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A B_{sister} MADS-box gene involved in ovule and seed development in petunia and Arabidopsis

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Summary

MADS-domain transcription factors are essential for proper flower and seed development in angiosperms and their role in determination of floral organ identity can be described by the 'ABC model' of flower development. Recently, close relatives of the B-type genes were identified by phylogenetic studies, which are referred to as B_{sister} (B_s) genes. Here, we report the isolation and characterization of a MADS-box B_s member from petunia, designated *FBP24*. An *fbp24* knock-down line appeared to closely resemble the Arabidopsis B_s mutant *abs* and a detailed and comparative analysis led to the conclusion that both *FBP24* and *ABS* are necessary to determine the identity of the endothelial layer within the ovule. Protein interaction studies revealed the formation of higher-order complexes between B_s–C–E and B_s–D–E type MADS-box proteins, suggesting involvement of these specific complexes in determination of endothelium identity. However, although there are many similarities between the two genes and their products and functions, interestingly *FBP24* cannot replace *ABS* in Arabidopsis. The results presented here demonstrate the importance of the comparative analysis of key regulatory genes in various model systems to fully understand all aspects of plant development.

Keywords: endothelium, ovule, petunia, B_{sister} MADS-box gene, seed, development.

Introduction

An essential step in the survival of land plants is the formation of seed. The seed contains the two fertilization products, the embryo and endosperm, which are surrounded by the seed coat or testa. The seed coat protects the embryo against adverse environmental conditions and influences seed dormancy, germination and longevity (Debeaujon *et al.*, 2000). The seed develops from a fertilized ovule, which in angiosperms is located inside the female reproductive organ, the pistil (Gasser and Robinson-Beers, 1993). Ovules are initiated from the placental region of the inner surface of the ovary. The ovule primordium arises as a fingerlike structure that further develops into the nucellus, one or two integuments and the funiculus, which forms the connection to the placenta. The nucellus is the site of megasporogenesis and megagametogenesis, which produce the female gametophyte or embryo sac. As sporogenesis and gametogenesis occur, the inner and outer integuments develop to finally envelop the embryo sac. Inside the embryo sac, the egg cell becomes fertilized by one of the two pollen sperm cells to produce the zygote, which subsequently becomes the embryo. The second sperm cell fertilizes the central cell to produce the endosperm (Angenent and Colombo, 1996; Chaudhury *et al.*, 1998; Gasser and Robinson-Beers, 1993; Grossniklaus and Schneitz, 1998; Reiser and Fischer, 1993; Robinson-Beers *et al.*, 1992; Schneitz *et al.*, 1995; Skinner *et al.*, 2004).

The integuments are of sporophytic maternal origin and finally form the seed coat. In some species, e.g. petunia, the ovule contains a single integument, while in others, such as Arabidopsis two integuments are formed. The outer integument of Arabidopsis ovules contains two cell layers and, initially, the inner integument consists of two cell layers as

well. Around the four-nuclear embryo sac stage, the inner layer of the inner integument gives rise to a third layer, referred to as the integumentary tapetum or endothelium, which is in direct contact with most of the embryo sac. The endothelium is characterized by cells with a compact appearance, which are regularly cube-shaped, have very little or no vacuolization and distinct staining properties (Bowman *et al.*, 1991; Haughn and Chaudhury, 2005; Robinson-Beers *et al.*, 1992; Schneitz *et al.*, 1995). Functionally, the endothelium is thought to play a role in protection and nourishment of the embryo (Kapil and Tiwari, 1978).

In the last decade, several mutants have been identified affecting ovule formation, identity and integument development (for reviews see Angenent and Colombo, 1996; Gasser *et al.*, 1998; Grossniklaus and Schneitz, 1998; Schneitz *et al.*, 1998; Skinner *et al.*, 2004). The molecular control of ovule identity is well-studied in petunia and Arabidopsis (reviewed in Skinner *et al.*, 2004). In petunia, the MADS-box genes *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11*, probably fully redundant, are necessary for ovule identity (Angenent *et al.*, 1995; Colombo *et al.*, 1995) and proper seed development (Colombo *et al.*, 1997). Simultaneous downregulation of *FBP7* and *FBP11* by co-suppression resulted in plants with carpeloid structures in positions where normally ovules develop. Furthermore, the less severe *fbp7*/*fbp11* knock-down lines revealed premature degeneration of endothelial cells during late seed development, which resulted in degeneration of the endosperm, though still producing viable seeds (Colombo *et al.*, 1997). All these observations have led to the postulation of an extended ABC model of floral organ development (Coen and Meyerowitz, 1991) with a D-function for ovule formation (Colombo *et al.*, 1995). In Arabidopsis, the D-function is represented by *SEEDSTICK* (*STK*), which promotes ovule identity redundantly with the *SHATTERPROOF* (*SHP1/2*) genes (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003). Ovule and seed development was completely disrupted in the *stk shp1 shp2* triple mutant and some ovules were converted to leaf-like or carpel-like structures (Pinyopich *et al.*, 2003). Furthermore, it was concluded that the C-type gene *AG* acts partially redundantly with *STK* in ovule identity (Pinyopich *et al.*, 2003). Previous experiments also suggested the involvement of *AG* in ovule identity, because in *ap2* mutants ectopic ovule formation was observed, and some of these ovules developed into carpeloid structures (Western and Haughn, 1999). The number of ovules converted into carpeloid structures increases in *ap2 ag* double mutants and even more in *ap2 stk ag* triple mutants, with the strongest loss of ovule identity in *ap2 stk shp1 shp2* quadruple mutants (Pinyopich *et al.*, 2003). Furthermore, carpeloid structures never showed ovule formation in the *ag-3* mutant upon ectopic *STK* expression (Favaro *et al.*, 2003). Furthermore, in the *SEP1*/*sep1 sep2 sep3* mutant affecting the E-function, ovules revealed homeotic transformations as seen in *stk shp1 shp2* mutants (Favaro *et al.*, 2003). Based on protein interaction studies it was proposed that AG and SEP form a stable higher-order complex together with STK or one of the SHP proteins, and that each of these complexes is probably sufficient to promote ovule identity (Favaro *et al.*, 2003).

Recently, the *transparent testa16* (*tt16*) mutant was described (Debeaujon *et al.*, 2003; Nesi *et al.*, 2002), in which the MADS-box gene encoding ARABIDOPSIS BSISTER (ABS) was disrupted (Becker *et al.*, 2002). The *tt16*/*abs* mutant was identified based on altered seed pigmentation and revealed a lack of proanthocyanidin (PA) accumulation in the endothelium. Furthermore, endothelial cells showed an abnormal cell shape, which led to the conclusion that *TT16*/*ABS* is involved in endothelium development (Nesi *et al.*, 2002). The *ABS* gene is a member of the B_{sister} (B_s) subfamily of MADS-box genes, which was designated as B_{sister} after phylogenetic studies (Becker *et al.*, 2002). This subfamily is related to the B-type floral homeotic genes, such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in Arabidopsis, which are required for petal and stamen identity (Goto and Meyerowitz, 1994; Jack *et al.*, 1992). It has been suggested that the B and B_s gene lineages were generated by a duplication of an ancestral gene before the divergence of gymnosperm and angiosperm lineages 300 Ma (million years ago), but after the separation of the fern lineage 400 Ma (Becker *et al.*, 2002; Stellari *et al.*, 2004). Interestingly, B_s genes are predominantly expressed in female floral organs, in contrast to the B-type genes (Becker *et al.*, 2002).

