The Plant Journal (2006) **47**, 501–518 doi: 10.1111/j.1365-313X.2006.02800.x

Petaloidy and petal identity MADS-box genes in the balsaminoid genera *Impatiens* and *Marcgravia*

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Received 30 March 2006; accepted 7 April 2006.

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Summary

***Impatiens* and *Marcgravia* have striking morphological innovations associated with the flowers. One of the sepals in *Impatiens* is spurred and petaloid, while in *Marcgravia* the petals are fused into a cap and nectary cups are associated with the inflorescence. Balsaminaceae (*Impatiens*) and Marcgraviaceae have surprisingly been shown to be closely related, since both belong to the balsaminoid clade of Ericales (basal asterids). However, several thorough morphological studies thus far have not revealed shared derived characters (synapomorphies) that support a close relationship between these families. In the balsaminoid clade, transitions from entirely green flowers to flowers with heterotopic petaloid organs can be observed. The primary role of class B genes in core eudicots is to specify the identity of petal and stamen floral organs. E-class genes, of which *SEP3* is a representative, have been identified as redundant mediators that confer transcriptional activation potential on protein complexes that specify organ identity. Given the conserved function of organ-identity MADS-box genes in model plants, but the rapid molecular evolution in angiosperms, it remains controversial whether these genes have been involved in shaping floral diversity. We have identified a *SEP3*-like gene and a total of five class B genes from *Impatiens hawkeri* and *Marcgravia umbellata* and report their quantitative expression in the floral organs. In *Impatiens*, two *AP3/DEF*-like genes were identified with strongly divergent C-terminal domains, one truncated and one unusually long. Both genes show a gradual decrease in expression towards the outer perianth organs, but no *GLO*-like gene expression is observed in the petaloid sepal. Remarkably, *SEP3*-like gene expression in the *Impatiens* perianth is absent from the green sepals but present in the petaloid sepal and in the petals. Dimeric protein interactions of the cloned *Impatiens* genes were studied in yeast and by using gel retardation. In *Marcgravia*, strong overlapping class B gene expression is limited to the stamens, but a *SEP3*-like gene is strongly expressed in the *Marcgravia* nectary, indicating that both *Impatiens* and *Marcgravia* show heterotopic expression of a *SEP3*-like gene. We discuss several candidate mechanisms for heterotopic petaloidy involving modified gene expression and protein interaction of *SEP3*-like and class B genes.**

Keywords: SEPALLATA3, class B genes, petal identity, MADS-box genes, *Impatiens*, *Marcgravia*.

Introduction

Angiosperm flowers show a bewildering structural and functional diversity, but little is known about the numerous evolutionary events that have created this diversity. A remarkable pair of plant families illustrating this phenomenon are the closely related core eudicot families Balsaminaceae and Marcgraviaceae. They belong to the balsaminoid clade in the order Ericales, which is placed at the base of the asterids. The only other members of the balsaminoid clade belong to the family Tetrameristaceae (Angiosperm Phylogeny Group, 1998, 2003; Figure 1).

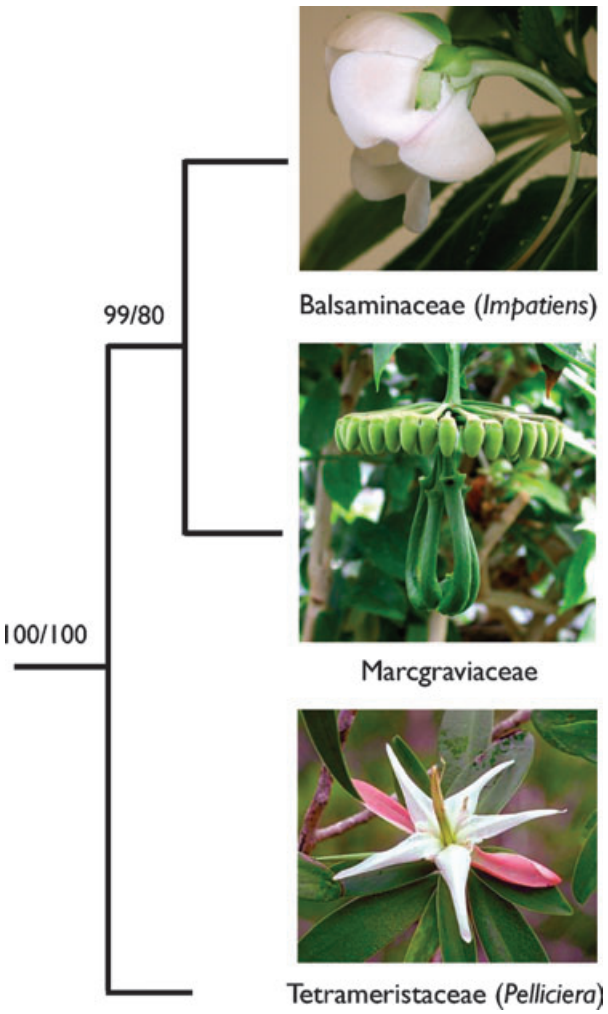

Figure 1. Proposed phylogeny of the balsaminoid clade.
Numbers above branches indicate Bayesian posterior probabilities in mixed model analysis with best-approximating models/next-best approximating models (Geuten *et al.*, 2004). Picture of *Pelliciera rizophorae* used with permission of J. Zarucchi. Picture of *M. umbellata* used with permission of A. J. Höggemeier.

Balsaminaceae and Marcgraviaceae show a sister group relationship in phylogenetic trees based on molecular data (Geuten *et al.*, 2004; but see also Schönenberger *et al.*, 2005). However, striking morphological differences exist between the two families and no morphological characters support their relationship (Geuten *et al.*, 2004; Janssens *et al.*, 2005; Lens *et al.*, 2005).

An intriguing feature in these families is the heterotopic petaloidy that can be observed (Albert *et al.*, 1998). In angiosperms in general, organs other than petals that display color are an abundant variation on the floral theme of green sepals, showy and colored petals, stamens and carpels (Endress, 1994). In the Balsaminaceae genus *Impatiens*, the perianth consists usually of three sepals, of which two are small and greenish and one is enlarged and secretes nectar in a spur. This enlarged sepal is usually very similar to the petals in color and tissue texture (Figure 2a (2)).

Marcgraviaceae are woody vines from the neotropics (Dressler, 2004). In members of the genus *Marcgravia*, conspicuous or contrasting color is most often limited to the stamens (Dressler, 1997). This is certainly the case for the type species *Marcgravia umbellata* L., where nectaries, pedicels, bracteoles, sepals and corolla caps are greenish (Figure 2b). However, in other genera of Marcgraviaceae, the nectaries are usually brightly colored in various reddish to yellowish tints (Dressler, 2004). These nectaries can be interpreted as modified bracts, since several intermediate forms, from regular bract to cup-shaped nectaries can be observed in inflorescences of Marcgraviaceae (de Roon, 1975). Heterotopic petaloidy also occurs in Tetrameristaceae: *Pelliciera* has a set of two bracts close to the flower, five smaller sepals and five larger petals; bracts, sepals and petals all have a white to pinkish color (Figure 1). *Tetramerista* on the other hand has green flowers (Kobuski, 1951; Maguire *et al.*, 1972; Melchior, 1925).

Petal identity in higher eudicot flowers is established through the combinatorial activity of class A and B organ identity genes, as illustrated in the well-known ABC model. In addition, the class A genes alone establish sepal identity, an overlap between class B and C gene expression specifies male reproductive organ identity, and the C function alone establishes female reproductive organ identity (Coen and Meyerowitz, 1991; Schwarz-Sommer *et al.*, 1990). Based on this model for what is known about flower development in model plants, it has been proposed that heterotopic petaloidy could be caused by an associated heterotopic expression of class A and B genes, or of class B genes alone in petaloid sepals (Albert *et al.*, 1998; Bowman, 1997; van Tunen *et al.*, 1993). This hypothesis is supported by experiments on constitutive expression in Arabidopsis. When *AP3* and *PI*, the Arabidopsis class B genes, are ubiquitously expressed, the first whorl sepals become petaloid but vegetative leaves remain untransformed, indicating that there are missing factors for establishing petal identity in these vegetative organs. When, in addition to the class B genes, the class A gene *AP1* is overexpressed, a single cauline leaf becomes petaloid. An even more severe phenotype with petaloid vegetative leaves results when *SEP3* is co-expressed with the B function genes (Honma and Goto, 2001). The protein products of all the above-mentioned genes belong to the MADS-domain family of transcription factors. The MADS-domain proteins involved in floral organ identity have been shown to interact with each other to regulate downstream genes (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). A current model depicting experimentally demonstrated and predicted interactions is the floral quartet model (Theißen, 2001). In this model of Arabidopsis floral organ development, sepal identity is established by a protein quartet of SEP–SEP–AP1–AP1 and

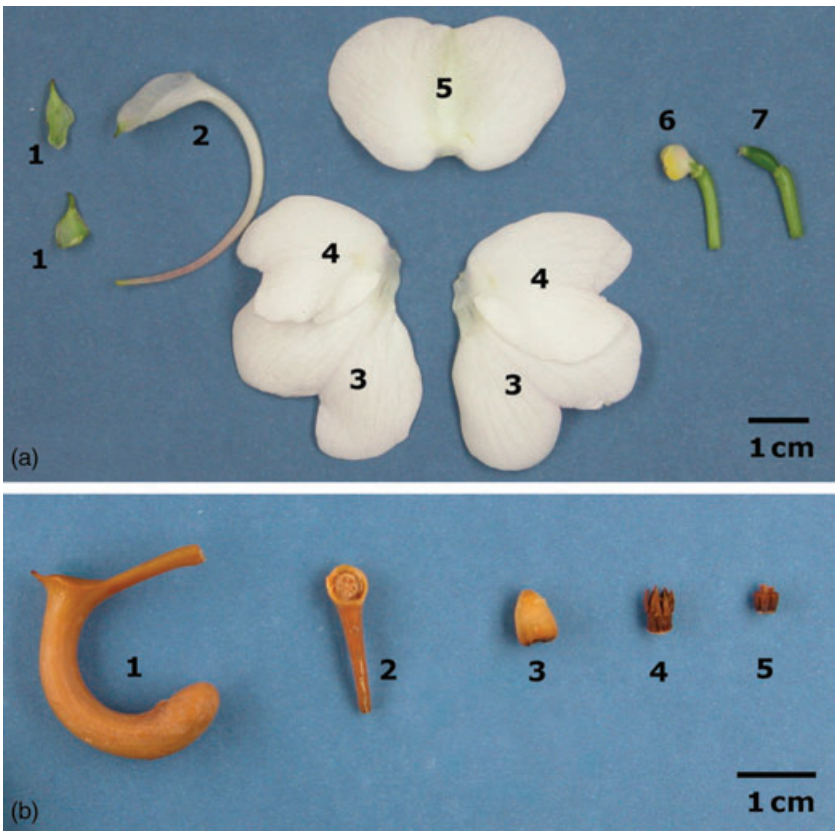

Figure 2. Floral organs of *I. hawkeri* and *M. umbellata*.
(a) Floral organs of *I. hawkeri*: 1, lateral sepals; 2, petaloid lower sepal; 3, lower lateral petals; 4, upper lateral petals; 5, dorsal petal; 6, stamens; 7, ovary.
(b) Fixed floral organs of *M. umbellata*: 1, inflorescence nectary; 2, bracteoles and sepals attached to the receptacle; 3, petal calyptra; 4, stamens; 5, ovary.

petal identity is established by a SEP–AP1–PI–AP3 quartet. Similar to AP1, the SEPALLATAs and mainly SEPALLATA3 are thought to confer transcriptional activation potential to quaternary MADS-domain protein complexes (Cho *et al.*, 1999; Honma and Goto, 2001; Kaufmann *et al.*, 2005; Pelaz *et al.*, 2001a,b).

In addition to the MADS-domain proteins, several other cloned genes have been shown to be able to control petaloidy in the Arabidopsis flower. One such gene is *UFO*, encoding an F-box protein which presumably functions in petal identity by targeting for degradation a negative regulator of class B organ identity genes, thus positively regulating class B genes in an indirect way (Lee *et al.*, 1997; Samach *et al.*, 1997; Wilkinson and Haughn, 1995).

