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BIGPETALp, a *bHLH* transcription factor is involved in the control of *Arabidopsis* petal size

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In *Arabidopsis*, APETALA1, PISTILLATA, APETALA3 and SEPALLATA interact to form multimeric protein complexes required to specify petal identity. However, the downstream events that lead to petal specific shape and size remain largely unknown. Organ final size can be influenced by cell number or cell expansion or both. To date, no gene that specifically limits petal size by controlling postmitotic cell expansion has been identified. Here we have identified a novel petal-expressed, basic helix-loop-helix encoding gene (*BIGPETAL*, *BPE*) that is involved in the control of petal size. *BPE* is expressed via two mRNAs derived from an alternative splicing event. The *BPEub* transcript is expressed ubiquitously, whereas the *BPEp* transcript is preferentially expressed in petals. We demonstrate that *BPEp* is positively regulated downstream of *APETALA3*, *PISTILLATA*, *APETALA1* and *PISTILLATA3* and is negatively regulated downstream of *AGAMOUS*. Plants that lack the petal-expressed variant *BPEp* have larger petals as a result of increased cell size, showing that *BPEp* interferes with postmitotic cell expansion. BPEp is therefore a part of the network that links the patterning genes to final morphogenesis.

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Introduction

During flower development, many proteins interact as components of the molecular networks that control biological processes such as the floral meristem emergence and the acquisition of flower organ identity (Krizek and Fletcher, 2005). Once a plant has been induced to flower, the floral organs (sepals, petals, stamens and carpels) develop sequentially on the flanks of the floral meristem under the combinatory action of four classes of organ identity genes (A, B, C and E) following the ABCE model (Krizek and Fletcher, 2005). In this model, A + E specify sepals, A + B + E petals, B + C + E stamens and C + E carpels. Over the past few years, these homeotic genes have been shown to be master regulatory genes that trigger the developmental programs required for flower organogenesis (Jack, 2004; Krizek and Fletcher, 2005). In *Arabidopsis thaliana*, these genes are *APETALA1* (*AP1*) and *APETALA2* (*AP2*) for the A class, *PISTILLATA* (*PI*) and *APETALA3* (*AP3*) for the B class; *AGAMOUS* (*AG*), the only known class C gene and *SEPALLATA 1, 2, 3, 4* (*SEP1*, *SEP2*, *SEP3*, *SEP4*) for the E class (Krizek and Fletcher, 2005). All of these genes (except for *AP2*) encode MADS-box transcription factors (TFs) that have been proposed to interact and form four different tetrameric complexes according to the 'quartet model' (Honma and Goto, 2001; Theissen and Saedler, 2001). This directly links the action of these four different tetrameric complexes to floral organ identity. AP3, PI, SEP3 and either AP1 or AG have been shown to interact to form multimeric protein complexes required to specify petal (in whorl 2) or stamen (in whorl 3) identity, respectively (Honma and Goto, 2001; Theissen and Saedler, 2001). In whorl 2, PI, AP3, AP1 and SEP presumably regulate a set of downstream structural genes (so called 'realizators') that encode proteins required for the cell division and differentiation events that lead to petal organogenesis. These downstream proteins must function in a timely, coordinated way, leading to a constant final organ shape and size. Thus, understanding petal development and morphogenesis requires the identification of those genes whose expression is regulated downstream organ identity genes. Efforts have been made to perform reverse genetic screens to identify petal-expressed genes using microarray approaches (Zik and Irish, 2003; Wellmer *et al*, 2004). These studies identified only a very small number of genes that were petal specific. To date, only a few genes have been clearly shown to be involved in petal development. *NAP*, whose expression is controlled by AP3/PI, has been suggested to function in the transition between growth by cell division and cell expansion in petals and stamens (Sablowski and Meyerowitz, 1998). *RABBIT EARS*, whose expression is under the control of *AP1*, is involved in second whorl organ development (Takeda *et al*, 2004). *PETAL LOSS* has been shown to be involved in petal organ initiation and orientation (Griffith *et al*, 1999). Very recently, an E3 ubiquitin ligase-encoding gene, *BIG BROTHER (BB)*, has been shown to limit plant organ size by controlling cell proliferation (Disch *et al*, 2006).

In this study, we used cDNA-AFLP-differential display (DD) to identify genes that act downstream of petal organ identity genes in *A. thaliana*. We identified a basic helix-loop-helix TF encoding gene (*BIGPETAL or BPE*) that is expressed via two mRNA transcripts derived from an alternative splicing event (*BPEp* and *BPEub*). We demonstrate that (i) *BPEp* is preferentially expressed in petal and is derived from a

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ubiquitously expressed gene via an alternative splicing event, (ii) *BPEp* acts downstream of petal organ identity genes and (iii) that *BPEp* regulates the size of *A. thaliana* petals by restricting cell expansion.

Results

Identification and expression analysis of petal-expressed genes

We carried out a cDNA-AFLP-DD to search for genes that are expressed specifically in petals. This screen was performed by comparing gene expression profiles in inflorescences of two *A. thaliana* floral homeotic mutants, *pistillata* (flowers composed of sepals and carpels) and *agamous* (flowers composed of sepals and petals) (Bowman *et al*, 1989). Genes upregulated in *agamous* flowers, in comparison to *pistillata* flowers, are therefore expected to be petal-expressed. The nucleotide sequences of the cDNA-AFLP bands that were differentially amplified in *agamous* corresponded to 13 *A. thaliana* genes.

RT–PCR analysis of the expression pattern of each of these 13 genes showed that eight were expressed specifically or preferentially in flowers compared to leaves (Figure 1). Based on their putative protein sequences, these genes are predicted to encode a basic helix-loop-helix TF (that we named *BPE* for *BIGPETAL*, see below), an F-box protein (F-box), a Histone2A (His2A), a Phosphatase 2C (Phos 2C), a Chalcone Synthase (ChS), a Receptor-Like-protein Kinase (RLK), a endo-β-1, 4-glucanase (β-gluc) and a PolyA-Binding protein (PABP), respectively. Moreover, *BPE*, His2A and F-box-encoding genes were expressed in *agamous* and wt flowers, but showed no or background level expression in *pistillata* flowers (lack petals), indicating that they are likely to be petal-expressed. The remaining five genes, which encode a putative translationally controlled tumor protein (TCTP), an early-responsive to dehydration stress protein-3 (ERD-3), a photosystem I subunit XI precursor, a vacuolar ATPase or α tonoplast intrinsic protein, showed ubiquitous expression (Figure 1 and data not shown).