In this study, we report the isolation and characterization of a MADS-box B_s member from petunia, designated *FBP24*. An *fbp24* knock-down line obtained by co-suppression, closely resembles the Arabidopsis B_s *abs* mutant. A detailed and comparative analysis is presented, which led to the conclusion that both *FBP24* and *ABS* are necessary for proper endothelium development. Nevertheless, *FBP24* failed to complement the Arabidopsis *abs* mutant, indicating divergence of the supposed orthologous genes in these two angiosperm species.

Results

Petunia FBP24 *isolation and sequence analysis*

A petunia ovary-specific cDNA expression library was screened by the yeast two-hybrid GAL4 system with FBP11 (Angenent *et al.*, 1995; Colombo *et al.*, 1995) as bait to identify interacting partners for this ovule-specific MADS-domain protein (Immink *et al.*, 2002). One of the interacting partners identified was a truncated MADS-domain protein, designated FLORAL BINDING PROTEIN 24 (FBP24). Subsequently, the full-length open reading frame (ORF) of *FBP24* was cloned by PCR, using plasmid DNA from the ovary cDNA library as template. The *FBP24* cDNA encodes a putative protein of 268 amino acids that, based on phylo-

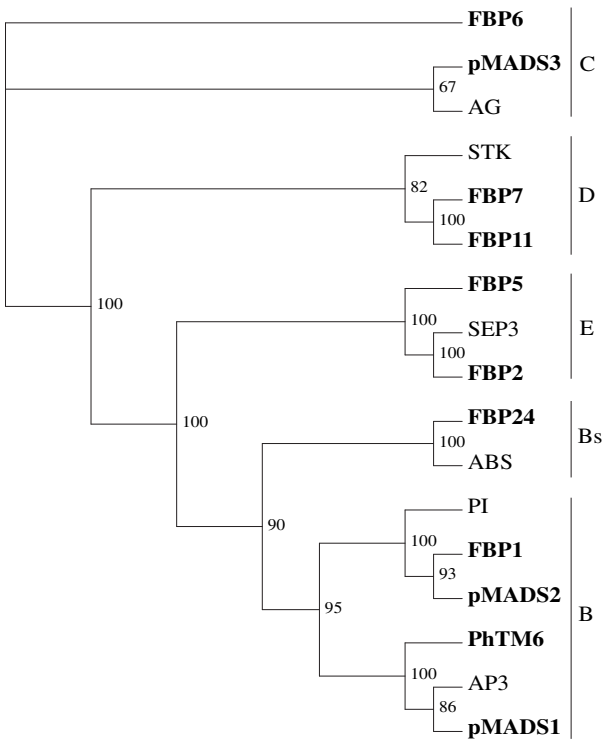

Figure 1. Phylogenetic tree based on an alignment of full-length MADS-domain proteins from *Petunia hybrida* and *Arabidopsis thaliana*.
Petunia proteins are given in bold and bootstrap values are given next to the branches. The various classes of homeotic genes are indicated with the relevant letter, according to the ABC model.

genetic analysis, belongs to the B_{sister} (B_s) subfamily of MADS-domain proteins (Figure 1), as described previously (Becker *et al.*, 2002).

FBP24 *expression analysis in wild-type petunia*

Northern blot hybridization experiments were performed to analyze the expression of *FBP24* in wild-type petunia plants (variety W115). RNA was isolated from various organs and hybridized to a specific *FBP24* cDNA fragment lacking the conserved MADS-box region. *FBP24* expression was detected in the ovary of wild-type petunia only, and its expression decreases slowly after pollination (Figure 2a,b). No expression was detected in vegetative tissues or other floral organs. In addition, *in situ* hybridization experiments were performed to obtain a more detailed picture of the *FBP24* expression pattern. A specific hybridization signal was visible in very young ovule primordia (Figure 2c), and slightly later in development in the nucellus and the integument of the developing ovule (Figure 2d). During late ovule and seed development *FBP24* expression was restricted to the endothelium (Figure 2e,f). This expression closely resembles the expression patterns of the D-type ovule identity genes *FBP7* and *FBP11*, which are also MADS-domain family members

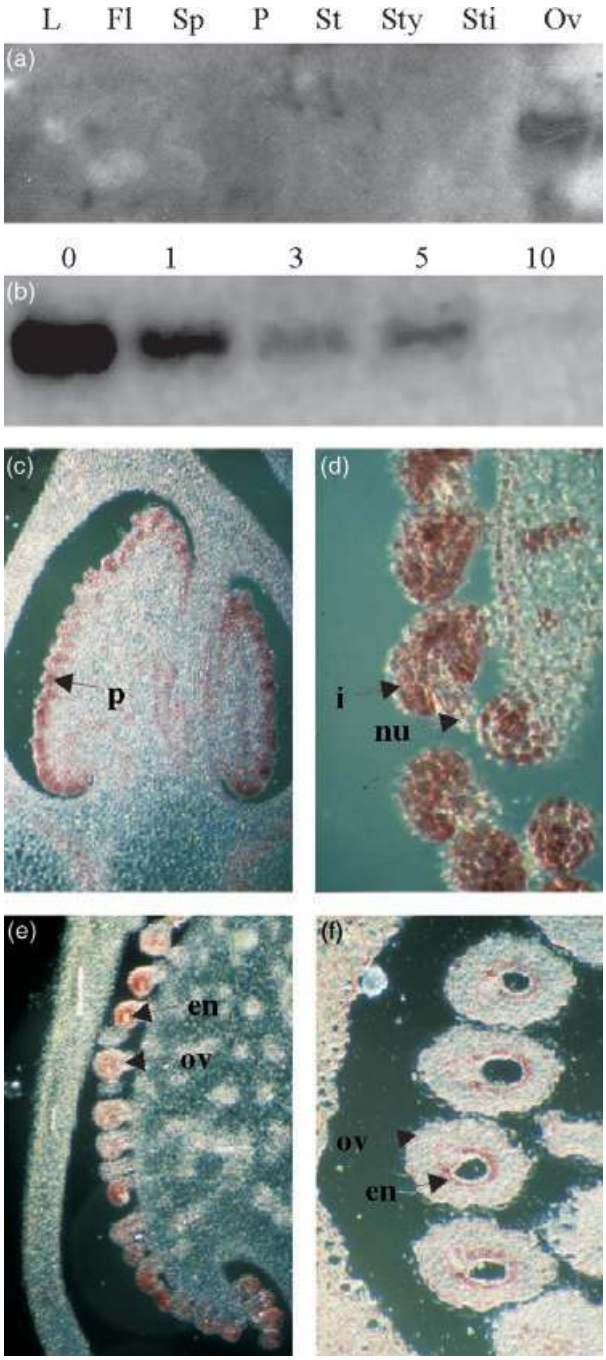

Figure 2. *FBP24* expression analyses by Northern blot (a, b) and *in situ* hybridization (c–f) in wild-type petunia W115 plants.
(a) *FBP24* expression analyzed in different tissues: L, leaf; FL, flower; Sp, sepal; P, petal; St, stamen; Sty, style; Sti, stigma; Ov, ovary.
(b) *FBP24* expression analyzed in ovaries at different time points (0, 1, 3, 5 and 10 days) after pollination (DAP).
(c)–(f) *FBP24* expression during ovule development: (c) expression in young ovule primordia, (d) nucellus and integument expression, (e, f) expression becomes restricted to the endothelium in mature ovules. p, ovule primordia; i, integument; nu, nucellus; ov, ovule; en, endothelium.

and are necessary for proper ovule development, but deviate with respect to the early nucellus expression (Angenent *et al.*, 1995; Colombo *et al.*, 1997). The observed expression pattern of *FBP24* and the similarity with the expression patterns of *FBP7* and *FBP11* suggest a role for *FBP24* in ovule development.