Most of the well-studied MADS-box genes to date belong to the type II class and encode proteins with a conserved domain structure, termed MIKC, in which a MADS-domain (M), an intervening domain (I) a keratin-like domain (K) and a C-terminal domain can be discerned (Münster *et al.*, 1997). As is evident from its evolutionary history, the family of MIKC-type genes, despite considerable gene loss, has proliferated in angiosperms through numerous duplications, with subsequent sub- and neofunctionalizations. In this way super- and subfamilies of genes with related functions arose (Becker and Theißen, 2003; Purugganan *et al.*, 1995). Sub- and neofunctionalization can be caused by changes in the *cis*-regulatory regions of a gene, resulting in different upstream regulatory control and sometimes modified expression (Zhang, 2003). However, functional evolution of MADS-domain proteins also results from changes in their protein sequence (Kramer *et al.*, 2003; Lamb and Irish, 2003; Vandenbussche *et al.*, 2003). The C-terminal region in particular has been implicated in functional diversification (Vandenbussche *et al.*, 2003). The C-terminal domain is most variable in length and sequence and subfamilies of MADS-box genes are characterized by small conserved amino acid motifs in the C-terminal domain, suggesting a role in determining functional specificity (Krizek and Meyerowitz, 1996; Lamb and Irish, 2003; Riechmann and Meyerowitz, 1996; Riechmann *et al.*, 1996). The C-terminal domain is thought to play a central role in proliferation of the MADS-box gene family (Lamb and Irish, 2003; Vandenbussche *et al.*, 2004), but its specific function is still not well resolved.

We used *Impatiens* and *Marcgravia* as core eudicot model systems to study modifications of the ABC and floral quartet systems of specification of floral organ identity in relation to heterotopic petal identity as proposed by Albert *et al.* (1998).

We report on putative organ identity genes in *Impatiens hawkeri* (Balsaminaceae) and *M. umbellata* (Marcgraviaceae) and show that the expression pattern of their class B

and *SEP3*-like genes is modified when compared with model plants.

To interpret our findings, we give an overview of possible molecular mechanisms that may have caused the modifications of floral organ identity and development in the two species.

Results

Floral organ epidermal morphology

Epidermal cell shape is a character that is frequently used to determine floral organ identity (Endress, 1994; Jaramillo and Kramer, 2004; Krizek *et al.*, 2000). R2R3 MYB transcription factors direct the formation of conical-papillate cells in the petal epidermis of Arabidopsis and are probably under the transcriptional control of B-function genes (Martin *et al.*, 2002; Perez-Rodriguez *et al.*, 2005). To correlate floral organ appearance in *M. umbellata* and *I. hawkeri* with epidermal cell shape of the respective organs, the surface structure of some of the outer floral organs of the two species was analyzed employing scanning electron microscopy (SEM).

In *I. hawkeri* (Figure 3), the abaxial and adaxial surfaces of the sepal (Figure 3a–d) consist of normal, non-papillate cells. Importantly, the large spurred sepal (Figure 3c,d), showy and petaloid at first glance, has no epidermal cell modifications. In contrast, the adaxial surface of all petals is covered with dome-shaped papillate cells (Figure 3f,h,i). The abaxial surface of the *I. hawkeri* petals has no modified epidermal cells (Figure 3e,g).

We also observed the morphology of the abaxial and adaxial surfaces of *M. umbellata* (Figure 4). The nectariferous bracts, both on the inside and the outside, are completely covered with elongated cells (Figure 4a–e), for which we prefer not to use the term conical-papillate as their appearance markedly differs from the cells observed in *I. hawkeri*.

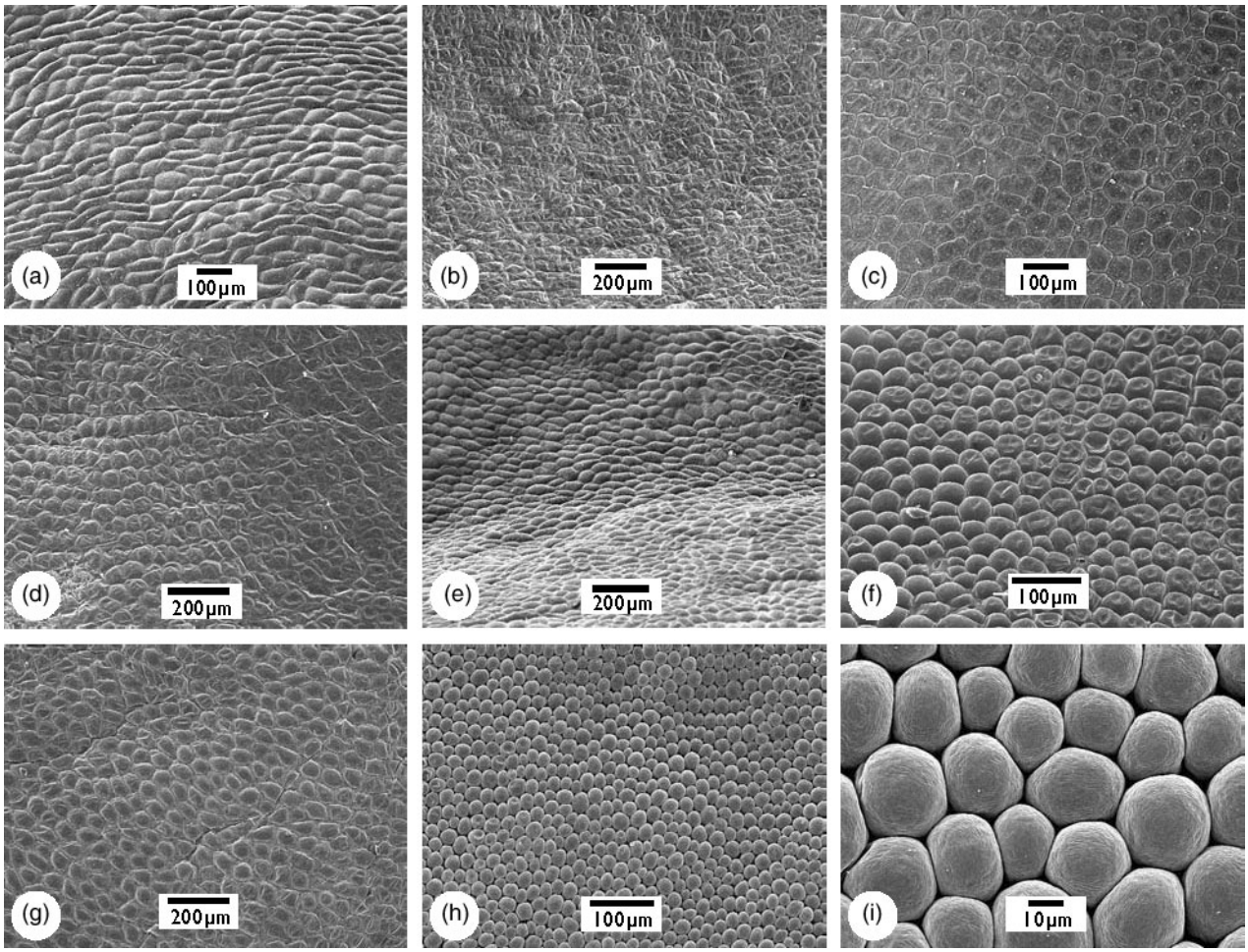

Figure 3. SEM observations of the epidermal structure of *I. hawkeri*.
(a) Upper sepal, abaxial, (b) upper sepal, adaxial, (c) spurred and petaloid lower sepal, abaxial, (d) lower sepal, adaxial, (e) dorsal petal, abaxial, (f) dorsal petal, adaxial, (g) lateral petal, abaxial, (h) lateral petal, adaxial, (i) detail of dome-shaped conical cells on the adaxial surface of a lateral petal.

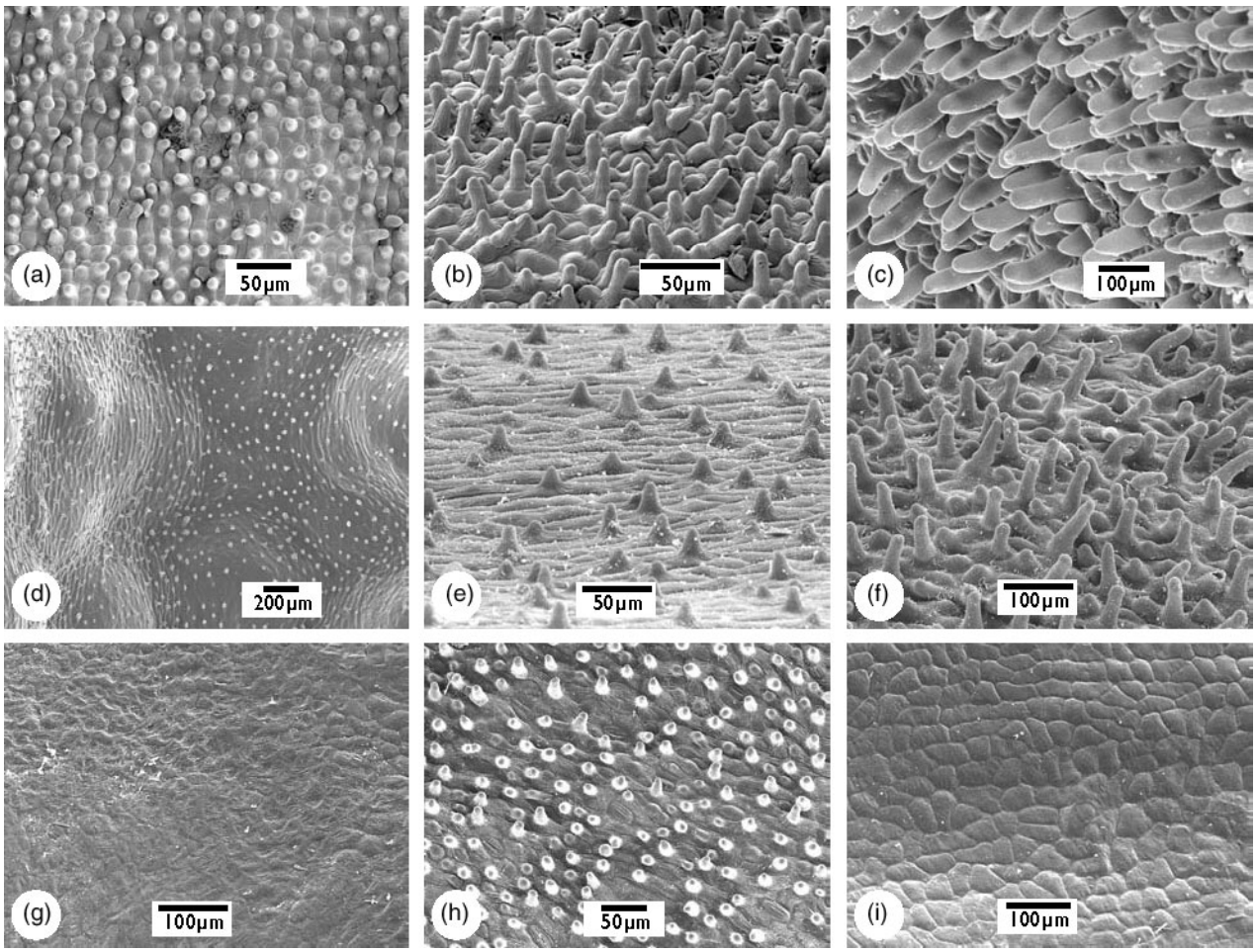

Figure 4. SEM observations of epidermal structure of *M. umbellata*.
(a) Papillae in a developing nectary. (b, c) Papillae can elongate during development: (b) upper nectary opening, (c) lower inside of nectary. (d) In older developmental stadia, papillae are further apart by stretching of cells. (e) Papillae on the outer side of the nectary are comparable to the inside, sometimes longer. (f) Calyx, abaxial (g) Calyx, abaxial. (h) Corolla, abaxial—not necessarily a different type in comparison to the calyx; a texture of long papillae as in (f) could also be observed on the abaxial surface of the corolla. (i) Corolla, adaxial.