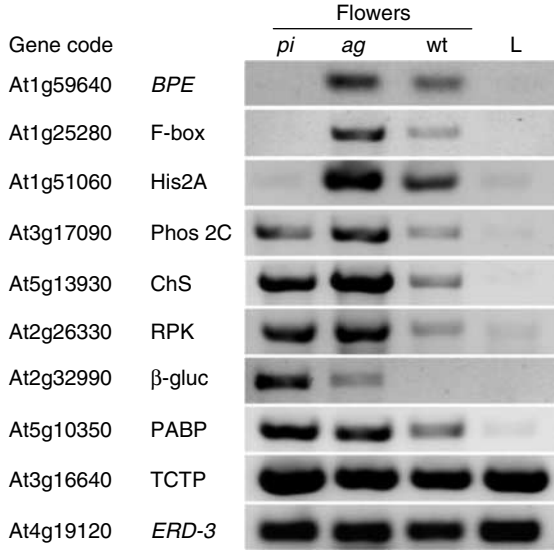

Figure 1 RT–PCR expression analyses of selected identified genes in flowers of wt, *pistillata (pi)* and *agamous (ag)* and in leaves (L) of wt *A. thaliana*. F-box, putative F-box protein; His2A, putative Histone2A; Phos 2C, putative phosphatase 2C protein; ChS, Chalcone Synthase; RPK, putative receptor-like protein kinase; β-gluc, similar to endo-β-1,4-glucanase; PABP, putative polyA-binding protein; TCTP, translationally controlled tumor protein; ERD-3, early-responsive to dehydration stress protein-3. Gene identifiers are listed.

BIGPETAL encodes two basic helix-loop-helix TFs

Sequence similarity searches showed that the *BPE* corresponds to *At*bHLH031 (*At*1g59640) which belongs to subgroup XII of the large family of basic helix-loop-helix TFs in *Arabidopsis* (Heim *et al*, 2003). Cloning of *BPE* cDNAs revealed that this gene encodes two transcripts (Figure 2A and B): a short transcript of 1215 nucleotides that we named *BPEub* (*ub* for ubiquitous, see below) and a longer, transcript of 1623 nucleotides, *BPEp* (*p* for petal-expressed). Both transcripts have identical 5′ nontranslated regions of 136 nucleotides. *BPEp* and *BPEub* differ in their 3′ coding and nontranslated regions because of an alternative splicing event that leads to the retention of the 408 nucleotide fifth intron in the *BPEp* transcript and a change in the reading frame. Thus, *BPEub* and *BPEp* are predicted to encode two different proteins of 29 and 38 kDa, respectively. The first 221 amino acids of the two proteins are identical and contain the highly conserved bHLH domain, known to be involved in DNA binding and in protein–protein interactions. The 43 amino acids corresponding to the C-terminal domain of BPEub are replaced by a stretch of 122 amino acids in BPEp that is translated from intron 5. These two C-terminal regions do not show any similarity to each other, nor to other known protein domains. BPEp and BPEub proteins fused to the GAL4-binding domain were able to activate transcription of a reporter gene in yeast (EV & MB, unpublished data) consistent with their predicted functions as transcription factors.

The two BPE transcripts have different expression profiles

We designed primers to discriminate between the *BPEp* and *BPEub* transcripts, in RT–PCR analyses. The primers 12–5 and 12–3 (Figure 2A) were used to specifically detect *BPEp* transcript and allow amplification of DNA fragment of 905 bp (note that these are the primers we used to detect *BPE* in Figure 1). As there is no nucleotide sequence that discriminates the shorter *BPEub* transcript from the *BPEp* transcript, the assay for the former was based on the size difference of the PCR products (by amplifying a region spanning intron 5). The primers 12-5 and 3C (Figure 2A) allow the amplification of DNA fragments of 804 or 1212 bp from cDNA template corresponding to *BPEub* or *BPEp*, respectively. As shown in Figure 2C, the *BPEub* transcript was expressed in flowers, stems and in cauline and rosette leaves of wt *A. thaliana* and this transcript was expressed at similar levels in flowers of both *pistillata* and *agamous* (Figure 2D). Thus *BPEub* is ubiquitously expressed. In contrast, the *BPEp* transcript accumulated in wt *A. thaliana* inflorescences, and flower buds, but no or very weak expression was observed in inflorescence stems and in rosette and cauline leaves. Figure 1 shows that the *BPEp* transcript accumulated in *agamous* flowers but not in *pistillata* flowers. These results indicate that *BPEp* has a petal- or petal and stamen-specific expression profile. To quantify the

