the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) **60**, 1–9 doi: 10.1111/j.1365-313X.2009.03917.x

The petunia *AGL6* gene has a *SEPALLATA*-like function in floral patterning

Anneke S. Rijpkema, Jan Zethof, Tom Gerats* and Michiel Vandenbussche*

Plant Genetics, IWWR, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands

Received 7 February 2009; revised 13 April 2009; accepted 20 April 2009; published online 15 June 2009.
*For correspondence (fax +31 24 3553450; e-mail T.Gerats@science.ru.nl or M.Vandenbussche@science.ru.nl).

SUMMARY

***SEPALLATA* (*SEP*) MADS-box genes are required for the regulation of floral meristem determinacy and the specification of sepals, petals, stamens, carpels and ovules, specifically in angiosperms. The *SEP* subfamily is closely related to the *AGAMOUS LIKE6* (*AGL6*) and *SQUAMOSA* (*SQUA*) subfamilies. So far, of these three groups only *AGL6*-like genes have been found in extant gymnosperms. *AGL6* genes are more similar to *SEP* than to *SQUA* genes, both in sequence and in expression pattern. Despite the ancestry and wide distribution of *AGL6*-like MADS-box genes, not a single loss-of-function mutant exhibiting a clear phenotype has yet been reported; consequently the function of *AGL6*-like genes has remained elusive. Here, we characterize the *Petunia hybrida AGL6* (*PhAGL6*, formerly called *PETUNIA MADS BOX GENE4*/*pMADS4*) gene, and show that it functions redundantly with the *SEP* genes *FLORAL BINDING PROTEIN2* (*FBP2*) and *FBP5* in petal and anther development. Moreover, expression analysis suggests a function for *PhAGL6* in ovary and ovule development. The PhAGL6 and FBP2 proteins interact in *in vitro* experiments overall with the same partners, indicating that the two proteins are biochemically quite similar. It will be interesting to determine the functions of *AGL6*-like genes of other species, especially those of gymnosperms.**

Keywords: MADS-box, Petunia, AGL6, SEPALLATA, E-function.

INTRODUCTION

The integrated description of a set of classical mutant phenotypes two decades ago led to the formulation of the textbook 'ABC' model, which describes how three functionally defined groups of genes (A, B and C) together regulate the specification of floral organ identity in angiosperms: A alone specifies sepals, A and B together petals, B and C stamens, and finally C alone specifies carpels (Schwarz-Sommer *et al.*, 1990; Coen and Meyerowitz, 1991). Later, two additional functional classes were added, D (ovule specification; Angenent *et al.*, 1995) and E (Angenent *et al.*, 1994; Pelaz *et al.*, 2000; named the E-function first in Theissen, 2001). All but one of the ABCDE genes are MIKC-type MADS-box genes. MIKC-type MADS-box genes fall into different clades with often subfamily-specific functions in flower development (like the B-, C-, D- or E-function) (Becker and Theissen, 2003; Parenicova *et al.*, 2003). E-function genes belong to the *SEPALLATA* gene clade (*SEP*, formerly called the *AGAMOUS LIKE2* or *AGL2* clade); they are required for the regulation of floral meristem determinacy and play a role in the specification of sepals, petals, stamens, carpels and ovules (Pelaz *et al.*, 2000; Vandenbussche *et al.*, 2003b; Ditta *et al.*, 2004). Floral organ identity is proposed to be regulated by multimeric complexes of ABCDE proteins (Theissen and Saedler, 2001). In these complexes the B-, C- and D-function proteins are thought to be important for organ-specific gene regulation, while the SEP and SQUAMOSA (SQUA) clade proteins might provide a transcription-activation domain, and act as the 'bridge proteins' that enable the formation of the floral higher-order protein complexes (Honma and Goto, 2001; Theissen and Melzer, 2007; Melzer *et al.*, 2009).

In phylogenetic reconstructions the *SEP* subfamily groups together with the *SQUA* (Schwarz-Sommer *et al.*, 1990) and *AGAMOUS LIKE6* (*AGL6*) subfamilies (Ma *et al.*, 1991) to form a highly supported clade (e.g. Purugganan, 1997; Nam *et al.*, 2003; Zahn *et al.*, 2005). While even the most primitive angiosperms contain *SEP*, *SQUA* and *AGL6* gene copies (Kim *et al.*, 2005), no *SEP*- or *SQUA*-like genes have so far been found in extant gymnosperms (Becker and Theissen, 2003; Theissen and Melzer, 2007). This is remarkable, as gymnosperms thus seem to miss the 'bridge proteins' that in angiosperms are thought to enable the formation of higher-order

protein complexes that regulate flower development. Gymnosperms do, however, contain the closely related *AGL6* genes. Despite their wide distribution and ancestry, the function of *AGL6* genes has remained elusive, since no mutant phenotype has yet been described. Therefore, a functional characterization of the *AGL6* subfamily (genes) might provide more insight into the evolution of the MADS-box gene family and the implication of *AGL6* subfamily genes in flower development.