Functional analysis of FBP24

To investigate the function of *FBP24* in petunia we analyzed a *dTph1* transposon insertion mutant, which was identified in a reverse-genetics screen (Vandenbussche *et al.*, 2003). The transposon insertion was located 56 nucleotides downstream the start codon in the MADS-box, and due to this various in-frame stop codons were introduced. Phenotypic analysis of this presumed knock-out mutant did not reveal any alterations in development, which can probably be explained by functional redundancy. Therefore, an overexpression/co-suppression approach was taken, to gain more insight in the function of *FBP24*. With this aim, a construct was made with the full-length *FBP24* cDNA under control of the 35S CaMV promoter, which was introduced in the *Petunia hybrida* line W115 by *Agrobacterium*-mediated transformation (Angenent *et al.*, 1993). Seventeen independent transgenic petunia plants were generated and examined for morphological alterations and *FBP24* expression levels. Northern blot analysis with total RNA from leaf tissue revealed only one plant (pGD614#13) with ectopic *FBP24* expression (Figure S1), although this plant did not show any obvious morphological alterations. However, two other plants (pGD614#2 and pGD614#8) produced seed pods with a dramatic reduction in seed number and the few seeds produced were not fully round and had an altered seed color (Figure 3a–f). These two plants were subjected to more detailed molecular and phenotypic analyses. Northern blot analysis was performed with RNA isolated from ovaries of plants #2 and #8, and instead of increased *FBP24* expression no *FBP24* hybridization signal was detectable (Figure S1). This indicates that silencing had occurred in these plants most likely due to co-suppression, which possibly caused the poor seed-set phenotype. Subsequently, segregation analyses were performed using offspring plants of these two independent *fbp24* knock-down lines and a perfect linkage was observed between the reduced seed-set phenotype and silencing of the *FBP24* gene (Figure S1). Notably, these phenotypic changes were not observed in the *fbp24 dTph1* insertion line, which suggests that *FBP24* functions redundantly with other genes that are simultaneously suppressed in the *fbp24* knock-down lines. However, a Southern blot hybridization under low-stringency conditions revealed that *FBP24* is present as a single copy gene and that very similar MADS-box genes do not exist in the petunia genome (Figure S2). Because we used a full-length construct to generate the knock-down lines, the phenotype could also be the result of suppression of other related MADS-box containing genes. Therefore, we analyzed, by Northern hybridization, whether the expression levels of other MADS-box genes were altered in the *fbp24* knock-down lines. Taking the complete ORF of *FBP24* into account, the E-type gene *FBP2* (Angenent *et al.*, 1994; Ferrario *et al.*, 2003) and the D-type genes *FBP7* and *FBP11* (Angenent *et al.*, 1995; Colombo *et al.*, 1997), are most similar in DNA sequence among the known petunia MADS-box genes, and these genes are strongly expressed in ovules. However, phylogenetic analysis classifies *FBP24* as a B_s gene (Becker *et al.*, 2002) and hence, the B-type genes are also good candidates for downregulation in the *fbp24* knock-down lines. Of the petunia B-type genes *PhTM6* is the most closely related to *FBP24* at DNA sequence level and this gene is strongly expressed in the fourth whorl (Vandenbussche *et al.*, 2004). Despite the similarity in sequence between *FBP24* and *FBP2*/*FBP7*/*FBP11* and the close phylogenetic relation between *FBP24* and *PhTM6*, no clear differences in expression levels of these genes were visible in the *fbp24* knock-down lines compared to the wild-type control (Figure S3). This demonstrates that these genes are not substantially affected, and therefore it is not likely that the obtained phenotype is related to the functioning of these genes. Based on the available sequence data, there is no other obvious candidate gene that is related to *FBP24* and that could act in a redundant fashion with *FBP24.*

Detailed analysis of the fbp24 *knock-down lines in petunia*

To investigate whether the observed phenotype in the *fbp24* knock-down lines is due to a male or female defect we performed reciprocal crosses. When *fbp24* mutant pollen was used to pollinate wild-type petunia plants normal seed-set was observed. However, when wild-type pollen was used to pollinate the *fbp24* knock-down line a reduced seed-set was observed, demonstrating that the *fbp24* knock-down line is maternally impaired. Subsequently, we performed analine staining for callose to see whether unfertilized ovules of pGD614#2 and pGD614#8 plants develop normally and whether wild-type pollen tube growth was affected upon pollination of the knock-down plants. These analyses revealed that the ovules had a normal callose deposition and that pollen tubes grew normally and were able to reach the ovules (data not shown). Nevertheless, only a few ovules developed into a viable seed, while the majority degenerated shortly after pollination. To unveil the cause of ovule degeneration, we performed histological analysis on mature ovules from the *fbp24* knock-down lines and compared them to the wild type (Figure 3g–i). Mature ovules [0 day after pollination (DAP)] from wild-type petunia plants contain an endothelial layer, which is the innermost layer of the single integument, and is thought to serve as a feeding layer. The endothelial cells are regularly shaped and appear dark blue

