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Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of *Antirrhinum majus* flowers

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Summary

Petal tissue comprises several different cell types, which have specialised functions in pollination in different flowering plant species. In *Antirrhinum majus*, the MIXTA protein directs the formation of conical epidermal cells in petals. Transgenic experiments have indicated that *MIXTA* activity can also initiate trichome development, dependent on the developmental timing of its expression. *MIXTA* is normally expressed late in petal development and functions only in conical cell differentiation. However, an R2R3 MYB transcription factor very similar to MIXTA (AmMYBML1), which induces both trichome and conical cell formation in transgenic plants, is expressed very early during the development of the ventral petal. Its cellular expression pattern suggests that it fulfils three functions: trichome production in the corolla tube, conical cell development in the petal hinge epidermis and reinforcement of the hinge through differential mesophyll cell expansion. The *DIVARICATA (DIV)* gene is required for ventral petal identity. In *div* mutants, the ventral petal assumes the identity of lateral petals lacking these three specialised cell types, and expression of *AmMYBML1* is significantly reduced compared with wild type, supporting the proposed role of AmMYBML1 in petal cell specification. We suggest that *AmMYBML1* is regulated by DIV in association with the B-function proteins DEFICIENS and GLOBOSA, and, consequently, controls specification of particular cells within the ventral petal which adapt the corolla to specialised functions in pollination.

Key words: MYB transcription factor, *AmMYBML1, DIVARICATA,* Trichome, Conical cell, Petal

Development

Introduction

Within a plant organ, there may be several different types of specialised cell. Even within a single tissue, such as epidermis, there are usually morphologically distinct cell types. The distribution and types of specialised cell depend on the specific function of particular organs. In the snapdragon, *Antirrhinum majus*, the zygomorphic flower is defined largely by the specialised structure of the corolla that is formed from five fused petals (two dorsal, two lateral and one ventral) that have distinct morphologies (Fig. 1A).The corolla is adapted for bee pollination. The lobes provide a brightly coloured visual signal to attract pollinators. The ventral petal, which is supported by the lateral petals, is bent at the hinge region between the tube and the lobe tissue and is reinforced to form a landing platform for bees. There are specialised conical cells on the adaxial surface (inner epidermis) of the petal lobes. The specialised shape of these cells optimises light capture by the pigments, causing the petal to appear deeper in colour, which increases the attractiveness of the flower to pollinating bees (Glover and Martin, 1998; Comba et al., 2000). Petals have other epidermal cell types, including multicellular hairs (trichomes). In *Antirrhinum*, trichomes are particularly numerous in the corolla tube, where they trap pollen from the surface of pollinators, act as nectar-guides for pollinators and produce scent to attract pollinators (Almeida et al., 1997; Kolosova et al., 2001). The processes regulating the differentiation of these specialised cell types constitute an important part of floral development.

In *A. majus*, the *MIXTA* gene regulates the differentiation of conical epidermal cells on the petal lobes. *MIXTA* encodes a MYB-related transcription factor that is necessary for the development of conical cells on the inner epidermis of the petals (Noda et al., 1994). *MIXTA* expression in *Antirrhinum* is limited to a narrow window of time relatively late in development of the petal epidermis and confined to adaxial petal epidermal cells (Glover et al., 1998). Ectopic expression of the *MIXTA* gene in tobacco results in the formation of two distinct cell types on leaves (Glover et al., 1998). All plants ectopically expressing *MIXTA* produce outgrowths on their leaf epidermal cells, which resemble conical cells. Some lines also produce ectopic trichomes on their leaves and petals. These experiments show *MIXTA* to be necessary and sufficient for conical cell formation, but also indicate that two specialised cell forms, trichomes and conical cells, can be induced by

MIXTA expression and therefore probably share at least part of a common developmental programme in tobacco. The same common developmental pathway is triggered by MIXTA in *A. majus*, as ectopic *MIXTA* expression also promotes formation of both cell types in its species of origin (Martin et al., 2002). Expression of *MIXTA* early relative to the commitment of cells to further rounds of division results in the formation of multicellular trichomes, while later expression gives rise predominantly to conical epidermal cells (Glover et al., 1998). In *Antirrhinum*, *MIXTA* is expressed in the petal epidermis only after cell division has ceased, explaining the observation that it is involved only in conical cell formation (Glover et al., 1998). *mixta* mutants develop floral trichomes normally, suggesting that other genes are responsible for the formation of trichomes in *Antirrhinum*.

Trichomes develop in specific regions of the petals of *Antirrhinum*, including the inner epidermis of the corolla tube and the outer epidermis of the petal lobes (Fig. 1A). Inside the mouth of the flower, in the throat of the corolla tube, a mass of trichomes forms that collects pollen from the undersides of pollinators. Beyond this mass, extending down the ventral/lateral petal border to the base of the anther filaments, are two strips of yellow trichomes that act as nectar guides for bees (Raman, 1990; Galego and Almeida, 2002). Analysis of *graminifolia* mutant flowers, in which the petals do not fuse to form the corolla tube, shows that the mass of trichomes in the throat of the tube forms largely on the ventral petal. The strips of yellow trichomes, which serve as nectar guides, are determined in part by the ventral petal and in part by the lateral petals (C.M., unpublished). The glandular heads of the trichomes are one of the sites of scent (methyl benzoate) production (Kolosova et al., 2001). Given that *Antirrhinum* flowers produce numerous multicellular glandular trichomes, but that *MIXTA* function is confined to the induction of conical cells on the inner petal epidermis, we investigated whether other MYB transcription factors related structurally to MIXTA might be involved in controlling trichome formation.

The *AmMYBML1* (*Antirrhinum majus MYB MIXTA LIKE 1*) gene encodes a protein structurally very similar to MIXTA. Here, we describe the functional characterisation of *AmMYBML1*. Ectopic expression of the gene in tobacco shows that it can induce the production of both trichomes and conical cells on floral tissues. In *Antirrhinum*, the *AmMYBML1* gene is expressed only in the ventral petal. Within the ventral petal its expression is localised to the conical epidermal cells of the hinge, the expanded mesophyll of the hinge and the inner (adaxial) epidermis, including trichomes, within the corolla tube. Floral expression is determined by the promoter of *AmMYBML1*. Loss of function of the *DIVARICATA* (*DIV*) gene, which specifies ventral petal identity (Galego and Almeida, 2002), results in a large reduction in *AmMYBML1* transcript levels and loss of the three specialised cell types in which *AmMYBML1* is highly expressed. Weak alleles of *DEFICIENS* (*DEF*), which encodes one of the B-function MADS-domain transcription factors required for petal identity, also result in reduced expression of *AmMYBML1* in petals, and cause similar losses in the three specialised cell types as mutants of *DIV*. These data suggest that *AmMYBML1* is involved in the differentiation of three distinct specialised cell forms and so contributes to petal form and function; this role is dictated, in large part, by the specific expression pattern of *AmMYBML1*, which is controlled by DIV in association with B-function MADS-domain proteins.

Materials and methods

In situ hybridisation

RNA in situ hybridisation was performed on sections of *Antirrhinum* buds according to Jackson et al. (Jackson et al., 1991) and Bradley et al. (Bradley et al., 1993). An *AmMYBML1* cDNA fragment (GenBank Accession Number AJ006292) and a *MIXTA* cDNA fragment, each cloned in pBluescript, were used as templates for riboprobe synthesis. The vector containing the *Cyclin D3b* gene from Antirrhinum has been described by Forbert et al. (Forbert et al., 1996).