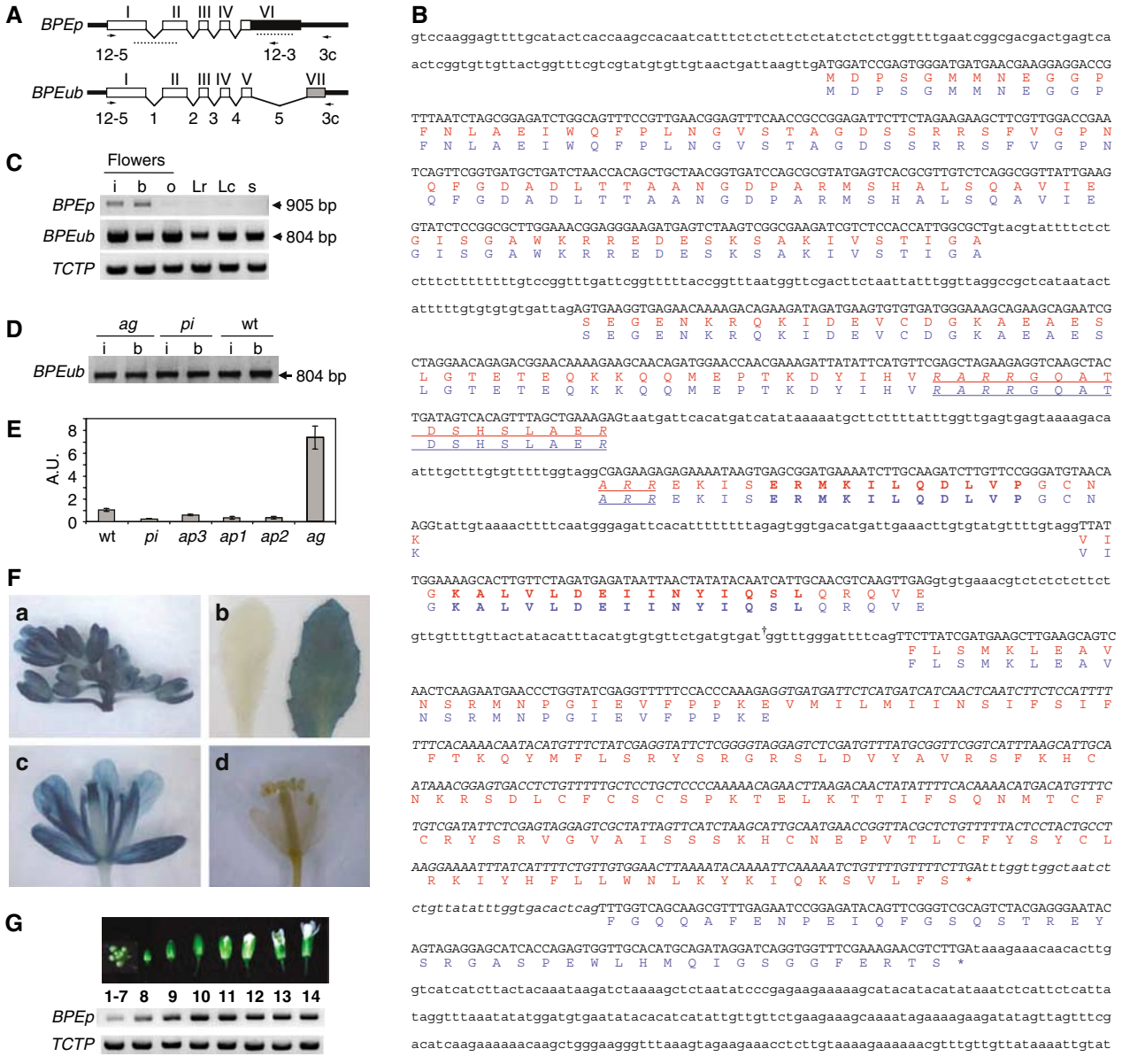

Figure 2 (**A**) Genome organization of *BPE* transcripts. Exons are presented as boxes numbered in roman characters. Introns are indicated in arabic numbers. Identical amino-acid sequences in both proteins are marked as white boxes. Amino-acid sequence present in BPEp only is shown as a black box and that present in BPEub by a gray box. Dashed lines show the position of the two regions used to generate RNAi constructs. Positions of primers used in RT–PCR experiments are indicated by arrows. (**B**) Nucleotide sequence of the pre-messenger RNA of *BPE*. The 5′ and 3′ nontranslated regions as well as introns are presented in lower case characters, exons in capital characters. The differentially spliced region that is expressed in *BPEp* and not in *BPEub* is marked in italicized capital characters. Stop codons are marked by stars. BPEp and BPEub proteins are shown in red and blue, respectively. The basic region is underlined and the helices of the helix-loop-helix region are marked in bold characters. The site of the T-DNA insertion in the *BPE* knockout line is indicated by '†'. *BPEub* and *BPEp* sequences have been submitted to the DDBJ/EMBL/GeneBank databases under accession numbers AM269753 and AM269754, respectively. (**C**) RT–PCR analysis of *BPEp* (top) and *BPEub* (middle) mRNA accumulation in wt *A. thaliana* inflorescence (i), flower buds (b), open flowers at the onset of petal senescence (o), rosette leaves (Lr), cauline leaves (Lc) and inflorescence stem (s). The constitutively expressed gene TCTP (bottom) was used as a control. (**D**) Analysis of *BPEub* mRNA accumulation using RT–PCR in flowers of mutant *pistillata (pi)* and *agamous (ag)* and of wt *A. thaliana*. (**E**) Expression analysis of the *BPEp* transcript in flowers of wt and mutant *A. thaliana* (lines *pi, ag, apetala1 [ap1]*, *ap2,* and *ap3*) using RT-QPCR. A.U., arbitrary units. (**F**) *BPE* promoter activity as monitored in the *Promoter*^{BPE}*:GUS*-expressing line. GUS staining in (**a**) the inflorescence, (**b**) the rosette leaves (right: *promoter*^{BPE}*:GUS* plant, left: wild-type control plant) and (**c**) in a flower at development stage 13. (**d**) GUS staining in wild-type flower. (**G**) Analysis of *BPEp* expression during early (1–7), mid (8–12) and at open flower development stages (13 and 14).

abundance of the *BPEp* transcript, we performed real time-quantitative PCR (RT-QPCR; Figure 2E). This sensitive assay detected low levels of *BPEp* transcripts in *pistillata* and *apetala3* flowers and a very high level of *BPEp* transcript in *agamous* flowers compared to wt flowers, thus agreeing with the RT–PCR results (Figure 1). Similar experiments showed that *BPEp* was very weakly expressed in *apetala1* or *apetala2* flowers (which possess stamens but lack petals; Figure 2E). Taken together, these results indicate that *BPEp* preferentially accumulates in petals.

Global gene expression during development in diverse organs of *A. thaliana* has been investigated using the Affymetrix ATH1 arrays (Zimmermann *et al*, 2004; Schmid *et al*, 2005). These microarray experiments indicate a ubiquitous pattern of expression for BPE, they do not agree with our data in as far as they indicate that *BPE* expression in petals and stamens is stronger than in other plant organs, with particularly high levels in petals (Zimmermann *et al*, 2004). These microarray data do not allow discrimination between the two splice variants *BPEp* and *BPEub* because the probe sets corresponding to *BPE* target both *BPEub* and *BPEp*, but it is very unlikely that the high level of expression detected in petals is owing to *BPEp* accumulation because this transcript is significantly less abundant than BPEub, even in petals (Figure 2C). Therefore, in contrast to our observations, the Affymetrix data suggest that *BPEub* is expressed to a higher level in petals and stamens compared to other organs.