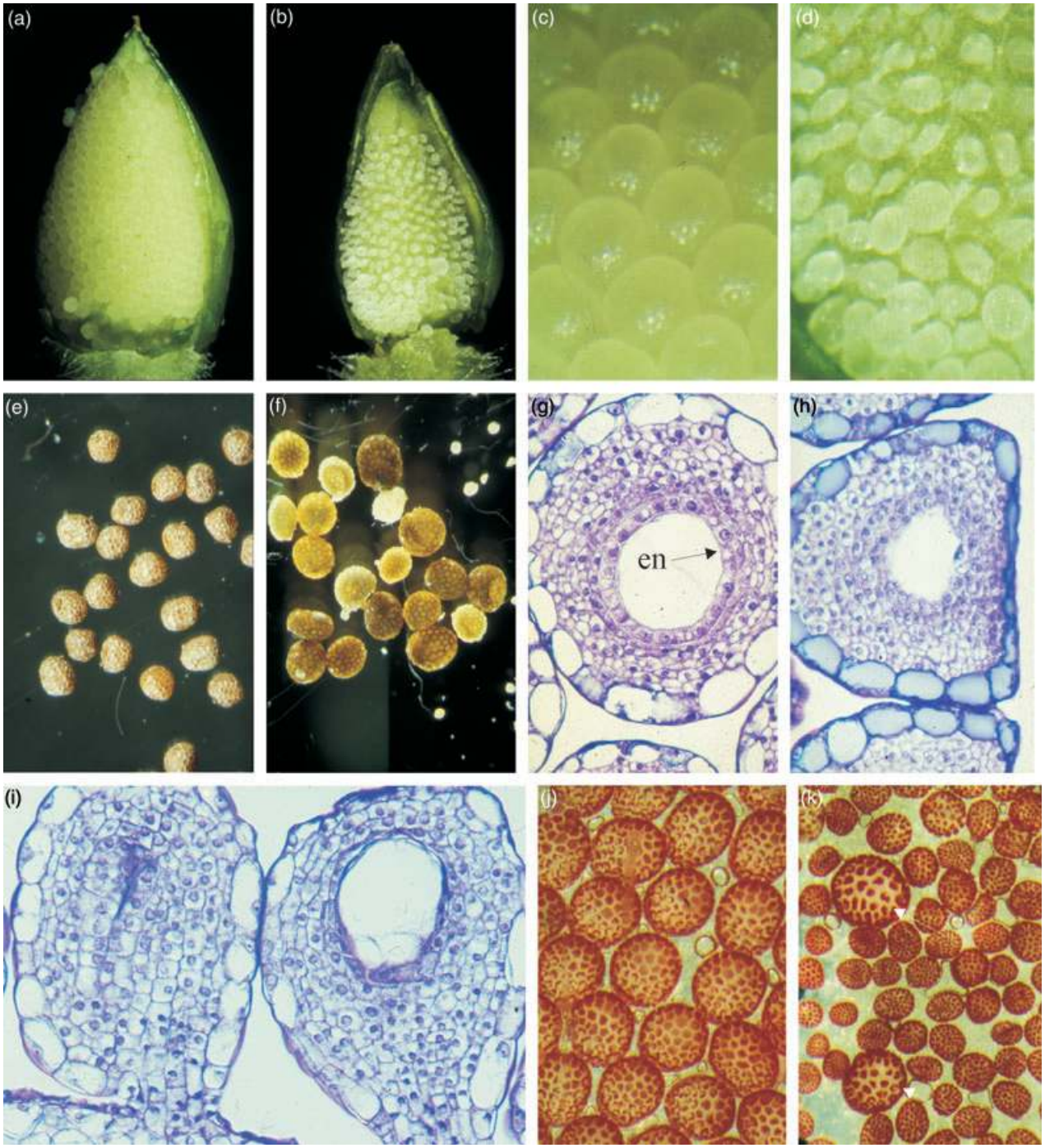

Figure 3. Morphological and histological analyses of the *fbp24* knock-down line versus wild-type petunia W115 plants.
(a, c) Phenotype of a wild-type petunia ovary with developing seeds (8 DAP) versus the altered phenotype of the *fbp24* knock-down line (8 DAP) (b, d).
(e) Seed phenotype of wild-type petunia plants versus the altered phenotype of the *fbp24* knock-down line (f).
(g) Sections of wild-type petunia ovules at 4 DAP versus sections of ovules of the *fbp24* knock-down line at 4 DAP (h).
(i) Ovules of the *fbp24* knock-down line at 0 DAP. In the left ovule the embryo sac is completely absent.
(j) Detection of proanthocyanidins with vanillin staining in developing seeds (6 DAP) of a wild-type petunia plant versus the *fbp24* knock-down line (k); arrows indicate normal developing seeds in the *fbp24* knock-down line. en, endothelium.

upon staining with toluidine blue, which marks the presence of phenolic compounds. In contrast, ovules of the *fbp24* knock-down line did not contain this regularly shaped inner cell layer (the endothelium) and hence lacked the dark blue staining pattern. Moreover, the embryo sac is occasionally completely absent, which resulted in less round-shaped

ovules (Figure 3i). This effect on embryo sac formation might be indirect and caused by aberrant endothelium development, eventually giving rise to completely degenerated ovules.

In Arabidopsis, PAs accumulate in the endothelium, which becomes part of the seed coat. During seed maturation, these compounds give rise to the brown color after oxidation (Devic *et al.*, 1999). To detect the presence of PAs in ovules of wild-type and the *fbp24* knock-down lines, vanillin staining was performed. Vanillin staining results in a dark red color when PAs are present (Gardner, 1975; Kristensen and Aastrup, 1986). An entire ovary of the *fbp24* knock-down line was assayed and revealed the presence of PAs. Interestingly, there was no visible difference in the staining pattern between the degenerated ovules or the few developing seeds of the *fbp24* knock-down lines and wild-type ovules and seeds (Figure 3j,k). This suggests that the endothelial layer in petunia is not the only layer where PAs accumulate; this in contrast to Arabidopsis where PAs accumulate exclusively in the endothelium (Debeaujon *et al.*, 2001; Devic *et al.*, 1999). Furthermore, it suggests that *FBP24* does not have an effect on PA accumulation in other cell layers of the petunia ovule/seed.

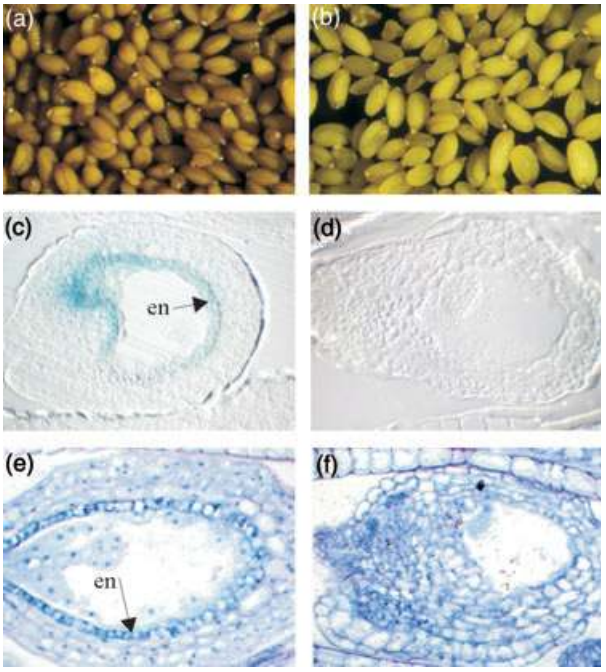

Figure 4. Morphological and histological analyses of the *abs* mutant versus wild-type Arabidopsis plants.
(a) Seed color phenotype of a wild-type Arabidopsis plant versus the homozygous *abs* mutant (b).
(c) GUS detection of the ET447 enhancer detector line in a developing seed of a wild-type Arabidopsis plant versus the homozygous *abs* mutant (d).
(e) Toluidine stained section of a developing seed of a heterozygous *abs* mutant versus a homozygous *abs* mutant (f). en, endothelium.

Detailed analysis of the Arabidopsis abs *mutant*

Because the previously described B_s MADS-box *tt16*/*abs* mutant (Nesi *et al.*, 2002) has a phenotype closely resembling that of the *fbp24* knock-down lines described here, and because of the high level of similarity at sequence level (Figure 1), we decided to reanalyze the *abs* mutant in more detail. For these analyses we used a new allele for the *abs* mutant (see Experimental procedures), called *tt16-6* according to conventional nomenclature (Kaufmann *et al.*, 2005; Nesi *et al.*, 2002). The *abs* mutant showed an altered seed pigmentation pattern due to a lack of PAs in the endothelium and, furthermore, the inner cell layer of the inner integument showed an abnormal cell shape (Figure 4), which confirms previously reported data (Debeaujon *et al.*, 2003; Nesi *et al.*, 2002).

