Therefore, if this sequence is translated, the amino acid sequences of exon 2 could be changed in the type IX sequence, compared to the normal *Eu.be.AP3* sequence.

Southern Blot Analysis

To obtain an additional estimate of the number of copies of *AP3* in *Eupomatia*, we conducted Southern blot analyses. One to three hybridizing bands were observed in each lane, indicating that there are probably no more than three *AP3*-like genes in the *E . bennettii* genome (fig. 7). This finding agrees with those of our cloning and sequencing studies, in which we detected two different *AP3* homologues in *Eupomatia*. This result also confirms that the additional transcripts we detected are not from different genes but result from the alternative splicing of transcripts from just a few genes.

B-Class Gene Expression

The expression patterns of B-class homologues in *Eupomatia* from R-Q-RT PCR are different from those in *Arabidopsis*. For *Eu.be.PI*, no signal was detected in either the calyptra or leaves, whereas strong signal was detected in stamens and staminodes, and intermediate signal was detected in carpels (fig. 8). In contrast, *Eu.be.AP3* was expressed in all floral organs and also in the leaf and calyptra (fig. 8). A particularly strong signal was detected in stamens, staminodes, and carpels, and relatively low signal was detected in the calyptra and leaf tissue. An additional product that was longer than other *Eu.be.AP3* transcripts was detected only in stamens (fig. 8). When we excised this band from an agarose gel and sequenced it, the sequence contained 80 additional bp 3′ of splicing site 5 and 112 additional bp 3′ of splicing site 6. These inserted sequences exactly matched the intron sequences that we determined from genomic DNA. Therefore, this signal is from the same or a similar type of alternatively spliced sequence as type III (fig. 6). Expression of *Ma.gr.PI* was restricted to tepal and stamen tissue. No signal was detected in spathaceous bracts, carpels, or leaves. Expression of *Ma.gr.AP3* was similar to that of *Ma.gr.PI*. However, weak signal of *Ma.gr.AP3* was detected in spathaceous bracts.

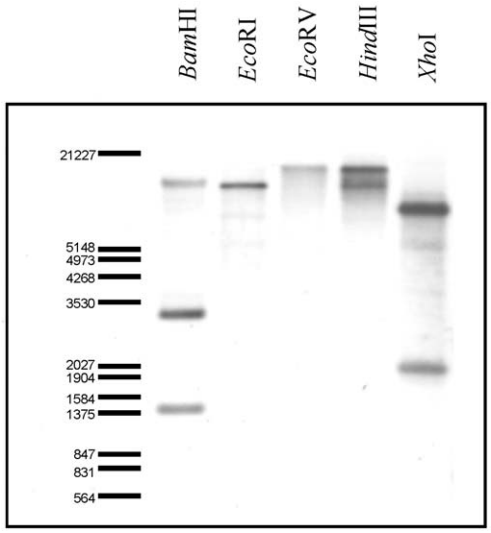

Fig. 7 Southern blot analysis of *Eu.be.AP3* in *Eupomatia*. Each lane contains 2.5 μg of total DNA digested with one of five restriction enzymes, *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, or *Xho*I.

The pattern of expression of *Eu.be.PI* in each floral part, including the calyptra, based on real-time PCR is similar to the results obtained using R-Q-RT PCR (fig. 9): strong signal in stamens and staminodes, intermediate signal in carpels, and very low signal (vs. no signal with R-Q-RT PCR) in calyptra and leaves (fig. 9). In *Eu.be.AP3*, the pattern of expression with real-time PCR was slightly different from that with R-Q-RT PCR in the calyptra and leaves: all tissues, including calyptra and leaves, showed high expression with real-time PCR and somewhat weaker expression with R-Q-RT PCR (cf. figs. 8 and 9).

In situ hybridization results show that the *Eu.be.PI* probe hybridized to target sequences in the primordia of stamens, staminodes, and carpels (fig. 10*A*, 10*B*). The *Eu.be.AP3* probe also hybridized to targets in these floral tissues, as well as to targets in the calyptra and the bract subtending the flower (fig. 10*C*, 10*D*). Negative controls of both experiments showed no signal (fig. A2). These results are in close agreement with the results of the R-Q-RT PCR and real-time PCR experiments and indicate that *Eu.be.PI* is expressed in reproductive organs and their primordia, whereas *Eu.be.AP3* is expressed more broadly.