To investigate these conflicting results further, we fused 1317 bp of the *BPE* promoter sequence to the *GUS* reporter gene and examined X-gluc staining in transgenic plants carrying this fusion. Nine independent *Promoter*^{BPE}*:GUS* lines were generated and showed similar GUS staining patterns. *BPE* expression was detected ubiquitously in vegetative organs and in flowers at different developmental stages, with no evidence of a higher level of expression of *BPEub* in petals compared to other floral organs or leaves (Figure 2F). Hence, the GUS-fusion data corroborated the RT–PCR experiments (Figure 2C and D) and we therefore propose that the *BPEub* transcript is expressed ubiquitously.

Attempts to investigate the *BPEp* expression pattern by *in situ* hybridization failed, most likely owing to the low level of expression of this transcript. Instead, we analyzed its expression at different flower development stages (Smyth *et al*, 1990); Figure 2G). A very low level of *BPEp* expression was observed at early development stages (1–7, corresponding to floral meristem determination, organ identity and initial development). The *BPEp* transcript accumulated from stage 8 and a very high relative abundance was observed at stage 10 and above (at which stage cell differentiation occurs in petals). *BPEp* expression decreased and was at a background level in flowers at the onset of petal senescence (Figure 2C).

In summary, *BPE* encodes two transcripts whose relative abundance is regulated at the post-transcriptional level, with *BPEub* showing ubiquitous expression and *BPEp* exhibiting an expression in petals mainly at flower development stages above 10.

BPEp expression is upregulated downstream of PI/AP3, SEP2, SEP3 and *AP1*

As *BPEp* was negatively regulated in *pistillata* and in *apetala3* flowers, we investigated its expression in *A. thaliana* lines that overexpressed PI (*35S:PI*) or AP3 (*35S:AP3*). In flowers of both lines, we observed a five-fold increase in the abundance of *BPEp* compared to wt flowers (Figure 3A). These results indicate that *BPEp* expression is positively regulated downstream of the B class genes *PI* and *AP3*.

It has been shown that, to mediate their function as organ identity proteins, PI, AP3 and SEP3 interact with AP1 in whorl 2 to specify petal identity and with AG in whorl 3 to specify stamen identity (Honma and Goto, 2001). We therefore investigated the expression of *BPEp* in lines that overexpress AP1, SEP3 or SEP2. The latter two genes are known to function redundantly (Pelaz *et al*, 2000, 2001b). Interestingly, *BPEp* abundance increased by about 10-fold or 6-fold in flowers of the *35S:SEP3* or the *35S:SEP2* lines, respectively (Figure 3B). To investigate the role of AP1 in *BPEp* regulation, we followed the latter's expression in an *apetala1* line that carried an inducible AP1 sequence fused to the steroid-binding domain of the rat glucocorticoid receptor (GR; line *35S:AP1-GR*; Yu *et al*, 2004). Following treatment with the GR substrate dexamethasone (DEX) to activate AP1, an approximately six-fold increase in *BPEp* expression was observed in flowers within 6 h after treatment (Figure 3C). These data indicate that *BPEp* is positively regulated by AP1. To investigate whether this activation of *BPEp* by AP1 is direct or indirect, inflorescences of the *35S:AP1-GR* line were simultaneously treated with DEX and with cycloheximide (CHX, a protein synthesis inhibitor). At 6 h post-treatment, no accumulation of the *BPEp* transcript was observed, indicating a requirement for intermediate protein synthesis (Figure 3C). Hence, AP1 is a positive regulator of *BPEp* accumulation and this regulation is indirect, requiring *de novo* protein synthesis. A small increase in *BPEp* transcript accumulation was also observed in leaves of lines ectopically expressing PI, AP3, SEP2 or SEP3 (Figure 3A and B). Taken together, these results indicate that *BPEp* is activated downstream of the petal organ identity PI, AP3, AP1 and SEP MADS-box TFs.

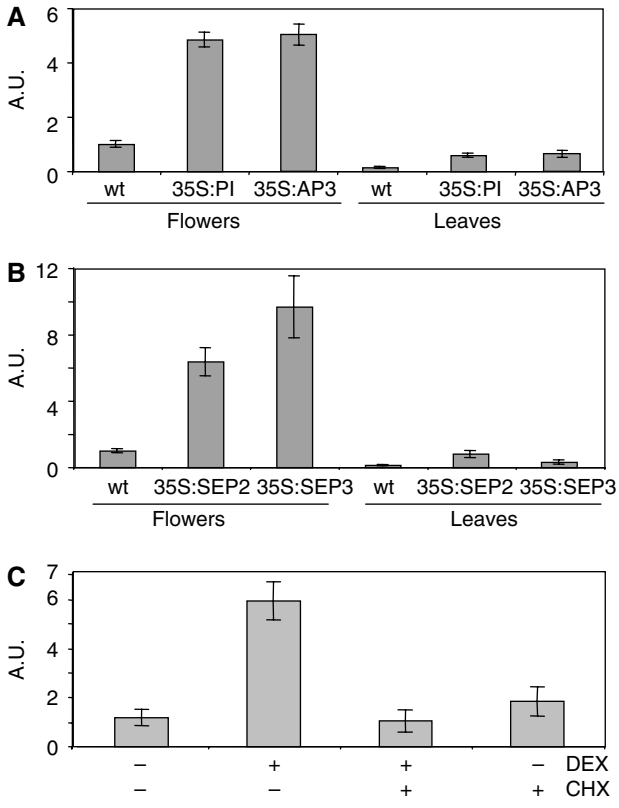

Figure 3 RT-QPCR analysis of *BPEp* mRNA expression in flowers and leaves of *A. thaliana* lines overexpressing (**A**) the B class proteins PI (line *35S:PI*) or AP3 (line *35S:AP3*); (**B**) the E class proteins SEP2 (line *35S:SEP2*) or SEP3 (line *35S:SEP3*). (**C**) *BPEp* expression in flowers of line *35S:AP1-GR* that overexpress an inducible activity of the A class protein AP1. Flower samples were collected at 6 h post-treatment with DEX or with CHX or simultaneously with DEX and CHX. A.U., arbitrary units.