On the abaxial surface of the sepals (Figure 4f), very similar cells are present; the adaxial surface (Figure 4g), however, consists of unmodified epidermal cells. The petals, fused in a calyptra, have the same epidermal cell surface as the sepals with a modified cell type only present on the abaxial surface (Figure 4h,i). We can conclude that all external parts of the flower of *M. umbellata* and the nectariferous bracts are covered with elongated cells.

Class B MADS-box genes from I. hawkeri *and* M. umbellata

For *Impatiens*, two putative *AP3/DEF*-like genes were cloned, named *IhDEF1* and *IhDEF2*. *IhDEF1* encodes a protein characterized by an unusually long C-terminal domain in which a strongly modified PI-derived motif can be recognized and a euAP3 motif is present. The *IhDEF2* sequence is coding for a MADS-domain protein with an unusually short C-terminal domain, lacking the PI-derived motif and the euAP3 motif. In addition, a putative *PI/GLO*-like gene was cloned, in which the PI motif is easily recognizable (Figure 5). The 3′ rapid amplification of cDNA ends (3′-RACE) experiments on *M. umbellata* floral bud mRNA resulted in the identification of *MuDEF1*, a putative *AP3/DEF*-like gene and *MuGLO1*, a putative *PI/GLO*-like gene. Both have the expected motifs in their protein sequence. The phylogenetic relationships of the sequences were further tested in a phylogenetic analysis (Figure 6), confirming that *IhDEF1*, *IhDEF2* and *MuDEF1* are within the clade of *AP3/DEF*-like genes *sensu stricto*. These genes are thus putative orthologs of *AP3* from Arabidopsis and *DEF* from Arabidopsis, rather than *TM6*-like genes. *IhGLO1* and *MuGLO1* are identified as *PI/GLO*-like genes.

The phylogenetically closest relative of *Impatiens* and *Marcgravia*, for which class B genes have been cloned and sequenced, is *Hydrangea macrophylla* (Kramer *et al.*, 1998),

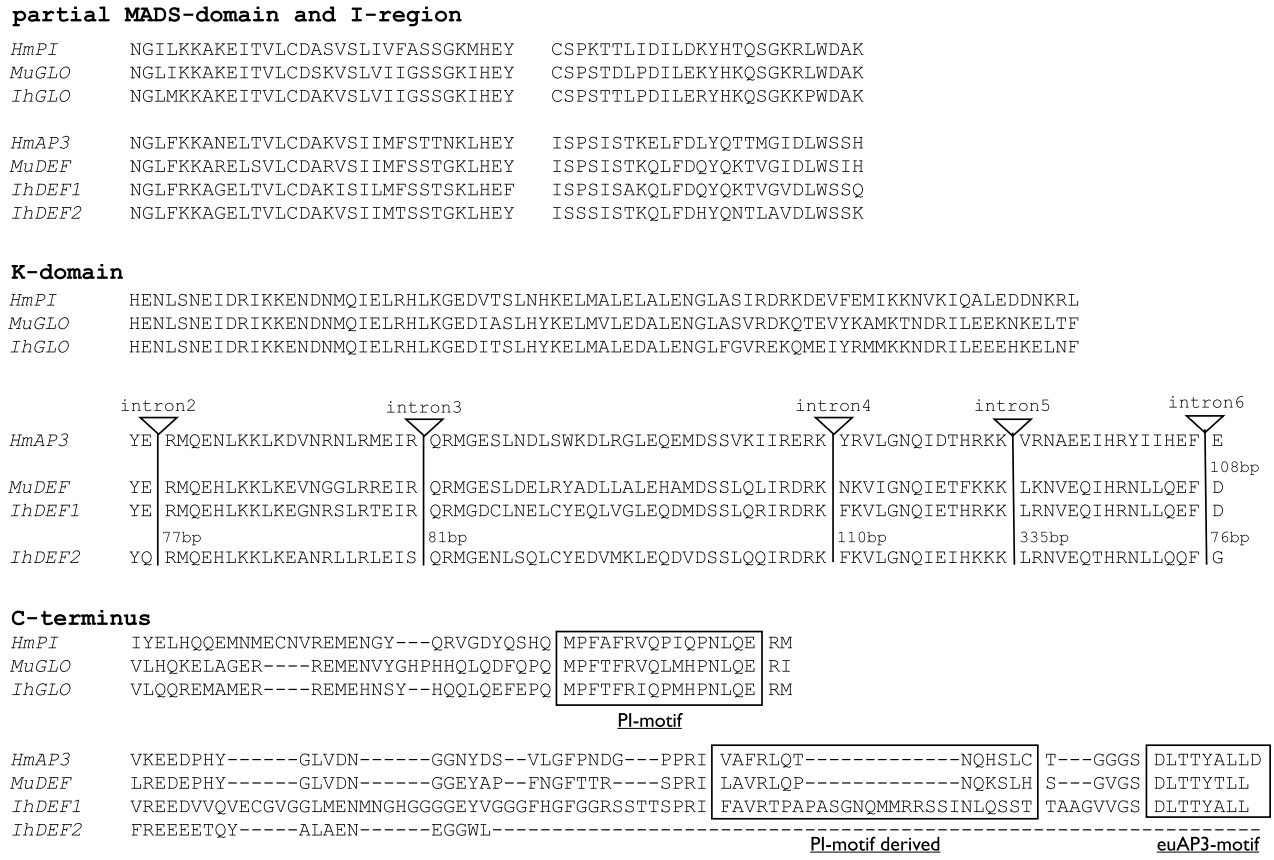

Figure 5. Alignment of *HmPI* and *HmAP3* from *H. macrophylla*, *MuDEF1* and *MuGLO1* from *M. umbellata* and *IhDEF1*, *IhDEF2* and *IhGLO1* from *I. hawkeri*. An asterisk indicates the stop codon of IhDEF2. The length of the sequenced introns of *IhDEF2* with their position and the length and position of intron 6 of *MuDEF1* is indicated. The PI motifs, the PI-derived motifs and the euAP3 motifs are also indicated.

which belongs to the small basal asterid order Cornales (Xiang *et al.*, 2002). An alignment of the more divergent K-domain and C-terminal domain of the cloned MADS-domain proteins from *Impatiens* and *Marcgravia* with the translated cDNA sequences from *H. macrophylla* is shown in Figure 5.

To check whether the unusual premature stop codon in the *IhDEF2* cDNA sequence is actually coded by the genome or is a result of differential splicing or a PCR or sequencing artifact, we cloned and sequenced part of the genomic locus of *IhDEF2*, encoding the K-domain and the C-terminal domain. In this region, we found five introns in the K-box. The sequenced introns are indicated with their length in Figure 5. The C-terminal end sequence of IhDEF2, including the early stop codon, was confirmed in the genomic DNA sequence, demonstrating that *IhDEF2* represents a structurally derived gene rather than an alternative splicing product or an experimental artifact.

To investigate the presence of additional recent duplicates of the cloned *AP3/DEF*-like genes or *PI/GLO*-like genes, genomic DNA hybridization was used. A minimum of two bands per lane and the genomic sequence with known restriction sites are consistent with two copies of *AP3/DEF*-like genes in the genome of *I. hawkeri* (Figure 7a). The *M. umbellata* Southern blot (Figure 7b) shows at least two hybridized bands per lane, suggesting the presence of two *AP3/DEF*-like genes in *M. umbellata*.

The phylogenetic reconstruction indicates that *IhDEF1* and *IhDEF2* together are orthologs ('co-orthologs') of *MuDEF1* with respect to the Balsaminaceae–Marcgraviaceae split. A second *AP3/DEF*-like gene either originated after the Balsaminaceae–Marcgraviaceae split or before that split, and was lost in the Balsaminaceae lineage. The fact that the bands in each lane of Figure 7(b) are of almost equal intensity makes the first scenario much more likely.

The Southern blot to test for the copy number of *PI/GLO*-like genes in *I. hawkeri* shows two or three bands per lane (Figure 8a). Sequencing of the partial genomic locus did not reveal restriction sites in introns. This indicates that in addition to *IhGLO1*, probably one, but possibly two additional duplicates are present in the genome of *I. hawkeri.* Genomic hybridization using a *MuGLO1* probe to investigate the number of recent duplicates of *PI/GLO*-like genes in

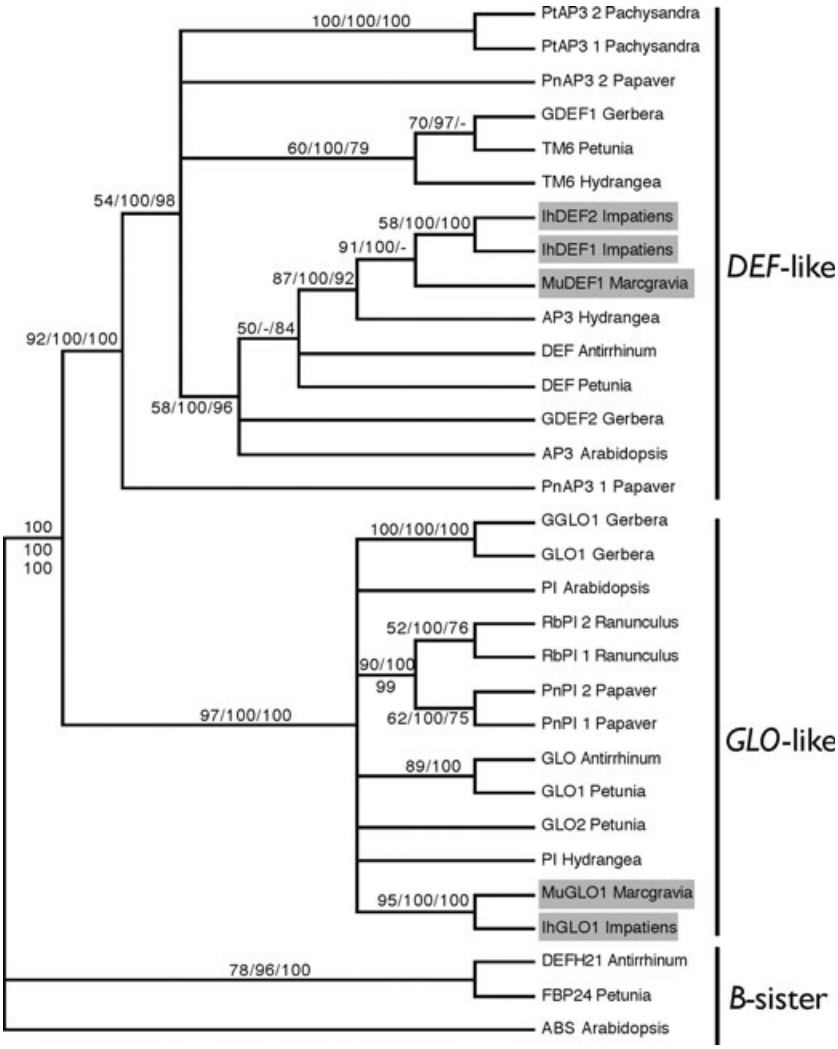

Figure 6. Phylogenetic identification of class B genes of *I. hawkeri* and *M. umbellata*.
The depicted topology is a parsimony bootstrap consensus tree, numbers above branches indicate, parsimony bootstrap values/Bayesian posterior probabilities (as percentages)/neighbor joining bootstrap values.