Construct for ectopic expression of *AmMYBML1* in tobacco

A sense construct for ectopic expression of *AmMYBML1* in tobacco was created by excising the whole *AmMYBML1* cDNA from pCR2.1 using *SnaB*I and *Bam*HI and inserting it into the *Hin*cII and *Bam*HI sites of pJIT60 (Guerineau and Mullineaux, 1993). A cassette containing the cDNA driven by the double CaMV 35S promoter and with the CaMV terminator sequence was excised using *Sst*I and *Bam*HI sites, and inserted into the *Sst*I and *Bgl*II sites of the pBin19 binary vector (Bevan, 1984).

AmMYBML1 Promoter::*GUS* reporter constructs

The genomic sequences of *AmMYBML1* were identified by screening a genomic library from *A. majus* in lambda EMBL4 (a gift from Hans Sommer, MPI Koln). Primers were designed to amplify a large region of promoter sequence (1.6 kb), and a selection of shorter promoter fragments, each with *Bam*HI and *Xba*I restriction sites on the end. These PCR products were digested and ligated into a *Bam*HI/*Xba*I-digested pGREEN-GUS binary vector (Hellens et al., 2000). The *AmMYBML1* promoter sequence has been submitted to EMBL/GenBank (AY661653), along with the *AmMYBML2* and *AmMYBML3* cDNA sequences (AY821655 and AY661654, respectively).

Plant transformation

Constructs were transformed into *Agrobacterium tumefaciens* LBA4404 using electroporation (Mattanovich et al., 1989). Tobacco leaf discs were transformed using the method of Horsch et al. (Horsch et al., 1985).

Expression analysis

RNA was extracted and transcript levels were monitored by northern analysis, using the method of Martin et al. (Martin et al., 1985). Quantitative RT-PCR was undertaken using the method of Jin et al. (Jin et al., 2000) using oligonucleotide primers G3540 (5′-ATTT-GGTGCTGAGGTTGAGA-3′) and G3543 (5′-ACAACTGACTCCA-GCAAACG-3′) for the *ubiquitin* cDNA, and F4266 (5′-CATTG-TACGTACGTTCATCTTTAGTTAGCTTC-3′) and F4267 (5′-GGGC-GCGCCAGCTTCCATGACCATGTTCTT-3′) for the *AmMYBML1* cDNA. Twenty cycles were used to amplify the *ubiquitin* cDNA within the linear range and 15 cycles for *AmMYBML1*.

Histochemical GUS assays

Tissue samples were immersed in GUS staining solution (40 mg/ml X-Gluc in 100 mM NaH_2PO_4, 10 mM Na_2EDTA, 0.5 mM K ferrocyanide, 0.5 mM K ferricyanide, 0.1% Triton X-100, pH 7) and incubated at 37°C for 3 hours. Samples were destained with 70% ethanol and examined using a light microscope.

Protoplast transfection assays for transcriptional regulators of the *AmMYBML1* promoter

Transformation of tobacco (*Nicotiana tabacum* cv Samsun)

protoplasts was performed as described by Negrutiu et al. (Negrutiu et al., 1987). Plasmid DNA (10 μg) containing the *AmMYBML1* promoter region (1571 bp) fused to the GUS gene was used for each transfection. Different combinations of 4 μg of plasmids containing cDNA sequences encoding DIV, DEF, GLO and DEF-VP16 under the control of the double CaMV35S promoter were used to test expression from the *AmMYBML1* promoter. Varying amounts of the plasmid pJIT60 were used to ensure that equal total amounts of DNA were introduced for each transfection and to control for any possible effects of the CaMV35S promoter in titrating out transcription factors. After transfection, protoplast extracts were assayed for β-glucuronidase (GUS) activity according to Jefferson (Jefferson, 1987). All transfections were performed in duplicate, and GUS assays were performed in duplicate for each transfection. Similar results were obtained in at least two independent experiments for each effector combination. GUS activity was calculated as nmol methylumbelliferone per mg protein per minute.

Scanning electron microscopy

Plant tissue was examined under a CamScan mark IV scanning electron microscope with a Hexland cryostage.

Results

Cloning of AmMYBML1, AmMYBML2 and AmMYBML3

To identify *MIXTA*-like genes that might control trichome formation in Antirrhinum flowers, a cDNA library made from RNA from lobe tissue of the *mixta* mutant line was screened for clones encoding proteins structurally related to MIXTA, by probing with the full-length *MIXTA* cDNA (Glover et al., 1998) and washing at low stringency (3×SSC, 0.5% SDS, 65°C). Transcripts encoding two distinct R2R3 MYB proteins closely related to MIXTA were identified. Two full-length clones of *AmMYBML1* and two full-length clones of *AmMYBML2* were isolated from 100,000 pfu screened. During subsequent screening of a genomic library in bacteriophage lambda EMBL4 with the *AmMYBML1* cDNA, a third, highly related, gene was identified (*AmMYBML3*). The predicted open reading frames in this genomic clone were identified and the sequence of the transcript was confirmed by cDNA isolation by RT-PCR (Frohman et al., 1988).

Phylogenetic analysis of AmMYBML1, AmMYBML2 and AmMYBML3

The amino acid sequences of the three MIXTA-like proteins, AmMYBML1, AmMYBML2 and AmMYBML3, were compared with the primary sequences of other R2R3 MYB proteins using CLUSTALW. The amino acid sequence in the two MYB domains of R2R3-MYBs is highly conserved, and phylogenetic analysis places AmMYBML1, AmMYBML2 and AmMYBML3 in subgroup 9 of the 24 subgroups that have been identified among the 125 *Arabidopsis* R2R3 MYB proteins (Stracke et al., 2001). Subgroup 9 contains the *MIXTA-LIKE MYB* genes, which are defined by both the conservation of their DNA-binding domains and by motifs present in the C-terminal domains (Kranz et al., 1998; Stracke et al., 2001). The functions of only two members of this group have been characterised. *PhMYB1* from *Petunia hybrida* plays a similar role to *MIXTA* in conical petal cell development (Avila et al., 1993; van Houwelingen et al., 1998).

AmMYBML2 is most closely related to PhMYB1 from *Petunia hybrida* (Fig. 1B) (Avila et al., 1993). AmMYBML2 and AmMYBML3 are also closely related to AtMYB16 and AtMYB106. In fact, AmMYBML2 appears to be orthologous to PhMYB1 from our alignments, and MIXTA and AmMYBML1 appear to be products of more recently derived gene duplications of a subgroup 9 ancestral gene. We were interested in whether the developmental function of these related genes had radiated along with the changes in floral morphology that adapt members of the *Scrophulariaceae* to specific pollinators. Consequently, we examined the function of *AmMYBML1* in *Antirrhinum* in greater detail.

Analysis of *AmMYBML1* expression by RNA gel blots

Previous analysis had indicated that *AmMYBML1* is expressed in petals, early in tissue development (Glover et al., 1998). Investigation of *AmMYBML1* expression in other plant organs showed the *AmMYBML1* transcript to be restricted to the floral tissues; no expression was detected in leaves or roots (Fig. 1C). Petal tissues express *AmMYBML1* only during the early stages of development, with maximum transcript found in buds of length 0-5 mm. Analysis of expression in young, medium and old leaves indicated that *AmMYBML1* is not expressed in leaves, irrespective of developmental stage.

In situ hybridisation to determine cell specific expression of *AmMYBML1*

In situ hybridisation of *Antirrhinum* flower buds showed that *AmMYBML1* expression is limited to the ventral petal, and is absent from the other floral whorls and the lateral and dorsal petals (Fig. 2A,B). Probing equivalent sections with the cell cycle marker *Cyclin D3B*, showed that *AmMYBML1* is expressed in the ventral petal while its cells are still competent for further division (Fig. 2B,C). In a double mutant of *A. majus* (*cyc,dich*) that has radially symmetrical flowers with only ventral petals (Luo et al., 1996) *AmMYBML1* is expressed in all the petals, confirming that its expression is specific to the ventral petal (Fig. 2D).