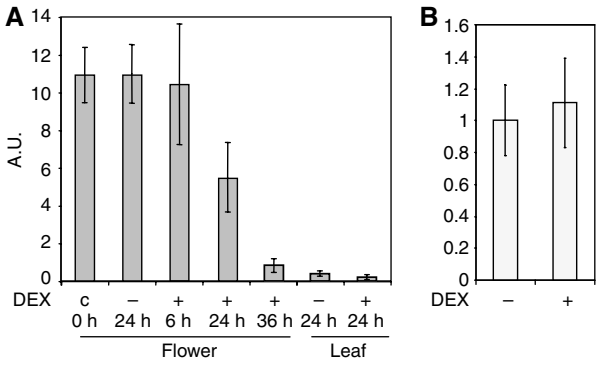

Figure 4 Expression analysis of *BPEp* (**A**) and *BPEub* (**B**) transcripts in flowers and leaves of *A. thaliana* line *35S:AG-GR* without or with DEX treatments using RT-QPCR. Flower samples were collected at 0, 6, 24 or at 36 h post-treatment and leaves at 24 h post-treatment. 'c', nontreated control flower samples. '−', control samples treated with the buffer alone and harvested at 24 h post-treatment. A.U., arbitrary units.

BPEp is negatively regulated downstream of AGAMOUS

BPEp accumulation was 8–10-fold higher in *agamous* flowers compared to wt flowers (Figure 2E), suggesting that *BPEp* is negatively regulated by *AG*. To further investigate this regulation process, we followed the expression of *BPEp* in an *agamous* line that overexpresses an inducible AG fused to GR (*35S:AG-GR*) (Ito *et al*, 2004). As expected, in the absence of DEX treatment, high levels of *BPEp* were detected, similar to those found in the *agamous* mutant (Figure 4A). When inflorescences of line *35S:AG-GR* were treated with DEX, a significant decrease in *BPEp* mRNA expression was observed between 6 and 24 h post-treatment. *BPEp* expression continued to decrease at 36 h post-treatment. As expected, DEX treatments did not modify *BPEp* accumulation in leaves (Figure 4A). These findings supported our previous observation that *BPEp* mRNA accumulation is negatively regulated downstream of *AG*. Interestingly, DEX treatment did not modify *BPEub* expression in leaves indicating that *BPEp* regulation of expression is likely to be post-transcriptional (Figure 4B). One way to investigate whether repression of *BPEp* by AG is direct or indirect, would be to simultaneously treat the inflorescences of the *35S:AG-GR* line with DEX and CHX. However, 24 h of CHX treatment (corresponding to the time at which we saw a significant repression of *BPEp* by AG) induces severe physiological phenotypes (toxic effects) and, therefore, this test was not possible (data not shown).

BPEp limits petal organ size

We identified a line (*bigpetal*-1 or *bpe-1* allele) carrying a T-DNA insertion in the fourth intron of the *BIGPETAL* gene. No *BPEp* nor *BPEub* transcripts were detected, using RT–PCR, in plants homozygous for the *bpe*-1 allele (data not shown). In parallel, we generated two RNA interference (RNAi) lines by expressing inverted repeats of nucleotide sequences corresponding to two different regions in the *BPE* transcripts under the control of the CaMV 35S promoter (Figure 2A). The target nucleotide sequence for the first RNAi (RNAi-*bpe*) spans the first and second exons that are present in both *BPE* transcripts, whereas the second RNAi (RNAi-*bpep*) is within intron 5. As intron 5 is spliced out of *BPEub* but retained in *BPEp*, *RNAi-bpep* should specifically target the latter. No or very little, *BPEub* and *BPEp* transcripts were detected in *RNAi-bpe*-expressing lines (data not shown). However, in the *RNAi-bpep*-expressing lines, some plants exhibited repression of both transcripts (e.g. plant 2 in Figure 5A) whereas other plants showed no accumulation of *BPEp* transcript while the *BPEub* transcript was not affected (e.g. plant 1 in Figure 5A). The latter plants are referred to as RNAi-*bpep*/*BPEub*.

Phenotypic analyses of RNAi and *bpe-1* loss-of-function mutants showed that petals in these plants were significantly increased in size compared to wild-type petals (Figure 5B, panels a–c). The other flower organs did not exhibit any phenotypic modifications (Figure 5B, panel b). Petal length and width (distal region of petal blade) measurements showed that in the mutant lines, petals were about 24% larger in size compared to the wt (Figure 5C). A Student's *t*-statistical test confirmed that the increase in petal size was statistically significant ($P < 0.05$; Figure 5C). The RNAi-*bpep*/*BPEub* plants also showed an increased petal size similar to that observed in the *bpe-1* line, demonstrating the role of the petal-expressed splice variant *BPEp* (and not *BPEub*) in influencing petal size.

BPEp limits final cell size

To check whether the increase in petal size associated with reduced *BPEp* expression is a result of failure to maintain correct cell proliferation or cell expansion or both, petals epidermis cell size and number per surface area were analyzed and compared to the wild type. Scanning electron microscopy (SEM) analyses showed that, in the *bpe*-1 line and in *RNAi-bpe*-expressing line, the elongated rectangular cells in the proximal region of petals retained their longitudinal form and orientation (Figure 5D, panels d–f). However, these cells appeared to be more irregular with a more pronounced surface curvature compared to the wild type. SEM visualization of the adaxial petal epidermis revealed that in the *bpe*-1 line and in the *RNAi-bpe*-expressing line, the conical differentiated cells were larger than the wild-type cells, but retained their conical form and tight packing (Figure 5D, panels a and b). The RNAi-*bpep*/*BPEub* plants also showed a similar increase in the size of petal conical cells (Figure 5B, panel c), in agreement with the role of *BPEp* in the control of petal size.

In the RNAi-expressing plants, the petal adaxial epidermis cells were about 30% larger compared to the wt (Figure 5E, panel a). This increase in cell size corresponded to a reduction in cell number over the same surface area in the RNAi mutant compared to the wt (Figure 5E, panel b). These data are in agreement with a role for BPEp in limiting petal size by restricting postmitotic cell expansion. No significant difference in the sizes of leaves was observed between the wt and the knockout or the RNAi mutant plants (data not shown).