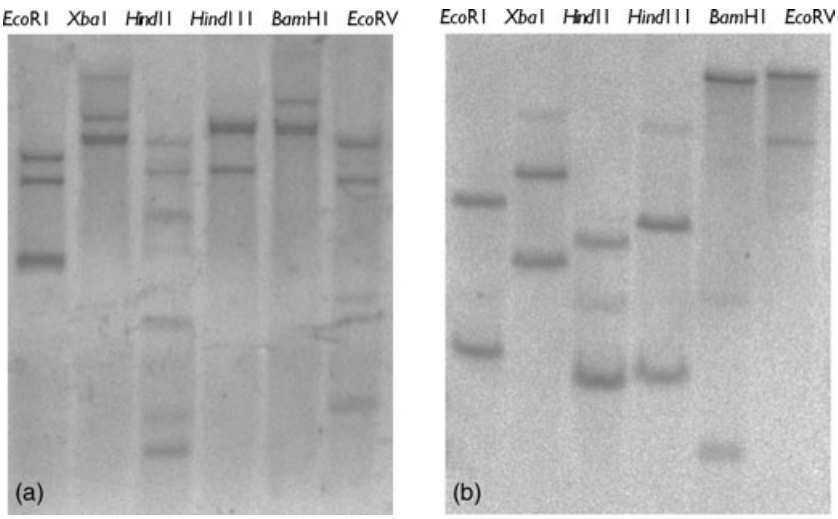

Figure 7. Southern blots to check copy number of *AP3/DEF*-like genes of *Impatiens hawkeri* and *Marcgravia umbellata*.
(a) Southern blot on genomic DNA of *I. hawkeri* using the coding sequence of the K- and C-terminal domains of *IhDEF2* as a probe.
(b) Southern blot on genomic DNA of *M. umbellata* using the K- and C-terminal domains of *MuDEF1* as a probe.

M. umbellata indicates a single band in the lane with *Xba*I restriction product (Figure 8b). In the other lanes, however, a second band of lower intensity is present. This shows that probably only a single *PI/GLO*-like gene is present in the genome of *M. umbellata*, but a second copy cannot be strictly excluded.

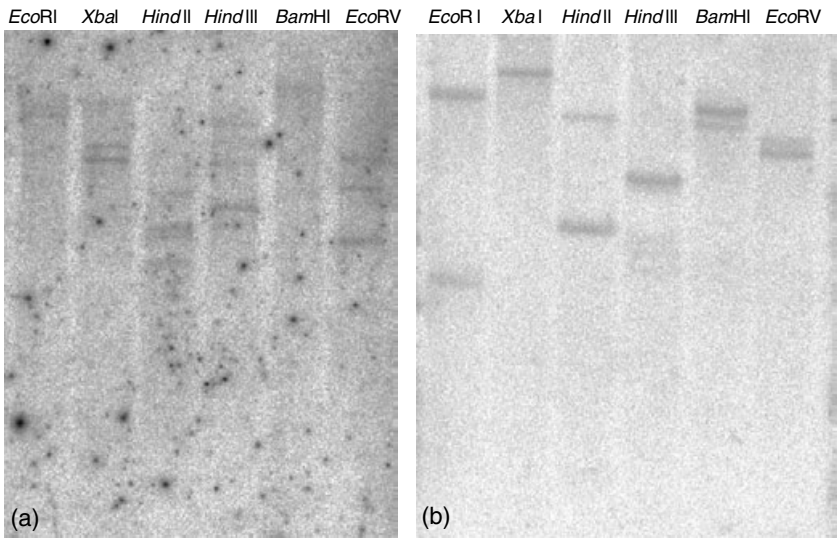

Figure 8. Southern blots to check copy number of *Pi/GLO*-like genes of *Impatiens hawkeri* and *Marcgravia umbellata*.
(a) Southern blot on genomic DNA of *I. hawkeri* using the coding sequence of the K- and C-terminal domains of *IhGLO1* as a probe.
(b) Southern blot on genomic DNA of *M. umbellata* using the K- and C-terminal domains of *MuGLO1* as a probe.

Quantification of Impatiens *and* Marcgravia *class B gene expression*

We analyzed the expression of the identified class B genes by real-time PCR in order to test the hypothesis that the expression domain of class B genes is correlated to petaloidy in the flowers of *Impatiens* and *Marcgravia*. The dissected floral organs are depicted in Figure 2. In *I. hawkeri*, the spatial expression pattern of the two *AP3/DEF*-like genes *IhDEF1* (Figure 9a) and *IhDEF2* (Figure 9b)

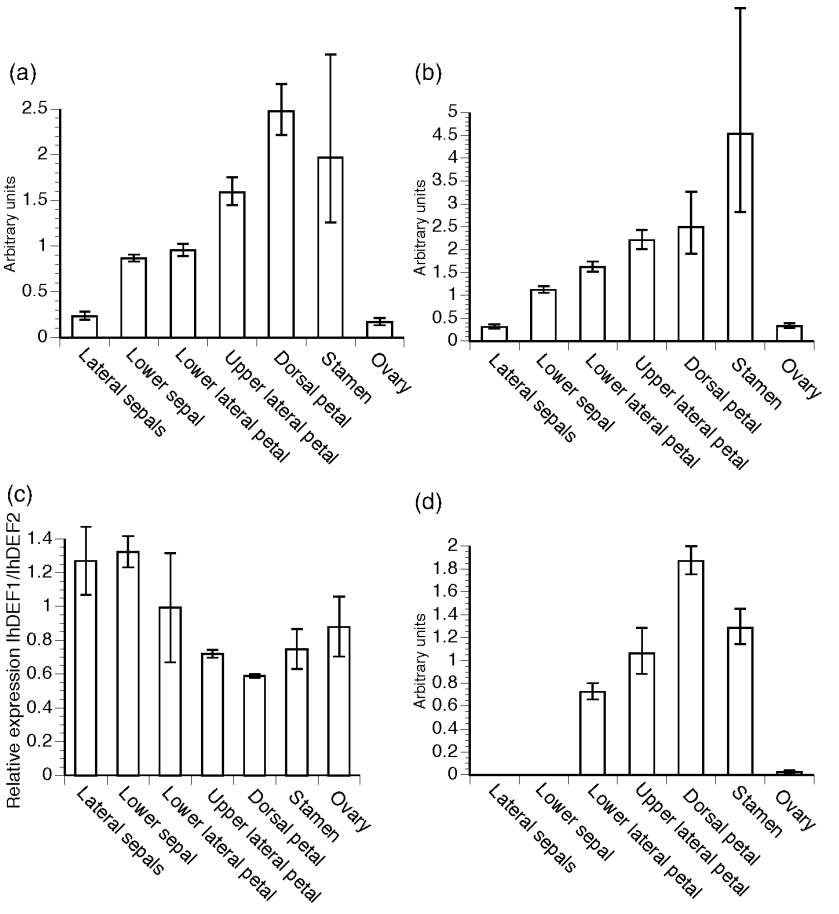

Figure 9. Analysis of quantitative expression in the floral organs of *I. hawkeri*.
(a) *IhDEF1*.
(b) *IhDEF2*.
(c) Analysis of relative gene expression of *IhDEF1/IhDEF2*.
(d) *IhGLO1*.

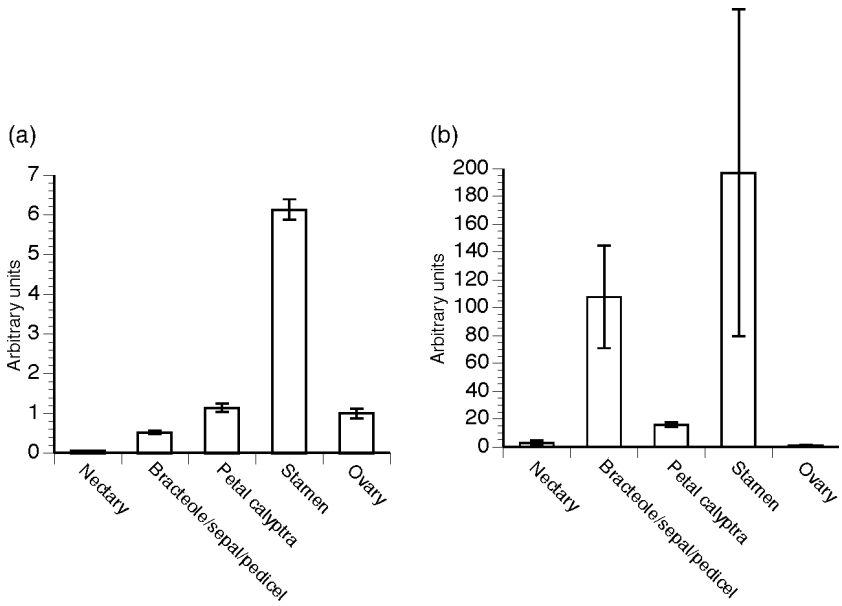

Figure 10. Analysis of quantitative expression in the floral organs of *M. umbellata*.
(a) *MuDEF1*.
(b) *MuGLO1*.

is quite similar (see also Figure 9c). Both are expressed at a very low level in the lateral sepals and the ovary, and they both show a gradual increase in expression from lower sepal to lower lateral petal, upper lateral petal to dorsal petal. Expression of *IhDEF1* in the dorsal petal is stronger than in the stamen in contrast to *IhDEF2*, which shows a stronger stamen expression when compared with the dorsal petal. The expression level of *IhDEF1* and *IhDEF2* is similar in the two sepal types (Figure 9c). *IhGLO1* lacks expression in sepals and ovary and shows a gradual increase in expression from lower lateral petal to upper lateral and dorsal petals. Like *IhDEF1*, *IhGLO1* shows a weaker expression in stamens than in the dorsal petal (Figure 9d).

The expression of the *M. umbellata MuDEF1* mRNA is mainly limited to the stamens (Figure 10a). Relative to the bracteole/sepal/pedicel tissue, there is a somewhat higher expression of *MuDEF1* in the green petal caps and in the ovaries.

Expression of *MuGLO1* is similarly low in the petal calyptra. Hardly any expression is present in the nectaries (bracts) or ovaries. Somewhat surprisingly, in addition to expression in the stamens, there is also clear expression in the bracteoles/sepals attached to the pedicel.

Heterotopic expression of IhSEP3 *and* MuSEP3

Expression of SEPALLATA3-like genes is generally restricted to the three inner whorls of core eudicot flowers (Malcomber and Kellogg, 2005). In *I. hawkeri*, however, strong expression is present in the petaloid spurred lower sepal, while expression in the green sepals is comparatively low (Figure 11a).

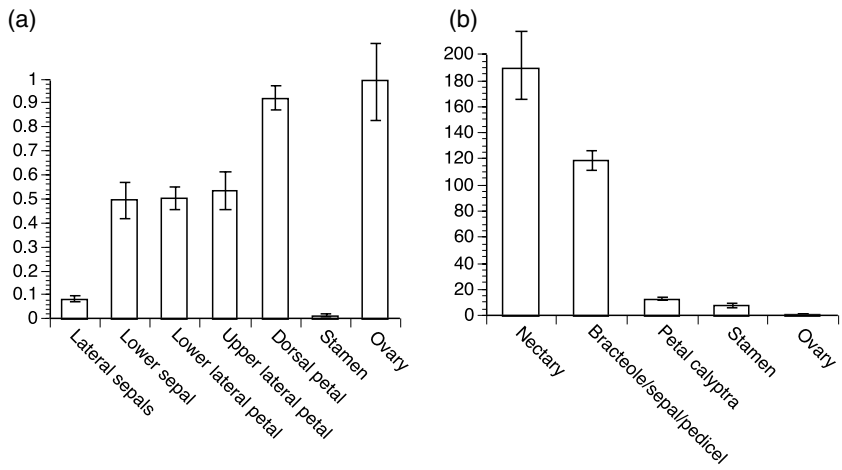

Figure 11. Analysis of quantitative expression of *SEPALLATA3*-like genes in the floral organs of *I. hawkeri* and *M. umbellata*.

In the petals, a similar transcript level is present in the upper and lower lateral petals, while *IhSEP3* is more strongly expressed in the dorsal petal. This expression of *IhSEP3* is thus correlated to the presence of petaloidy in the *Impatiens* perianth. In the stamens, *IhSEP3* expression is almost completely absent but strong expression is present in the ovary. The expression level of *MuSEP3* in the *M. umbellata* floral organs is mainly restricted to the nectariferous bracts and the sepals/bracteoles/receptacle sample (Figure 11b). In contrast to the case in *I. hawkeri*, no expression is present in the ovary and expression is very reduced in the petals.

