Control of cell and petal morphogenesis by R2R3 MYB transcription factors

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Petals of animal-pollinated angiosperms have adapted to attract pollinators. Factors influencing pollinator attention include colour and overall size of flowers. Colour is determined by the nature of the pigments, their environment and by the morphology of the petal epidermal cells. Most angiosperms have conical epidermal cells, which enhance the colour intensity and brightness of petal surfaces. The MYB-related transcription factor MIXTA controls the development of conical epidermal cells in petals of Antirrhinum majus. Another gene encoding an R2R3 MYB factor very closely related to MIXTA, AmMYBML2, is also expressed in flowers of A. majus. We have analysed the roles of AmMYBML2 and two MIXTA-related genes, PhMYB1 from Petunia hybrida and AtMYB16 from Arabidopsis thaliana, in petal development. The structural similarity between these genes, their comparable expression patterns and the similarity of the phenotypes they induce when ectopically expressed in tobacco, suggest they share homologous functions closely related to, but distinct from, that of MIXTA. Detailed phenotypic analysis of a phmyb1 mutant confirmed the role of PhMYB1 in the control of cell morphogenesis in the petal epidermis. The phmyb1 mutant showed that epidermal cell shape affects petal presentation, a phenotypic trait also observed following re-examination of mixta mutants. This suggests that the activity of MIXTA-like genes also contributes to petal form, another important factor influencing pollinator attraction.

KEY WORDS: Petal, Cell shape, Petunia, Antirrhinum, MYB transcription factor

INTRODUCTION
The function of the petals of most animal-pollinated flowers is to attract pollinators by providing strong, recognisable visual signals. For example, bees are attracted by the size of flowers and by the colour signals they provide. Size is particularly important in recognising flowers from a distance, and there are numerous examples of flower size being positively correlated with pollinator attention (Ashman, 2000; Ashman et al., 2000; Moller, 1995; Spaethe et al., 2001; Stanton and Preston, 1988; Totland, 2004; Young and Stanton, 1990). Indeed, in some plants the size of the visual signal is effectively increased by the clustering of flowers on inflorescences (Ohara and Higashi, 1994). The colour signal provided (usually by petals) influences the distance at which flowers of a particular size can be recognised (Menzel et al., 1997; Spaethe et al., 2001). The colour signal is determined not only by the composition of different pigments produced in the petals, but also by the patterns of pigmentation, colour intensity, colour saturation and colour brightness (Lunau, 2000). In flowers that are coloured by anthocyanin pigments (blue, purple and red pigments), colour hue, intensity and brightness are determined not only by the chemical nature of the pigments that accumulate in the vacuoles of petal epidermal cells, but also by their environment (pH, presence of metal ions, presence of flavonoid co-pigments) and by the morphology of the epidermal cells themselves. An estimated 80% of angiosperms have specialised petal epidermal cells that are conical or papillate in shape, particularly on the epidermal surfaces that face prospective pollinators (Kay, 1988; Kay et al., 1981). Conical cells enhance the colour intensity and brightness of petal surfaces by reflecting a higher proportion of incident light into the epidermal cells, where more is absorbed by the vacuolar pigments, relative to flat petal epidermal cells (which appear pale and dull in comparison) (Kay, 1988; Kay et al., 1981; Noda et al., 1994). Flowers of Antirrhinum majus with conical epidermal cells are more attractive to pollinating bees than those with flat petal epidermal cells (Comba et al., 2000; Glover and Martin, 1998).

The activity of the MIXTA gene in petal epidermal cells of the snapdragon, A. majus, controls the development of conical cell shape (Noda et al., 1994). The activity of the MYB-related transcription factor encoded by MIXTA appears to be both necessary and sufficient to drive the formation of conical epidermal cells from the default flat epidermal cells (Glover and Martin, 2002). MIXTA-like genes, encoding MYB-related transcription factors that are structurally closely related to MIXTA, have been identified in a number of other plant species and include the PhMYB1 gene from Petunia hybrida (van Houwelingen et al., 1998) and the AtMYB16 gene (also referred to as AtMIXTA) of Arabidopsis thaliana (Romero et al., 1998). However, three genes that encode R2R3 MYB transcription factors that are very closely related to MIXTA (AmMYBML1, AmMYBML2 and AmMYBML3) (Perez-Rodriguez et al., 2005) are also expressed in petals of A. majus, showing that there are multiple genes encoding proteins belonging to this subclass of transcription factors expressed in the same tissues of one species. These genes are not functionally redundant, as evident by the clear phenotype of the mixta mutant. Indeed, discrete functions have already been established for AmMYBML1 as compared with MIXTA, the former controlling trichome, conical cell and mesophyll cell morphogenesis in the ventral petal of Antirrhinum flowers (Perez-Rodriguez et al., 2005).
Emerging data from the study of transcription factors belonging to large families of structurally related proteins suggest that very similar members of phylogenetically-clustered subgroups usually share closely related functions, even though functions may have diverged over the entire family. Structural similarity has led to claims of orthology and functional equivalence between MIXTA, PhMYB1 and AtMYB16 (van Houwelingen et al., 1998; Romero et al., 1998). However, to achieve a general understanding of the control of morphogenesis of petal epidermal cells, the function of new genes needs to be assayed and compared with that of the prototype, MIXTA. In addition, the relevance of these genes to cell shaping and, specifically, to their roles in adapting petals for pollinator attraction, needs to be established in different angiosperm species.