RESULTS

AGL6-like genes are more closely related to *SEP*-like genes than to *SQUA* genes at the sequence level, as in most phylogenies *AGL6*-like genes are sister to the *SEP*-like genes while *SQUA*-like genes are sister to these two groups (Purugganan, 1997; Nam *et al.*, 2003; Zahn *et al.*, 2005). Moreover, the C-terminal motifs of AGL6 proteins more closely resemble those of SEP proteins (Figure S1 in Supporting Information and Vandenbussche *et al.*, 2003a); and SEP and AGL6-clade proteins share the same PRODOM domain ID for K3 (PD352768), whereas SQUA-like proteins possess a different one (Veron *et al.*, 2007). Within the *AGL6* subfamily, genes group according to the phylogeny of the species they were isolated from, and different lineages for the major plant groups can easily be distinguished (eudicots, monocots, magnoliids and gymnosperms; Figure 1). In contrast to the *SQUA* and *SEP* subfamily, where gene duplications generated considerably more paralogs, diversity between *AGL6*-like genes seems to have been generated mainly upon speciation. In petunia, one *AGL6*-clade gene has been identified: *PETUNIA MADS BOX GENE4* (*pMADS4*) (Tsuchimoto *et al.*, 2000). For reasons of clarity and to acknowledge its position within the *AGL6* subfamily, we have renamed *pMADS4* as *Petunia hybrida AGL6* (*PhAGL6*). Here we describe the functional characterization of *PhAGL6* using mutants, gene expression studies and protein–protein interaction analyses.

The expression pattern of *PhAGL6* is similar to that of *SEP*-like genes

PhAGL6 is not expressed during the vegetative phase, but it is expressed in different floral tissues (Figure 2e). *PhAGL6* mRNA is present at high levels in developing petals and ovaries (Figure 2a,b,e), at lower levels in young developing anthers (Figure 2a) and at much lower levels in sepals and older anthers (Figure 2e). Initially *PhAGL6* is expressed throughout the developing ovary, where its expression becomes restricted to the (developing) ovules at later developmental stages (Figure 2a–d).

When comparing the expression pattern of *PhAGL6* with that of other petunia MADS-box genes it appears most similar to that of the *SEP*-like genes *FBP2*, *FBP5*, *pMADS12* and *FBP23* (Ferrario *et al.*, 2003; Immink *et al.*, 2003). *FBP2* is expressed at high levels in petals, carpels and ovules, and at slightly lower levels in stamens. Like *PhAGL6*, *FBP2* is also specifically expressed in developing flowers (Figure 2f). The expression levels of *FBP2* relative to those of the constitutive genes *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) and *ACTIN* are about a hundred-fold higher than those of *PhAGL6* relative to the same constitutive genes (notice the different scales in Figure 2e,f).