Interaction of I. hawkeri *organ identity proteins in yeast*

In this study, we found expression of two *DEF*-like genes in the *I. hawkeri* petaloid sepal, but no expression of a *PI/GLO*-like gene could be shown in this organ. Furthermore, the corresponding conceptual DEF-like proteins have a strongly divergent C-terminal domain: IhDEF1 has an unusually long and strongly modified PI-derived motif, while IhDEF2 has a truncated C-terminal domain. This makes it conceivable that the proteins have a modified interaction specificity. To test this hypothesis we performed yeast two-hybrid analysis of the interactions between IhDEF1, IhDEF2, IhGLO and IhSEP3 (Figure 12). We found that both IhDEF1 and IhDEF2 have retained the capacity to interact strongly with IhGLO1. This is the case in both directions, with IhGLO either in the activating or in the binding domain vector. However, the shorter IhDEF2 protein can interact individually with IhSEP3, while IhDEF1 does not. Modified C-termini have been shown to be involved in homodimerization of LMADS1, a lily AP3/DEF-like protein (Tzeng *et al.*, 2003). Because of the modified C-terminal domains of the *I. hawkeri AP3/DEF*-like proteins, we tested the hypothesis that the two DEF-like proteins could interact in yeast, but we could find no evidence for this.

In vitro *protein interaction studies using gel retardation*

To test the hypothesis that a IhSEP3–IhDEF2 dimer could be involved in establishing the modified identity of the petaloid spurred sepal of *I. hawkeri*, we investigated its DNA-binding capacity to a canonical CArG-box, as present in the second intron of the *Arabidopsis thaliana* AGAMOUS gene. As shown for Arabidopsis SEP3 (Figure 13a, lanes 2–5), we found that IhSEP3 can bind this CArG-box as a homodimer (Figure 13a, lanes 6, 7). We found no evidence of DNA binding by a IhSEP3–IhDEF2 heterodimer (Figure 13a, lanes 6, 7 and Figure 13b, lane 5). As a reference, we tested DNA binding of SEP3–AP3 in Arabidopsis, but, as expected, this dimer would not form in addition to a SEP3–SEP3 homodimer (Figure 13a, lanes 4, 5).

Because DNA binding could mediate dimerization of IhDEF1 and IhDEF2, we also investigated whether a IhDEF1–IhDEF2 heterodimer would form in the presence of a CArG-box sequence. We found no evidence to corroborate this hypothesis (Figure 13b, lane 4). Another hypothesis tested was whether the IhDEF1 or IhDEF2 proteins could bind DNA in a homodimeric configuration. However, such homodimers could not be demonstrated using our gel retardation system (Figure 13b, lanes 2, 3).

Discussion

Petaloidy and clues to organ identity in Impatiens *and* Marcgravia

The two-whorled perianth with a whorl of sepals and a whorl of petals is the condition in the majority of angiosperms and is most pronounced in eudicots. However, many variations exist on this theme (Endress, 1994; Takhtajan, 1997). Usually, the petals are the most attractive organs, but other organs can take over this function. Flowers have diversified through this regular 'transference of function', in this case

Activating D	Binding D	28°C -L/-T	22°C -L/-T/-H + 3 AT	28°C -L/-T/-H + 3 AT	28°C -L/-T/-H/-A + 3 AT	
IhSEP3	IhSEP3	++++	+++	+++	+++	Auto
IhSEP3	IhDEF1	++++	-	-	-	
IhSEP3	IhDEF2	++++	++++	++++	+++	Dimer
IhGLO	IhDEF1	++++	+++	++++	++	Dimer
IhGLO	IhDEF2	++++	++	+++	++	Dimer
IhDEF1	IhGLO	++++	++++	++++	++	Dimer
IhDEF2	IhGLO	++++	+++	+++	+++	Dimer
IhDEF1	IhDEF1	++++	-	-	-	
IhDEF2	IhDEF2	++++	-	-	-	
IhDEF1	IhDEF2	++++	-	-	-	
IhDEF2	IhDEF1	++++	-	-	-	
empty	empty	++++	-	-	-	

Figure 12. Table summarizing dimeric interactions in yeast two-hybrid between IhDEF1, IhDEF2, IhGLO and IhSEP3 proteins of *I. hawkeri*.

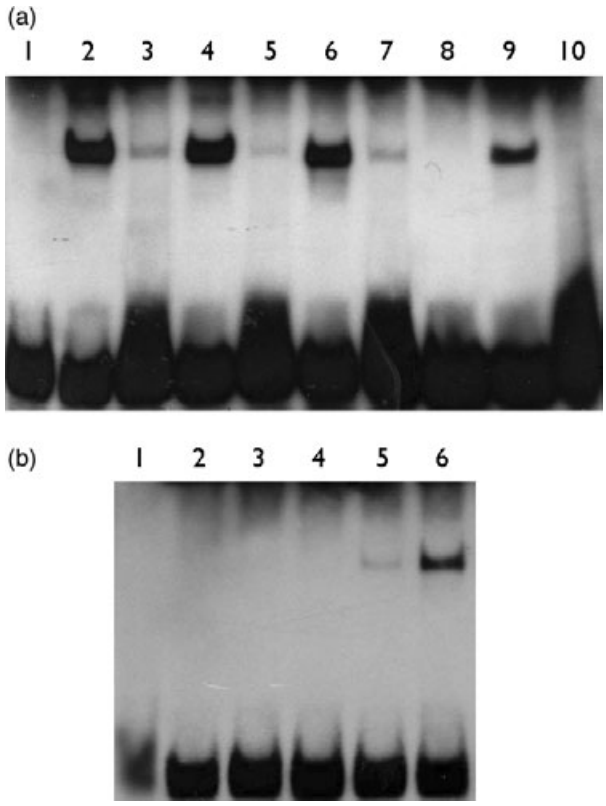

Figure 13. Electrophoretic mobility shift assays to investigate DNA-binding and protein interaction of *Impatiens* MADS-obtain proteins.
(a) Lane 1, lysate only; lane 2, Arabidopsis SEP3; lane 3, Arabidopsis SEP3 unlabeled probe competition; lane 4, Arabidopsis SEP3/AP3; lane 5, Arabidopsis SEP3/AP3 unlabeled probe competition; lane 6, IhSEP3; lane 7, IhSEP3 unlabeled probe competition; lane 8, IhDEF2; lane 9, IhDEF2/IhSEP3; lane 10, IhDEF2/IhSEP3 unlabeled probe competition.
(b) Lane 1, lysate only; lane 2, IhDEF1; lane 3, IhDEF2; lane 4, IhDEF1/IhDEF2; lane 5, IhSEP3/IhDEF2; lane 6, IhSEP3.

petaloidy, inwards to the stamens or carpels as well as outwards to the sepals, bracts or even entire inflorescences (Albert *et al.*, 1998; Baum and Donoghue, 2002; Corner, 1958; Endress, 1994). Because of the great diversity that exists in showy petaloid organs, there is no morphological character combination known at this moment that can conclusively verify an organ's nature as a petal or a sepal. This makes comparison within a larger taxonomic group necessary to homologize between perianth parts (Endress, 1994). However, in a two-whorled perianth, the inner whorl is usually believed to consist of petals, while the outer whorl consists of sepals. Apart from their relative position, petals can often be recognized by their size, shape and coloring. Additional criteria are ontogeny, vascularization and cell surface structure (Endress, 1994).

So how do these criteria apply in Balsaminaceae and Marcgraviaceae? Heterotopic petaloidy is probably an ancestral condition in Balsaminaceae. This can be inferred from the morphology of the single species that is sister to the genus *Impatiens*: *Hydrocera triflora* (Janssens *et al.*, 2006; Yuan *et al.*, 2004). *Hydrocera* has a full set of five sepals, and all are colored like the petals (Grey-Wilson, 1980a). Similar to *Impatiens*, the lower sepal is spurred, but in contrast to *Impatiens*, four larger lateral sepals are present in *Hydrocera*. In the ontogeny of *I. hawkeri*, first the two lateral sepals arise, then the petaloid spurred sepal develops and afterwards all five petal primordia develop simultaneously (Caris *et al.*, 2006). So in position and development, the petaloid sepal has the characteristics of a sepal. Earlier studies have shown that the vascularization of the showy sepal is also similar to the other sepals (Grey-Wilson, 1980b). The results presented here show that dome-shaped petal epidermal cells in *Impatiens* clearly distinguish between sepals and petals, as they were observed on the adaxial surface of all petals but not on the showy spurred sepal. However, in shape, size and coloring the petaloid sepal differs from the lateral sepals. Also for the dorsal petal in *Impatiens*, the answer to the question of organ identity is not straightforward. Most species of *Impatiens* have three sepals. But, in some species of *Impatiens*, as well as in *Hydrocera*, an extra pair of sepals is present (Grey-Wilson, 1980c). Evidence from floral anatomy (Ramadevi and Narayana, 1989) and floral ontogeny (Caris *et al.*, 2006) shows that in the species with only three sepals, like *I. hawkeri*, the two extra sepals are strongly associated with the dorsal petal, apparently creating a mixed sepal/petal organ. Rudiments of these extra sepals may or may not be present in the ontogeny, depending on the species (Caris *et al.*, 2006). This illustrates that homology between organs often can be established through comparative ontogenetic studies.

The probable function of the modified conical-papillate cells is optical, in the sense that the effect of a unidirectional light source is reduced and the brightness of the petals remains constant irrespective of the angle from which it is viewed (Kay *et al.*, 1981). The color and brightness of petals results from reflecting pigments in the petal epidermis.

Marcgravia umbellata has no colored pigment and therefore, the elongated cells will have a different function. The cells might provide tactile clues to pollinators visiting the green flowers (Kay *et al.*, 1981).

SEP3-*like dependent petaloidy of the* Impatiens *sepal spur?*

We investigated the expression of four genes in the floral organs of *I. hawkeri*: *IhDEF1*, *IhDEF2*, *IhGLO1* and *IhSEP3*. In the petaloid spurred sepal, we found overlapping expression of *IhDEF1*, *IhDEF2* and *IhSEP3*. *IhGLO1* expression is absent from this organ, although it seems likely that apart from *IhGLO1* an additional *PI/GLO*-like gene is present in the genome of this species. The most striking expression pattern in explaining petaloidy of the spurred lower sepal is that of *IhSEP3*, because expression of this gene in the perianth organs of *I. hawkeri* is correlated to petaloidy: *IhSEP3*

transcript is present in the showy lower sepal, but is absent or reduced in the green sepals.

An interesting analogy exists to the monocot *Lilium longiflorum LMADS3* gene, a putative ortholog of *SEP3*, which is expressed in the outer whorl tepals (Tzeng *et al.*, 2003).

It is an open question whether class B genes are involved in establishing the petaloidy of the spurred sepal. Although maybe unlikely at first, petaloidy independent of the B genes has already been convincingly shown for *Asparagus officinalis* (Park *et al.*, 2003, 2004). The outer and inner whorl of tepals of this species are petaloid and almost identical, but expression of *AoDEF*, *AoGLOA* and *AoGLOB* is limited to the tepals of the inner whorl.

Do model plant studies shed any light on *SEP3*-like gene-dependent petaloidy? The Arabidopsis *sep3* single mutant shows a partial conversion from petals to sepals, indicating that *SEP3* is required in establishing full petal identity. However, constitutive expression of *SEP3* alone does not result in a conversion of sepals to petals or in any modification of floral organ identity. This in turn indicates that *SEP3* is not sufficient for establishing petaloidy (Pelaz *et al.*, 2001a,b). The combined constitutive expression of *SEP3* and *LFY* or of *SEP3* and *UFO* results in the development of ectopic petals directly from the vegetative leaf rosette or from the stem (Castillejo *et al.*, 2005). Because both LFY and UFO have been shown to regulate class B genes in Arabidopsis (Parcy *et al.*, 1998), ectopic expression of class B genes was used to explain these phenotypes. However, only the expression of AP3 was investigated; its obligate dimerization partner, PI, was not studied (Castillejo *et al.*, 2005). Although activation of petal downstream genes by Arabidopsis SEP3 alone is not evident from the *35S::SEP3* phenotype, the ectopic heterologous expression in Arabidopsis of *BpMADS1*, the birch *SEP3* putative ortholog, does result in petaloid Arabidopsis sepals (Lemmetyinen *et al.*, 2004). This suggests that a mechanism in Arabidopsis sepals can act to repress (or not activate), *SEP3*-dependent petaloidy, which cannot act on heterologous birch *BpMADS3*.