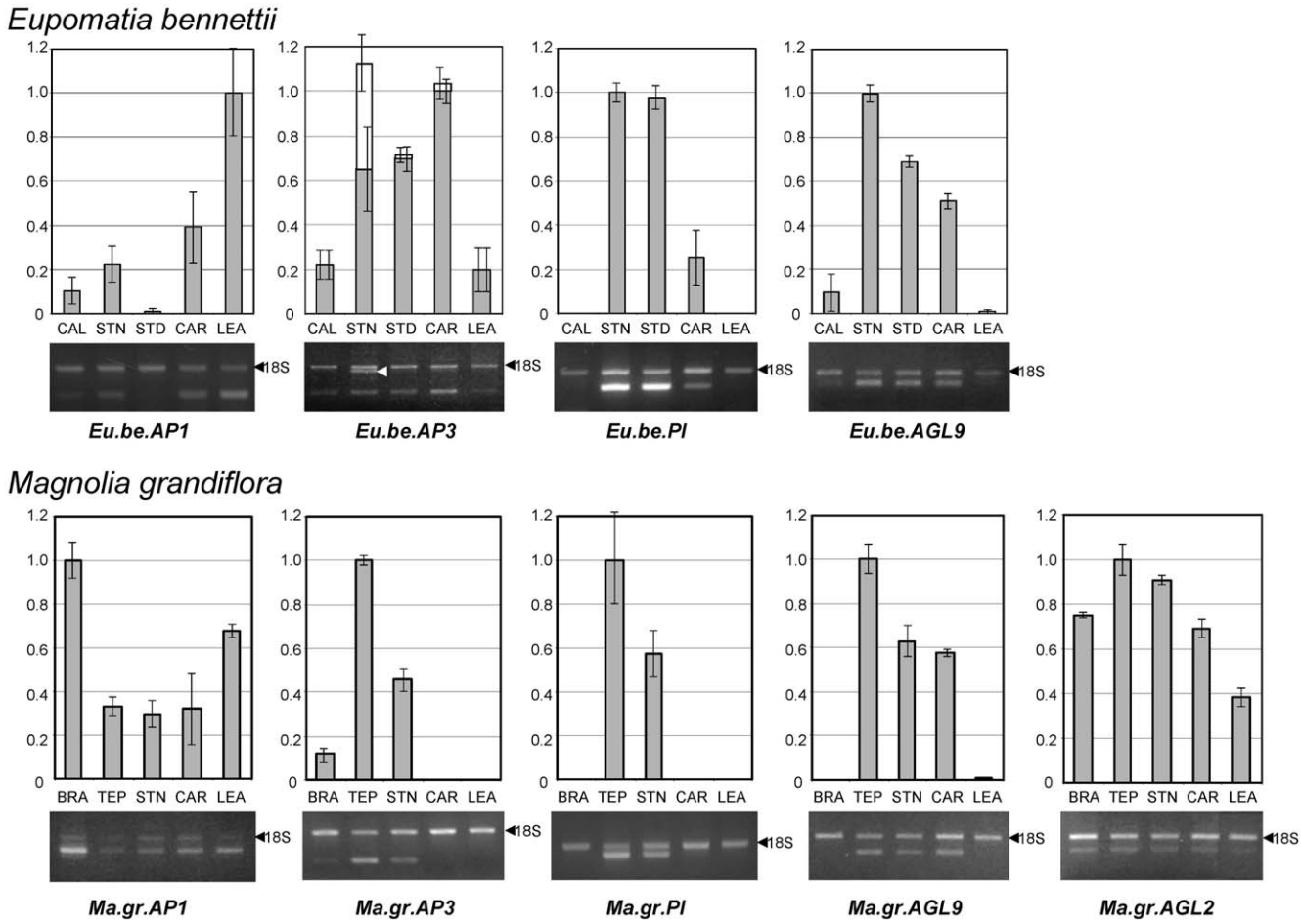

Fig. 8 Relative-quantitative-reverse transcriptase PCR result of A-, B-, and E-class homologues of *Eupomatia bennettii* and *Magnolia grandiflora* for floral tissues and leaves. Standard deviations (SDs) are indicated for each value. Arrowhead indicates a longer band than expected (see text). For *Eu.be.AP3*, expression signals of expected bands and the sum of signals of longer and expected bands were calculated separately: the SD to the right is for the former values, and the SD to the left is for the latter values. *CAL* = calyptra; *STN* = stamens; *STD* = staminodes; *CAR* = carpels; *BRA* = spathaceous bracts; *TEP* = tepals; *LEA* = leaves.