We have examined the function of three of the genes encoding proteins very closely related to MIXTA; PhMYB1 from Petunia hybrida, AmMYBML2 from A. majus and AtMYB16 from Arabidopsis thaliana. Structurally, these proteins are most closely related to each other and their genes are therefore orthologous. All three proteins promote directional cell expansion in a bioassy in tobacco. The similarities between the phenotypes induced by these proteins in this bioassy (their biochemical functions) and the similarities in their expression patterns in the three different species, suggest that these proteins have equivalent effects on cellular morphology (their developmental functions). More detailed analysis of the function of PhMYB1 in Petunia, using a transposon-induced unstable mutant, showed that PhMYB1 does indeed have a primary function in determining the degree of extension growth of the epidermal cells of the petal and its activity contributes to the final shape of these cells. Thus, MIXTA and PhMYB1 have overlapping developmental functions despite not being encoded by orthologous genes. The loss of PhMYB1 activity affects petal placement as well as petal cell shape. As a result of loss of PhMYB1 activity, petals are much more reflexed than in the wild type. Re-examination of the mixta mutant phenotype revealed it to have a similar effect on the angle of presentation of the dorsal petal lobes in A. majus. It would appear that cell shaping affects not only petal appearance, but also overall petal design. Both aspects of the activity of these MIXTA-like genes in angiosperm flowers might contribute positively to pollinator attraction.

MATERIALS AND METHODS
Phylogenetic methods

For the phylogeny shown in Fig. 1, protein sequences were manually aligned using MacClade 4.08 (D. R. Maddison and W. P. Maddison, Sinauer Associates). The protein sequences of the MYB domain (Kranz et al., 1998) plus 60 amino acids downstream, including the nuclear localisation signal and a conserved QWSAR motif (Kranz et al., 1998), were used to create the alignment (see Fig. S1 in the supplementary material). Phylogenetic analysis was performed with PAUP*4.0b10 (Swoford, 2001). An optimal tree according to the distance criterion (minimum evolution; mean character difference) was obtained with a heuristic search (TBR). One thousand bootstrapped data sets were used to estimate the confidence of each tree clade.

The following sequence accessions were used:
AmMYBML1: X79108.1 GI:485866 from Antirrhinum majus (snapdragon)
AmMYBML2: CAB43399.1 GI:4886264 from A. majus
AmMYBML3: AAV70655.1 GI:56069813 from A. majus
AmMYBML3: AUA13905.1 GI:51895758 from A. majus
OsMYB75: NT_107239.1 GI:50953764 from Orysa sativa (rice)
OsMYB44: NT_079863.2 OsJNBa0072F16.11 from O. sativa
AtMYB16: X98989.1 GI:1514443 from Arabidopsis thaliana
AtMYB17: APO06286.6 GI:3941423 from Arabidopsis thaliana
AtMYB106: NP_186763.2 GI:79386566 from Arabidopsis thaliana
PhMYB1: CAA78386.1 GI:20563 from Petunia hybrida
PpMYB1: X67051.1 GI:22639 from Physcomitrella patens (moss)

For the phylogeny of all R2R3 subgroup-9 members (at the time of going to press) shown in Fig. S2 (see Fig. S2 in the supplementary material), protein sequences were aligned using the ClustalW (version 1.83) program (Thompson et al., 1994). Phylogenetic analysis was performed with Phylip programs (version 3.63) using only the MYB domain and the adjacent MYB subgroup-9 motif (see Fig. S2 in the supplementary material) (Kranz et al., 1998). A distance matrix method employing the Jones-Taylor-Thornton model (Jones et al., 1992) was used to compare the sequences and a tree was built using the Neighbour-joining clustering method (Saitou and Nei, 1987). One thousand bootstrapped data sets were used to indicate the confidence of each tree clade.

Constructs for ectopic expression of PhMYB1, AmMYBML2 and AtMYB16

The full-length cDNA clone of PhMYB1 was a generous gift from Javier Paz-Ares (Centro Nacional de Biotecnologia, Madrid, Spain). The isolation of the AmMYBML2 cDNA has been described previously (Paz-Rodriguez et al., 2005). AmMYB16 (At5g15310) was amplified from first-strand cDNA prepared from RNA from seedlings of Arabidopsis thaliana ecotype Columbia. The primers used for amplification were 16ats (5’-GAC-CTCTCAAAACAACTGGGTAGATC-3’) and 16atas (5’-GAACATCGGTGAACTCGGCGGTAAG-3’). All three cDNAs were cloned in sense orientation into pPH60 (Guerrero and Mullineaux, 1993) for expression driven by the double CaMV 35S promoter and terminated by the CaMV 35S terminator sequence. The expression cassettes were excised with KpnI and XhoI and cloned into the KpnI and SalI sites of pBin19.

Plant transformation and growth conditions

The constructs in pBin19 were transferred into Agrobacterium tumefaciens strain LBA4404, and used for transformation of tobacco (Nicotiana tabacum var. Samsun) by the leaf disc method (Mittanovich et al., 1989; Horsch et al., 1985). Tobacco and Petunia plants were grown in a glasshouse at 22°C with 16 hours light. The binary vector expressing AtMYB16 under the control of the CaMV 35S promoter was also used for transformation of Antirrhinum (Colombia) as described by Jin et al. (Jin et al., 2000).

Petunia hybrida lines

The progeny of two plants of the unstable phmyb1 mutant line from Petunia hybrida (van Houwelingen et al., 1998), one showing a mutant phenotype with clear revertant sectors (line KB1/7) and the other showing a wild-type revertant phenotype (line KB1/11), were analysed. The offspring of the first plant (KB1/7) showed sectors like its parent. The wild-type revertant (KB1/11) was heterozygous for the dTph1 insertion, and progeny segregated – one mutant to three wild-type individuals, indicating that the unstable phmyb1 allele is recessive to the wild-type revertant allele. The absence of the dTph1 transposon insertion was screened in individual wild-type plants from seed of KB1/7 by PCR to identify homozygous, wild-type revertants. These plants were used to press) shown in Fig. S2 (see Fig. S2 in the supplementary material), to press) shown in Fig. S2 (see Fig. S2 in the supplementary material).