Because it was also shown that *35S::SEP3* is able to transcriptionally activate *AP3* in vegetative leaves (Castillejo *et al.*, 2005), the heterotopic expression of *IhDEF1* and *IhDEF2* in the petaloid lower sepal of *Impatiens* could be under transcriptional control of the heterotopic expression of *IhSEP3* in this organ.

Evolution of class B genes in Impatiens *and* Marcgravia

As an alternative to the hypothesis that IhSEP3 can establish the petaloid features of the *Impatiens* sepal spur, it is possible that class B genes could be involved. In the latter scenario, subfunctionalization after gene duplication of class B genes seems likely, either involving the *Impatiens AP3/DEF*-like genes or the *Impatiens PI/GLO*-like genes. It is thought that gene duplication with subsequent sub- or neofunctionalization can provide material for morphological evolution (Force *et al.*, 1999). Apart from the two major duplication events of class B genes, that resulted in the *euAP3*, *TM6* and *PI/GLO* lineages in core eudicots, the evolutionary importance of recurring duplication in the history of class B genes in angiosperms has been stressed by a number of recent investigations (e.g. Kim *et al.*, 2004; Kramer *et al.*, 2003; Stellari *et al.*, 2004; reviewed in Zahn *et al.*, 2005a,b).

The most thoroughly examined example of such a more recent duplication in class B genes can be found in *Petunia PI/GLO*-like genes. The different *Petunia* GLOs have redundant functions in specifying petal and stamen identity, but *PhGLO1* has an additional, non-redundant function in the fusion of petals and stamens (Vandenbussche *et al.*, 2004).

Even though one could imagine that *IhDEF1* and *IhDEF2* are fully redundant, this does not appear to be very plausible, for a number of reasons. First, the amino acid sequences of both copies are very different: IhDEF2 terminates early in the C-terminal domain and lacks the derived PI and the euAP3 motifs while IhDEF1 is longer than most *AP3/DEF*-like genes and contains both characteristic motifs. Second, although the expression patterns of the two genes are rather similar, their relative mRNA concentration in the floral organs differs (Figure 8c) and more detailed expression analysis might indicate more differences in the expression pattern of the duplicates. Third, not only in *I. hawkeri*, but also in nearly all other *Impatiens* species examined, we were able to amplify and sequence these two paralogs of *AP3/DEF*-like genes (SJ, submitted) certainly arguing in favor of a functional role of the duplicate pair. Fourth, our yeast-two-hybrid data show that the proteins have differences in interaction specificity.

When testing for interactions between class B proteins and IhSEP3 from *I. hawkeri*, we found that IhSEP3 has the capacity to interact strongly in yeast with IhDEF2, while interaction of IhSEP3 with IhDEF1 could not be shown. We also investigated whether this protein dimer has the capacity to bind DNA, as has been shown for the Arabidopsis SEP3–ABS interaction (Kaufmann *et al.*, 2005), but we could not find evidence for this. SEP3 from Arabidopsis has been shown to interact with the class B heterodimer AP3–PI in a ternary complex (Honma and Goto, 2001). However, a dimeric interaction of a SEP3-like protein with one of the class B proteins has not been shown so far (Malcomber and Kellogg, 2005). The data allow for two alternative explanations. First, truncated versions of MADS-domain proteins have been shown previously to interact more easily in yeast (e.g. Yang and Jack, 2004), and because IhDEF2 has a C-terminal domain of only 18 amino acids, more interaction could be detected in yeast, which is not necessarily functional *in vivo*. However, the constructs of Yang *et al.* (2003)

and Yang and Jack (2004) were MADS-domain deleted and not C-terminal deleted. Second, the IhSEP3–IhDEF2 interaction could be of functional importance. In this scenario, because we were not able to show DNA binding of IhSEP3–IhDEF2, the function of the strong dimer could be to mediate the formation of a higher-order protein complex.

If it is implausible that *IhDEF1* and *IhDEF2* are redundant, and both are expressed in the petaloid sepal but no *IhGLO1* expression can be shown, how could IhDEF1 and IhDEF2 function in establishing the petaloid sepal in *Impatiens*?

A number of factors prompted us to test whether a IhDEF1–IhDEF2 heterodimer or a IhDEF1–IhDEF1 or IhDEF2–IhDEF2 homodimer could be involved in specifying organ identity. Class B floral homeotic proteins have been shown to function as obligate heterodimers of AP3/DEF-like and PI/GLO-like proteins for the studied core eudicots (Riechmann *et al.*, 1996; Schwarz-Sommer *et al.*, 1992). However, this condition evolved from less stringent interaction specificity (Winter *et al.*, 2002). In monocots, class B genes seem to have retained their capacity to homodimerize and bind DNA, although the functional importance of the two dimerization modes has not been uncovered thus far (Winter *et al.*, 2002). Also for core eudicots, homodimerization and DNA binding of homodimers has been observed *in vitro* (Riechmann *et al.*, 1996; West *et al.*, 1998), although there seems to be no *in vivo* role associated with these complexes. However, we could find no evidence for a IhDEF1–IhDEF2 heterodimer in yeast, and also IhDEF1–IhDEF1 or IhDEF2–IhDEF2 homodimers did not form in yeast. We also investigated whether the possible dimers would form upon binding of a canonical CArG-box, but neither the IhDEF1 or IhDEF2 proteins, nor the IhDEF1, IhDEF2 proteins in combination could be shown to shift the labeled probe. Based on these results, it cannot yet be excluded that these dimers form *in vivo*. It has to be noted that specific CArG-box sequences could mediate the formation of any of these dimers or that a higher-order complex could form in yeast or bind DNA.

Apart from the hypotheses that, first, IhSEP3 without involvement of B genes, or second, a change in dimerization behavior of class B genes, could explain petaloidy of the lower sepal in *Impatiens*, an important third hypothesis is the presence of a duplicate *PI/GLO*-like gene in the genome of *Impatiens*. In this scenario, the additional *PI/GLO*-like gene would need to be expressed in the petaloid lower sepal and thus have a different expression pattern from IhGLO1. The presence of such a *PI/GLO*-like gene in the first whorl would allow the formation of a IhDEF–IhGLO dimer and a ternary complex of IhSEP3 with such a dimer. Because it is thought that conical-papillate cells are specified by class B genes, inferring such a complex could imply subfunctionalization of the two *IhGLO*-like genes, in which *IhGLO1* has retained a function in specifying the conical-papillate cells and the second copy has lost this function.

For *Marcgravia*, only a single member of the *AP3/DEF* and *PI/GLO*-like lineages was found, although the presence of additional members cannot be excluded. From the phylogeny of balsaminoid class B genes, it can be concluded that when two *AP3/DEF* genes are present in the genome of *M. umbellata*, the gene duplication that resulted in these two copies did not precede the Balsaminaceae–Marcgraviaceae separation.

Consistent with the absence of colored petaloidy in *Marcgravia*, relatively low expression of *MuDEF1* and *MuGLO1* can be observed in the petal calyptra and expression is completely absent from the nectaries. The petal calyptra of *M. umbellata* has a peculiar ontogeny, developing as a single ring primordium, remarkably different from usual petal development (P. Caris personal communication). Possibly, the expression level in this whorl is high enough to confer petal identity or petal development, but fails to activate the downstream genes that establish coloring. Alternatively, the color-producing genes are not transcriptionally activated in the petals, because the B genes do not bind their regulatory sequences.

Another possibility is that the biochemical pathways leading to pigment production in petals or nectaries are lost in *M. umbellata*, which might also be true for the genes responsible for the development of conical cell shapes. The lower expression levels of class B genes could be only sufficient to maintain activation of the remaining target genes in *M. umbellata* second-whorl organs.

Conclusion

Although there is ample evidence for a close relationship of the families in the balsaminoid clade, Balsaminaceae, Marcgraviaceae and Tetrameristaceae, there are currently no clear-cut morphological synapomorphies (shared derived characters) grouping these families (Geuten *et al.*, 2004). In this respect, the expression pattern of *MuSEP3* and *IhSEP3* is very informative. *SEP3*-like gene expression is usually limited to the three inner whorls of eudicot flowers (Mallcomber and Kellogg, 2005). Because *IhSEP3* in *I. hawkeri* and *MuSEP3* in *M. umbellata* are both heterotopically expressed in outer floral whorls, this is a first character relating the two families. In this way, our study illustrates the potential of evo–devo studies in clarifying relationships between morphologically distinct but closely related flowering plant families.

This also raises the issue of homology of morphological features and expression data in an evo–devo context. Homology of organs can be defined as 'structurally derived from the same ancestral structure' (Abouheif *et al.*, 1997; Nielsen and Martinez, 2003; Theißen, 2005; Wray and Abouheif, 1998). In the case of the *Impatiens* petaloid sepal and the *Marcgravia* nectariferous bracts, there is no single structure that we can imagine being present in the most

recent common ancestor from which the current structures would be derived. From a morphological viewpoint, we can test homology more explicitly by applying the criteria of Remane (1952). First, the two organs differ in position. Second, although the two organs share some structural features such as spur-like growth and petaloidy (in many Marcgraviaceae species), they differ in many other structural features. Third, there are no transitional forms between the petaloid sepal and the nectariferous bract. So we have little evidence from morphology that the two structures are homologous.

From the molecular viewpoint, it seems likely that heterotopic *SEP3*-like gene expression was present in the most recent common ancestor of Balsaminaceae, Marcgraviaceae and possibly even Tetrameristaceae (*Pelliciera*). A novel term describing the relationship between the *Impatiens* sepal and the *Marcgravia* bracts would be homocratic (Nielsen and Martinez, 2003). This term is coined for the description of possibly homologous structures that share the expression of the same gene, here *SEP3*. An evolutionary scenario can then be derived from the plausible phylogeny of the balsaminoid clade (Geuten *et al.*, 2004): a most recent ancestor of the clade could have extended expression of *SEP3*-like genes in both sepals and bracts, like the case in *Marcgravia* and possibly in *Pelliciera*, which has both petaloid sepals and bracts (Melchior, 1925). Subsequently, this condition has evolved into a more spatially restricted expression domain: the petaloid sepal of *Impatiens*. Investigating *SEP3*-like gene expression in *Pelliciera* and sister group species of the balsaminoid clade can test the latter hypothesis.

Our data illustrate that, without a comparative perspective, a strict definition of petal identity, which is not possible from a morphological point of view, is probably not maintainable from a molecular point of view, not even in core eudicots. Given the diverse and phylogenetically unrelated taxa in which heterotopic petaloidy can be observed, it does not seem very likely that all have arisen through the same mechanism. A narrowly defined 'sliding boundary' model (Albert *et al.*, 1998; Bowman, 1997; Kanno *et al.*, 2003; van Tunen *et al.*, 1993) as suggested for the monocot tepals, with heterotopic expression of both the *AP3/DEF* and the *PI/GLO* ortholog in the first whorl floral organs, does not seem applicable to all cases of heterotopic petaloidy. Even if extension of class B genes is observed in plant species showing heterotopic petaloidy, the reason for the expansion may still remain unclear, e.g. changes in the *cis*-regulatory regions or differences in regulatory protein activity. 'Transference of function' of organs does not necessarily mean taking on an existing organ identity, but could involve modifying previous identities or creating novel ones. In *Impatiens*, the conversion from sepal identity to petaloidy probably required the heterotopic expression of *IhSEP3*.

Experimental procedures

Material

Impatiens hawkeri (voucher no. PCV06) was grown by the first author at the Laboratory of Plant Systematics, K.U. Leuven or at the Genetics Department, F.S.U. Jena. *Marcgravia umbellata* was obtained from the greenhouse collection of the Botanic Garden Berlin-Dahlem (specimen no. 060-69-74-83). Voucher specimens are kept at the Institute of Botany and Microbiology, K.U. Leuven and the Botanic Garden Berlin-Dahlem, respectively.