Expression analyses revealed expression of *ABS* in reproductive organs, in buds, flowers and seed (Becker *et al.*, 2002; Nesi *et al.*, 2002; Parenicová *et al.*, 2003), and the *ABS* transcript appeared to accumulate during seed development (de Folter *et al.*, 2004; Hennig *et al.*, 2004). Nevertheless, its expression is very low or restricted, because *in situ* hybridization experiments failed to detect any expression (data not shown). Furthermore, a promoter GUS fusion did not result in any detectable expression under normal growth conditions (data not shown), which is consistent with the low expression levels of *ABS* reported previously (Becker *et al.*, 2002). However, based on the Arabidopsis *tt16/abs* phenotype (Nesi *et al.*, 2002) and expression of the B_s genes from antirrhinum (*DEFH21*, Becker *et al.*, 2002) and petunia (*FBP24*, this study) it seems that the *ABS* gene is specifically expressed in and is important for the endothelial layer.

To further investigate the endothelial cell layer, which is affected in the *abs* mutant, we used an enhancer detector line (ET447) that shows specific expression in the endothelium of ovules and developing seeds. Analysis of ET447 expression in the *abs* T-DNA insertion mutant showed that endothelial expression was lost in a homozygous *abs* background (Figure 4c,d). This strongly indicates that the endothelial layer is either absent or, alternatively, that the identity of this cell layer is changed. Subsequently, we determined the number of cell layers in the integuments to determine whether the endothelial layer is lost or transformed into a cell layer with another identity. The five integumental cell layers, with the innermost layer normally developing as the endothelium, were all present in the homozygous *abs* mutant, but they lacked the endothelial characteristics typical for the innermost layer. Determining the number of cell layers in the ovules of petunia is very difficult, but a similar change in identity of the endothelial layer as observed in the *abs* mutant also occurs in the *fbp24* knock-down lines.

Table 1 Higher-order complex formation for the petunia and Arabidopsis B_s proteins

Combination	pAD-GAL4	pRED	pBD-GAL4	Interaction	Complex
1	FBP24	FBP2ΔC	FBP7	±	B_s–E–D
	FBP24		FBP7	–	
		FBP2ΔC	FBP7	–	
2	FBP24	FBP2ΔC	FBP11	±	B_s–E–D
	FBP24		FBP11	–	
		FBP2ΔC	FBP11	–	
3	FBP24	FBP2ΔC	FBP6	–	
	FBP24		FBP6	–	
		FBP2ΔC	FBP6	–	
4	FBP24	FBP2ΔC	pMADS3	±	B_s–E–C
	FBP24		pMADS3	–	
		FBP2ΔC	pMADS3	–	
5	STK	SEP3	ABS-I	+	D–E–B_s
		SEP3	ABS-I	–	
6	STK	SEP3	ABS-II	+	D–E–B_s
		SEP3	ABS-II	–	
7	AG	SEP3	ABS-I	+	C–E–B_s
		SEP3	ABS-I	–	
8	AG	SEP3	ABS-II	+	C–E–B_s
		SEP3	ABS-II	–	
9	ABS-II	SEP3	SHP1	+	B_s–E–SHP
	ABS-II		SHP1	–	
		SEP3	SHP1	–	
10	ABS-II	SEP3	SHP2	+	B_s–E–SHP
	ABS-II		SHP2	–	
		SEP3	SHP2	–	

Nine different combinations of three proteins were tested in a yeast three-hybrid experiment. For each combination 1 or 2 control combinations were analyzed to either exclude that the growth of the yeast is caused by the interaction between two proteins instead of three, or to determine the level of autoactivation by the SEP3 protein. In case of petunia, the FBP2ΔC protein was used that showed no autoactivation but is still able to form higher-order complexes (Ferrario *et al.*, 2003). Therefore all these combinations were analyzed at 1 mM 3AT. In contrast, the full-length Arabidopsis SEP3 protein gives autoactivation allowing growth up to a concentration of 30 mM 3AT. Therefore all combinations with the Arabidopsis proteins were tested at a 3AT concentration of 60 mM.

Do ABS *and* FBP24 *interact with the same proteins?*

Protein interaction studies may help with functional analyses and inter-specific comparison of gene products. It has been shown that protein interactions are often conserved between plant species (Favaro *et al.*, 2002; de Folter *et al.*, 2005; Immink and Angenent, 2002). Recently, we have demonstrated that FBP24 specifically interacts with the E-type proteins FBP2 and FBP4, and was able to form a higher-order complex with the ovule-specific D-type proteins FBP7 or FBP11 in yeast (Nougalli Tonaco *et al.*, 2006). Moreover, by fluorescence resonance energy transfer–fluorescence lifetime imaging microscopy (FRET-FLIM) studies heterodimerization was shown for FBP24 and FBP2 *in planta*, and strong indications were found for higher-order complex formation of FBP24, FBP11 and FBP2 (B_s–D–E complex) *in planta* (Nougalli Tonaco *et al.*, 2006). In a comprehensive interaction study for all Arabidopsis MADS-domain proteins we determined that ABS interacts with the E-type SEP1/2/3 proteins (de Folter *et al.*, 2005), of which at least SEP3 is a functional equivalent of FBP2 from petunia (Ferrario *et al.*, 2003). Recently, higher-order complex formation was tested for ABS and revealed B_s–D–E complexes with STK (D) and SEP3 (E) proteins (Kaufmann *et al.*, 2005).

Interestingly, two splicing forms are reported for *ABS* (Nesi *et al.*, 2002), in contrast to a single form for *FBP24*. Furthermore, taking into account that the C-type *AG* gene has a redundant function in ovule identity (Pinyopich *et al.*, 2003), and that its expression becomes restricted to the endothelium as the ovule matures (Bowman *et al.*, 1991; Sieburth and Meyerowitz, 1997), we tested all the Arabidopsis proteins from the C-/D-type clade in a yeast three-hybrid screen in combination with the ABS-I and ABS-II proteins. A similar screen was performed for FBP24 with the equivalent petunia proteins (Table 1). ABS-II lacks the last five amino acids at the end of the K-box, which are present in the splicing form ABS-I. Although the K-box is involved in protein–protein interactions (Fan *et al.*, 1997; Yang *et al.*, 2003), no difference in higher-order complex formation was observed between the products of the two ABS splicing

variants. In the case of petunia, the complex B_s–C–E (FBP24–pMADS3–FBP2) was found in addition to the previously reported B_s–D–E complex (FBP24–[FBP7/11]–FBP2) (Nougalli Tonaco *et al.*, 2006). In summary, the higher-order complexes that have been tested in yeast are conserved between petunia and Arabidopsis.