Discussion

We used a cDNA-AFLP-DD strategy to identify genes involved in petal organogenesis downstream of organ identity genes. Three of the genes we identified encode putative petal-, or petal and stamen-specific proteins. Our results are in agreement with previous studies using microarray strategies, in the sense that a relatively small number of genes were found to be petal upregulated or petal-specific (Zik and Irish, 2003;

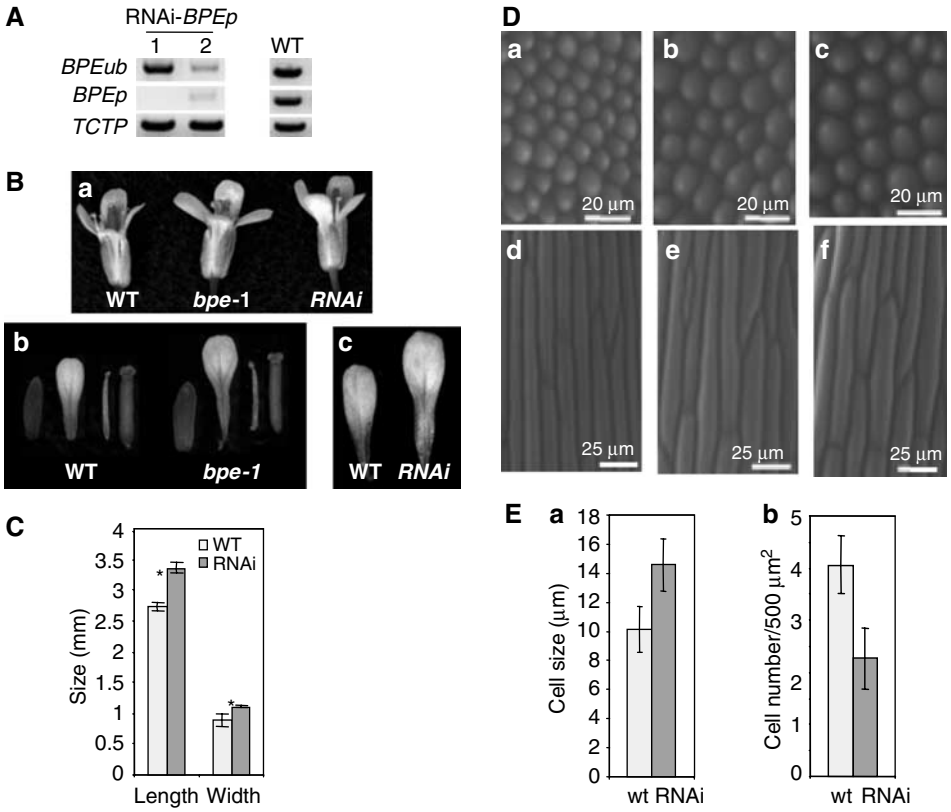

Figure 5 (**A**) Expression of *BPEp* and *BPEub* in two individual plants of an *RNAi-bpep*-expressing line and of wt control. TCTP was used as control. (**B**) Flower phenotype in the KO line *bpe-1* and in lines expressing RNA interference constructs directed against *BPE* transcript. (**a**) Flowers of wt, *bpe-1* and RNAi-expressing lines. (**b**) Flower organs of the *bpe-1* line compared to those of wt plants. Note the larger size of petals in the *bpe-1* line. (**c**) Petals of the RNAi lines compared to those of wt plants. (**C**) Petal length and width in RNAi-*BPEp* plants was measured and compared to wt petals. The data are means ± s.d. A total of 60 flowers from 12 plants were examined for each genotype. *, Significantly different from wt at $P<0.05$. (**D**) SEM views of the petal adaxial surface cells from wt (**a** and **d**), *RNAi-bpe* (**b** and **e**) and *RNAi-bpep/BPEub* (**c** and **f**) plants at flower developmental stage 15. (**E**) (**a**) Means ± s.d. of cell size in the distal adaxial surface of petals in the RNAi-*bpep* and wt plants. (**b**) Cell number per 500 μm^2 surface area in the distal adaxial surface of petals in the RNAi-*bpep* and wt plants.

Wellmer *et al*, 2004). A possible explanation would be because the anatomy of the petal is less complex, composed of a smaller number of cell types compared to stamens or carpels, petal-specific genes may be less abundant. Alternatively, many of the structural genes involved in petal organogenesis may also have roles in developmental processes in other floral or vegetative organs and would thus not be identified by a differential screen based on gene expression. However, it should be noted that none of the genes we identified in the present study were found in the microarray screens and among the petal upregulated genes identified in the microarray screens, only one (GDLS-motif lipase/hydrolase like encoding gene) was identified in both studies (Zik and Irish, 2003; Wellmer *et al*, 2004). Therefore, it is likely that these studies have identified only a subset of the petal-expressed genes and hence underestimate the number of genes required for petal organogenesis.

In this work, we identified *BPE* that encodes two bHLH TFs (BPEp and BPEub) originating from an alternative splicing event (intron retention in *BPEp*). This post-transcriptional event is correlated with differential expression profiles of these two transcripts: *BPEp* shows a petal-expression pattern, while *BPEub* is ubiquitously expressed. The presence of a ubiquitous *BPEub* transcript explains why the *BPE* gene was not identified in previous microarray studies (Zik and Irish, 2003; Wellmer *et al*, 2004).

BPEp mRNA accumulates to equally high relative abundance in flowers of both *35S:PI* and *35S:AP3* lines. It is known that PI and AP3 form a heterodimer and work together in an autoregulatory loop to maintain each other's expression (Goto and Meyerowitz, 1994; Jack *et al*, 1994; Krizek and Meyerowitz, 1996; Riechmann *et al*, 1996; Tilly *et al*, 1998). Ectopic expression of *AP3* results in persistent fourth whorl expression of the endogenous *PI* gene. Similarly, in plants overexpressing *PI* (*35S:PI*), AP3 autoactivates its expression in whorl 1 and in other regions where *PI* is active (Goto and Meyerowitz, 1994; Jack *et al*, 1994; Krizek and Meyerowitz, 1996). Therefore, *BPEp* presumably acts downstream of the *PI/AP3* heterodimer during petal development. It should be noted that the endogenous *AP3* and *PI* were highly expressed in flowers, but expressed at very lower levels in leaves of the *35S:PI* and *35S:AP3* lines, respectively (data not shown), in agreement with previously reported work (Goto and Meyerowitz, 1994; Jack *et al*, 1994; Krizek and Meyerowitz, 1996). This explains why a small but significant increase in *BPEp* accumulation was observed in leaves compared to wt. These data provide additional evidence that PI and AP3 are able to induce *BPEp* accumulation.