Scanning electron microscopy

The material was fixed in FAA solution (70% ethanol, 40% formaldehyde, acetic acid in proportion 90:5:5). Sepals and petals of *I. hawkeri* and nectariferous bracts, calyx and corolla of *M. umbellata* were washed twice for 5 min with 70% ethanol, for a further 5 min with a mixture (1:1) of 70% ethanol and dimethoxymethane (DMM) and eventually placed in pure DMM for 20 min. The material was critical point dried using liquid CO_2 in a BAL-TEC CPD030 (BAL-TEC AG, Balzers, Liechtenstein). The material was mounted onto stubs using Leit-C and gold-coated with a sputter coater (SPI Supplies, West Chester, PA, USA). Observations of the abaxial and adaxial sides of all organs were made using a JEOL JSM-6360 microscope or a JEOL JSM-5800 LV microscope at the National Botanic Garden of Belgium (JEOL Ltd, Tokyo, Japan) at the Laboratory of Plant Systematics, K.U. Leuven.

Cloning

Total RNA from flowerbuds of different developmental stages was isolated with the INVISORB kit (Invitek, Berlin, Germany) and checked for quality with denaturing agarose gel electrophoresis. Poly-A RNA was isolated from the total RNA with the NUCLEOTRAP mRNA kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using OMNISCRIPT Reverse Transcriptase (Qiagen, Hilden, Germany) and an oligo-dT primer according to the manufacturer's protocol. From this cDNA pool, we amplified cDNA using the 3′-RACE technique (Frohmann *et al.*, 1988), with primers taken from the literature, designed to anneal at the conserved MADS-box (Kramer *et al.*, 1998; Winter *et al.*, 1999) with the temperature profile from Kramer *et al.* (1998).

An alignment of the *M. umbellata* sequence and the sequences of several cloned *PI/GLO* sequences from other Ericales species (TV, unpublished results) allowed the design of a specific Ericales *PI/GLO* primer, 5′-AATGGSMTCATRAAGAARGCCAARGAGAT-3′, to amplify cDNA of a *PI/GLO* gene from *Impatiens*. We used semi-nested PCR on previously obtained amplification products, with 35 cycles of 30 sec denaturing at 95°C, 30 sec annealing at 55°C and 60 sec extension at 72°C.

To clone *SEP3*-like genes from *I. hawkeri* and *M. umbellata*, we applied the approach and primers from Litt and Irish (2003).

We used the primers of White *et al.* (1990) to amplify the 18S nuclear ribosomal RNA from *I. hawkeri* and *M. umbellata*.

Amplification products were analyzed on agarose gel and bands with the expected length were cut from gel, purified and cloned into pGEM-T Vector System II (Promega, Madison, WI, USA). The resulting plasmid insertions were sequenced with an Applied Biosystems 310 capillary sequencer using the BIGDYE TERMINATOR 1.1 kit (Applied Biosystems, Foster City, CA, USA).

The cloned genes were named using an abbreviation of the species name *IhDEF1*, *IhDEF2* and *MuDEF1* for *AP3/DEF*-like genes

from *I. hawkeri* and *M. umbellata* respectively or *IhGLO1* and *MuGLO1* for *PI/GLO*-like genes.

Because of its strongly derived C-terminal domain, and to investigate the possibility of alternative splicing, we amplified the genomic coding region of the K- and C-terminal domain of IhDEF2 with primers 5′-AGAACACACTGGCGGTTGA-3′ and 5′-AGCATAAATATCGTGTGG-3′ and for comparison, part of the genomic locus of MuDEF1 was amplified with primers 5′-AGCTTGGTGCTACTAAAG-3′.

To obtain full-length clones that would allow for gel retardation experiments for genes assumed to be involved in establishing the identity of the petaloid sepal of *I. hawkeri*, we used 5′-RACE with the 5′/3′-RACE kit according to the manufacturer's protocol (Roche, Basel, Switzerland). Gene-specific primers for *IhSEP3* amplification were: SP1–IhSEP3, 5′-TGAAAGCCATCCTGGAAAGT-3′, SP2–IhSEP3, 5′-TGGGCTCACATTCCAATGGATGAA-3′ and SP3—IhSEP3, 5′-TGTTTCAAGGCCCTGTTTGCTTCA-3′; for *IhDEF2,* SP1–IhDEF2, 5′-GTGATTGATCAAGTCCCACACT-3′, SP2–IhDEF2, 5′-CGGTCACGGATTTGCTGCAAGG-3′ and SP3–IhDEF2, 5′-TGGAGCTTGCCTGTGCTTGAGG-3′; for *IhDEF1* amplification SP1–IhDEF1, 5′-CATTAATCCTCCAACTCCAC-3′, SP2–IhDEF1, 5′-CGCTGCAGGGAACTGTCCAT-3′ and SP3–IhDEF1, 5′-CCGAAATGGAAGGGCTGATG-3′.

Sequence analysis

Sequences were assembled in the Staden software package (Staden *et al.*, 1998) and submitted to GenBank and have accession numbers DQ493928–DQ493933. We identified the cloned partial cDNAs as belonging to the *AP3/DEF* or *PI/GLO* clades of class B MADS-box genes by aligning their conceptually translated protein sequence to those of previously cloned and characterized MADS-box genes and by including them in a phylogenetic analysis. Alignment of the protein sequences together with sequences from GenBank was done in ClustalX (Thompson *et al.*, 1997) and manually inspected. paup4b10 (Swofford, 2002) was used for parsimony bootstrap analysis and neighbor joining bootstrap analysis with respectively 1000 and 10 000 bootstrap replicates. In the parsimony analysis, for each bootstrap replicate the most parsimonious tree was searched for heuristically, using 10 random stepwise addition replicates and hold 2. MrBayes3.0b4 was used for Bayesian analysis with model averaging (Ronquist and Huelsenbeck, 2003). After an initial identification as class B genes (data not shown), we used a class B gene alignment to assign the *I. hawkeri* and *M. umbellata* sequences to the *PI/GLO*, *AP3/DEF* or *TM6* lineages utilizing B-sister proteins representing the outgroup.

IhSEP3 and *MuSEP3* genes were unambiguously identified as eudicot *SEP3*-like genes in a neighbor joining bootstrap analysis with members from *euAP1*, *euFUL*, *FUL*-like, *SEP3*, *SEP1/2*, *SEP4* and *AGL6* clades (data not shown).

Genomic hybridization

To test for the presence of the identified genes in the *I. hawkeri* and *M. umbellata* genome, to detect possible recent duplicates and to further investigate the genomic organization of the *AP3/DEF* homologs, we used genomic hybridization. Before harvesting fresh leaves for DNA isolation from *I. hawkeri*, we kept the plants in the semi-dark for several days. Frozen crushed leaves were then extracted with a lysis buffer containing 2% cetyl trimethyl ammonium bromide (CTAB) and 3% polyvinylpyrrolidone 40 (PVP-40). Several rounds of chloroform extraction and a subsequent ethanol–salt precipitation resulted in genomic DNA which was then digested by several restriction enzymes. The Plant DNA XL kit (Macherey-Nagel) was used to isolate genomic DNA from *M. umbellata*. The digested genomic DNA was blotted on a BRIGHTSTAR-PLUS membrane (Ambion, Austin, TX, USA). As a template for probe labeling, we amplified the partial cDNA of *IhDEF2* and *MuDEF1*, omitting the MADS-box. Probes were synthesized via random prime ^{32}P labeling using the HIGHPRIME Kit (Roche, Munich, Germany). Hybridization was in ULTRAHYB buffer (Ambion), under high-stringency conditions.

Real-time RT-PCR

Floral organs in various stages of their development from *I. hawkeri* and *M. umbellata* were dissected and snap-frozen in liquid nitrogen. Total RNA was isolated and each RNA sample was DNase treated using TURBO DNA-free (Ambion) and reverse transcribed using THERMOSCRIPT RT (Invitrogen, Carlsbad, CA, USA) with a combination of oligo-dT and random hexamer primers (Fermentas, Burlington, Canada). Primers for PCR amplification were designed with Primer Express software (Applied Biosystems) with the respective cDNA sequences used as a template and are available from the first author on request. Amplification and real-time detection was done in an ABI PRISM 7000 with the qPCR MASTERMIX PLUS for SYBR GREEN 1 (EuroGenTec, Liege, Belgium). Quantification of expression was calculated relative to 18S rRNA using the deltadeltaCt method. Gene expression in the different *I. hawkeri* floral organs is presented relative to the expression in whole flower buds. Gene expression in *M. umbellata* is presented relative to the ovary expression level. The bracteoles and sepals of *Marcgravia* are very small and firmly attached to the pedicel and thus were not investigated separately.

Yeast hybrid analysis

Yeast hybrid analysis essentially followed the procedures outlined in Kaufmann *et al.* (2005). 5′ partial cDNAs were amplified from plasmid or from a cDNA pool using Pfu polymerase (Fermentas) using primers with an *Eco*RI or *Bam*HI restriction site. Vectors pGBKT7 and pGADT and inserts were double restricted using the same set of enzymes. Inserts were ligated into vectors and correct in-frame ligation and sequence correctness was checked by sequencing using vector primers. The activation domain vector pGADT7 was always transformed into yeast strain Y187; the binding domain vector pGBKT7 was transformed into strain AH109.

To obtain double transformants, two single transformants were mated on non-selective YAPD medium (2% (w/v) peptone, 2% (w/v) glucose, 1% (w/v) yeast extract, 0.01% (w/v) adenine, pH 5.8) and subsequently transferred to selective SD/–Leu/–Trp medium (0.17% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and an appropriate dropout mix). To test for dimeric interactions under different stringency conditions, cells were grown under three different conditions: SD–Leu/–Trp/–His + 3 mM 3-AT (3-amino-1,2,4-triazole) at 22°C, SD–Leu/–Trp/–His + 3 mM 3-AT at 28°C, SD–Leu/–Trp/–Ade/–His + 3 mM 3-AT at 28°C. Experiments were done with one repetition and interactions were tested in both directions, except in the case of autoactivation.

Gel retardation

Gel retardation followed the procedures described in Winter *et al.* (2002) and Kaufmann *et al.* (2005). The IhSEP3, IhDEF2, IhDEF1 coding sequences were cloned into pSPUTK vector (Stratagene, La Jolla, CA, USA) using *Nco*I and *Eco*RI restriction sites. Biotin labeled

oligos with the sequence of the canonical CArG-box of the second *AGAMOUS* intron (Gómez-Mena *et al.*, 2005) were annealed and used as a probe for band-shift. After electrophoresis, the gels were blotted to positively charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) and signal detection was done using the chemiluminescent nucleic acid detection module (Pierce, Rockford, IL, USA).

Acknowledgements

We would like to thank B. Leuenberger and the Berlin-Dahlem Botanic Garden for help with the material of *M. umbellata*. Thanks to J. Zarucchi and the Missouri Botanical Gardens for use of the *Pelliciera rizophorae* picture. Thanks to A. J. Höggemeier and the Botanischer Garten Bochum for use of the *M. umbellata* picture. Thanks to Dr F. Stolz, M. De Jonge and Professor J. Thevelein (VIB10) for support with real-time PCR experiments. Thanks to Anja Vandeperre, Eef Vroom and Marcel Verhaegen for technical assistance. This research was supported by a grant from the Research Council of the K.U. Leuven (OT/05/35) and the Fund for Scientific Research-Flanders (Belgium) (G.0268.04;1.5.003.05N).

References

Abouheif, E., Akam, M., Dickinson, W.J., Holland, P.W.H., Meyer, A., Patel, N.H., Roth, V.L. and Wray, G.A. (1997) Homology and developmental genes. *Trends Genet.* **13**, 432–433.

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

Angiosperm Phylogeny Group (1998) An ordinal classification for the families of flowering plants. *Ann. Mo. Bot. Gard.* **85**, 531–553.

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

Baum, D.A. and Donoghue, M.J. (2002) Transference of function, heterotopy and the evolution of plant development. In *Developmental Genetics and Plant Evolution* (Cronk, Q.C.B., Bateman, R.M. and Hawkins, J.A., eds). London: Taylor and Francis, pp. 52–69.

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.

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

Caris, P.L., Geuten, K.P., Janssens, S.B. and Smets, E.F. (2006) Floral development in three species of *Impatiens* (Balsaminaceae). *Am. J. Bot.* **93**, 1–14.

Castillejo, C., Romera-Branchat, M. and Pelaz, S. (2005) A new role of the *Arabidopsis* SEPALLATA3 gene revealed by its consitutive expression. *Plant J.* **43**, 586–596.