Can FBP24 *replace* ABS *in a mutant complementation experiment?*

The *abs* mutant and *fbp24* knock-down lines show similarities, but also exhibit minor differences. Based on the yeast interaction studies, it cannot be concluded that *ABS* and *FBP24* have diverged in function during evolution. Therefore, we performed a mutant complementation experiment with the *abs* mutant, which should reveal whether *ABS* can be replaced by *FBP24* in Arabidopsis. The *FBP24* cDNA under control of the constitutive 35S CaMV promoter (pGD614) was introduced into the Arabidopsis *abs* mutant by crossing. The 35S::FBP24 transgenic line was generated in ecotype Columbia and the expression of *FBP24* in leaves was confirmed by Northern analysis (results not shown). In parallel, we produced 35S::ABS (pGD 797) lines and crossed it into the *abs* genetic background in a similar way as was done for 35S::FBP24. This resulted, however, in a lack of complementation in the case of 35S::FBP24, while the *ABS* gene under the control of the 35S CaMV promoter (pGD797) was able to complement the *abs* mutant as was reported previously by Nesi *et al.* (2002) (results not shown). Despite the fact that *FBP24* failed to complement the *abs* mutant, ectopic expression of *FBP24* caused curly leaves and altered flower and silique development in Arabidopsis in a similar way as 35S::ABS does, which indicates that *FBP24* is ectopically expressed, and that a protein is produced. However, these ectopic expression phenotypes are suggested to be caused by a dominant-negative effect (Kaufmann *et al.*, 2005). Taking into consideration that the 35S CaMV activity in ovules is low in comparison to other tissues (Figure S4), we repeated the complementation experiment, making use of the petunia *FBP7* promoter. This promoter of the petunia D-type *FBP7* MADS-box gene is highly expressed in ovules and seeds (Colombo *et al.*, 1997). Firstly, its expression pattern in Arabidopsis was tested by a fusion with the β-glucuronidase (GUS) reporter gene. This experiment showed that the *FBP7* promoter drives high expression in Arabidopsis ovules as well (Figure S4). Besides the *FBP24* coding region (pARC675), we also generated constructs for both splicing forms of *ABS*, *ABS-I* (or *ABS*; pARC677) and *ABS-II* (shorter version; pARC676), under the control of the *FBP7* promoter. All three constructs were introduced in the homozygous *abs* mutant by the floral dip method (Clough and Bent, 1998). In the case of complementation, the seeds of the T_1 plants (primary transformants) are expected to give brown colored seeds, instead of yellow seeds. We first examined the expression levels of the introduced genes by Northern blot analysis for at least nine individual plants of each line (Figure S5). Ribonucleic acid was isolated from mature pistils in which the *FBP7* promoter is highly active in Arabidopsis (Figure S4). After ripening, mature seeds were harvested from all Arabidopsis plants. Strikingly, all seeds from *abs* mutant plants expressing the introduced *FBP24* gene (five plants) still had a yellow color, which indicates that the petunia *FBP24* gene expressed in the ovule was not able to complement the Arabidopsis *abs* mutant. In contrast, *abs* mutant plants with the introduced *ABS-I* gene revealed several plants that produced brown seeds. The molecular analysis revealed that all transgenic plants with *ABS-I* expression driven by the *FBP7* promoter (five plants) produced brown colored seed, whereas plants in which no *ABS-I* expression was detected by Northern blot analysis gave only yellow seeds. This demonstrated that the *ABS-I* gene under control of the *FBP7* promoter is able to complement the *abs* mutant, as was shown for the *35S::ABS-I* construct. Surprisingly, seeds from *abs* mutant plants with the introduced splicing variant *ABS-II* were still yellow, indicating that expression of this cDNA failed to complement the mutant (nine plants with expression).

Discussion

MADS-domain proteins are important factors that fulfill a plethora of diverse biological functions in plant development (Ng and Yanofsky, 2001; Riechmann and Meyerowitz, 1997; Theissen *et al.*, 2000). Tightly linked to MADS-domain proteins are floral organ formation and identity, which led to the postulation of the ABC model (Coen and Meyerowitz, 1991; Ferrario *et al.*, 2004). Here we describe the identification and functional analysis of the petunia B_s gene *FBP24* and its comparison with the Arabidopsis B_s gene *ABS*.

The petunia *FBP24* and the Arabidopsis *ABS* genes are closely related at the sequence level, but differ in their expression levels and patterns. *FBP24* is highly and specifically expressed during ovule formation and, at later developmental stages, it is restricted to the innermost layer of the integument, the endothelium. In contrast, *ABS* is expressed at a very low level and is not detectable by *in situ* hybridization analysis. Reverse transcriptase-PCR experiments revealed expression in reproductive organs and during seed development (Becker *et al.*, 2002; de Folter *et al.*, 2004; Hennig *et al.*, 2004; Nesi *et al.*, 2002; Parenicová *et al.*, 2003). Phenotypical analysis of an *fbp24* knock-down line and an *abs* T-DNA insertion mutant revealed similarities but also striking differences. The petunia mutant ovules degenerated immediately after pollination and only a few developed into viable seeds. In contrast, viable but yellow colored seeds are produced by the Arabidopsis *abs* mutant. Histological analyses suggest that both mutants lack the endothelial layer due to a change in identity of the cells in

this layer. Apparently, the absence of this endothelial layer has a different effect in the two species. Arabidopsis appeared to be able to produce viable seeds without an endothelium, while in petunia the endothelial layer appears to be indispensable. Ablation of the endothelial layer in Arabidopsis also resulted in normal seeds, and this has been shown to have no effect on embryo or endosperm development (Debeaujon *et al.*, 2003). Interestingly, there exists a structural difference between petunia and Arabidopsis ovules with respect to the number of integuments. Arabidopsis ovules contain a double integument (bitegmic ovules), like most angiosperms, while petunia ovules have only one integument (unitegmic ovules) (Angenent and Colombo, 1996). It is tempting to speculate that an endothelial layer is more important when a plant species has only one integument, although evidence for this hypothesis is lacking.

The yellow seed phenotype in the Arabidopsis *abs* mutant, as previously reported for the *tt16*/*abs* mutant (Nesi *et al.*, 2002), is due to a lack of PAs that normally accumulate in the endothelial layer only (Devic *et al.*, 1999). The altered pigmentation is probably a secondary effect due to a change in identity of the endothelial cells. In petunia, these PAs still accumulate in the *FBP24* knock-down lines, in which the endothelial layer is dramatically affected, indicating that these pigmentation molecules are also produced in other layers of the integument and seed coat.

It is important to note that the *fbp24* knock-down lines are most likely not affecting a single gene because the *fbp24* knock-out mutant did not show any altered phenotype. Therefore, it is likely that at least one more gene, which may act redundantly with *FBP24* to control development of the endothelium, is simultaneously suppressed in the *fbp24* knock-down lines. The most likely candidates for a redundant action with *FBP24* were tested and appeared to not to be affected in the knock-down lines. Therefore, other petunia MADS-box genes that have not yet been identified may be involved in development of the endothelium. The two paralogs *FBP7* and *FBP11*, which were not downregulated in the mutant, are important factors for proper endothelial development (Colombo *et al.*, 1997). Simultaneous suppression of *FBP7* and *FBP11* resulted in precocious degeneration of the endothelium during late seed development in weak *FBP7*/*FBP11* co-suppression lines, which subsequently resulted in the degeneration of the endosperm. Nevertheless, embryo development was not dramatically affected and seeds were still viable. These observations indicate that besides *FBP24* the D-type genes *FBP7* and *FBP11* are also required for development of the endothelium. This is consistent with their predominant expression in the endothelial layer and is further supported by the fact that they are components of a higher-order complex formed in yeast and in plant cells (Nougalli Tonaco *et al.*, 2006), which suggests that they perform this role in concert with FBP24 and potentially other factors.