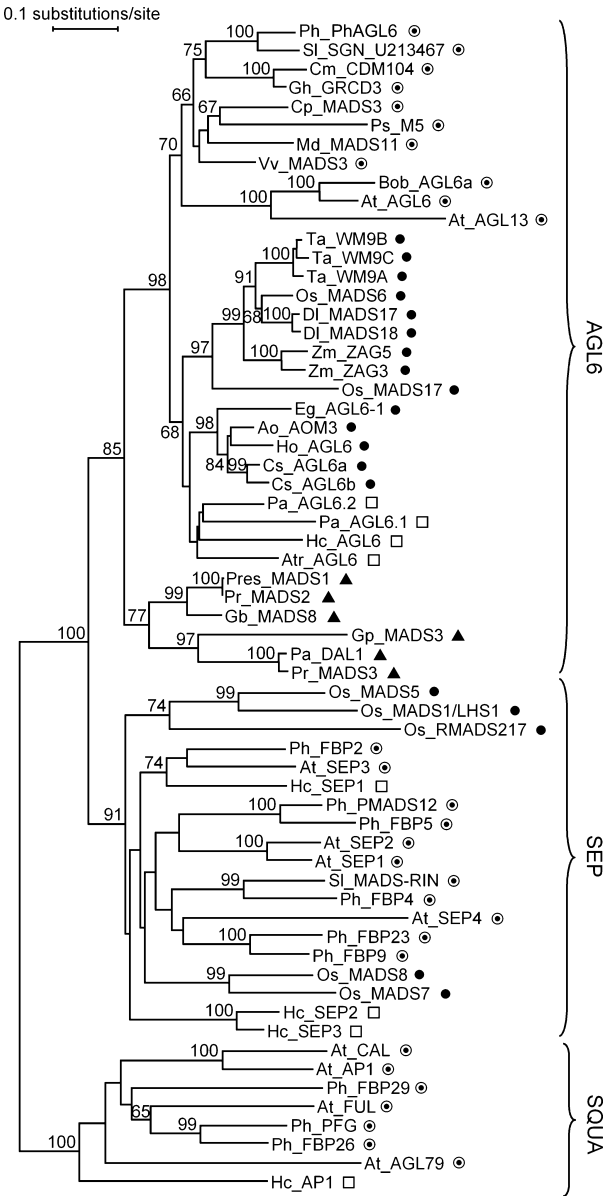

Figure 1. Neighbor-joining tree of a selection of the known AGL6 clade proteins and SEP plus SQUA clade proteins from petunia, Arabidopsis, and several other species. Eudicots are indicated with open circles with inner filled circles, monocotyledons with filled circles, magnoliophyta with open squares and gymnosperms with filled triangles. One thousand bootstrap samples were generated to assess support for the inferred relationships. Local bootstrap probabilities are indicated for branches with >60% support. The alignment is depicted in Figure S1. Species names are abbreviated as indicated in Table S2 and accession numbers are listed in Table S3.