Localisation of dTph1 insertion in the PhMYB1 gene

Genomic DNA was extracted from two young leaves or from one pair ofrophylls as described by Souer et al. (Souer et al., 1995). The entire coding region of the PhMYB1 gene was amplified using gene-specific primers Ph1-A (5’-GGTGGATTTTTCCTCAAATGGG-3’) and Ph1-B (5’-AAT-TCAACATCGGATCACTAG-3’), subcloned into pGEM-T-easy (Promega) and sequenced.

RNA extraction and expression analysis

Total RNA was extracted from petals of Petunia (wild-type revertant line KB1/11) and RNA gels were run and blotted as described by Martin et al. (Martin et al., 1985). Equivalent loading of RNA (20 μg per lane) was confirmed by staining the membrane with a Methylene Blue solution (0.02%
Methylene Blue, 0.3 M Na acetate pH 5.5. A gene-specific probe for PhMYB1 was used which consisted of a 782 bp DNA fragment from the 3′ end of the gene. This was obtained by PCR amplification using the PhMYB1 cDNA from plasmid pJAM1354, primers Phmyb1-Probe5 (5′-CGAGCCGAAGCTCGACTAG-3′) and Phmyb1-Probe3 (5′-GAATCTGAGGTGGAAGAATTCAC-3′), and labelled by random priming with [32P]dCTP.

Total RNA was isolated from different tissues of wild-type Antirrhinum (Stock J17) or petals of A. majus, A. barrelieri and A. australis according to Martin et al. (Martin et al., 1985). Poly(A+) RNA was prepared using poly(A)+ columns (Promega). Poly(A) RNA (5 μg per lane) was separated on denaturing gels and blotted onto nitrocellulose (Martin et al., 1985). The levels of RNA loaded were checked by probing the membrane with a UBIQUITIN gene fragment. Probes for MIXTA and AmMYBML2 were prepared from full-length cDNA clones as described by Perez-Rodriguez et al. (Perez-Rodriguez et al., 2005).

RNA in situ hybridisation

In situ hybridisation with digoxigenin-labelled antisense RNA was performed on 7 μm sections of Petunia hybrida and A. majus flowers as described previously (Perez-Rodrigues et al., 2005). RNA probes were generated using T7 polymerase. To produce PhMYB1 sense and antisense RNA probes, the 3′ end of the gene was amplified using primers Phmyb1-Probe5 and Phmyb1-Probe3 and subcloned into pGEM-T vector in both orientations. The probe for AmMYBML2 was the full-length cDNA (Perez-Rodriguez et al., 2005).

Scanning electron microscopy

Plant tissue was frozen in nitrogen slush at −190°C. Ice was sublimed at −95°C, and the specimens were then sputter-coated with platinum and examined in a Philips XL 30 FEG scanning electron microscope (SEM) fitted with a cold stage. For freeze-fractures, frozen samples were warmed to −100°C prior to fracture. Cell counts and cell size measurements were made from SEM micrographs at 400× magnification.

RESULTS

PhMYB1 is more closely related to AmMYBML2 than to MIXTA

R2R3 MYB proteins have been divided into distinct subgroups according to the degree of similarity between their primary amino acid sequences (Kranz et al., 1998; Stracke et al., 2001; Jiang et al., 2004). Among the 126 members of the R2R3 MYB family in Arabidopsis thaliana, there are 24 subgroups, characterised by their highly conserved DNA-binding domain as well as conserved amino acid motifs in their C-terminal domains (Kranz et al., 1998; Stracke et al., 2001). The PhMYB1 protein from Petunia hybrida falls into subgroup 9, which also contains the MIXTA and MIXTA-LIKE MYB proteins AmMYBM1, AmMYBML1 and AmMYBML3 from A. majus, and AtMYB16 (At5g15310) and AtMYB106 (At3g01140) from Arabidopsis thaliana (Fig. 1) (Perez-Rodriguez et al., 2005).

PhMYB1 is thought to encode the structural and likely functional counterpart of MIXTA in Petunia (Avila et al., 1993; van Houwelingen et al., 1998). However, the isolation of AmMYBM1, AmMYBML2 and AmMYBML3 cDNAs from A. majus suggests that PhMYB1 is, in fact, orthologous to AmMYBML2 rather than to MIXTA (Fig. 1; see Figs S1 and S2 in the supplementary material) (Perez-Rodriguez et al., 2005). This raised the question of whether these structurally related proteins share homologous functions, and whether the functions of MIXTA and AmMYBML1 have diverged from those of AmMYBML2 and PhMYB1.

PhMYB1 is the only MIXTA-like gene in Petunia hybrida

We first undertook experiments to identify R2R3 MYB genes belonging to subgroup 9 in Petunia hybrida. First-strand cDNA, made from RNA from developing flower buds, was amplified by 3′ RACE and by RT-PCR, using oligonucleotides from regions of the MIXTA sequence that encode the amino acid sequences conserved in subgroup-9 MYB proteins (Kranz et al., 1998; Stracke et al., 2001). No transcripts were amplified, even at low annealing temperatures. In addition, genomic DNA from Petunia was digested with different restriction enzymes, blotted onto nitrocellulose and probed with labeled DNA fragments encoding the different parts of the MIXTA protein. Blots were washed at low stringency. No hybridizing bands were detected, despite DNA fragments corresponding to PhMYB1 being readily detectable on the same blots (see Fig. S3 in the supplementary material). We concluded that the Petunia genome does not contain genes equivalent to the MIXTA gene of A. majus. PhMYB1 was the only MIXTA-like gene we could detect in Petunia.