A- and E-Class Gene Expression

A-class homologues of *Eupomatia* and *Magnolia* showed similar expression patterns. Both *Eu.be.AP1* and *Ma.gr.AP1* were strongly expressed in leaves, whereas the expression of both was intermediate in stamens and carpels. Expression of *Eu.be.AP1* was very weak or undetectable in the calyptra and staminodes. *Ma.gr.AP1* was strongly expressed in spathaceous bracts and showed intermediate signal in tepals (fig. 8).

The E-class homologue of *Eupomatia* (*Eu.be.AGL9*) identified in this study grouped with *Ma.gr.AGL9* and *AGL9* in the MP tree (fig. 3). Using R-Q-RT PCR, strong signal of *Ma.gr.AGL9* was detected in all floral organs in *Magnolia* but not in leaves and spathaceous bracts (fig. 8). For *Eu.be. AGL9*, strong signal was detected in stamens, staminodes, and carpels, but very weak or no signal was observed in the leaves and calyptras examined (fig. 8). In contrast, *Ma.gr. AGL2*, a paralogue of *Ma.gr.AGL9* in the *AGL2*-like clade, showed similar expression across all floral organs, as well as in spathaceous bracts; an intermediate level of expression was detected in the leaves (fig. 8).

Discussion

Applicability of the ABC Model to Eupomatia *and* Magnolia

Expression patterns of B-class genes of *Eupomatia* inferred from R-Q-RT PCR, real-time PCR, and *in situ* hybridization are very similar. This strong similarity exists despite the fact that alternatively spliced sequences are present and that each method may have different sensitivities to detecting these forms. Hence, these results reinforce the conclusions described below for B-class and other floral organ identity genes.

Following the ABC model derived from studies in *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz 1991; Meyerowitz et al. 1991; Schwarz-Sommer et al. 1992;

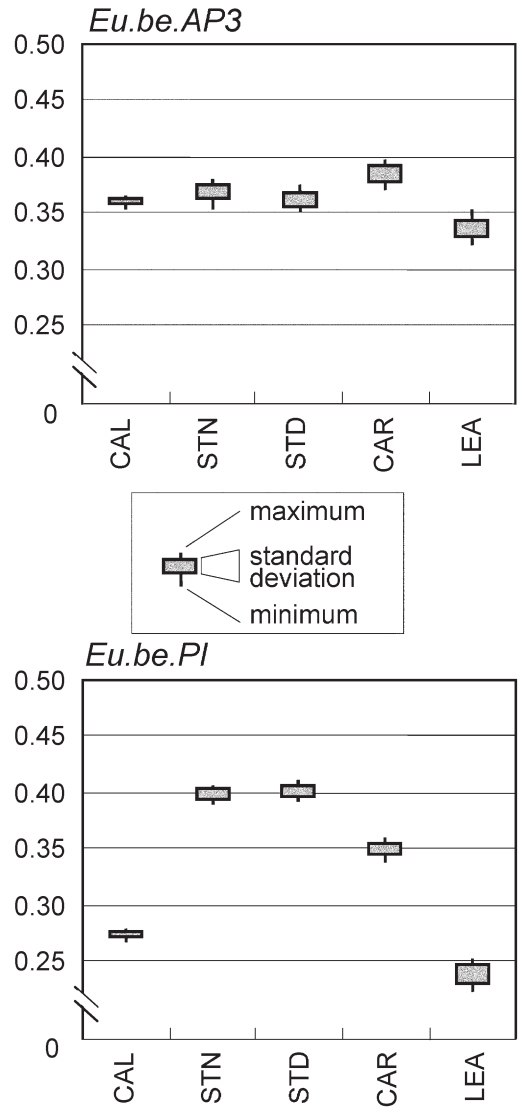

Fig. 9 Results of real-time PCR for B-class homologues in *Eupomatia*. The Ct value of the 18S rDNA control was divided by those of *Eu.be.AP3* and *Eu.be.PI*.