Investigation of the localisation of *AmMYBML1* transcript within ventral petal tissue at slightly later developmental stages identified three key regions of expression. *AmMYBML1* was found to be expressed in the epidermal cells and in the trichomes which line the throat of the corolla tube and act to collect pollen from the undersides of pollinating bees (Fig. 2E). Transcript was also found in the adaxial epidermal cells overlying the hinge of the petal, which is the folded region of the ventral petal between tube and lobes (Fig. 2E,F). *AmMYBML1* expression was also detected in the mesophyll cells of the hinge region, but restricted to those cells on the adaxial side of this tissue (Fig. 2E,F). The expression of *AmMYBML1* in mesophyll cells is in direct contrast to the expression pattern of *MIXTA*, which is restricted in its expression to the adaxial epidermal cells (Fig. 2G).

Activity of *AmMYBML1* ectopically expressed in *Nicotiana tabacum*

To test the biological function of *AmMYBML1,* and to compare it with *MIXTA,* a construct containing the *AmMYBML1* cDNA under the control of the double CaMV35S promoter was used for transformation of tobacco. Expression of *AmMYBML1* in regenerated tobacco plants was confirmed by RNA gel blot analysis. Eight independent lines with detectable levels of

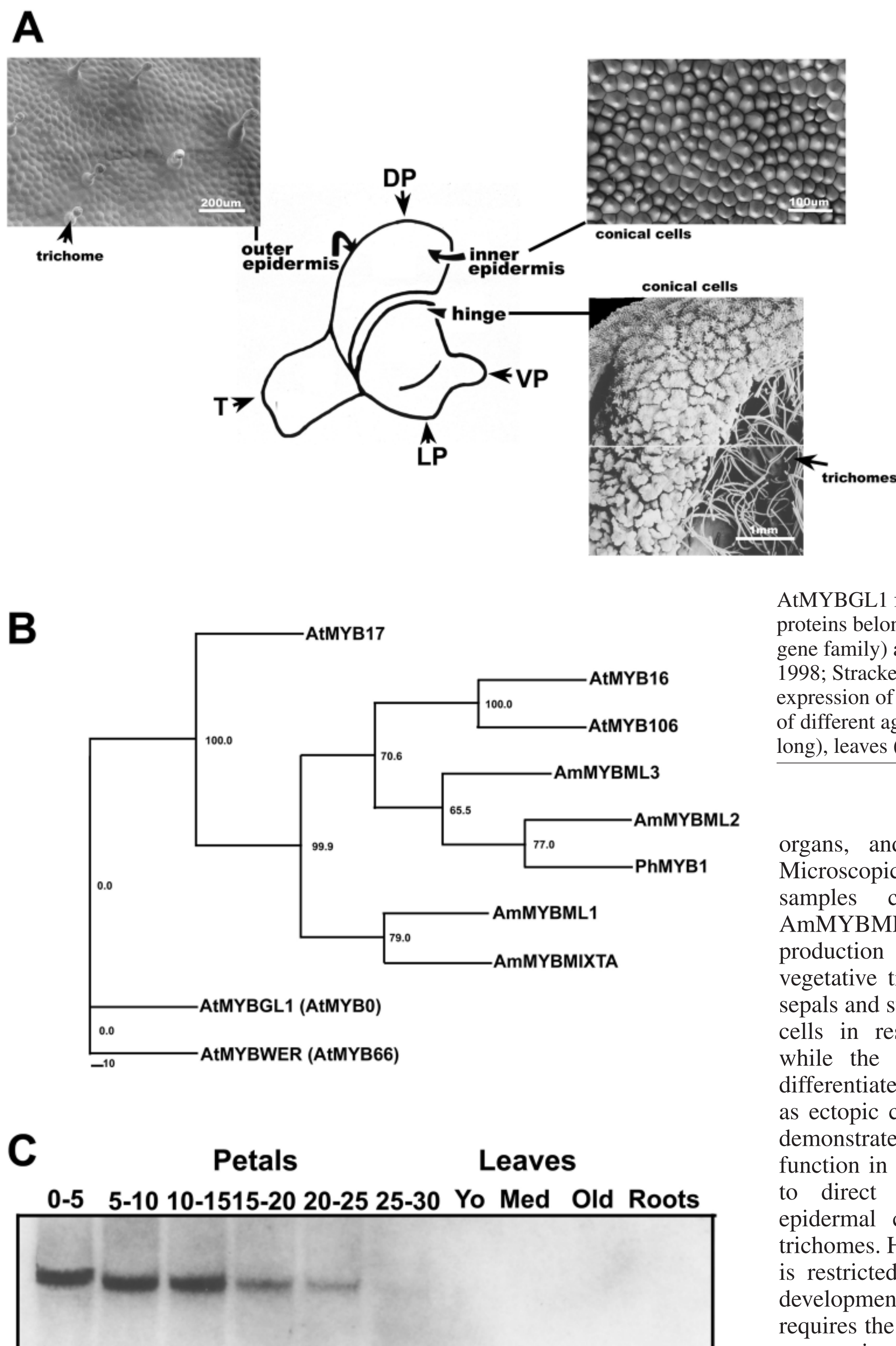

Fig. 1. Cell types in *Antirrhinum* petals and AmMYBML1 structure. (A) Trichomes are present on both epidermal surfaces of the tube and on the outer epidermis of the petal lobes. The inner epidermis of the petal lobes comprises conical cells. The sheet of conical epidermal cells is highly folded in the region of the hinge to give an undulating surface. T. tube; DP, dorsal petal; VP, ventral petal; LP, lateral petal. (B) Phylogenetic relationships between the protein sequences encoded by R2R3 MYB proteins of subgroup 9. AtMYB16, 106 and 17 are from *Arabidopsis thaliana* (Kranz et al., 1998; Strake et al., 2001), PhMYB1 is from *Petunia hybrida* (Avila et al., 1993), AmMYBMIXTA, AmMYBML1, AmMYBML2 and AmMYBML3 are from *Antirrhinum majus* (Noda et al., 1994; Glover et al., 1998) (this paper). The sequences of AtMYBWER and AtMYBGL1 from *Arabidopsis* (which are R2R3 MYB proteins belonging to subgroup 15 of the R2R3 MYB gene family) are included as outliers (Kranz et al., 1998; Stracke et al., 2001). (C) RNA gel blot showing expression of *AmMYBML1* in corollas of flower buds of different ages (between 0-5 mm and 25-30 mm long), leaves (young, yo; medium, med; old) and roots.

AmMYBML1 expression in leaves were identified and used for phenotypic characterisation (Fig. 3H). In the vegetative tissues, the phenotypes of the plants expressing *AmMYBML1* were identical to those of wild-type tobacco plants. However, significant differences were observed in floral tissues. Scanning electron microscopy showed that conical cells and trichomes developed ectopically in the epidermis of several floral organs. The strongest phenotype was observed in carpel epidermal tissue, but the epidermal cells of the other floral organs, and the bracts, were also affected. Microscopic analysis of leaf and stem epidermal samples confirmed that, unlike MIXTA, AmMYBML1 was not able to regulate the production of conical cells or trichomes in vegetative tissues (Fig. 3A,B). However, bracts, sepals and stamens were found to develop conical cells in response to *AmMYBML1* expression, while the petals and carpels were found to differentiate full multicellular trichomes, as well as ectopic conical cells (Fig. 3C-G). These data demonstrate that the AmMYBML1 protein can function in the same way as the MIXTA protein to direct the differentiation of specialised epidermal cell forms, both conical cells and trichomes. However, the activity of AmMYBML1 is restricted to the reproductive phase of plant development, suggesting that AmMYBML1 requires the presence of additional factors, which are restricted to floral tissues, to induce cellular differentiation. As tobacco provides an excellent model for testing gene function in *Antirrhinum*, as evidenced by the identical effects of ectopic expression of *MIXTA* in the two species (Martin et al., 2002), these data indicate that AmMYBML1 can promote both conical cell and trichome formation in *Antirrhinum*.