DNA gel blots of Petunia genomic DNA, probed with fragments of the PhMYB1 gene encoding the C-terminal domain of the protein, gave a single DNA fragment when washed at either high or low stringency (see Fig. S2 in the supplementary material). This suggested that PhMYB1 is a single-copy gene and that the Petunia genome does not contain additional genes highly homologous to PhMYB1.
The *Antirrhinum* genome contains genes encoding three members of R2R3 MYB subgroup 9: *AtMYB16*, *AtMYB17* and *AtMYB106* (Kranz et al., 1998). *AtMYB16* and *AtMYB106* are structurally very similar to *PhMYB1*, *AmMYBML2* and *AmMYBML3* (Fig. 1). No orthologue of *MIXTA* is encoded by the *Arabidopsis* genome.

**High-level expression of *PhMYB1*, *AmMYBML2* and *AtMYB16* modify the shape of tobacco epidermal cells**

We used high-level expression in tobacco plants as a bioassay to determine whether the three most structurally similar genes in subgroup 9 from different plant species – *PhMYB1* (*Petunia hybrida*), *AmMYBML2* (*A. majus*) and *AtMYB16* (*Arabidopsis thaliana*) – share similar functions, and to compare their functions with that of *MIXTA*. Constructs with the cDNAs encoding *AmMYBML2*, *PhMYB1* or *AtMYB16*, driven by the strong constitutive double CaMV 35S promoter, were transformed into tobacco and the expression of each of the transgenes confirmed by RNA blots. At least ten independent lines with comparable transcript levels in leaves were used for phenotypic characterisation. Tobacco plants overexpressing *AmMYBML2*, *PhMYB1* or *AtMYB16* showed identical phenotypes; changes induced by the activity of all three genes were almost exclusively restricted to floral organs. Scanning electron microscopy revealed that petal cells of transgenic tobacco plants expressing any one of the three genes grew to a greater length than in wild type. In shape, modified cells of the inner petal epidermis resembled skittles rather than cones (Fig. 2A-D). The activity of each of the three genes also induced the formation of outgrowths on the cells of the carpel (Fig. 2E,F), and occasionally outgrowths were observed on the epidermal cells of leaves that were borne on the inflorescence (see Fig. S4 in the supplementary material). However, unlike *MIXTA*, which is able to induce changes in cell shape in all epidermal cells of aerial organs of tobacco (Glover et al., 1998), neither *AmMYBML2*, *PhMYB1* nor *AtMYB16* induced the formation of outgrowths of epidermal cells on organs other than flowers and inflorescence leaves (see Fig. S4 in the supplementary material). The ectopic outgrowths induced by 35S:*PhMYB1*, 35S:*AtMYB16* and 35S:*AmMYBML2* were clearest on the carpel surface. The outgrowths never developed into multicellular trichomes, unlike the response to high ectopic expression of *MIXTA* or *AmMYBML1* (see Fig. S5 in the supplementary material) (Glover et al., 1998; Martin et al., 2002; Perez-Rodriguez et al., 2005). Freeze-fractured leaves and petals also showed that ectopic expression of these genes had no effect on cell layers other than the epidermis (not shown). These data suggested that *AmMYBML2*, *PhMYB1* and *AtMYB16* are functionally homologous and are involved in the control of cell shape, but function in a manner distinct from that of *MIXTA* and *AmMYBML1*.

We also ectopically expressed *AtMYB16* under the control of the double 35S promoter in *Arabidopsis*. Five independent transgenic lines were analysed. The inner epidermal cells of the petals grew to a greater length and changed from cone-shaped to bullet-shaped (Fig. 2G,H). No other changes in cell morphology in flowers were observed in these lines.

These data from high-level expression in tobacco demonstrated that *PhMYB1*, *AmMYBML2* and *AtMYB16* have equivalent biochemical functions. They can, in certain tissues, promote the formation of outgrowths, but their primary function seems to be to promote unidirectional cell expansion once an outgrowth has been initiated. However, developmental function, particularly that of transcription factors, is also dependent on the cellular context in which proteins are active (Lee and Schiefelbein, 2001).

**Expression patterns of *PhMYB1*, *AmMYBML2* and *AtMYB16***

The expression of the *PhMYB1* gene in different organs of *Petunia* has been reported by Avila et al. (Avila et al., 1993). The gene is highly expressed in flowers, but is also expressed in sepals and at a low level in leaves. No expression was detected in roots. We analysed the timing of expression of the *PhMYB1* gene, relative to different stages of flower development, by RNA gel blots. *PhMYB1* transcript levels peaked at stage 3 (Fig. 3A). In situ hybridisation showed *PhMYB1* to be expressed in both the inner and outer epidermal cells of the corolla limb (Fig. 3B).

For comparison, we analysed the expression of *AmMYBML2* in *Antirrhinum*. *AmMYBML2* was expressed relatively late in petal development, being maximally expressed in the corollas of flowers which had just opened. The peak of *AmMYBML2* expression was significantly later during petal development than...
the expression of MIXTA. AmMYBML2 was expressed in leaves, particularly leaves undergoing expansion growth and mature leaves. Expression was also detected in roots (Fig. 3C). In situ hybridisation showed that AmMYBML2 was expressed at an apparently low level in both epidermal layers of the petals of A. majus (Fig. 3D).

