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

Koen Geuten1,∗, Annette Becker2,†, Kerstin Kaufmann2,‡, Pieter Caris1, Steven Janssens1, Tom Viaene1, Günter Theißen2 and Erik Smets1,3

1Laboratory of Plant Systematics, K.U. Leuven, Leuven, Belgium,
2Genetics Department, Friedrich Schiller University, Jena, Germany, and
3Nationaal Herbarium Nederland, Leiden University Branch, Leiden, The Netherlands

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∗For correspondence: (fax +32 163 21 955; e-mail koen.geuten@bio.kuleuven.be).
†Present address: Evolutionary Developmental Genetics Group, University of Bremen, Bremen, Germany.
‡These authors contributed equally to the manuscript.

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).
Balsaminaceae and Marcgraviaceae show a sister group relationship in phylogenetic trees based on molecular data (Geuten et al., 2004; but see also Schönberger 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...
petal identity is established by a SEP–AP1–PI–AP3 quartet. Similar to AP1, the SEPALLATA s 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).

![Figure 2. Floral organs of I. hawkeri and M. umbellata.](image)

(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.
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 (Martín 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.*](image-url)
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 nectary 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),
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 I. hawkeri.
**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 XbaI 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.
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 petalloidy 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)
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 \textit{IhDEF1} in the dorsal petal is stronger than in the stamen in contrast to \textit{IhDEF2}, which shows a stronger stamen expression when compared with the dorsal petal. The expression level of \textit{IhDEF1} and \textit{IhDEF2} is similar in the two sepal types (Figure 9c). \textit{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 \textit{IhDEF1}, \textit{IhGLO1} shows a weaker expression in stamens than in the dorsal petal (Figure 9d).

The expression of the \textit{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 \textit{MuDEF1} in the green petal caps and in the ovaries.

Expression of \textit{MuGLO1} is similarly low in the petal calytra. 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 bracteole/sepals attached to the pedicel.

\textit{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 \textit{I. hawkeri}, however, strong expression is present in the petaloid spurred lower sepal, while expression in the green sepals is comparatively low (Figure 11a).
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...
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 l. 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 l. 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 nor 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. SEPs-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 hetero- topically expressed in outer floral whors, 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
of petaloidy probably required the heterotopic expression of monocot tepals, with heterotopic expression of both the novel ones. In necessarily mean taking on an existing organ identity, but activity. 'Transference of function' of organs does not cis-extend of class B genes is observed in organs, does not seem applicable to all cases of heterotopic expression domain: the petaloid sepal of this condition has evolved into a more spatially restricted petaloid sepals and bracts (Melchior, 1925). Subsequently, et al. through the same mechanism. A narrowly defined 'sliding observed, it does not seem very likely that all have arisen unrelated taxa in which heterotopic petaloidy can be maintainable from a molecular point of view, not even in group species of the balsaminoid clade can test the latter hypothesis.

From the molecular viewpoint, it seems likely that heterotopic SEPII-like gene expression was present in the most recent common ancestor of Balsaminaceae, Marcgraviaceae and possibly even Tetrameristataceae (Pellliciera). 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 SEPII. 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 SEPII-like genes in both sepal and bracts, like the case in Marcgravia and possibly in Pellliciera, which has both petaloid sepal and bracts (Melchior, 1925). Subsequently, this condition has evolved into a more spatially restricted expression domain: the petaloid sepal of Impatiens. Investigating SEPII-like gene expression in Pellliciera 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 new ones. In Impatiens, the conversion from sepal identity to petaloidy probably required the heterotopic expression of IhSEPII.
from *I. hawkeri* and *M. umbellata* respectively or *IhGLO1* and *MuGLO1* for *P/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′-AGACACACGTCTGGGATGGA-3′ and 5′-AGCATAATATCGTGGTGGA-3′ and for comparison, part of the genomic locus of *MuDEF1* was amplified with primers 5′-AGCTTGGTCTGACTA-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′-TGAAGAAGCCACCTGGAAAGT-3′, SP2-IhSEP3, 5′-TGAGCTCACATTCAATGGATGAA-3′ and SP3-IhSEP3, 5′-TTCTCAAGGCCCCTGTGTCCA-3′; for *IhDEF2*, SP1-IhDEF2, 5′-GTGATTGATCGTCCACACT-3′, SP2-IhDEF2, 5′-CGCTCCACGATTTGTGCAAG-3′ and SP3-IhDEF2, 5′-TGGAGCTTGGCTACTGAGG-3′. For *IhDEF1* amplification SP1-IhDEF1, 5′-CAGCTCGAGGAACTGCTCCA-3′; for *IhDEF1* amplification SP2-IhDEF1, 5′-CAGCTCGAGGAACTGCTCCA-3′; for *IhDEF1* amplification SP3-IhDEF1, 5′-CCGAATAGTTGAAGGCTGATG-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 *P/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.

**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 *EcoRI* or BamHI restriction site. Vectors pGBK7 and pGADT and inserts were double restricted using 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 pGAS MER-TERMIX 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* and *M. umbellata* 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.

**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% polvinylypyrrolidone 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 32P 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 pGQ PCR MASTERMIX PLUS for SYBR GREEN 1 (EuroGenTec, Liege, Belgium). Quantification of expression was calculated relative to 18S rDNA using the delta delta Ct 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.
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).

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