Cho, S., Jang, S., Chae, S., Chung, K.M., Moon, Y.H., An, G. and Jang, S.K. (1999) Analysis of the C-terminal region of *Arabidopsis thaliana* APETALA1 as a transcription activation domain. *Plant Mol Biol.* **40**, 419–429.

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

Corner, E.J.H. (1958) Transference of function. *Bot. J. Linn. Soc.* **56**, 33–40.

Dressler, S. (1997) *Marcgravia umbellata*, Marcgraviaceae. *Curtis. Bot. Mag.* **14**, 130–136.

Dressler, S. (2004) Marcgraviaceae. In *Families and Genera of Flowering Plants*, Vol. 6 (Kubitzki, K., ed.). Berlin: Springer-Verlag, pp. 258–265.

Egea-Cortines, M., Saedler, H. and Sommer, H. (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO J.* **18**, 5370–5379.

Endress, P.K. (1994) *Diversity and Evolutionary Biology of Tropical Flowers*. Cambridge: Cambridge University Press.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L. and Postlethwait J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*, **151**, 1531–1545.

Frohmann, M.A., Dush, M.K. and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA*, **85**, 8998–9002.

Geuten, K., Smets, E., Schols, P., Yuan, Y.-M., Janssens, S., Kupfer, P. and Pyck, N. (2004) Conflicting phylogenies of balsaminoid families and the polytomy in Ericales: combining data in a Bayesian framework. *Mol. Phylogenet. Evol.* **31**, 711–729.

Gómez-Mena, C., de Folter, S., Costa, M.M, Angenent, G.C. and Sablowski, R. (2005) Transcriptional program controlled by the floral homeotic gene *AGAMOUS* during early organogenesis. *Development*, **132**, 429–438.

Grey-Wilson, C. (1980a) *Hydrocera triflora*, its floral morphology and relationship with *Impatiens*. Studies in Balsaminaceae: V. *Kew Bull.* **35**, 213–219.

Grey-Wilson, C. (1980b) Some observations on the floral vascular anatomy of *Impatiens*: studies in Balsaminaceae: VI. *Kew Bull.*, **35**, 221–227.

Grey-Wilson, C. (1980c) Impatiens *of Africa*. Rotterdam: Balkema.

Honma, T. and Goto, K. (2001) Complexes of MADS-box genes are sufficient to convert leaves into floral organs. *Nature*, **25**, 469–471.

Janssens, S., Vinckier, S., Lens, F., Dressler, S., Geuten, K. and Smets, E. (2005) Palynological variation in Balsaminoid Ericales. II. Balsaminaceae, Tetrameristaceae Pellicieraceae and general conclusions. *Ann. Bot.* **96**, 1061–1073.

Janssens, S., Geuten, K., Yuan, Y.-M., Song, Y., Küpfer, P. and Smets, E. (2006) Phylogenetics of *Impatiens* and Hydrocera using chloroplast atpB-rbcL spacer sequences. *Syst. Bot.* **31**, 171–180.

Jaramillo, A.M. and Kramer, E.M. (2004) APETALA3 and PISTILLATA homologs exhibit novel expression patterns in the unique perianth of Aristolochia (Aristolochiaceae). *Evol. Dev.* **6**, 449–458.

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.

Kaufmann, K., Melzer, R. and Theißen, G. (2005) MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene*, **347**, 183–198.

Kay, Q.O.N., Daoud, H.S. and Stirton, C.H. (1981) Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.* **83**, 57–84.

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: evolutionary and functional implications of a 260-million-year-old duplication. *Am. J. Bot.* **91**, 2102–2118.

Kobuski, C.E. (1951) Studies in the Theaceae – XXIII. The genus *Pelliciera*. *J. Arn. Arb.* **32**, 256–262.

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 Schlüter, P.M. (2003) Complex patterns of gene duplication in the APETALA3 and PISTILLATA lineages of the Ranunculaceae. *Int. J. Plant Sci.* **164**, 1–11.

Krizek, B.A., and Meyerowitz, E.M. (1996) Mapping the protein regions responsible for the functional specificities of the Arabidopsis MADS domain organ-identity proteins. *Proc. Natl Acad. Sci. USA*, **93**, 4063–4070.

Krizek, B.A., Prost, V. and Macias, A. (2000) AINTEGUMENTA promotes petal identity and acts as a negative regulator of AGAMOUS. *Plant Cell*, **12**, 1357–1366.

Lamb, R.S. and Irish, V.F. (2003) Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. *Proc. Natl Acad. Sci. USA*, **100**, 6558–6563.

Lee, I., Wolfe, D.S., Nilsson, O. and Weigel, D. (1997) A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. *Curr. Biol.* **7**, 95–104.

Lemmetyinen, J., Hassinen, M., Elo, A., Porali, I., Keinonen, K., Makela, H. and Sopanen, T. (2004) Functional characterization of SEPALLATA3 and AGAMOUS orthologues in silver birch. *Physiol. Plant.* **121** 149–162.

Lens, F., Dressler, S., Jansen, S., van Evelghem, L. and Smets, E. (2005) Relationships within balsaminoid Ericales: a wood anatomical approach. *Am. J. Bot.* **92**, 941–953.

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

Maguire, B.C., de Zeeuw, C., Huang, Y.-C. and Clare, C.C., Jr (1972) Bonnetiaceae. In *The botany of the Guyana Highland – Part IX. Mem. N. Y. Bot. Gard.*, **23**, 131–165.

Malcomber, S.T. and Kellogg, E.A. (2005) SEPALLATA gene diversification: brave new whorls. *Trends Plant Sci.* **10**, 427–435.

Martin, C., Bhatt, K., Baumann, K., Jin, H., Zachgo, S., Roberts, K., Schwarz-Sommer, Z., Glover, B., Perez-Rodrigues, M. (2002) The mechanics of cell fate determination in petals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 809–813.

Melchior, H. (1925) Theaceae. In *Die Natürlichen Pflanzenfamilien*, 2nd edn, Vol. 21 (Engler, A. and Prantl, E., eds). Leipzig: W. Engelmann, pp. 109–154.

Münster, T., Pahnke, J., Di Rosa, A., Kim, J.T., Martin, W., Saedler, H. and Theißen, G. (1997) Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. *Proc. Natl Acad. Sci. USA*, **94**, 2415–2420.

Nielsen, C. and Martinez, P. (2003) Patterns of gene expression: homology or homocracy?. *Dev. Gen. Evol.* **213**, 149–154.

Parcy, F., Nilsson, O., Busch, M.A., Lee, I. and Weigel, D. (1998) A genetic framework for floral patterning. *Nature*, **395**, 561–566.

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 dioecious 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.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R. and Yanofsky, M.F. (2001a) Conversion of leaves into petals in *Arabidopsis*. *Curr. Biol.* **11**, 182–184.

Pelaz, S., Gustafson-Brown, C., Kohalmi, S.E., Crosby, W.L. and Yanofsky, M.F. (2001b) APETALA1 and SEPALLATA3 interact to promote flower development. *Plant J.* **4**, 385–394.

Perez-Rodriguez, M., Jaffe, F.W., Butelli, E., Glover, B.J. and Martin, C. (2005) Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of *Antirrhinum majus* flowers. *Development*, **132**, 359–370.

Purugganan, M.D., Rounsley, S., Schmidt, R.J. and Yanofsky, M.F. (1995) Molecular evolution of flower development: Diversification of the plant MADS-box regulatory gene family. *Genetics*, **140**, 345–356.

Ramadevi, D. and Narayana, L.L. (1989) Floral anatomy of Balsaminaceae. *Plant Sci. Res. India*, **12**, 707–713.

Remane, A. (1952) *Die Grundlagen des natürlichen Systems, der vergleichenden Anatomie und der Phylogenetik*. Leipzig: Akademische Verlagsgesellshaft, Geest Portig K.-G.

Riechmann, J.L., Wang, M. and Meyerowitz, E.M. (1996) DNA-binding properties of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. *Nucleic Acids Res.* **24**, 3134–3141.

Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

de Roon, A.C. (1975) Contributions towards a monograph of the Marcgraviaceae. Dissertation RUU, Utrecht, The Netherlands.

Samach, A., Kohalmi, S.E., Motte, P., Datla, R. and Haughn, G.W.W. (1997) Divergence of function and regulation of class B floral organ identity genes. *Plant Cell*, **9**, 559–570.

Schönenberger, J., Anderberg, A.A. and Sytsma, K.J. (2005) Molecular phylogenetics and patterns of floral evolution in the Ericales. *Int. J. Plant Sci.* **166**, 265–288.

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. and Sommer, H. (1990) Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science*, **250**, 931–936.

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 *Arabidopsis* floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11**, 251–263.

Staden, R., Beal, K.F. and Bonfield, J.K. (1998) The Staden Package. In *Computer Methods in Molecular Biology. Volume 132.* (Misener, S., Krawetz, S., eds). Totowa, NJ: The Humana Press Inc., pp. 115–1130.

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

Swofford, D.L. (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland, MA: Sinauer Associates.

Takhtajan, A. (1997) *Diversity and classification of flowering plants*. Columbia University Press, New York.

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

Theißen, G. (2005) Birth, life and death of developmental control genes: new challenges for the homology concept. *Theory Biosci.* **124**, 199–212.

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

van Tunen, A.J., Eikelboom, W. and Angenent, G.C. (1993) Floral organogenesis in TULIPA. *FNL*, **116**, 33–38.

Tzeng, T.-Y., Liu, H.-C. and Yang, C.-H. (2003) The C-terminal sequence of LMADS1 is essential for the formation of homodimers for B function proteins. *J. Biol. Chem.* **12**, 10747–10755.

Vandenbussche, M., Theißen, G., Van de Peer, Y. and Gerats, T. (2003) Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. *Nucleic Acids Res.* **31**, 4401–4409.

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

West, A.G., Causier, B.E., Davies, B. and Sharrocks, A.D. (1998) DNA binding and dimerisation determinants of *Antirrhinum majus* MADS-box transcription factors. *Nucleic Acids Res.* **26**, 5277–5287.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., Sninsky, J. and White, T., eds). San Diego, CA: Academic Press, pp. 315–322.

Wilkinson, M.D. and Haughn, G.W. (1995) Unusual floral organs controls meristem identity and organ primordia fate in *Arabidopsis*. *Plant Cell*, **7**, 1485–1499.

Winter, K.U., Becker, A., Münster, T., Kim, J.T., Saedler, H. and Theißen, G. (1999) MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. *Proc. Natl Acad. Sci. USA*, **96**, 7342–7347.

Winter, K.U., Weiser, C., Kaufmann, K., Bohne, A., Kirchner, C., Kanno, A., Saedler, H. and Theißen, G. (2002) Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. *Mol. Biol. Evol.* **19**, 587–596.

Wray, G.A. and Abouheif, E. (1998) When homology is not homology. *Curr. Opin. Gen. Dev.* **8**, 675–680.

Xiang, Q.-Y., Moody, M.L., Soltis, D.E., Fan, C.-Z. and Soltis, P.S. (2002) Relationships within Cornales and circumscription of Cornaceae – matK and rbcL sequence data and effects of outgroups and long branches. *Mol. Phylogenet. Evol.* **24**, 35–57.

Yang, Y. and Jack, T. (2004) Defining subdomains of the K domain important for protein–protein interactions of plant MADS proteins. *Plant Mol. Biol.* **55**, 45–59.

Yang, Y., Fanning, L. and Jack, T. (2003) The K domain mediates heterodimerization of the *Arabidopsis* floral organ identity proteins, APETALA3 and PISTILLATA. *Plant J.* **33**, 47–60.

Yuan, Y.M., Song, Y., Geuten, K., Rahelivololona, E., Wohlhauser, S., Fischer, E., Smets, E. and Kupfer, P. (2004) Phylogeny and biogeography of Balsaminaceae inferred from ITS sequences. *Taxon*, **32**, 391–403.

Zahn, L.M., Leebens-Mack, J., DePamphilis, C.W., Ma, H. and Theißen, G. (2005a) 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.

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

Zhang, J. (2003) Evolution by gene duplication: an update. *Trends Ecol. Evol.* **18**, 292–298.