The expression patterns of AtMYB16 and AtMYB106 were established by consultation of the microarray data stored in GENEVESTIGATOR (Zimmermann et al., 2004b). Both AtMYB16 and AtMYB106 are most highly expressed in flowers, particularly petals, but expression of both genes was also detectable in leaves, particularly younger rosette leaves. These data confirmed previous findings reported by Kranz et al. (Kranz et al., 1998) and Stracke et al. (Stracke et al., 2001). Together with the bioassay analyses these data showed that PhMYB1, AmMYBML2 and AtMYB16 have the same biochemical functions and are expressed in the same tissues, implying that they probably play equivalent developmental roles. These proteins are functionally distinct from MIXTA in terms of their biochemical functions and expression patterns. This separation of function is also clear in Antirrhinum, where AmMYBML2 is expressed in the petals of mixta mutants but cannot rescue the conical cell phenotype that is lost with loss of MIXTA activity (C.M. and M.P.-R., unpublished).

**phmyb1 mutants have paler petals than wild type**

To determine the precise developmental functions of PhMYB1, AmMYBML2 and AtMYB16, we looked for mutations affecting these genes. Although we identified insertions in AtMYB16 in the ZIGGIA collection of Antirrhinum insertion mutants, these had no discernable phenotype. This might have been due to the activity of the very closely related gene, AtMYB106, which is probably functionally redundant with AtMYB16. Searches for insertion mutants of AmMYBML2 amongst the transposon-insertion collection of A. majus were also unsuccessful. The phmyb1 mutant from Petunia hybrida is therefore the only mutant reported to date for any of these orthologous MIXTA-like genes (van Houwelingen et al., 1998). To define in more detail the developmental role of
PhMYB1 in Petunia hybrida and to relate its activity to that of MIXTA. AmMYBML1 and AmMYBML2 in A. majus, we analysed the changes in cell shape caused by mutation of the PhMYB1 gene.

phmyb1 is a somatically unstable mutant of Petunia resulting from the insertion of an endogenous 284 bp transposable element, dTph1 (van Houwelingen et al., 1998), in the PhMYB1 gene. dTph1 is able to undergo excision, both somatic and germinal, to generate two types of derivative allele; stable mutant alleles (when the transposon leaves a footprint upon excision) and stable, wild-type revertant alleles (where the excision event restores the wild-type sequence) (Gerats et al., 1990; Kroon et al., 1994). When derivative alleles are created in germinal cells, these events give rise to stable derivative alleles in the progeny.

The typical petal phenotype of the unstable phmyb1 mutant consists of darker revertant sectors on a background of paler mutant cells (Fig. 4A). The size of the sectors varies depending on the timing of excision of the dTph1 transposon. This petal phenotype is very similar to that of the unstable mixta mutant of A. majus, where the difference in colour is due to a difference in shape of the cells of the petal epidermis (Fig. 4B) (Noda et al., 1994; van Houwelingen et al., 1998). No obvious phenotypes were seen in any tissues of phmyb1 mutants other than in the petal epidermis.

dTph1 is inserted into the coding region of PhMYB1

The exact position of the transposon insertion in the PhMYB1 gene was determined by PCR of the mutant allele using genomic DNA from the phenotypically unstable line. Two PCR products were obtained, differing in size by approximately 300 bp (Fig. 5A). Sequencing of the larger DNA fragment established that dTph1 was inserted in the third exon of PhMYB1, a region encoding part of the C-terminal domain that is conserved among members of R2R3 MYB subgroup 9 (Kranz et al., 1998). In subgroup-9 MYB proteins, the C-terminal domain comprises two conserved motifs that are 10 and 22 amino acids long; dTph1 was inserted in the region encoding the second one, where it introduced a stop codon into the coding sequence (Fig. 5B). The smaller PCR band from genomic DNA corresponded to the products of somatic reversion, as confirmed by sequencing.

Changes in cell shape resulting from the phmyb1 mutation

Differences in cell shape induced by the mutation in the PhMYB1 locus were observed only in the flower petal epidermis. SEM micrographs showed that cells of the inner epidermis of wild-type Petunia plants had a conical-papillate shape, with a polygonal or hexagonal base, appearing very similar to those found in Antirrhinum (Fig. 6A,B). However, unlike Antirrhinum, the cells of the outer epidermis of the petals were conical, although they grew out less than those of the inner epidermis and were irregularly shaped at their base (Fig. 6A,C). The somatically unstable phmyb1 line of Petunia allowed us to compare the cellular morphology of revertant wild-type sectors with the background mutant cells, at exactly the same stage of development and in a uniform genetic background. Fig. 7 shows SEM micrographs of the inner petal epidermis of the unstable line. phmyb1 mutant cells still developed as small cones, although they were not as conical as wild-type revertant cells (Fig. 7A-C). This was in contrast to the effect of MIXTA in Antirrhinum petals, because mixta mutant cells of the inner epidermis are flat (see Fig. S6 in the supplementary material) (Noda et al., 1994). In the outer epidermis of Petunia, phmyb1 mutant cells were completely flat and

![Fig. 5. The dTph1 transposon is inserted in the coding region of the PhMYB1 gene. (A) Agarose gel stained with ethidium bromide showing the bands obtained by PCR amplification of the PhMYB1 sequence from genomic DNA of six unstable phmyb1 lines. The difference in size between the lower and the upper bands corresponds to the size of dTph1. (B) Diagram of the phmyb1 allele showing the position of the transposon insertion. Hatched boxes represent introns. The blue boxes correspond to the R2R3 MYB domain and the stippled box corresponds to the conserved region in the C-terminal domain that identifies subgroup 9 within the R2R3 MYB gene family. This region comprises two conserved stretches of 10 and 22 amino acids; dTph1 (purple box) is inserted in the second one, where it introduces a stop codon into the phmyb1 open reading frame.](image)