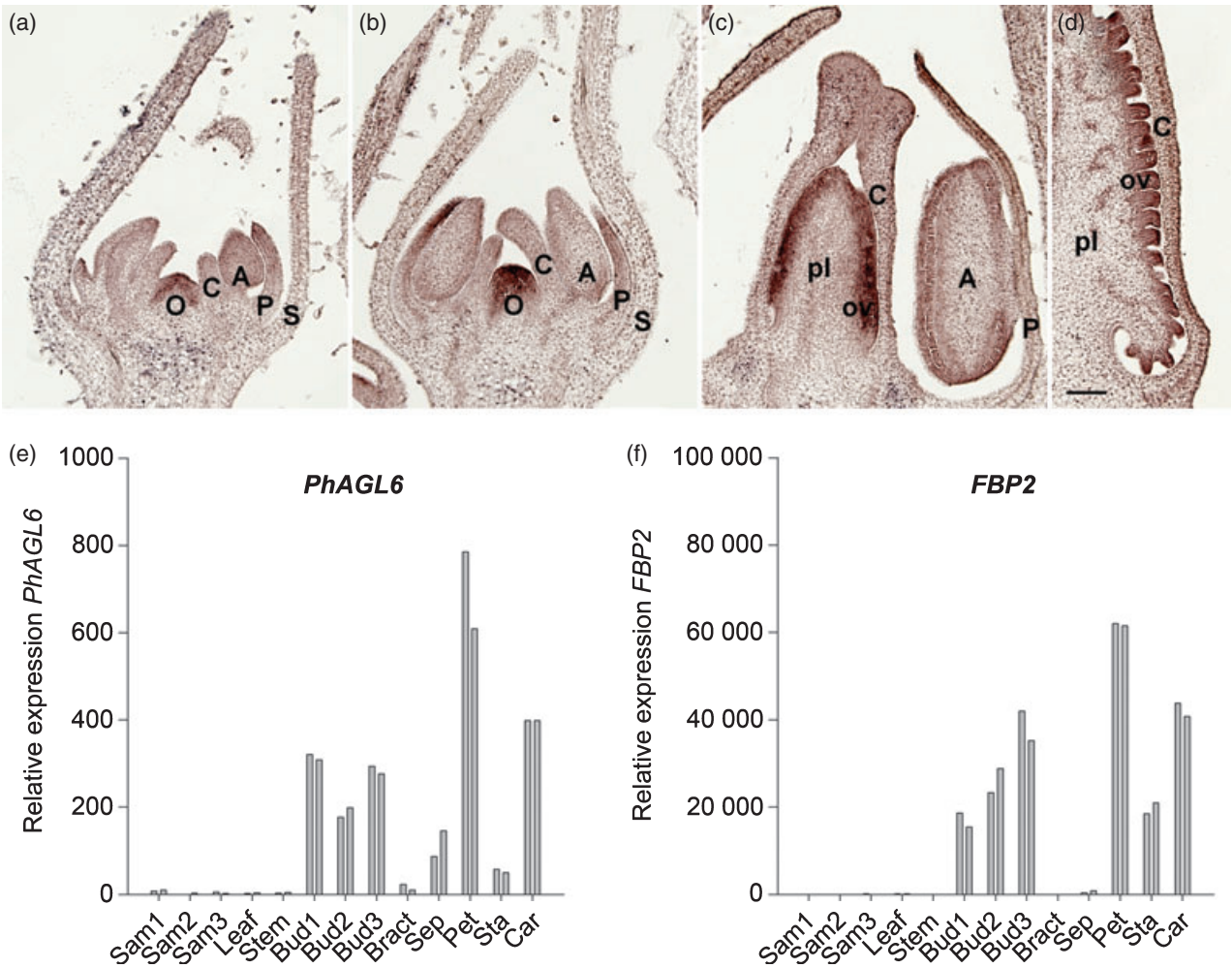

Figure 2. Expression of *FBP2* and *PhAGL6*, using quantitative RT-PCR analysis and *in situ* hybridization.
(a–d) Longitudinal sections of wild-type *Petunia hybrida* flowers of young developing flowers (a) to flowers at anthesis (d), hybridized with *PhAGL6* antisense probes. The scale bar in panel (d) represents 100 μm; all pictures (a)–(d) were taken at the same magnification. S, sepal; P, petal; A, anther; C, carpel wall; O, developing ovary; ov, ovule; pl, placenta.
(e, f) Quantitative RT-PCR on *PhAGL6* (e) and *FBP2* (f) transcripts in different wild-type *P. hybrida* tissues. The two bars per sample represent the relative expression values determined for the two biological replicates. sam1, shoot apical meristem of a 2-weeks-old seedling; sam2, shoot apical meristem of a 3-weeks-old seedling; sam3, shoot apical meristem of a 4-weeks-old seedling; buds of three different stages: bud1, 0.2 cm long; bud2, 0.7 cm long; bud3, 1.2 cm long; sep, sepal; pet, petal; sta, stamen; car, carpel.

The petunia *AGL6* gene has a *SEP*-like function

Despite the wide distribution of *AGL6*-like MADS-box genes amongst the gymnosperms and the angiosperms, not a single loss-of-function mutant with an obvious phenotype has been described; consequently the function of *AGL6*-like genes remains elusive. The only loss-of-function mutants that have been reported were those of the two Arabidopsis *AGL6* clade genes *AGL6* and *AGL13*. The Arabidopsis *AGL13* gene represents a more diverged *AGL6* subfamily member and lacks part of the usually strongly conserved AGL6 C-terminal motif (Vandenbussche *et al.*, 2003a). Neither *agl6* nor *agl13* null mutants exhibit an abnormal phenotype, possibly due to redundancy (Schauer *et al.*, 2007, 2008). Only one *AGL6* gene has been identified in petunia (Tsuchimoto *et al.*, 2000; Vandenbussche *et al.*, 2003b), and we used a reverse genetics strategy (Vandenbussche *et al.*, 2008) to identify a likely loss-of-function mutation in this gene. A single individual was found with a *dTph1* transposon inserted in the first exon, disrupting the MADS-domain, which probably represents a null mutation. Moreover, expression analysis shows that *PhAGL6* transcripts are strongly reduced in both homozygous *phagl6-2* mutants and plants homozygous for a derived footprint allele (*phagl6-2*^{ft7}) which differs from wild-type transcripts by a 7-bp insertion in the MADS-domain (Figure S2). Even though the *phagl6-2* mutant is a null mutant, no morphological abnormalities could be observed upon close inspection of flower

organs and ovules, and seed-set was not impaired. No change in flowering time was observed. These results indicate that *PhAGL6* might function in a redundant fashion, a phenomenon common among plant MADS-box genes.

Given their close phylogenetic relationship and similar expression patterns, the most likely candidates to act redundantly with *PhAGL6* are *SEP* subfamily genes. The four Arabidopsis *SEP* genes act largely redundantly with each other in mediating organ identity and control of determinacy (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). Of the six petunia *SEP* genes, the most extensively studied is the *SEP3* ortholog *FBP2*. A *35S::FBP2* construct has been shown to fully complement s*ep1 sep2 sep3* mutants in Arabidopsis (Pelaz *et al.*, 2000; Ferrario *et al.*, 2003); and unlike single *sep* mutants in Arabidopsis (Pelaz *et al.*, 2000) and single mutants for the other petunia *sep* genes, *fbp2* mutants exhibit a clear mutant phenotype (Vandenbussche *et al.*, 2003b). For this reason, we analyzed possible redundancy between *PhAGL6* and *FBP2* by generating *phagl6 fbp2* double mutants (Figure 3).