Further exploration of the function of AmMYBML1

The detection of *AmMYBML1* expression in the adaxial epidermis and adaxial mesophyll cells of the hinge region of

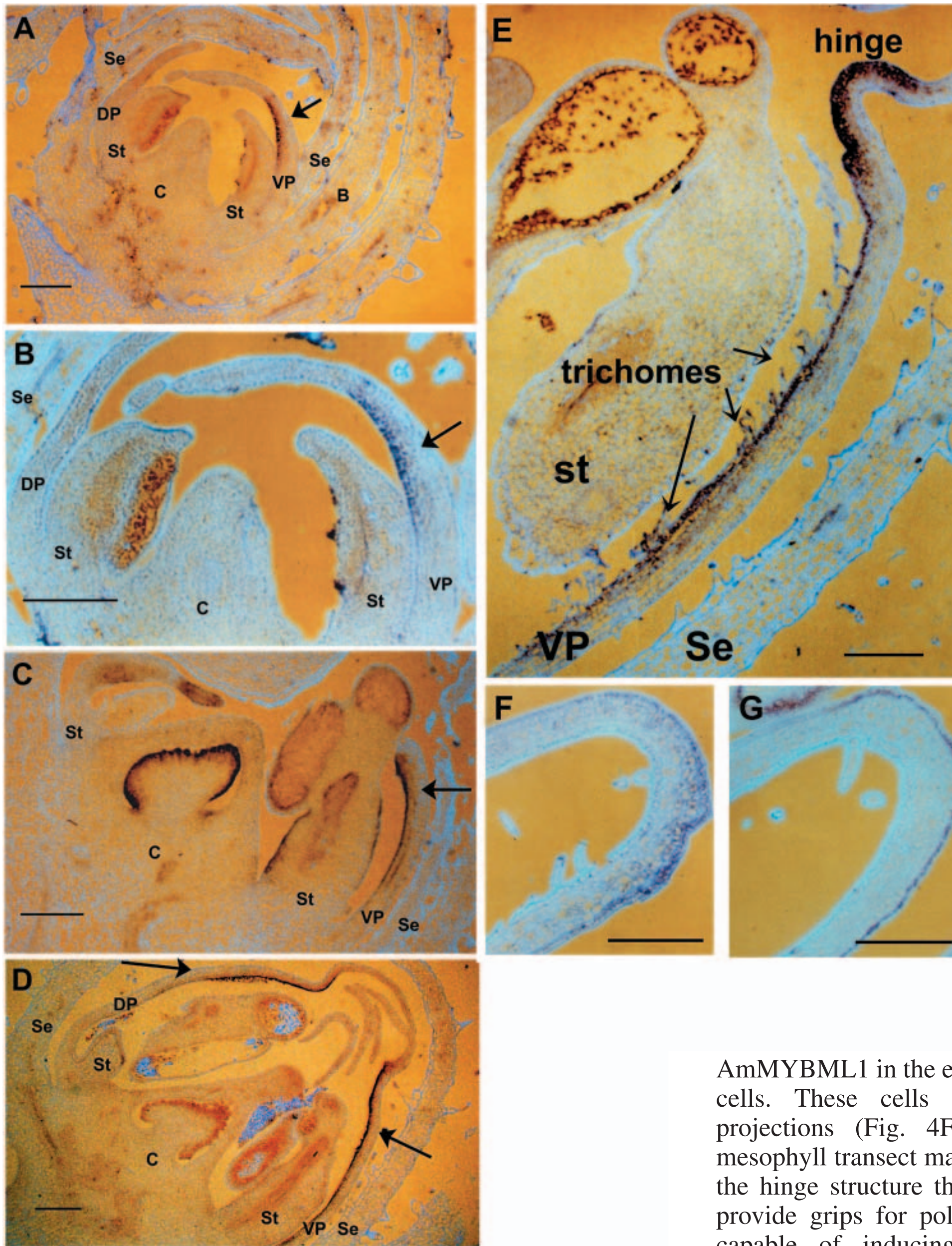

Fig. 2. In situ hybridisation of *AmMYBML1* in *Antirrhinum* flower buds. (A) Entire bud probed with *AmMYBML1*. Expression was observed only in the ventral petal. Se, sepal; DP, dorsal petal; St, staminal filament; C, carpel; VP, ventral petal; B, bract; arrow indicates expression. (B) Higher magnification view to show *AmMYBML1* expression on the adaxial side of the ventral petal. (C) Flower bud of equivalent age (to B) probed with *Cyclin D3b*. There is strong labelling in ovules, anthers and in the ventral petal of the corolla (arrowed). (D) Flower bud from *cyc dich* double mutant showing *AmMYBMl1* expression in the developing petals. All the petals in this line have ventral identity, and *AmMYBML1* is expressed (arrowed) in the petals that lie in the dorsal position (DP) as well as in the ventral position (VP). Scale bars: 100 μm. (E) In situ hybridisation of bud (15-25 mm) showing expression of *AmMYBML1* in the ventral petal. Labelling was observed in the adaxial epidermis of the tube, in the trichomes of the tube, and in the hinge but not in the main part of the petal lobe epidermis. (F) Expression of *AmMYBML1* in the ventral petal. In the hinge region the gene is expressed in the adaxial (inner) epidermis and in mesophyll cells on the adaxial side. (G) In contrast to B, *MIXTA* is expressed only in epidermal cells, even in the hinge region. Scale bars: 100 μm.

the ventral petal (Fig. 2E,F) led us to investigate the possible function of the protein in this region of the *Antirrhinum* petal. Fig. 4A shows a freeze fracture section through the hinge of a *mixta* mutant petal, which has flat epidermal cells on the inner epidermis of the petal lobes. In the region of the hinge, however, a few conical cells (indicated by arrows in Fig. 4A) are formed in the mutant line, presumably induced by the expression of *AmMYBML1* as the transposon insertion inactivates the *MIXTA* gene entirely (Noda et al., 1994). Conical cells in the hinge region were also observed in a second stable *mixta* mutant allele derived by EMS mutagenesis (C.M. and Z.S.-S., unpublished). These cells (indicated by arrows) can be seen in surface view in Fig. 4B.

Freeze fracture sections of the petal hinge indicated that the mesophyll cells in this region are tightly packed on the adaxial side (Fig. 4D). This contrasts with the loosely packed cells of the petal lobe mesophyll (Fig. 4E) and suggests a role for AmMYBML1 in the expansion of the adaxial hinge mesophyll cells. These cells contact each other through surface projections (Fig. 4F). Differential expansion across the mesophyll transect may result in the generation of the folds of the hinge structure that strengthen the landing platform and provide grips for pollinating bees. AmMYBML1 is indeed capable of inducing tissue thickening, because ectopic expression throughout the petal mesophyll in tobacco resulted in petals with much thicker mesophyll (Fig. 4H) than controls (Fig. 4G). Measurements of the thickness of equivalent regions of petal tissue gave a mean value of 251±119 μm for control tobacco flowers and 374±79 μm for flowers from the *35S-AmMYBML1* tobacco lines, values that were different with 95% confidence limits. Interestingly, flowers of tobacco plants expressing *MIXTA* under the control of the CaMV35S promoter (Glover et al., 1998) did not show such pronounced thickening of the petal mesophyll (mean value 330 μm) and *Antirrhinum* flowers ectopically expressing *MIXTA* retained the folds of tissue in the hinge region of the flower (see Fig. S1 in the supplementary material). These observations suggested that *MIXTA* is less effective than *AmMYBML1* at promoting the expansion of petal mesophyll cells.