AP3 is expressed in sepals but *PI* is not. As a result, the first whorl organs of a *35S:PI* line have more functional AP3/PI than *35S:AP3* plants and are mosaic petal–sepal organs

(Krizek and Meyerowitz, 1996). As there is more functional AP3/PI in the first whorl organs of the *35S:PI* line compared to the *35S:AP3* line (Krizek and Meyerowitz, 1996), we expected to have more of the splice variant *BPEp* in *35S:PI* than in *35S:AP3*. However, our data show that *BPEp* expression is as highly induced in both overexpressing lines. Although, we do not have a clear explanation for this observation, the data suggest that other regulatory factors may be involved. The fact that in the *35S:PI* line we observe a five-fold greater accumulation of the *BPEp* transcript in flowers compared to leaves represents another argument that other factors whose expression is restricted to flowers are needed for efficient activation of *BPEp* expression.

Analyses of *A. thaliana* plants overexpressing SEP3 or SEP2 demonstrated an increase of *BPEp* expression in flowers, indicating the involvement of SEP in *BPEp* activation. To our knowledge, there are no known targets of SEP proteins downstream of flower organ identity genes. The only known targets of SEP3 are the organ identity genes *AP3* and *AG* (Castillejo *et al*, 2005). Therefore, it is possible that (i) *BPEp* is activated downstream of SEP3 via AP3 or (ii) that *BPEp* accumulation is activated downstream of the petal organ identity complex formed by AP3, PI, SEP and AP1 (Honma and Goto, 2001; Theissen and Saedler, 2001). However, although ectopic expression of *SEP3* is sufficient to highly activate AP3 in leaves (Castillejo *et al*, 2005), we did not observe a strong accumulation of *BPEp* transcript in leaves of the *35S:SEP2* or *35S:SEP3* lines (in contrast to the situation in flowers; Figure 3B). Thus, the second possibility involving regulation via the MADS protein complex is likely to be correct. Moreover, we found that *BPEp* expression is activated by AP1 in an indirect manner. During early floral stages, AP1 is required to specify floral meristem identity. At later floral stages, AP1 is required to specify the identity of sepals and petals by activating B class genes via the *UNUSUAL FLORAL ORGANS* gene (UFO) (Mandel *et al*, 1992; Ng and Yanofsky, 2001; Durfee *et al*, 2003). Therefore, *BPEp* may be activated downstream AP1 via AP3 and PI. As the tissues used in this experiment were mostly from flower buds older than stages 5 and 6, *BPEp* accumulation is most likely to be controlled by the protein complex formed by AP3, PI, SEP and AP1 during petal organogenesis. These data taken together indicate that the regulation of *BPEp* accumulation is likely to occur downstream of floral organ identity MADS protein complexes.

As *BPEp* is positively regulated downstream of *AP3*, *PI* and *SEP3*, we would expect it to be highly expressed in the second and third whorls (petals and stamens, respectively). However, the *BPEp* transcript accumulated to a low level in flowers of *apetala1* and *apetala2* mutants which possess stamens and lack petals. This low level of expression in stamens could be explained by the analyses of *BPEp* expression in the flowers of *agamous* and *35S:AG-GR* plants which showed that *BPEp* is negatively regulated by *AG*. It must be noted that at 24 h post-DEX treatment, the flowers do not show any phenotypic difference from the non-DEX-treated plants: flowers with repeated sepal–petal–petal. This is in agreement with the timing of flower organ development (Smyth *et al*, 1990). It takes more than 7 days from the identity specification of petals to stage10 at which *BPEp* starts to be highly expressed. Taking this into account, it is likely that the activation of AG in petal cells leads to the almost immediate negative regulation of *BPEp* observed. Our results demonstrate the involvement of AG in the negative regulation of a petal preferentially expressed transcript. These results are in agreement with a previous report of an *AG*-dependent inhibitory pathway where an unknown factor produced in whorl 3 has been proposed to block petal development (Durfee *et al*, 2003). It is therefore possible that the negative regulation of *BPEp* by AG is mediated by this unknown factor.

BPEp is produced by an alternative splicing event, thus involving control at the post-transcriptional level. Such regulation is likely to occur via intermediate proteins acting downstream of PI, AP3, SEP and/or AP1 in agreement with the fact that regulation downstream *AP1* requires *de novo* protein synthesis. Alternative splicing is a major mechanism for expanding functional diversity of animal and plants genes (Kriventseva *et al*, 2003; Ner-Gaon *et al*, 2004). Thus, *BPEp* represents another example of functional diversification by means of alternative splicing, and the elucidation of its origin at the evolutional level would be interesting.

All loss-of-function mutants (*bpe-1* and *RNAi-bpe* lines where both *BPEp* and *BPEub* expression were affected) showed a significant increase in petal size compared to wt plants. The *RNAi-bpep/BPEub*-expressing plants (which exhibit downregulation of *BPEp*, whereas *BPEub* is not effected) showed increased petal size compared to the wt. These results demonstrated clearly the role of the petal-expressed splice variant *BPEp* in influencing petal size. In the *BPE* loss-of-function mutants, the flower organs (other than petals) exhibit no size phenotypic modifications. These data constitute another argument toward the specificity of *BPEp* involvement during petal development. Organ growth is a well-coordinated process. Therefore, cellular proliferation and expansion must be tightly controlled to give a remarkably constant final size of petals. Failure in maintaining correct cell proliferation and/or expansion will therefore result in organ size modification. To date, loss-of-function analyses have identified few genes involved in plant floral and/or vegetative organ morphogenesis. In one recent example, the E3 ubiquitin ligase-encoding gene *BIG BROTHER* was shown to limit plant organs size by controlling cell proliferation (Disch *et al*, 2006). *AINTEGUMENTA* (*ANT*) gene has been shown to control organ growth by promoting cell proliferation, thus in an antagonist manner to *BIG BROTHER* (Krizek, 1999; Mizukami and Fischer, 2000). Similarly, *JAGGED* and *FRILL* genes have been described to control organ growth mainly by affecting cell cycle activity, by controlling either the mitotic state or the endo-reduplication processes, respectively (Hase *et al*, 2000; Dinneny *et al*, 2004).

Petals of the *BPEp* loss-of-function mutants possess less cells for the same surface area compared to the wt. These results demonstrate that BPEp controls *A. thaliana* petal size by restricting cell growth. Therefore, BPEp is the first protein that specifically limits petal organ size by controlling the postmitotic rate of cell growth and expansion. These data are in agreement with the expression profile of *BPEp* starting at stages when cell differentiation occurs (maximum expression at stages 10 and above; Figure 2G).