Tröbner et al. 1992), high levels of stable expression of B-class genes are expected in petals and stamens. However, deviations from these expectations have been reported in other basal angiosperms and in basal eudicots. For example, in the basal eudicots poppy (Papaveraceae) and various Ranunculaceae, *AP3* and *PI* homologues are expressed throughout the perianth, as well as in stamens and staminodes (Kramer et al. 2003). In the monocot tulip, *AP3* and *PI* homologues (*TGDEFA*, *TGDEFB*, and *TGGLO*) are expressed in outer tepals, inner tepals, and stamens (Kanno et al. 2003), and weak signal of the *PI* homologue (*TGGLO*) is also detected in the leaf and carpel. In the basal angiosperms *Amborella* and *Nuphar* (Nymphaeaceae), expression of *AP3* and *PI* homologues is detected throughout the perianth, as well as in stamens and staminodes (S. Kim et al., unpublished data). Our findings for the *AP3* homologue of *Eupomatia* also differ from predictions of the ABC model: weak signal of *Eu.be.AP3* was detected in the calyptra and leaves, in addition to strong expression in all floral organs. However, the role of the weak extrafloral expression in *Eupomatia* is not clear, because in both *Arabidopsis* and *Antirrhinum*, B-function genes are also expressed weakly outside the petal and stamen primordia but not in leaves (Jack et al. 1992; Schwarz-Sommer et al. 1992; Tröbner et al. 1992; Goto and Meyerowitz 1994). The role of *Eu.be.AP3* in structures other than stamens and staminodes requires further study.

Recent phylogenetic analyses of *AP1*-like genes have led to the proposal that a duplication event occurred at the base of the core eudicots to give rise to the *euAP1* and *euFUL* gene lineages (Litt and Irish 2003). Previously reported *euAP1* genes are most strongly expressed in sepals and petals (*AP1*: *Arabidopsis*, Mandel et al. 1992; *SQUA*: *Antirrhinum*, Huijser et al. 1992; *PEAM4*: *Pisum*, Taylor et al. 2002; *SLM4*: *Silene*, Hardenack et al. 1994), as predicted by the ABC model (reviewed by Irish 2003). In contrast to *euAP1* genes, high levels of expression of *euFUL* genes were found in carpels and bracts (*FUL*: *Arabidopsis*, Gu et al. 1998; Mandel et al. 1992; *DEFH28*: *Antirrhinum*, Muller et al. 2001). A basal angiosperm A-class homologue from *Nuphar* (*Nu.ad.SQUA*) was also expressed in carpels and leaves (S. Kim et al., unpublished data). *AP1* homologues from *Eupomatia* and *Magnolia* also showed this *euFUL*-like expression pattern in this study (fig. 8). It seems that the A-function of *euAP1*-like genes is restricted to the *euAP1* lineage, and the expression of *AP1* homologues from *Eupomatia* and *Magnolia* is not correlated with the formation of typical first and second floral whorls.

Phylogenetic analyses have been performed for *AGL2*-like genes (E-class) (Hasebe and Banks 1997; Becker and Theissen 2003; Zahn et al., forthcoming), although these analyses could not address the divergence and duplication of this gene group because of the limited number of genes in these studies. In our study, two *Magnolia AGL2*-like genes showed redundant expression in all floral organs, a pattern similar to that in *Arabidopsis* for *AGL2*, *AGL4*, and *AGL9* (Flanagan and Ma 1994; Savidge et al. 1995; Mandel and Yanofsky 1998).

Although C-class genes have yet to be analyzed and functional studies are needed in the species examined here, we propose that the ABC model may not be strictly applicable in Magnoliales, based on our observations of gene expression patterns of A-, B-, and E-class homologues in *Eupomatia* and *Magnolia*. Floral genes of *Eupomatia* and *Magnolia* show a broader range of expression across floral whorls than core eudicots and derived monocots (Irish 2003), similar to those for other basal angiosperms and some monocots, such as tulip (Kanno et al. 2003). Our data join a growing body of data indicating that increased functional specialization of these floral genes occurred in core eudicots and derived monocots.

Evolutionary Origin of the Calyptra

There has been controversy regarding whether the expression of floral genes can be used to address organ identity (Albert et al. 1998; Buzgo et al. 2004). Over large phylogenetic distances it is probably inappropriate to use patterns of expression to assess organ identity. However, among closely related taxa where clear expectations have been established