![Fig. 6. Cell shapes in wild-type Petunia hybrida flower petals as seen by SEM. (A) A freeze-fracture across a wild-type petal showing the presence of conical cells in both the inner/adaxial and outer/abaxial epidermis. ie, inner epidermis; m, mesophyll; oe, outer epidermis. (B) Inner epidermal cells of wild-type petals. (C) Cells of the outer epidermis. The base shapes of one cell in the inner and outer epidermal layers are outlined in pink. t, trichome. Scale bars: 50 μm.](image)
had an irregular shape, like the pavement epidermal cells of leaves (Fig. 7D), whereas the revertant cells were conical but still had their lobed base shape. In the inner epidermis, the developmental function of PhMYB1 is distinct from, although related to, that of MIXTA in *A. majus*. The developmental function of PhMYB1 in the inner epidermis is probably more closely aligned to that of AmMYBML2. By contrast, in the outer epidermis, the role of PhMYB1 appears to be equivalent to that of MIXTA. The *phmyb1* mutation had no effect on the development of the multicellular trichomes that formed in the outer epidermis of flower petals (Fig. 7D).

**Changes in epidermal cell shape affect the overall shape of petals**

In somatically unstable mutants such as *phmyb1*, the size of revertant sectors depends on the timing of transposon excision. If the transposon excises early during development, the revertant sectors obtained are larger. We analysed the phenotypes of unstable *phmyb1* flowers where the excision of the dTph1 transposon occurred early during flower development, giving rise to sectors occupying a surface equivalent to 10-50% of the corolla limbs (Fig. 8A). A difference in curvature of the petals was observed between regions of the petals composed of mutant epidermal cells and the wild-type revertant sectors. Whereas revertant sectors had the straight presentation of petals observed in wild-type flowers, regions of the corolla containing mutant epidermal cells were reflexed (curved backwards and downwards). This difference in petal presentation was observed for all large, wild-type sectors when compared with adjacent mutant tissue (Fig. 8A). Strongly reflexed petals were also observed in lines homozygous for the *phmyb1* mutant allele, where somatic revertant sectors were small or where no somatic reversion was observed.

To understand the basis for this *PhMYB1*-dependent difference in petal presentation, the number of epidermal cells in a given revertant or mutant area was counted (Fig. 8B). In the inner epidermis, there were more wild-type cells (14% increase on average) than mutant cells (Fig. 8B). These cell counts showed that, on average, *phmyb1* mutant cells occupied a greater surface area than equivalently positioned wild-type cells in the inner epidermis. However, in the outer epidermis, there was no significant difference in the number of cells per unit area between mutant and wild-type revertant sectors.

**Effect of mutation of the MIXTA gene on petal presentation in *A. majus***

The effect of the *phmyb1* mutation on petal presentation was an unexpected phenotypic consequence of its effects on cell shape. To determine whether cell shaping affected the presentation of petals in other species, we re-examined *mixta* mutants of *A. majus* and compared them with wild-type lines. Differences in petal presentation were observed only for the dorsal petals of the corolla. In freshly opened flowers of wild-type lines with conical inner epidermal cells, the dorsal petal lobes were relatively straight and presented at an angle to the direction of approach of prospective pollinators (Fig. 9). In a somatically stable *mixta* mutant line caused by the insertion of the Tam4 transposon (Noda et al., 1994), and in an independent EMS-generated *mixta* allele (Perez-Rodriguez et al., 2005), the dorsal petals were reflexed, as compared with wild-type lines, in a manner analogous to the *phmyb1* mutant phenotype (Fig. 9).

**Cell shape and petal presentation in species of *Antirrhinum***

Fig. 7. Differences in the shape of wild-type and *phmyb1* mutant cells in petals of the unstable *phymb1* mutant. (A) SEM micrograph of the inner epidermis in which a revertant sector can be seen on a background of mutant cells that retain a flatter conical shape. (B, C) Freeze-fracture across one sector of the inner epidermis showing in detail the shape of revertant wild-type cells (C), which grow to a greater length than mutant cells (B). (D) The outer epidermis of unstable *phmyb1* lines, showing sectors of revertant conical cells and flat mutant cells, both with lobed base shapes. Trichomes (t) also develop in the outer epidermis of *Petunia hybrida* petals but are unaffected by the inactivation of the *PhMYB1* gene. Scale bars: 100 μm in A,D; 20 μm in B,C.
In some of the wild species of the genus *Antirrhinum* there are marked differences in the presentation of the dorsal petals. In *A. barrelieri*, for example, the dorsal petals are erect with no reflexing (Fig. 10H), whereas in *A. australis* the petals are highly reflexed (Fig. 10I) so that the area visible (looking at the flowers straight on) is only about 50% of the total area of the dorsal lobes. We examined the petal epidermal cells in *A. barrelieri* and *A. australis* by SEM and found that although the cells in *A. barrelieri* were significantly smaller than those in *A. majus* and *A. australis*, they were steeply conical (Fig. 10B,E). By contrast, the cells of *A. australis* were very shallow cones (Fig. 10C,F). RNA gel blot analysis showed MIXTA to be more highly expressed in *A. barrelieri* than in *A. majus* and much more highly expressed in *A. australis*. *AmMYBML2* was expressed equally in *A. barrelieri* and *A. australis*, and at higher levels than in equivalent flowers of *A. majus* (Fig. 10J). This suggested that differences in the degree of petal reflexing between *Antirrhinum* species are probably a function of the differences in extension growth of the inner epidermal cells of the dorsal petals which, in turn, are a function of the relative activity of the MIXTA-like genes in the different species.

**DISCUSSION**

*MIXTA*, *AmMYBML2* and *AtMYB16* are able to induce changes in epidermal cell shape, but in a different way to MIXTA.