Fbp2 single mutants exhibit conversion of petals to green sepaloid tissue only at the edges of the petals and in the areas surrounding the main veins at the abaxial side (Vandenbussche *et al.*, 2003b). In contrast, *phagl6 fbp2* double mutants had completely green petals, and a corolla that was significantly reduced in size (Figure 3c,d). Scanning electron microscopy analysis of *phagl6 fbp2* petals revealed the conversion of conical petal epidermal cells into flatter and more jigsaw-shaped cells, similar to those found in the sepal epidermis, over the entire surface, while in *fbp2* single mutants this conversion was only observed at the petal edges (Figure 3c,d). Moreover, the stamens of *phagl6 fbp2* double mutants often showed sepal/petal-like structures and occasionally stigma-like structures on top of the anthers (Figure 3m). The carpels were morphologically indistinguishable from the wild type. Remarkably, *phagl6 fbp2*/+ plants were also easily recognizable: the petals were slightly more indented than wild-type petals, and were greenish, especially around the major veins (Figure 3k,l). Apparently, in *phagl6 fbp2*/+ plants the level of *FBP2* mRNA was not sufficient to allow the formation of normal flowers in the absence of *PhAGL6*. To confirm the *phagl6 fbp2* mutant phenotypes, we generated *PhAGL6* RNAi lines (Figure S2a). Like *phagl6* single mutants, the *PhAGL6* RNAi lines were indistinguishable from the wild type. However, the phenotypes of the different *PhAGL6* RNAi *fbp2*/+ and *PhAGL6* RNAi *fbp2* lines were identical to those of the combinations with the *phagl6* mutant allele (Figure 3e–h), demonstrating that the phenotypic differences between *fbp2* and *phagl6 fbp2* mutants can be fully attributed to loss of *PhAGL6* function.

Another important petunia *SEP*-clade gene is *FBP5*. The *fbp5* single mutant is morphologically indistinguishable from the wild type. Flowers of *fbp2 fbp5* mutants, however, display an enhanced phenotype compared to *fbp2* flowers: the green areas at the petal edges extend slightly further towards the center; sepal-like structures appear on top of the anthers and the carpel walls become more sepal-like. Inside the carpel, ovules are replaced by leaf-like organs (Vandenbussche *et al.*, 2003b). To uncover yet other possible redundant functions between the *PhAGL6* gene and *SEP*-clade genes, we generated *phagl6 fbp2 fbp5* triple mutants. The *phagl6 fbp2 fbp5* triple mutant phenotype was further enhanced compared with that of the *fbp2 fbp5* and *phagl6 fbp2* double mutants (see Figure 3d,i,j). The clearest change was observed in the stamens: the sepal-like structures on top of the anthers were larger and more leaf-like than those present in *fbp2 fbp5* double mutants, while the remaining antheroid structures were smaller (Figure 3m). In addition, the conversion of petal epidermal cells to sepal-like cells was more prominent than in *phagl6 fbp2* mutants (Figure 3j).

Taken together, these data indicate that *PhAGL6* functions redundantly with the *SEP*-like genes *FBP2* and *FBP5* in the specification of petal and stamen identity, while the high levels of expression in the developing ovary and in ovules (Figure 2) indicate a role in those organs as well.

PhAGL6 and FBP2 interact with similar MADS-box proteins

The results of our mutant analysis indicate that PhAGL6 functions in a similar manner to SEP proteins. To investigate this further, we compared their ability to interact with other MADS-box proteins in a yeast two-hybrid experiment. The results of this analysis show that PhAGL6 and FBP2 both interact with SOC1-clade proteins, C- and D-function proteins and some other SEP-clade proteins (Table 1). Neither FBP2 nor PhAGL6 interacts with the individual B-function proteins PhDEF, PhTM6, PhGLO1 or PhGLO2 (data not shown). The fact that FBP2 and PhAGL6 share similar interaction partners in this GAL4 yeast two-hybrid experiment, indicates that the two proteins are biochemically quite similar.

DISCUSSION

We have demonstrated that the petunia *AGL6* subfamily gene *PhAGL6* functions in specification of flower organ identity, and acts redundantly with at least the *SEP*-like genes *FBP2* and *FBP5*. Mutant analysis shows that *PhAGL6* is involved in petal and anther development. Moreover, the expression pattern of *PhAGL6* hints at a role in ovary, ovule and/or gametophyte development, especially because high expression levels of *AGL6*-like genes in ovules seem to be quite conserved amongst different species (this study and Moon *et al.*, 1999; Favaro *et al.*, 2002; Schauer *et al.*, 2007, 2008). Despite this, no obvious changes were observed in the ovules of *phagl6* mutant plants, and these seemed as fertile as those of wild-type plants. Also, in *phagl6 fbp2* double mutants the ovules appear normal, though in these plants, as in *fbp2* single mutants, fertility is impaired.

Figure 3. Phenotypes as observed in *phagl6* mutants, *PhAGL6* RNAi lines, and various mutant combinations with *fbp2* and *fbp5*, compared with wild-type.
(a–l) Top and/or side view of wild-type (WT) and various mutant *Petunia hybrida* flowers. For the side views, half of the sepals, petals and carpel wall were removed to reveal the inner organs. Genotypes are as indicated. Flowers shown in (e)–(h) and (k) are in the transformable Mitchell-W138 hybrid genetic background; all others are in the W138 background. Cryo scanning electron microscope (cryo-SEM) pictures of part of the adaxial petal epidermis, as indicated by the white rectangles, are shown in the top right corner of (a), (b), (c), (d), (i) and (j); a cryo-SEM picture of the adaxial leaf epidermis is shown in (n). Scale bars equal 50 μm.
(m) Stamens of wild-type (1), *phagl6* (2), *fbp2* (3), *phagl6 fbp2*, (4) *fbp2 fbp5* (5) and *phagl6 fbp2 fbp5* (6) flowers.