The two predicted BPE-encoded proteins possess C-terminal stretches that do not show similarities to each other. The significance of these sequences to the biological function of these two putative TFs is yet to be determined. Three TFs belonging to the No Apical Meristem (NAM) family, namely NAM, CUC and NAP (act downstream AP3/PI), and one

bHLH (SPATULA) have also been shown to be involved in flower organogenesis (Souer *et al*, 1996; Aida *et al*, 1997; Sablowski and Meyerowitz, 1998; Heisler *et al*, 2001). Hence, bHLH and NAM TFs may be important components in the genetic control of flower organogenesis downstream of the organ identity genes. In turn, BPEp and NAP could be intermediate TFs responsible for regulating the downstream structural genes that directly mediate petal and/or stamen morphogenesis. It is worth noting that published studies suggest that certain bHLHs interact with MYB TFs and WD40 repeat proteins to form complexes involved in multiple cellular differentiation pathways leading to diverse cell fates (Ramsay and Glover, 2005). In a similar way, the bHLHs involved in flower organ development may act in complexes with other proteins (MYB, WD40, and/or other proteins) to activate the downstream structural genes. Identifying, on the one hand the interacting proteins and on the other hand the target genes of BPEp and NAP will, therefore, help to unravel the network of structural genes (realizators) involved in petal morphogenesis.

Materials and methods

Constructions and plant lines

Arabidopsis mutants *agamous*-1, *pistillata*-1, *apetala3*-3, *apetala1* and *apetala2* and the lines overexpressing PI (*35S:PI*) or AP3 (*35S*:*AP3*) or SEP2 (*35S*:*SEP2*), SEP3 (*35S*:*SEP3*), AG (*35S*:*AG-GR*) or AP1 (*35S*:*AP1-GR*) have been described (Bowman *et al*, 1989; Jack *et al*, 1992; Mandel *et al*, 1992; Jack *et al*, 1994; Jofuku *et al*, 1994; Krizek and Meyerowitz, 1996; Pelaz *et al*, 2001a, b; Ito *et al*, 2004; Yu *et al*, 2004).

bigpetal-1 (*bpe*-1) T-DNA insertion knockout line for *BPE* was identified in a screen of Wisconsin *A. thaliana* mutant collections. The two RNA interfering lines (*RNAi-bpe* and *RNAi-bpep*) were generated by expressing inverted repeats of nucleotide sequence corresponding to two different regions (Figure 2A) in the target gene under the control of the CaMV 35S promoter in the vector pK7GWIWG2 (Karimi *et al*, 2002).

The 1317 bp DNA sequences up to and including the *BPE* start codons (corresponding to nucleotides 62 136–61 453 in the *A. thaliana* genomic sequence, GenBank accession AC009317) was PCR amplified and cloned upstream of the *GUS* sequence in the pMDC162 vector (Curtis and Grossniklaus, 2003). Resulting constructs harboring RNAi or *Promoter*^{BPE}*:GUS* expression cassette were transformed into *A. thaliana* Col-0 plants. GUS staining was performed as previously described (Hill *et al*, 1998). All plants were kept in growth chambers with a condition of 16/8 h day/night at 22°C and 70 μE/m^2/s light.

Treatment of plants with DEX and CHX

Plants were treated with 10 μM (*35S*:*AG-GR*) or with 1 μM (*35S*:*AP1-GR)* DEX solution containing 0.01% DMSO, 0.1% (v/v) ethanol and 0.01% (v/v) Silwet-L77. The same solution without DEX was applied to control plants under the same conditions. CHX treatment was performed by adding 10 μg/ml of CHX to the DEX solution.

RNA preparation and cDNA synthesis

Total RNA was prepared using the Trizol reagent (Invitrogen, Groningen, NL) according to the manufacturer's instructions. Contaminating DNA was removed using the DNA-free™ kit (Ambion, Cambridgeshire, UK). Total RNA was used for cDNA synthesis using a Revert Aid M-MuLV Reverse transcriptase (Fermentas, Ontario, Canada) according to the manufacturer's recommendations.

cDNA-amplified fragment length polymorphism-differential display (AFLP-DD)

The cDNA-AFLP-DD was performed using the DisplayPROFILE™ kit (Q-BIOgene, Irvine, CA) following the manufacturer's guidelines. Amplified reaction products were separated side-by-side on a 6% polyacrylamide gel under denaturing conditions. DNA bands were excised from the gel, cloned, their nucleotide sequence was determined and their corresponding gene was identified in the *A. thaliana* genomic sequence.

Mapping of the 5′ and 3′ ends of the BIGPETAL mRNA transcripts

The Marathon™ cDNA amplification method was used to map the exact 5′ and 3′ ends of *BPEp* and *BPEub* mRNA from wt *A. thaliana* Columbia flowers, according to manufacturer's recommendations (Clontech, Mountain View, CA).

Gene expression analysis

Primers (available upon request) specific to each gene were used for expression analysis by RT–PCR and RT-QPCR. RT-QPCR was performed with the qPCR Core Kit for SYBR Green I Quick Gold Star (Eurogentec, Liege, Belgium) using the Gene Amp 5700 Sequence Detector System (Applied Biosystems, Foster City, CA). Reactions were run in duplicate and quantified against a relative standard curve made from a serially diluted stock cDNA containing the target sequence. Data collection and analysis were performed using GeneAmp 5700 SDS Software package (Applied Biosystems). Results were expressed using the $\Delta\Delta C_t$ calculation method in arbitrary units by comparison to the control (flower of a wild-type plant). The housekeeping gene *TCTP* we identified as constitutively expressed (this study), was used as a control in all gene expression analyses.

SEM and organ and cell size measurements

Petals from flowers at stage 14–15 (maximum expansion (Smyth *et al*, 1990) were carefully dissected and mounted flat on their abaxial surface. Samples were imaged using a Hitachi S 3000N scanning electron microscope.

Two adjacent petals were removed from flowers at developmental stage 14 (Smyth *et al*, 1990) and their length and width (distal region of petals blade) were measured using digital images. For cell size measurements, petals were cleared overnight in a solution containing 86% ethanol–14% acetic acid followed by two times 4 h incubation in ethanol 70%. Digital images of the distal region of the cleared petals were used to measure the adaxial epidermis cell size. Cell number was calculated per 500 μm^2 surface area using SEM images and digital images of the cleared petals.

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