An important question is whether the B-sister genes from petunia and Arabidopsis are functionally interchangeable, as this may provide clues about functional conservation across different angiosperm lineages. Protein–protein interactions are conserved among species and, therefore, may help to predict protein functions that have been conserved during evolution and to identify functionally equivalent proteins (Favaro *et al.*, 2002; de Folter *et al.*, 2005; Immink and Angenent, 2002). Yeast protein interaction screens revealed that FBP24 and ABS interacted with a similar set of floral organ identity proteins. The higher-order protein complexes B_s–C–E and B_s–D–E were identified for both petunia and Arabidopsis. Interestingly, for petunia the B_s–C–E complex could only be identified for the combination FBP24–pMADS3–FBP2 and not in combination with the second putative C-type protein FBP6. This preference for one of the duplicated genes encoding a putative C-type protein was reported previously for petunia for the higher-order complexes of FBP2, with specific combinations of B-type and C-type proteins (Ferrario *et al.*, 2003). It was speculated that these complexes are recruited for slightly different functions. Furthermore, the petunia higher-order protein complex B_s–D–E (FBP24–FBP11–FBP2) was recently reported *in planta* by FRET-FLIM analyses, as well as in yeast (Nougalli Tonaco *et al.*, 2006). Taking all these results together, and consistent with the proposed 'quartet model' of MADS-domain transcription factor function (Theissen, 2001; Theissen and Saedler, 2001), we propose that the conserved higher-order protein complex B_s–D–E is necessary to determine endothelial identity, at least in petunia and Arabidopsis. Despite the similarities in protein interactions and ternary complexes formed with ABS and FBP24, the mutant complementation experiments revealed that *FBP24* cannot replace *ABS* in Arabidopsis. This lack of complementation by *FBP24* cannot be explained by the failure to form heterologous higher-order protein complexes, because FBP24 was able to form complexes with STK and SEP3 in yeast (SdF, RGHI and GCA, unpubl. results). Possibly, the heterologous protein complex possesses a different affinity for target DNA sequences or, alternatively, the target DNA sequences have evolved differently in the two species. Another explanation could be that a specific co-factor is missing or unable to bind, and hence the complex is not able to act on the downstream target genes. A recent study with the C-type genes in antirrhinum and comparison with Arabidopsis demonstrated that paralogs may evolve differently in different species. The antirrhinum ortholog of *AG* is *FARINELLI* (*FAR*), but the paralog *PLENA* (*PLE*) is the equivalent of AG from a functional point of view (Causier *et al.*, 2005). In a similar way, the presumed orthologous B-sister genes *FBP24* and *ABS* may have deviated slightly after the divergence of the Asterids (petunia) and Rosids (Arabidopsis).

Experimental procedures

Plant growth

Petunia hybrida, *Arabidopsis thaliana*, transgenic and mutant plants were grown under normal greenhouse conditions (22°C, long-day light regime). The *fbp24* transposon insertion mutant was in a petunia W138 background and all other petunia experiments were conducted in the W115 background. The Arabidopsis *abs* mutant was in the ecotype Wassilevskija-3 (Ws-3), the ET447 enhancer detector line in Landsberg *erecta* (L*er*), and ectopic expression studies were performed in Columbia-0 (Col-0).

Sequence analyses

Full-length amino acid sequence comparisons were performed using the multiple sequence alignment program CLUSTALX (v. 1.5b) (Thompson *et al.*, 1997). A phylogenetic tree was generated using the neighbor-joining method in CLUSTALX and bootstrap analysis was performed on 100 data sets, and branches with values of <50 were collapsed. The tree was visualized using the software program TREEVIEW (v. 1.6.6) (Page, 1996). Sequences included in the phylogenetic analysis were from Arabidopsis (AG, X53579; STK, U20182; SEP3, AF015552; ABS, AJ318098; PI, D30807; AP3, M86357) and from petunia (FBP6, X68675; pMADS3, X72912; FBP7, X81651; FBP11, X81852; FBP5, AF335235; FBP2, M91666; FBP24, AF335242; FBP1, M91190; pMADS2, X69947; PhTM6, AY532264; pMADS1, X69946).

Construction of binary vectors and plant transformation

The *FBP24* (AF335242) ORF was PCR amplified and subcloned into pGEM®-T Easy (Promega, Madison, WI, USA). In parallel, the expression cassette of the pGD120 vector (Immink *et al.*, 2002; Nougalli Tonaco *et al.*, 2006) was cloned as a *Asc*I–*Pac*I fragment into the pBINPLUS vector (van Engelen *et al.*, 1995), giving the binary expression vector pGD121. Subsequently, the *FBP24* coding region was transferred by *Xba*I/*Xho*I digestion into the binary pGD121 vector, resulting in the construct pGD614. Cloning of the *ABS-I* (AJ318098) and *ABS-II* (AY141212, shorter version) ORFs was described previously (de Folter *et al.*, 2005; Parenicová *et al.*, 2003). The genomic *ABS* locus was amplified by PCR in a similar way as the previously described *ABS* ORFs and recombined into pDONR-207 (Invitrogen, Carlsbad, CA, USA) to generate a Gateway™ entry clone. Subsequently the obtained *ABS* clone was recombined into the binary Gateway™ overexpression vector pGD625, under the control of the double 35S CaMV enhancer, resulting in the pGD797 construct. The pGD625 Gateway™ destination vector was made by changing the orientation of the expression cassette in pGD120 by digestion with *Eco*RI, followed by ligation, resulting in pGD622. The pGD622 vector was digested with *Xba*I, treated with Klenow fragment, followed by ligation of the Gateway™ RF-A cassette (Invitrogen), resulting in pGD624. Finally, this vector was digested with *Asc*I/*Pac*I to transfer the complete expression cassette to an *Asc*I/*Pac*I-digested pBIN19 binary vector (Bevan, 1984). The binary pFBP7:GUS reporter construct (pFBP202/pARC443) was described previously (Colombo *et al.*, 1997). Constructs pARC675, 676 and 677, expressing *FBP24*, *ABS-II* and *ABS-I* under control of the *FBP7* promoter, respectively, were obtained as follows. Initially, the *FBP7* promoter fragment (*pFBP7*) was PCR amplified, using the binary FBP7:GUS construct (pARC443) as template, and adding a 5′-*Asc*I site and a 3′-*Nco*I site to the promoter. The obtained fragment was cloned into pGEM®-T Easy (Promega). In parallel *ABS-I*, *ABS-II* and *FBP24* full-length coding regions were amplified by PCR, adding a *Nco*I site to the 5′-end and a *Sac*I site to the 3′-end. All three fragments were cloned into pGEM®-T Easy (Promega). Subsequently, all the obtained pGEM®-T Easy vectors were partially digested with *Nco*I/*Sac*I, and the MADS-box gene fragments were cloned behind the *pFBP7* fragment. In the next step the three '*pFBP7*-MADS-box gene' fragments were cloned as *Asc*I/*Sac*I fragments into the binary pGD121 vector. In the latter step the 35S CaMV promoter of pGD121 is replaced by *pFBP7*. The final constructs were analyzed by sequencing (DYEnamic™ ET Terminator Cycle Sequencing Kit; Amersham Biosciences, Piscataway, NJ, USA) and designated pARC675, pARC676 and pARC677.

abs *mutant identification*

The T-DNA insertion in the *AGL32/ABS/TT16* locus (*At5g23260*) was identified in the T-DNA population of the Knockout Facility of Wisconsin (Sussman *et al.*, 2000). The position of the T-DNA insert was determined by PCR and sequencing and was localized in the 3′ region of the first intron, 51 nucleotides upstream of the start of the second exon. This novel *abs* allele was designated as *tt16-6*, in accordance with the nomenclature used by Nesi *et al.* (2002) and Kaufmann *et al.* (2005).