Table 1 Interactions of FBP2 and PhAGL6 with other MADS-box proteins. Yeast two-hybrid study using the GAL4 system. Interactions are scored ++ when interactions were clear in both directions (PhAGL6 or FBP2 fused with the GAL4 AD-domain tested against other MADS-domain proteins fused with the BD-domain, and vice versa, are both positive). Interactions are scored + when interactions are positive only in one direction; – indicates no growth at all

	AG		AGL11		SEPALLATA						AGL6	SOC1			
	pMADS3	FBP6	FBP7	FBP11	FBP2	FBP5	pMADS12	FBP4	FBP9	FBP23	PhAGL6	UNS	FBP21	FBP22	FBP28
PhAGL6	+	+	++	++	+[b]	+[a]	++	+[b]	+[a]	+[a]	–	+	+	++	++
FBP2	++	+	++	++	++[b]	–[a]	+	+[b]	+[a]	+[a]	+	+	++	+	++

[a]Interactions could only be tested in one direction due to autoactivation of the GAL4 reporter genes by intrinsic transcription activation domains in these proteins.
[b]Truncated FBP2ΔC–BD and FBP4ΔC–BD constructs (Ferrario *et al.*, 2003) were used to abolish autoactivation.

Several of the petunia *SEP* genes are also highly expressed in developing ovules (Immink *et al.*, 2003). The *SEP* genes *FBP2* and *FBP5* were shown to be required for ovule identity, as ovules are converted to leaf-like structures in *fbp2 fbp5* double mutants (Vandenbussche *et al.*, 2003b). Given the redundant role of *PhAGL6* and these *SEP* genes in petal and stamen development, it is conceivable that *PhAGL6* plays a similar role during ovule development as well. Despite the strong indication the expression pattern of *PhAGL6* gives for a function in ovule development, we have for the moment no clear results from mutant analyses confirming this.

In our yeast two-hybrid experiment the PhAGL6 and FBP2 proteins interact overall with the same partners, which demonstrates that the two proteins are biochemically quite similar. The set of proteins that interact with PhAGL6 and FBP2 in the yeast two-hybrid experiment is very similar to the set of proteins that was shown to interact with the Arabidopsis AGL6 and SEP proteins in an extensive yeast two-hybrid screening experiment performed under identical testing conditions (de Folter *et al.*, 2005). Like PhAGL6 and FBP2, the Arabidopsis SEP-proteins and AGL6 interact with (amongst others) AG-clade, SOC1-clade and SEP-clade proteins (de Folter *et al.*, 2005). Differences do exist, however, between the results from our GAL4 yeast two-hybrid experiment and a previously published CytoTrap yeast two-hybrid study on interactions between different petunia MADS-box proteins (Immink *et al.*, 2003). For FBP2 the majority of interacting partners found are identical to our findings. However, for PhAGL6 Immink *et al.* (2003) only found interactions with SOC1-clade proteins. We suspect these differences might be explained by the use of two different yeast two-hybrid systems (GAL4 versus CytoTrap), as our results did agree with results from the GAL4 yeast two-hybrid screening for Arabidopsis MADS-box proteins (de Folter *et al.*, 2005). The results obtained with the CytoTrap or GAL4 yeast two-hybrid systems might differ due to differences between the two systems: the proteins that are tested for are fused to different protein fragments and are expressed in different yeast strains grown under different growing conditions. Future research will have to show whether the interactions determined in these yeast two-hybrid experiments actually take place *in planta*.

Besides the interactions mentioned above, Arabidopsis AGL6 also interacts with quite a few MADS type I proteins (de Folter *et al.*, 2005). This makes AGL6 one of the few MIKC-type proteins that interacts with several type I MADS-domain proteins. This is interesting, as several of the type I genes seem to be involved in the development of ovule and gametophyte and several *AGL6* genes are expressed during development of ovary and ovule (this study and Favaro *et al.*, 2002; Portereiko *et al.*, 2006; Schauer *et al.*, 2007; Bemer *et al.*, 2008; Steffen *et al.*, 2008).