Regulation of *AmMYBML1* expression

The *AmMYBML1* promoter, 1610 bp upstream from the

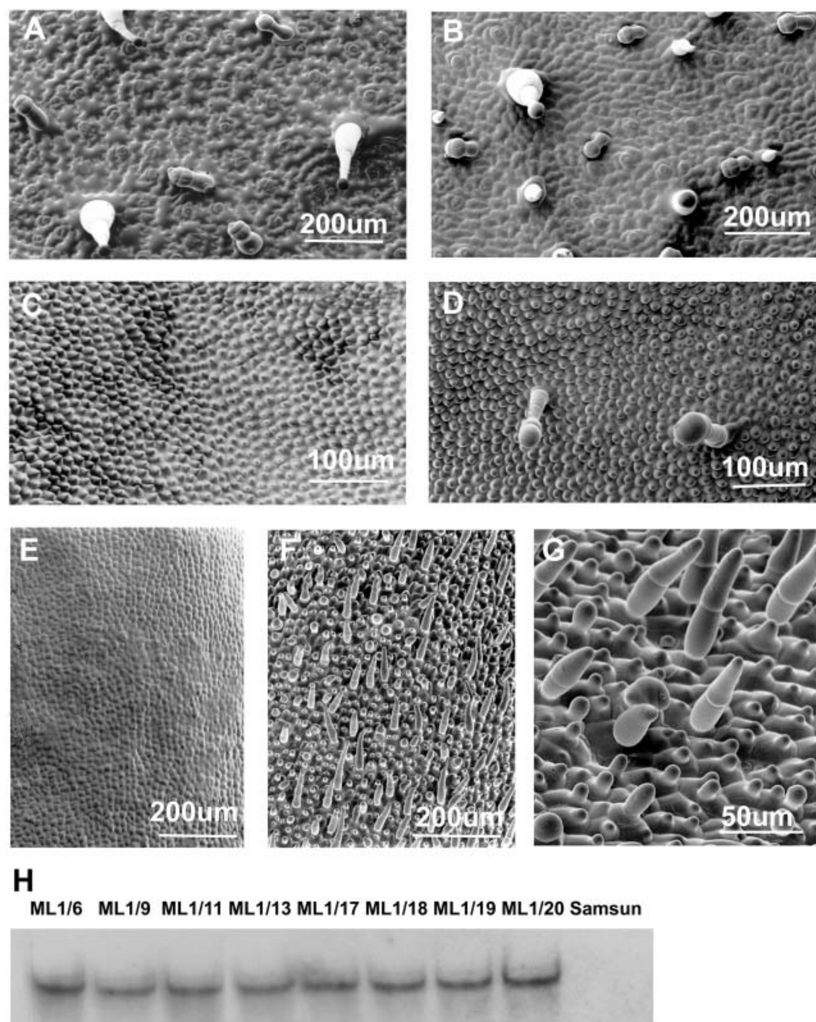

Fig. 3. Morphological consequences of strong constitutive expression of *AmMYBML1* in transgenic tobacco. (A) Scanning electron micrograph (SEM) of control (*N. tabaccum* var. Samsun) leaf, upper epidermis. (B) Tobacco leaf of plant expressing *AmMYBML1* under the control of the CaMV 35S promoter (*35S-AmMYBML1* plant), upper epidermis. (C) Petal inner epidermis of control. (D) Inner petal epidermis of 35S-*AmMYBML1* plant; note glandular trichomes. (E) Carpel of control. (F) Carpel of line expressing *AmMYBML1*. (G) Higher magnification SEM of carpel epidermis of 35S-*AmMYBML1* line; note ectopic production of both conical cells and trichomes. (H) RNA gel blot of transgenic tobacco lines carrying 35S-*AmMYBML1* or a control line (Samsun) probed with the complete *AmMYBML1* cDNA sequence.

initiating ATG codon, was sequenced and analysed using databases of transcription factor binding sites. Fig. 5A shows the *AmMYBML1* promoter with key regions highlighted. The putative TATA box is located between 171 and 162 bp upstream of the initiating ATG codon. Two motifs of interest were identified between –364 and –358 bp and between –1113 and –1109 bp upstream of the ATG. These contained putative I-box motifs, which is interesting because these sequences have been shown to be bound by proteins similar to DIVARICATA (DIV) (Rose et al., 1999; Galego and Almeida, 2002). *DIV* influences specifically the growth of ventral and lateral petals (Almeida et al., 1997; Galego and Almeida, 2002). In *div* mutant plants, each half of the ventral petal becomes a mirror image of the adjacent part of the lateral petal (Almeida et al., 1997), resulting in a loss or significant reduction of the hairs in the throat of the tube that collect pollen and in the size of the strips of hairs that pass down the corolla and mark the junction between ventral and lateral petals (Almeida et al., 1997). The DIV protein is a MYB-related transcription factor which has two MYB domains, which are separated in the primary sequence of the protein. Structurally, it is most similar to LeMYB1 from tomato (Rose et al., 1999). LeMYB1 binds the I-box sequence GATAAG, while StMYB1 (a related protein from potato) binds to a similar sequence, GGATAAG, or to a core sequence, GGATA (Baranowskij et al., 1994; Rose et al., 1999). The putative I-box at –364 in the *AmMYBML1* promoter is AGATAAA. As A and G are both purines, and therefore functionally similar, the sequence AGATAAA may be similar enough to GGATAAG to be bound by similar proteins. Alternatively, between –1113 and –1109 bp the sequence GGATA, the core for StMYB1 binding, was found.

The promoter region of *AmMYBML1* was used to produce four promoter-GUS fusion constructs of different sizes: 1571 bp, 1260 bp, 884 bp and 223 bp in length. Promoter activity was analysed by transfer of these reporter constructs into tobacco and histochemical assays of GUS activity in T1 plants. The 1610 bp and 1260 bp promoters drove GUS expression in outgrowing cells of the floral organs. This included all types of trichomes covering the floral organs, both glandular trichomes and conical petal cells (Fig. 5B,C). The reduced promoter (884 bp) and the short promoter (223 bp) conferred GUS expression in trichomes in all aerial organs (Fig. 5D,E) but also gave expression in the vascular tissue of the plant (Fig. 5D). No expression was apparent with any *AmMYBML1* promoter-GUS constructs in root tissue of tobacco seedlings.

The expression pattern determined by the 223 bp promoter fragment showed that this sequence is all that is required for expression in trichomes. Consequently, this region is likely to include the important cis-acting elements regulating trichome-specific expression. Aside from the TATA box and one potential CAAT box, there are two GAMYB (MBSII) (Solano et al., 1995) binding motifs within this region.