DNA gel blot analysis

Total plant DNA was isolated from petunia leaf material according to Koes *et al.* (1986). Ten micrograms was digested with either *Eco*RI, *Hin*dIII, *Bam*HI or *Sal*I, seperated on a 0.8% agarose gel in 1× Tris-acetate–EDTA (TAE) buffer, and followed by blotting onto Hybond-N + membrane (Amersham Biosciences) in 0.4 M NaOH solution. The full-length *FBP24* cDNA probe was labelled with the RadPrime DNA Labeling System (Invitrogen) and the blot was hybridized as described by Angenent *et al.* (1992). The blot was washed under low-stringent conditions at 55°C with 2× SSC, 1% SDS.

RNA gel blot analysis and in situ *hybridization*

Total RNA was isolated from frozen plant tissue according to Verwoerd *et al.* (1989). Five micrograms of each RNA sample was denatured by 1.5 M glyoxal, separated on a 1.2% agarose gel in 15 mM Na-phosphate buffer pH 6.5, checked for equal loading by staining with ethidium bromide, and followed by blotting onto Hybond-N + membrane (Amersham Biosciences) in 25 mM Na-phosphate buffer pH 6.5. Probes were labelled with the RadPrime DNA Labeling System (Invitrogen) and blots were hybridized as described by Angenent *et al.* (1992). Probes were prepared by amplification of specific 3′ regions with the following primers: *FBP24*, PRI112, 5′-CTCGTGCCGATTCGGCACGAGTG and PRI657, 5′-TGTCGACGATCAGTCA-TAACTAGGGCC, *ABS*, PRI668, 5′-CTCATTGACCGATACTTGCATACC and PRI416, 5′-CCGTCGACTTAATCAT TCTGGGCCGTTGGATC.

In situ hybridizations were performed as described by Cañas *et al.* (1994). Initially, a PCR was performed with a T7 primer and a FBP24-specific forward primer (PRI656, 5′-GATGATCATTATGGGGAGGGG), using the pBD-GAL4-FBP24 plasmid as template. Subsequently, 10 μl of the obtained PCR product was used to synthesize the digoxigenin-labelled RNA probe by T7 polymerase-driven *in vitro* transcription, according to the manufacturer's instructions (Boehringer Mannheim GmbH, Germany).

Microscopy

Petunia ovaries of the *fbp24* knock-down lines and wild-type W115 were fixed in a solution of three parts 50% ethanol with one part of acetic acid, and incubated twice for 10 min with vacuum applied at room temperature. The material was rinsed with 70% ethanol and incubated overnight, followed by dehydration in a series of alcohol solutions (70%, 96% and 100% ethanol) for 15 min each. The material was embedded in hydroxy-ethylmethacrylate Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions, and as described previously (Angenent *et al.*, 1993). Sections (5–8 μm) were made on a rotary microtome (Reichert-Jung 2040; Leica, Rijswijk, the Netherlands) and stained for 1 min with a solution of 1% toluidine blue and 1% sodium tetraborate (w/v) in distilled water.

Whole-mount vanillin staining was performed on ovules and developing seeds in a solution of 1% (w/v) vanillin (Sigma-Aldrich, St. Louis, MO, USA) in 6 M HCl at room temperature for 10 min. Clearing of ovules and developing seeds was obtained by incubation in Hoyer's solution (7.5 g Arabic gum, 100 g chloral hydrate, 5 ml glycerol, and 60 ml water) (Liu and Meinke, 1998) for a few hours to overnight at room temperature.

To detect GUS activity (Jefferson *et al.*, 1987), Arabidopsis inflorescences and siliques were fixed in 90% ice-cold acetone for 1 h at −20°C, followed by three rinses with 0.1 M Na-phosphate buffer pH 7.0 containing 1 mM potassium ferrocyanide. The three rinse steps in total took 1 h and during the first rinse step vacuum was applied for about 15 min. Finally, the substrate was added to the samples, containing 50 mM Na-phosphate buffer pH 7.0, 1 mM EDTA, 0.1% (v.v) Triton X-100, 1 mM potassium ferrocyanide and 1 mM X-Gluc (Duchefa, Haarlem, the Netherlands), and vacuum was applied for 5 min, followed by incubation from overnight to several days at 37°C in the dark. Chlorophyll was removed by first 1 h incubation in 96% ethanol and then transfer to 70% ethanol. After GUS detection and chlorophyll removal, Arabidopsis tissue used for embedding was transferred to 96% ethanol, followed by the manufacturer's instructions for Technovit 7100 embedding (Heraeus Kulzer). All samples were observed with a Nikon Optiphot microscope equipped with Normaski optics or a Zeiss Stemi SV8 stereo microscope (Carl Zeiss, Oberkochen, Germany).

Protein–protein interactions

All yeast two-hybrid experiments were performed as described by Immink *et al.* (2003) and all three-hybrid experiments as described by Ferrario *et al.* (2003). Higher-order complex formation was scored after incubation of the yeast at 20°C for at least 5 days. The gene encoding for the third protein was cloned into the pREDnIsA vector (Ferrario *et al.*, 2003). *FBP2* was cloned with a truncation at the 3′-end, to overcome the strong autoactivation by the encoded protein, as described (Ferrario *et al.*, 2003). The SEP3 protein gives autoactivation as well; however, this could be overcome by screening at a 3-amino-triazole (3AT) concentration above 40 mM. Therefore, the full-length *SEP3* ORF was cloned into the pREDnIsA vector, and used in the screens for higher-order complex formation at high concentrations of 3AT. For the interaction studies with Arabidopsis MADS-domain proteins the previously generated Gateway™ compatible yeast two-hybrid vectors were used (de Folter *et al.*, 2005), whereas for the petunia experiments, the vectors generated by Immink *et al.* (2003) and Nougalli Tonaco *et al.* (2006), were used.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Northern blot analysis of *FBP24* expression level in leaves and ovaries of transgenic petunia plants.

Figure S2. Southern blot analysis to determine the copy number of *FBP24* in the petunia genome.

Figure S3. Northern blot analyses of *FBP24*, *FBP2*, *FBP7*, and *PhTM6* expression levels in transgenic petunia plants with construct pGD614.

Figure S4. Reporter GUS expression in *Arabidopsis thaliana* (WS-3).

Figure S5. Northern blot analyses of *FBP24* and *ABS* expression levels in pistils of transgenic Arabidopsis *abs* mutant plants.

This material is available as part of the online article from http://www.blackwell-synergy.com

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