The question remains whether the *SEP*-like function we could assign to the petunia *AGL6*-clade gene *PhAGL6* is a general one. As mentioned, no extensive mutant analysis has so far been performed for *AGL6*-like genes. *AGL6*-like genes from the monocots hyacinth, orchid, bamboo and rice and the gymnosperm Norway spruce have, however, been studied functionally by constitutively expressing the genes in Arabidopsis and/or tobacco. All these heterologous experiments resulted in early flowering and dwarfed transformants, while curled leaves and loss of inflorescence determinacy could also often be observed (An, 1999; Hsu *et al.*, 2003; Carlsbecker *et al.*, 2004; Tian *et al.*, 2005; Fan *et al.*, 2007). In addition, constitutive expression of the hyacinth gene *HoAGL6* and the orchid *Oncidium* Gower Ramsey *OMADS1* gene in Arabidopsis resulted in homeotic transformations. Ectopic expression of *HoAGL6* in Arabidopsis led to upregulation of the floral organ identity genes *AG* and *SEP1*, a transformation of sepals, petals and leaves into carpel-like or ovary structures and disappearance or a reduction in the number of stamens (Fan *et al.*, 2007). Overexpression of the *Oncidium* Gower Ramsey *AGL6*-like gene, *OMADS1*, in Arabidopsis occasionally gave rise to homeotic transformations of sepals into carpel-like structures with stigmatic papillae and ovules (Hsu *et al.*, 2003). All these phenotypes are very similar to the phenotypes of Arabidopsis plants overexpressing the closely related *SEP* or *SQUA* clade genes (Chung *et al.*, 1994; Mandel and Yanofsky, 1995; Honma and Goto, 2001; Tzeng *et al.*, 2003;

Castillejo *et al.*, 2005). This is in accordance with the *SEP*-like function we found for petunia *AGL6*. The early flowering phenotypes of the *AGL6* gene overexpression studies seem to point in the direction of a role in flowering induction (e.g. Carlsbecker *et al.*, 2004). Even though in our *phagl6* mutants flowering time is not changed, we cannot exclude the possibility that *PhAGl6* is redundantly involved in regulating flowering time. However, it is clear that the petunia *AGL6*-clade gene acts redundantly with *SEP*-clade genes in flower organ formation. To fully understand the evolutionary significance of our findings it is important to determine the functions of *AGL6* genes of other species, especially those of gymnosperms.

EXPERIMENTAL PROCEDURES

Phylogenetic analyses

Using MUSCLE (Edgar, 2004) we aligned 62 protein sequences (representative AGL6 clade protein sequences and a selection of SQUA and SEP sequences). The alignment was then edited manually as described in Zahn *et al.* (2005). A neighbor-joining tree was computed as described previously (Vandenbussche *et al.*, 2003a). All protein sequences were full length, except for four protein sequences that missed a small part of the N-terminus but were also included in the alignment (Figure S1). Species names are abbreviated as indicated in Table S2 and accession numbers are listed in Table S3. One thousand bootstrap samples were generated to assess support for the inferred relationships. Local bootstrap probabilities (in percentages) are indicated for branches with >60% support.

Plant material and genotyping

In a large screening experiment aimed at identifying insertions into any member of the MADS-box gene family (Vandenbussche *et al.*, 2003b), a *dTph1* insertion was identified about 200 bp upstream of the ATG start codon of *PhAGL6* (*phagl6-1*, published as the *pmads4-1* allele, Vandenbussche *et al.*, 2003b). However, due to the small size of *dTph1* (284 bp), insertions outside the coding region rarely interfere with gene function. Therefore we screened a new insertion library (Nijmegen2005 library, 4096 plants) for insertions into the coding region of *PhAGL6*. In *phagl6-2* mutants the *dTph1* transposon is inserted at position +118 relative to the translational start, thereby disrupting the first exon encoding the MADS-domain. The *phagl6-2* derived footprint allele *phagl6-2*^{ft7} has a 7-bp footprint insertion at the same position, causing a frameshift and early stop codon. For the crosses described in this paper we have used *fbp2-1*^{ft7} and *fbp5-1*^{ft8} lines in which the *dTph1* transposons have excised, leaving behind a 7-bp and an 8-bp footprint, which cause a frameshift at amino acid positions 111 and 42 of the *FBP2* and *FBP5* coding sequences, respectively. These *fbp2-1* and *fbp5-1* derived footprint alleles induce an identical phenotype to the originally described alleles (Vandenbussche *et al.*, 2003b). Genotyping was performed as described previously (Rijpkema *et al.*, 2006). The primers used for genotyping the *fbp2*, *fbp5* and *phagl6* mutants are listed in Table S1.

Transgenic plants

Agrobacterium tumefaciens strain EHA105 was transformed using the freeze–thaw method (Chen *et al.*, 1994) with a pKGWIW2(I) (Karimi *et al.*, 2002) binary RNAi vector containing 334 bp of a non-conserved part of the coding sequence and 3′ untranslated region (UTR) of the petunia *PhAGL6* gene. This vector was constructed using a Gateway cloning strategy (Invitrogen, http://www.invitrogen.com/) as described previously (Rijpkema *et al.*, 2006). The primers used for amplification of the 334-bp gene fragment are listed in Table S1. Leaf disks from petunia F_1 progeny plants resulting from a cross between *fbp2-1*^{ft7} mutants and the easily transformable Mitchell variety were transformed as described (Horsch *et al.*, 1985). The T_1 progenies resulting from self-pollinations of the transgenic plants were genotyped for *fbp2-1*^{ft7}.