The 1260 bp promoter was enough to limit the expression of *AmMYBML1* to the trichomes of the flowers but the 884 bp promoter directed GUS expression in trichomes and vascular tissue in both floral and vegetative tissues. Thus, reducing the promoter length from 1260 bp to 884 bp resulted in GUS expression being directed outside the flower. These results suggest that negative regulatory elements silencing expression in vegetative tissues are located within the promoter of *AmMYBML1* between 1260 bp and 884 bp upstream of the ATG. Within this region there are at least four potential MADS domain protein-binding sites (CArG boxes; Fig. 5A). One of

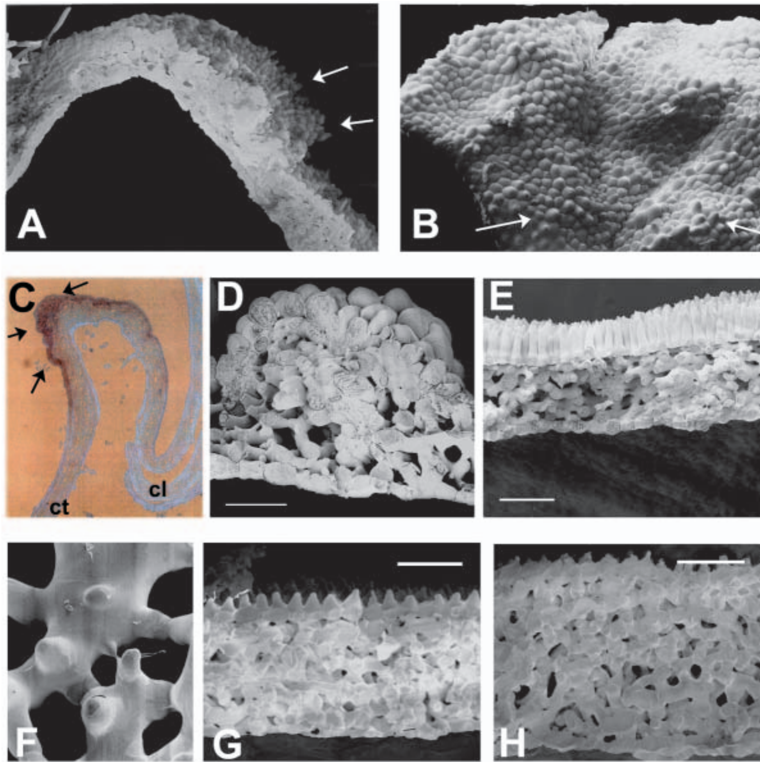

Fig. 4. Further exploration of the function of *AmMYBML1*. (A) Freeze fracture SEM through the corolla hinge of *A. majus*. This flower was taken from a *mixta* mutant, which has flat epidermal cells on the inner epidermis of the corolla lobes. However, a few conical cells are present in the hinge region (indicated by arrows), presumably as a result of *AmMYBML1* activity, which is expressed, in lobes, just in the hinge region. (B) Conical cells (indicated by arrows) in the hinge region of an EMS-induced *mixta* mutant line are shown in more detail. (C) In situ hybridisation showing the expression of *AmMYBML1* in the mesophyll tissue of the folds of the ventral petal (arrowed) on the adaxial side of the hinge. ct indicates the corolla tube tissues; cl the corolla lobe tissues. (D) The mesophyll of the folds of the petal hinge consists of compact and tightly packed cells, especially towards the adaxial side. (E) The mesophyll cells of the petal lobes are more loosely packed (compare with D). (F) The cells of the petal mesophyll have projections which contact other cells. (G) Transverse section through wild-type tobacco petal lobe for comparison with H. (H) Section through petal lobe of a tobacco line ectopically expressing *AmMYBML1*. Expression of *AmMYBML1* thickens the petal through increased expansion of the mesophyll cells (compare with G). Scale bars: 200 μm.

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the two putative I boxes is also located in this region, and may be involved in specifying ventral petal-specific expression in *Antirrhinum*.

Unfortunately, because tobacco flowers are radially symmetrical, we were unable to use expression in tobacco to identify sequences active in determining expression in the ventral petal only.

Comparison of *AmMYBML1* expression in wild type and *divaricata* mutant flowers

The observation that *AmMYBML1* transcript is restricted to the ventral petal in wild-type *Antirrhinum* flowers (Fig. 2A) led us to investigate its expression in the *div* mutant. The *div* mutant lacks ventralising signals in its flowers, and all five petals are consequently dorsalised (Galego and Almeida, 2002). The characteristic mass of trichomes in the throat of the tube in the wild-type flower is absent from this mutant (Fig. 6A-D). The mutant also fails to make the folds of tissue around the hinge region of the lower petals (Fig. 6A-D,F,G) and lacks conical cells in the region of the corolla epidermis just below the hinge (Fig. 6H). On the inner epidermis of the rest of the ventral petal lobe, conical cells develop normally (Fig. 6I,J), suggesting that MIXTA activity is unimpaired in *div* mutants (Fig. 6E). Because *DIV* encodes a MYB-related transcription factor responsible for determining ventral petal identity (Galego and Almeida, 2002), the restriction of *AmMYBML1* expression to the ventral petal suggested that it may be a target for transcriptional control by DIV, and that the phenotype of *div* mutants might reflect the loss of *AmMYBML1* expression as a consequence of the loss of activity of its upstream regulator. Gel blots of RNA from petals of wild-type and *div* mutant flowers showed that *AmMYBML1* was expressed in both lines, but the expression in wild-type petals was at least 10-fold higher than in the *div* mutant petals (Fig. 6E). *MIXTA* expression was unaffected by mutation of *DIV* (Fig. 6E).

Comparison of *AmMYBML1* expression in flowers of wild type and weak alleles of *DEFICIENS*

Because *AmMYBML1* expression is specific to petal tissue, it seemed likely that further regulation of *AmMYBML1* expression might operate through proteins determining petal identity. In *Antirrhinum*, these include the B-function MADS-domain proteins DEFICIENS (DEF) and GLOBOSA (GLO). One problem in examining the effect of these genes on expression of petal-specific target genes is that null mutants of *DEF* and *GLO* produce no petals. Two weak alleles of *DEF* do produce petals, however: the temperature sensitive allele *def101* (Zachgo et al., 1995) and *deficiens-chlorantha* (*def-chlor*), an allele with patchy *DEF* expression in petals resulting from an alteration in the promoter of the gene (Sommer et al., 1990; Schwarz-Sommer et al., 1992). Quantitative RT-PCR analysis was used to estimate the expression of *AmMYBML1* in the flowers of plants homozygous for the *def101* (grown at the permissive temperature of 15°C) and *def-chlor* alleles compared with wild type. A reduced level of *AmMYBML1* expression was found in both mutants, indicating that DEF activity influences the expression of *AmMYBML1* (Fig. 7E). This result is consistent with the presence of CArG motifs within the *AmMYBML1* promoter, as these are the recognition motifs of the B-function MADS-domain proteins (Schwarz-Sommer et al., 1992; West et al., 1998; Parenicova et al., 2003). Examination of the ventral petals of flowers of *def101* (15°C) revealed them to have a reduced mass of trichomes in the throat of the tube (Fig. 7A,B), reduced folds of tissue on the hinge and flat epidermal cells in the region of the hinge (Fig. 7C,D), features predicted as a consequence of the reduction in *AmMYBML1* expression.

Protoplast transfection assays were attempted with *35S:DIV*, *35S:DEF* and *35S:GLO*, and the *AmMYBML1* promoter