The ectopic expression of *MIXTA* in tobacco has been used to demonstrate that the *MIXTA* gene is not only necessary but also sufficient to induce the formation of conical cells in epidermal tissues (Glover et al., 1998). The *AmMYBML1* protein from *A. majus* can also induce the formation of ectopic conical cells and trichomes when overexpressed in tobacco (Perez-Rodriguez et al., 2005). The similarity of the tobacco phenotypes induced in the same bioassay by the activity of each of the three genes, *AmMYBML2*, *AtMYB16* and *PhMYB1*, suggests that these genes are functionally equivalent. It also suggests they are involved in controlling cell shape, but that their activity, although similar, is distinct from that of *MIXTA* and *AmMYBML1* (see Fig. S5 in the supplementary material). In fact, the phylogenetic analysis of the subgroup-9 proteins, and our failure to find a gene orthologous to *MIXTA* in *Petunia hybrida*, suggest that *PhMYB1*, *AmMYBML2* and *AtMYB16* are the more ancestral genes in dicotyledonous flowers and that *MIXTA* and *AmMYBML1* might have resulted from more recent gene duplications (Fig. 1 and see Fig. S2 in the supplementary material). Sub-functionalisation following gene duplication might have given rise to *MIXTA*, which has very strong activity in inducing cellular outgrowths, and to *AmMYBML1*, which promotes the formation of...
The phenotype of unstable *phmyb1* mutant petals resembles that of unstable *mixta* mutant lines: darker revertant sectors are visible on a pale background of mutant cells. However, whereas the mutation in the *MIXTA* gene results in flat inner epidermal cells (see Fig. S6 in the supplementary material), the inner epidermal cells in the *phmyb1* mutant are still able to develop into cones (Fig. 7B). The conical shape of *phmyb1* mutant cells is shallower than that of wild-type cells in *Petunia*. This result reinforces the view that *MIXTA* and *PhMYB1* are not functionally identical, and suggests that the formation of fully developed conical cells requires two distinct activities: one, conferred by *MIXTA* in *Antirrhinum*, might initiate the change in growth direction and direct the cells to grow in a polar manner, mainly along one axis; whereas the second activity, conferred by *PhMYB1* in *Petunia* and *AmMYBML2* in *Antirrhinum*, might be responsible for a second phase of elongation that leads to the formation of a complete cone. In *Antirrhinum*, where the first activity is conferred by *MIXTA*, and the second by *AmMYBML2*, the relative activity of these two genes might determine the final shape of the petal epidermal cells. In *Petunia*, the identity of the first activity is unknown; it could be another R2R3 MYB member of subgroup 9, very similar to PhMYB1 (although we could find no molecular evidence for the existence of additional genes encoding subgroup-9 proteins in *Petunia*), or it might not be a MYB protein at all, but rather the result of a mechanism for preparing cells for shape changes (A. Gouveia, PhD thesis, University of East Anglia, 2005).

Unlike the inner epidermal cells, mutant cells of the petal outer epidermis of the *phmyb1* mutant of *Petunia* are flat. This suggests that the formation of cones in the outer epidermis of *Petunia* petals is not controlled in exactly the same way as in the inner epidermal cell layer, as the formation of cones in the outer epidermis is completely dependent upon the activity of PhMYB1. Moreover, this

**PhMYB1 and MIXTA play related but distinct roles in petal conical cell development**

![Fig. 10. Comparison of petal reflexing, epidermal cell shape and the levels of MIXTA and AmMYBML2 transcripts in petals of recently opened buds of *A. majus*, *A. barrelieri* and *A. australe*. (A-C) Freeze-fracture SEM micrographs of sections across the mid-point of the left dorsal petal of *A. majus* (A), *A. barrelieri* (B) and *A. australe* (C). The cells in *A. barrelieri* are more conical than in *A. majus*. The cells in *A. australe* are the flattest. (D-F) This is supported by surface SEM views of these cells in *A. majus* (D), *A. barrelieri* (E) and *A. australe* (F). Scale bars: 50 μm. (G-I) The degree of reflexing of the dorsal petals is small in *A. majus* (G), minimal in *A. barrelieri* (H) and very strong in *A. australe* (I). RNA gel blots of poly(A)+ RNA from buds just prior to opening of *A. majus* (A.m), *A. australe* (A.a) and *A. barrelieri* (A.b), showing the relative expression of *MIXTA* and *AmMYBML2* in the different species of *Antirrhinum*. A cDNA fragment encoding ubiquitin was used to probe the RNA gel blots as a loading control. The ubiquitin transcript was polymorphic in *A. majus* (two bands). Only the larger transcript was detected in *A. australe*, whereas in *A. barrelieri* only the smaller transcript was detected.

trichomes and conical cells and tissue folding in the ventral petal of the corolla, a feature specific to the Scrophulariaceae (Perez-Rodriguez et al., 2005).