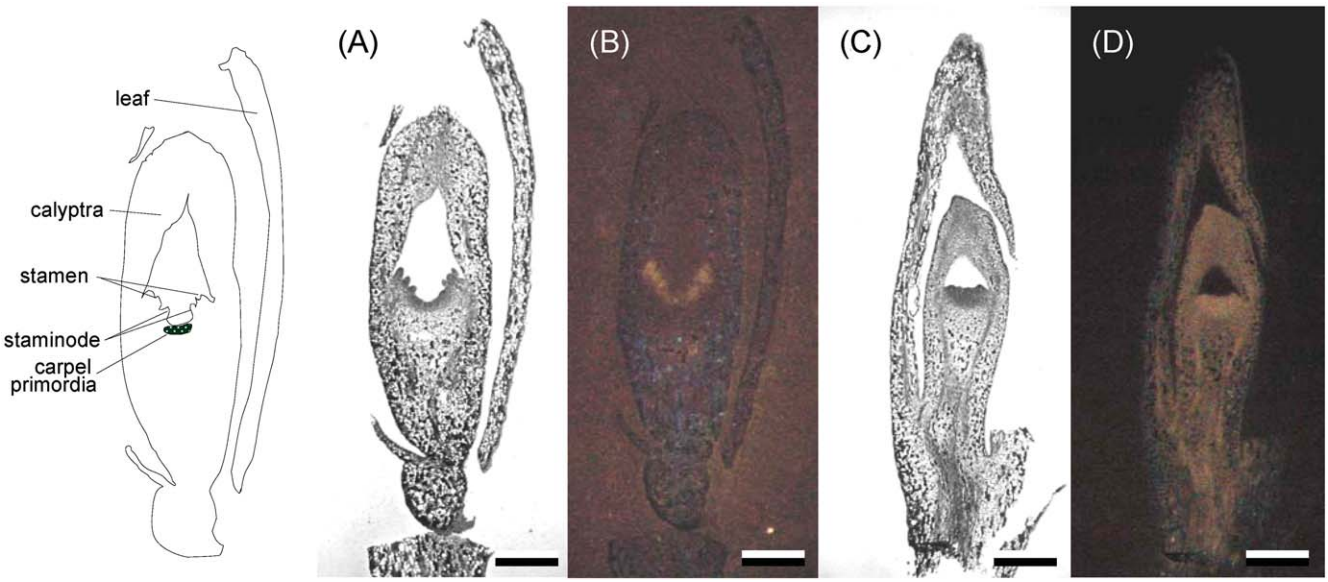

Fig. 10 *In situ* hybridization using *Eu.la.AP3* and *Eu.la.PI* gene probes on a longitudinal section of a developing *Eupomatia laurina* flower. *A*, *B*, Results with *Eu.la.PI* as probe; *C*, *D*, results with *Eu.la.AP3* as probe. *A*, *C*, Bright-field images; *B*, *D*, dark-field images. Scale bar = 0.5 mm.

in one or more reference taxa, it may be fruitful to compare patterns of expression in a unique or unusual floral organ with those of typical organs. We adopted this approach with *Eupomatia*, using data obtained for the closely related *Magnolia* as a reference genus.

Because the expression patterns of the homologues of the ABC genes are not conserved in Magnoliales relative to model organisms, we cannot use the expected expression patterns from *Arabidopsis* and *Antirrhinum* to infer the homology of the calyptra. For example, even though A-, B-, and E-class homologues are expressed in the calyptra of *Eupomatia*, as predicted if the calyptra were derived from sepals and petals (but note that the *PI* homologue is not expressed), the lack of a differentiated perianth in at least some Magnoliales and other basal angiosperms, coupled with less specific patterns of expression of floral regulators across the developing flower (S. Kim et al., unpublished data), makes application of this model inappropriate. The modified ABC model based on studies of lily and tulip also predicts A-, B-, and E-class gene expression in the calyptra, if the calyptra is derived from an undifferentiated perianth. The expression of A-, B-, and E-class homologues in the calyptra would seem to support an origin of the calyptra from the perianth. However, at least two factors argue against this hypothesis. First, the *PI* homologue is not expressed in the calyptra, as would be expected if the calyptra were homologous to the perianth. Second, A-, B-, and E-class homologues are also expressed in leaves of *Eupomatia* and in spathaceous bracts and leaves of *Magnolia grandiflora*, signifying that even the modified ABC model is not appropriate for evaluating homology of the calyptra. The hypothesis of a bracteate origin of the calyptra relative to derivation from the perianth requires comparison of expression patterns between the calyptra and vegetative organs: leaves of *Eupomatia* and leaves and spathaceous bracts of *M. grandiflora*.