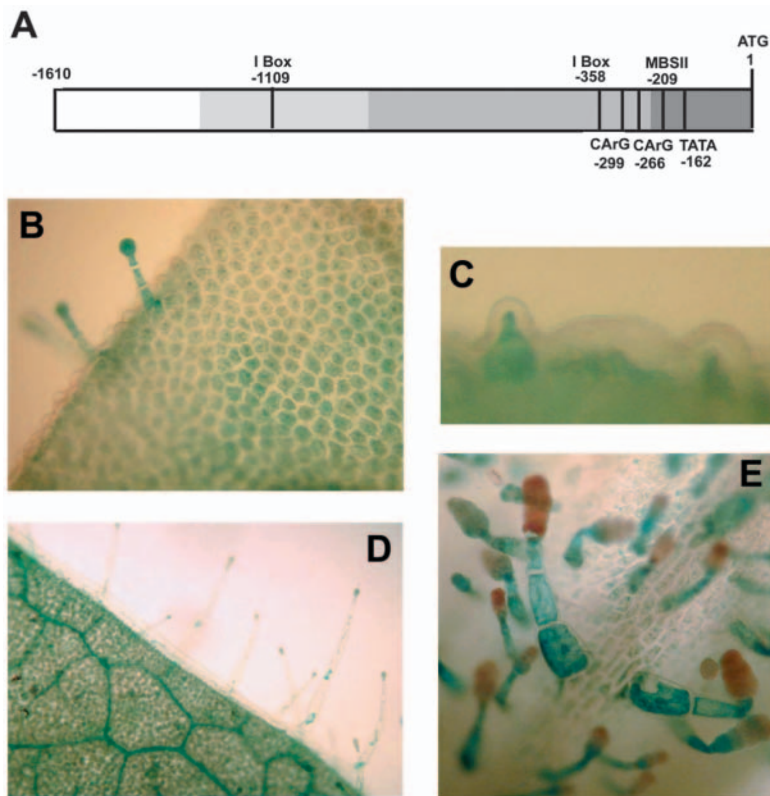

Fig. 5. Analysis of the *AmMYBML1* promoter. (A) The *AmMYBML1* promoter, showing the locations of the TATA box, putative I-boxes, CArG boxes and the MYB-binding site II (MBSII), recognised by proteins such as GAMYB (Solano et al., 1995). The different lengths of the promoter fragments tested in GUS reporter assays in tobacco are indicated in different shades of grey; 1571 bp fragment in white, 1260 bp fragment in very pale grey, 884 bp promoter in mid grey, 223 bp promoter in darker grey. (B) Expression of GUS driven by the full-length *AmMYBML1* (1571 bp) promoter in tobacco petal trichomes. (C) Expression of GUS driven by the full-length *AmMYBML1* promoter in tobacco petal conical cells. (D) Expression of GUS driven by the 884 bp *AmMYBML1* promoter in tobacco leaf vascular tissue. (E) Expression of GUS driven by the 884 bp *AmMYBML1* promoter in the trichomes of tobacco leaves.

driving GUS as a reporter. Results from these results suggested that DIV and DEF plus GLO can bind to the *AmMYBML1* promoter in plant cells. However, even in combination, their activity was not adequate to drive transcriptional activation of gene expression (see Fig. S2 in the supplementary material). Other interacting proteins, such as other MADS-domain proteins of the SEPALLATA and/or SQUAMOSA/AP1 classes, are probably required to provide a complex capable of transcriptional activation. Alternatively, because the transfection assays were performed with protoplasts

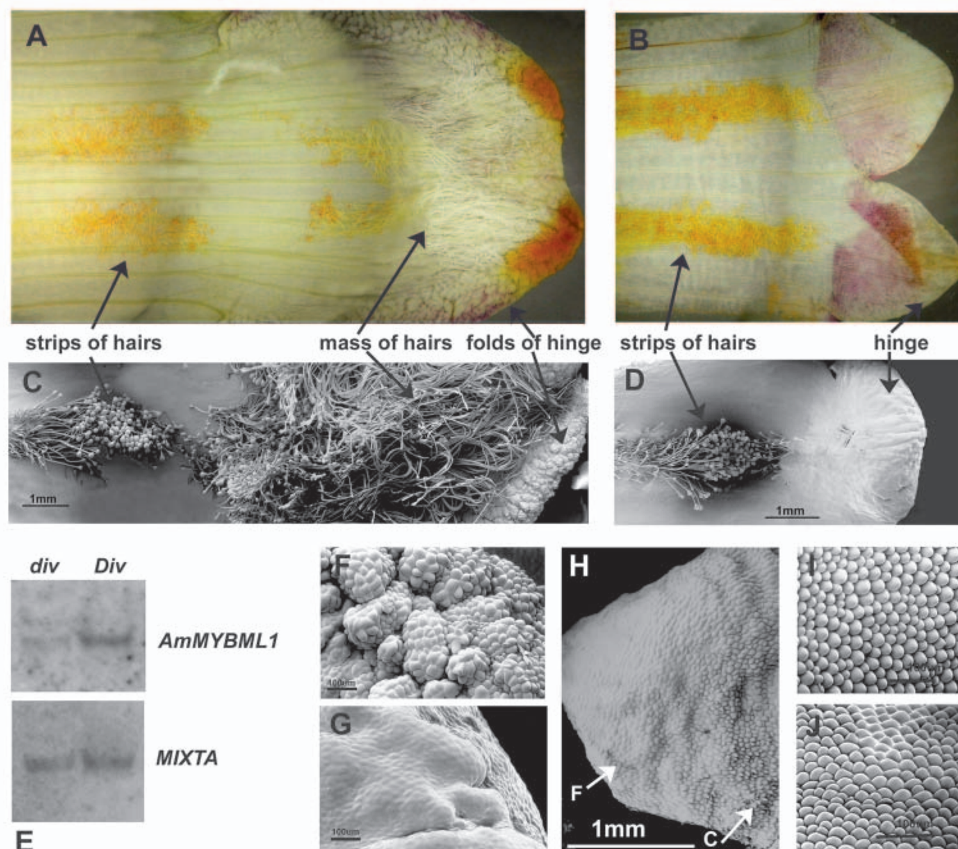

Fig. 6. Effects of mutation of the *DIV* gene on development of the ventral petal of *A. majus* and expression of *AmMYBML1*. (A) Light micrograph of the corolla tube of the ventral petal of a wild-type flower of *A. majus*, showing the mass of hairs in the throat of the tube that collect pollen, the strips of yellow hairs (nectar guides) and the folds of the ventral petal that reinforce the hinge. (B) Light micrograph of the corolla tube of the ventral region of a *div* mutant flower for comparison with A. The *div* mutant petal lacks the hairs in the throat of the tube and the folds around the hinge. (C) SEM of the corolla tube of the ventral petal of a wild-type flower. Scale bar: 1mm. (D) SEM of the corolla tube of the ventral region of the corolla of a *div* mutant flower. Scale bar: 1 mm. (E) RNA gel blots showing the effects of the *div* mutation on expression of *AmMYBML1* and *MIXTA*. *Div* and *div* represent RNA from wild-type corollas and *div* mutant corollas, respectively. (F) SEM of folds of ventral petal in the region of the hinge of a wild-type flower. Scale bar: 100 μm. (G) SEM of region equivalent to F around the hinge in *div* mutant flower. Only very slight undulations of tissue are observed compared with F. Scale bar: 100 μm. (H) SEM of surface of ventral petal lobe of a *div* mutant flower. The hinge is on the left. F indicates flat cells; C indicates conical cells. Scale bar: 1 mm. (I) SEM of petal epidermal cells of ventral petal lobe in a region more distal to the hinge (than in G) in a wild-type flower. Scale bar: 100 μm. (J) SEM of petal epidermal cells of petal lobe in the ventral position in a region more distal to the hinge (than in H) in a *div* mutant flower. Scale bar: 100 μm.