The spatial restriction of the ectopic expression phenotype of *AmMYBML2*, *AtMYB16* and *PhMYB1* to floral organs and leaves of the inflorescence (despite the expression of the genes in all organs, driven by the double CaMV 35S promoter) suggests that, in order to be active, these MIXTA-like proteins need different co-factors to those required by MIXTA, which induces outgrowths in all aerial epidermal tissues of tobacco (Glover et al., 1998) and *Antirrhinum* (Martin et al., 2001). Such co-factors might be present in epidermal tissues in a gradient, the maximum level being in flowers. In some cases, R2R3 MYB factors are known to interact with other proteins. For example, the C1 and Pl proteins of maize interact with bHLH proteins to induce the synthesis of anthocyanin (Goff et al., 1992; Lloyd et al., 1992), and the R2R3 MYB transcription factor GL1 interacts with the bHLH proteins GL3 and EGL3 and the WD40-repeat-containing protein TTG1 to promote the formation of trichomes in *Antirrhinum* leaves (Larkin et al., 1994; Payne et al., 2000; Schiefelbein, 2003; Zhang et al., 2003; Zimmermann et al., 2004a). However, the R2R3 MYB subgroup-9 proteins lack the consensus signature motif for interaction with bHLH partners (Grotewold et al., 2000; Zimmermann et al., 2004a; Serna and Martin, 2006), so interacting proteins that contribute to the functional specificity of this group of proteins are unlikely to be subgroup IIIf bHLH co-factors (Heim et al., 2003; Zimmermann et al., 2004a). An alternative explanation for the phenotypic differences between 2x35S::MIXTA and 2x35S::PhMYB1 is that, in the cells lacking a visible phenotype, other factors may negatively regulate responses to the specific MIXTA-like genes.
shows that although PhMYB1 is not orthologous to MIXTA, its function is closely related. Interestingly, the orthologous gene to PhMYB1, A. majus phmby1, is unable to induce conical cell formation in the outer epidermis of the petals of Antirrhinum, even though it is expressed in these cells. Perhaps it is not expressed at high enough levels to induce outgrowths of the outer epidermal cells.

The inner epidermal cell layer affects overall floral architecture

The phmby1 mutation not only influences the shape of single cells, but also influences the overall design of the corolla, affecting the curvature of the petals. This effect was seen very clearly upon comparing large sectors of revertant limb tissue with mutant tissue in the unstable phmby1 mutant. This observation raises the important question of how cell shape might influence petal form and the degree of curvature of petals. Our data show that the curvature of petals is strongly influenced by the morphogenesis of the cells of the epidermis. An explanation for the effects of mutation of PhMYB1 on the degree of petal curvature is that when cones do not expand fully, the cells of the adaxial epidermis of the petals expand more periclinally than those on the abaxial side (Fig. 11). No phenotypic effect of loss of PhMYB1 function is observed in cell layers other than the epidermis, suggesting that the petal curvature results exclusively from changes in epidermal cell shape. Cell counts showed that in the inner epidermis of the phmby1 mutant, cells occupied a larger surface area than cells of wild-type petals, but no difference was observed between wild-type and mutant cells of the outer epidermis. This suggests that the phmby1 mutation results in an increased lateral (pericllinal) expansion of inner epidermal cells, probably as a result of loss of competing expansion in the outward (anticllinal) direction, but these changes in cell expansion operate only in the inner epidermis of the petal (Fig. 11). This could result in reflexing of the petals, such that they bend backwards and downwards, and highlights the differential contribution of cell expansion by each epidermal layer to overall petal design. A similar effect on petal presentation is observed in the dorsal lobes of mixta mutants of A. majus. As MIXTA is expressed only in the inner epidermal cell layer, the effects of this gene are restricted to this single sheet of cells (Glover et al., 1998). Therefore, it is likely that the effects of MIXTA on petal presentation result from similar effects on pericllinal expansion of the inner epidermal cells relative to the expansion of the outer epidermal cells, as suggested for PhMYB1 in Petunia.

Three species of Antirrhinum show different degrees of reflexing of their dorsal petal lobes and the shapes of their cells in the inner epidermis differ significantly. A. barrelieri has the most upright dorsal petals and the most conical epidermal cells. A. majus has more-reflexed dorsal petals and less-steep cones on its inner epidermis. The highly reflected petals of A. australe have the flattest conical cells of the three species. The degree of steepness of the cones in the three species is inversely correlated with the degree of petal reflexing, but positively correlated with MIXTA transcript levels, emphasising the importance of epidermal cell shape in determining petal form.

The effect of loss-of-function of R2R3 MYB subgroup-9 proteins on petal curvature is likely to have appreciable effects on the attractiveness of the flowers to prospective pollinators. The petal reflexing, observed in both the phmby1 and the mixta mutants, causes an effective reduction in the diameter of the corolla, the parameter that is the principal visual signal identified by pollinators at a distance (Menzel et al., 1997). We estimate that the effect of the phmby1 mutation is to reduce the apparent diameter of the corolla by at least 20%. This would affect the distance at which the floral signal could be recognised. Extrapolation of empirical data for bees suggests the recognition distance might be reduced by as much as 12 cm (Menzel et al., 1997). Reflexing of the petals also affects the degree of colour saturation across the corolla (Eckert and Carter, 2000). Varying domains of colour saturation might provide pollinators with targeting information over shorter distances. Consequently, the effects of these mutations might extend to modifying these short-range signals as well as affecting recognition at distance through changing perceived corolla size. The shape of the petal epidermal cells also affects the perceived intensity of the colour signal from the petals and its brightness as a result of differences in the reflection and absorption of light by these differently shaped cells (Gorton and Vogelmann, 1996; Kay, 1988; Kay et al., 1981; Noda et al., 1994). Field trials have shown that flowers of A. majus with conical cells (Mixta+) are more attractive to bees than flowers with flat epidermal cells (mixta), particularly under conditions of low pollinator density (Comba et al., 2000; Glover and Martin, 1998). This might be due partly to the differences in the perceived colour intensity of the flowers, but pollinators showed preferences for flowers with conical epidermal cells even when no anthocyanin pigments were produced and the flowers were white. The explanation for this additional dimension to the positive signal provided by conical petal cells might be the effect of epidermal cell shaping on corolla reflexing and perceived corolla size. These aspects of petal design represent additional parameters under the control of members of the MIXTA-like family of MYB transcription factors that direct the morphogenesis of petal epidermal cells for their specialised functions in pollinator attraction.

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Supplementary material

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