Expression patterns of floral organ identity genes in the calyptra of *Eupomatia* clearly resembled those detected in leaves of *Eupomatia* and differed from expression patterns in the perianth of the close relative, *M. grandiflora*. Expression of two B-class homologues and an E-class homologue in the calyptra of *Eupomatia* was almost identical to that detected in leaves of *Eupomatia*. However, expression of the A-class homologue in the calyptra differs from that in leaves and from expression of A-class homologues in bracts of *Magnolia*. In sum, however, gene expression data for B- and E-class genes in the calyptra of *Eupomatia* are very similar (but not identical) to expression patterns in leaves of *Eupomatia* and leaves and bracts of *Magnolia* but very different from expression patterns in the perianth of *M. grandiflora*. These data therefore support the interpretation that the calyptra of *Eupomatia* is derived from a bract, in agreement with comparative developmental studies (Endress 1977, 2003) (fig. A3), and argue against its derivation from a perianth (Hiepko 1965; von Mueller 1862).

Introns and Alternative Splicing of Eu.be.AP3

On the basis of both the *Eu.be.AP3* genomic and cDNA alignment and the consensus donor/acceptor site sequences of *Arabidopsis* (AG/GTAAGT and TGCAG/G; Brown and Simpson 1998; Hebsgaard et al. 1996; Lorkovic et al. 2000), the putative acceptor and donor sites for the boundaries of introns 1, 2, 5, and 6 were determined (fig. 4). These sites exactly matched the putative splicing sites recognized in our previous study (Kim et al. 2004).

Two different types of nuclear introns have been reported: "GT-AG introns" (or U2-type) and "AT-AC introns" (U12-type) (Lorkovic et al. 2000). The name GT-AG intron is taken from the two strongly conserved nucleotides at the 5′ and 3′ ends of the intron, respectively. Most introns are GT-AG, and only ca. 0.1% of introns are AT-AC (Lorkovic et al.

2000). GT-AG sequences are conserved in the introns of *Eu.be.AP3*, with the exception of the 5′ end of exon 2: it has GC instead of GT (fig. 4). This is an exceptional case in GT-AG introns. In the GT-AG intron, G in the 5′ end and AG in the 3′ end were conserved in 100% of the "dicot" plants examined, and T in the 5′ end was conserved in 99% of the "dicots" (Lorkovic et al. 2000). The GC-AG intron was found in only 1.04% of the predicted introns in the *Arabidopsis* expressed sequence tag database (Zhu et al. 2003).

Alternative splicing has emerged as one of the most significant generators of functional complexity in several relatively well-studied animal genomes, but only recently has more attention focused on its importance in plants (Kazan 2003). According to recent estimates, ca. 40% of human genes are alternatively spliced (Modrek and Lee 2002). Four types of alternatively spliced transcripts were recognized for the flowering-time gene *FCA* in *Arabidopsis*, and the role of alternative processing on the floral transition was studied (Macknight et al. 2002). Examples of alternative splicing of MADS-box genes have recently been identified in *AGAMOUS* (Cheng et al. 2003) and B-class homologues in *Nymphaea* (*NymAP3*; Stellari et al. 2004), *Magnolia* (*Ma.gr.AP3*; S. Kim et al., unpublished data), and *Amborella* (*Am.tr.PI*; S. Kim et al., unpublished data). Furthermore, recent computational and experimental studies indicated that alternative splicing probably plays a far more significant role in the generation of proteome diversity in plants than was previously thought (Kazan 2003). Our findings for *AP3* homologues of *Eupomatia* suggest that alternative splicing may be very important in generating organ-specific sequences.

A critical question involves the functional implications of these alternatively spliced transcripts. Kramer et al. (2004) suggested two potential manners in which the production of alternative transcripts could negatively regulate a gene. If alternatively spliced sequences are not translated (possibly types I, II, III, IV, VIII, X, and XI in *Eu.be.AP3*), their generation could reduce the concentration of functional transcript. If the alternatively spliced transcripts are translated (possibly type V, VI, VII, and IX in *Eu.be.AP3*), the truncated products may be capable of functioning as dominant negative factors, similar to what has been found for truncated AG and AP3 proteins in *Arabidopsis* (Mizukami et al. 1996; Krizek et al. 1999). The clearest example we observed of strong expression of an alternatively spliced transcript involves *Eu.be.AP3* in stamen tissue. On the basis of observations of different expression patterns of alternatively spliced fragments in each floral organ (fig. 5), it seems that the acquisition of different functional roles by alternatively spliced forms may have occurred, perhaps influencing organ identity. The extent and roles of alternatively spliced variants of floral regulatory genes are open avenues for future research.

Acknowledgments

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