Protein–protein interaction studies

Two-hybrid analyses were performed using the GAL4 system. The pBD–GAL4 bait and pAD–GAL4 prey vectors containing a range of different MADS sequences, except for the PhAGL6, FBP13, FBP21, FBP22, FBP25 and FBP28 vectors, were provided by Richard Immink and described previously (Immink *et al.*, 2003). Full-length cDNA copies of *PhAGL6*, *FBP13*, *FBP21*, *FBP25* and *FBP28* were cloned into both pGBT9gate (Clontech vector adapted for Gateway cloning by insertion of a Gateway cassette) and pGAD424-gate (Clontech vector adapted for Gateway cloning by insertion of a Gateway cassette) vectors (Clontech, http://www.clontech.com/). All primers used for the cloning are listed in Table S1. Due to autoactivation of most SEP–BD fusion proteins, FBP4–BD, FBP9–BD and FBP23–BD were only tested in one direction, while for FBP2 and FBP4 the truncated FBP2ΔC–BD and FBP4ΔC–BD constructs (Ferrario *et al.*, 2003) were used. The GAL4 yeast two-hybrid analyses were performed as described previously (de Folter *et al.*, 2005), using yeast strain PJ69-4a (James *et al.*, 1996).

Quantitative real-time PCR analyses

For analysis of the expression levels of *PhAGL6* and *FBP2* in different plant tissues total RNA was isolated from shoot apical meristems of seedlings of three different stages (sam1: seedling 2 weeks; sam2: seedling 3 weeks; sam3: seedling 4 weeks), leafs, stem, buds of three different stages (bud1: 0.2 cm long; bud2: 0.7 cm long; bud3: 1.2 cm), bracts, sepals (bud stage 2), petals (bud stage 2), stamen (bud stage 2), and carpels (bud stage 2) of wild-type petunia plants. Every organ type was sampled twice independently. To examine the *PhAGL6* mRNA levels in *phagl6* mutants and knockdown lines, ovules of each of the lines to be tested were sampled (in duplicate). In this set of samples, leaf tissue was also included as representing (almost) zero expression of *PhAGL6*. The RNA extraction, cDNA synthesis and quantitative RT-PCR analyses were essentially performed as described previously (Rijpkema *et al.*, 2006). All primers used are listed in Table S1. Expression levels were corrected for PCR efficiencies and normalized to *GAPDH* and *ACTIN* expression levels, as described in Vandesompele *et al.* (2002). For the analysis of *PhAGL6* mRNA levels in mutants and RNAi plants (Figure S2) the highest expression value for wild-type ovules was set to 100, and all other values were plotted relative to the highest value on a linear *Y*-axis scale.

In situ hybridization

The 3′ gene-specific fragments of *PhAGL6* were generated by PCR using the primer pair that was also used for generating the RNAi construct (Table S1), and subsequently cloned into pGEM-T easy (Promega, http://www.promega.com/), containing T7 and SP6 transcription sites. Probe synthesis and *in situ* hybridization were performed as described previously (Canas *et al.*, 1994). Images were recorded using a Leica DM IRE2 microscope with a Leica DFC420C camera (Leica Microsystems, http://www.leica-microsystems.com/).

Electron microscopy

Samples for cryoscanning electron microscopy were first frozen in slush, prepared in an Oxford Alto 2500 cryosystem (Gatan, http://www.gatan.com/), and then analyzed in a JEOL JSM-6330F field emission electron scanning microscope (JEOL, http://www.jeol.com/).

ACKNOWLEDGEMENTS

We thank Walter Hendrickx, Yvette Evers, Harry van Zuijlen and Gerard van der Weerden for assistance with the plant work, Sandra Mansveld and Lorenz Boeckhorst for their help in the lab, Geert-Jan Janssen and Liesbeth Pierson for assistance with the microscopy and Richard Immink for kindly providing plasmids for the two-hybrid analysis. We also thank Barry Causier and Brendan Davies for their willingness to teach ASR the yeast two-hybrid technology (Marie Curie fellowship no. QLK3-GH-99-50575-16). The work of ASR is funded by Netherlands Organization for Scientific Research Grant 814.02.009.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of a selection of the known *AGL6* clade proteins; and *SEP* plus *SQUA* clade proteins from petunia, Arabidopsis and several other species.

Figure S2. *PhAGL6* mRNA levels in leaf tissue and in ovules of wild-type, mutant and transgenic plants.

Table S1. Primers used in this research.

Table S2. Abbreviations used for plant species in the phylogenetic analysis.

Table S3. NCBI accession numbers of protein sequences included in the phylogenetic analysis.

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