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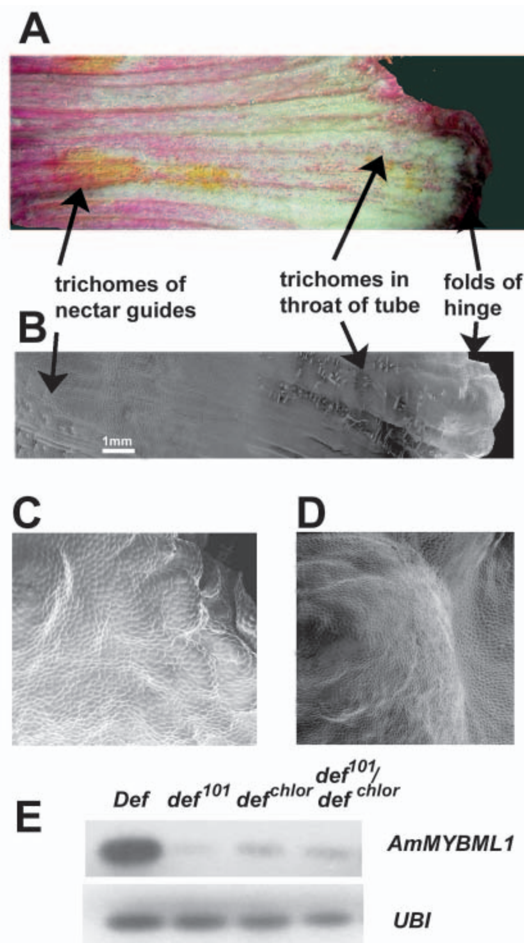

Fig. 7. Effects of weak alleles of the *DEF* gene on development of the ventral petal of *A. majus* and expression of *AmMYBML1*. (A) Light micrograph of the corolla tube of the ventral region of a *def101* mutant flower for comparison with wild type shown in Fig. 6A. The *def101* mutant petal has many fewer hairs in the throat of the corolla tube and less folds around the hinge. (B) SEM of the corolla tube of the ventral petal of a *def101* mutant flower. There are far fewer hairs in the throat of the tube and fewer folds around the hinge. Scale bar: 1 mm. (C) SEM of folds of ventral petal in the region of the hinge of a *def101* mutant ventral petal for comparison to the structure of this region in the wild-type corolla shown in Fig. 6F. (D) SEM of surface of the ventral petal lobe of a *def101* mutant flower in the region of the hinge. The hinge is on the left. All the epidermal cells are flat. This should be compared with the wild type shown in Fig. 6F. (E) Quantitative RT-PCR of *AmMYBML1* transcript levels in corollas of wild type (*Def*), *def101* mutant, def^{chlor} mutant and lines heterozygous for *def101* and def^{chlor}. Transcript levels of *AmMYBML1* should be compared with the levels of control gene, encoding ubiquitin (*UBI*) in the same tissues.

derived from leaves (a technical constraint) and the full-length *AmMYBML1* promoter is negatively regulated in vegetative tissues, the absence of transcriptional activation in response to DIV, DEF and GLO may be a consequence of over-riding transcriptional repression of the *AmMYBML1* promoter in vegetative tissues.

Discussion

The AmMYBML1 protein is capable of inducing the differentiation of both conical cells and trichomes

Previous studies have demonstrated that the MIXTA protein is sufficient for the formation of both conical cells and trichomes in tobacco, but necessary only for the formation of conical petal cells in its species of origin, *Antirrhinum majus*. These observations led us to suspect that other proteins, similar in structure to MIXTA, might promote trichome formation in *Antirrhinum* flowers.

The *AmMYBML1* gene encodes a protein with very strong structural similarity to MIXTA (Fig. 1B), such that both proteins belong to R2R3 MYB subgroup 9 (Stracke et al., 2001). On the basis of this structural similarity, the AmMYBML1 protein was predicted to play a role in conical cell and trichome formation. This was confirmed by ectopic expression of *AmMYBML1* in tobacco. AmMYBML1 induced the formation of both ectopic conical cells and trichomes on floral organs of tobacco. No phenotypic consequences of *AmMYBML1* expression were observed in vegetative tissues, in contrast to the conical cells and trichomes induced on leaves in response to ectopic *MIXTA* expression (Glover et al., 1998). Therefore, AmMYBML1 can function only in floral tissues. This restriction of AmMYBML1 function is most probably attributable to the interaction of AmMYBML1 with other transcription factors with activity localised to floral tissues.

Petal and carpel tissue were most responsive to *AmMYBML1* expression. Tobacco petals are already covered with conical cells, but in the transgenic lines, ectopic trichomes were formed among the conical cells (Fig. 3D). The wild-type carpel epidermal surface was smooth, but in the transgenic lines ectopic conical cells and trichomes were produced (Fig. 3E-G). The excess trichomes on both organs were multicellular and competent to form glandular heads. These data confirmed that the AmMYBML1 protein, as predicted from its structural similarity to MIXTA, is able to induce the formation of both conical cells and trichomes but, unlike MIXTA, only in floral organs.

AmMYBML1 expression is restricted to the ventral petal of the corolla

The endogenous role of a gene is dependent not only on the function of its encoded protein but also on its expression pattern. To investigate the role of *AmMYBML1* in *Antirrhinum*, its expression pattern was determined using northern analysis and in situ hybridisation. Northern analysis indicated that expression of *AmMYBML1* is restricted to floral tissues (Fig. 1C). From this evidence it can also be concluded that any role of AmMYBML1 in the differentiation of cells is restricted to the flower.

In situ hybridisation demonstrated that *AmMYBML1* expression was restricted to the second whorl of the flower and, within the corolla, to the ventral of the five petals (Fig. 2A,B). Confirming this, in floral symmetry mutants where all five petals adopt ventral identity (*dich, cyc*) all five petals expressed *AmMYBML1* (Fig. 2D). Several specialised cell types form on the ventral petal, including trichomes in the throat of the corolla tube, conical cells on the adaxial epidermis of the petal lobes and hinge region, and expanded adaxial mesophyll cells in the hinge region.

Analysis of the timing of *AmMYBML1* expression relative

to a predictive marker of cell division, *Cyclin D3b* (Forbert et al., 1996), indicated that *AmMYBML1* is expressed in the ventral petal while cells are still dividing (Fig. 2A-C). Previous studies have shown that that the ability of MIXTA to direct the formation of conical cells or trichomes is linked to the competence of cells for further division when the gene is expressed (Glover et al., 1998). The observation that *AmMYBML1* is expressed while cells are still competent for further division indicates that it may play a role in the formation of either trichomes or conical cells, or both.

AmMYBML1 expression is required for the development of specialised trichomes within the corolla tube

In situ hybridisation studies identified *AmMYBML1* expression in the trichomes of the ventral petal, especially those that form in a mass in the throat of the corolla tube and that collect pollen from pollinating bees (Fig. 2A, Fig. 6A,C). Expression in trichomes was confirmed by promoter::GUS analysis, which identified GUS staining in all floral trichomes (Fig. 5B). Deletions of the promoter expanded GUS expression to the trichomes of vegetative organs, suggesting that flower-specific expression is the result of negative regulation of *AmMYBML1* expression (Fig. 5E). The activity of the *AmMYBML1* promoter in trichomes was consistent with the observed ability of *AmMYBML1* to induce trichome formation on the petals and carpels of tobacco, when ectopically expressed (Fig. 3D,G). Taken together with the early expression of *AmMYBML1* relative to the progression of cell division (Fig. 2B,C) and the expression of *AmMYBML1* in the trichomes of the ventral petal (Fig. 2E), these data suggest strongly that AmMYBML1 directs the formation of specialised trichomes in the ventral petal.

AmMYBML1 initiates the development of some conical cells in the ventral petal epidermis

In situ hybridisation experiments also identified *AmMYBML1* transcript in cells of the inner epidermis of the hinge of the ventral petal (Fig. 2E,F). This epidermis develops conical cells, similar to those of the main expanse of the petal lobes. Observation of transcript in this region, in combination with the ability of *AmMYBML1* to direct conical cell formation when ectopically expressed, led us to investigate further the role of this gene in the differentiation of these specialised cells. Previous studies had indicated the importance of MIXTA in the differentiation of petal conical cells in *Antirrhinum* and suggested that in *mixta* mutants, no conical cells are produced on the adaxial epidermis. Re-investigation of *mixta* mutant plants, which have flattened epidermal cells on the main expanse of their petal lobes, did reveal a role for *AmMYBML1*. The epidermis of the ventral petal in the region of the hinge still produced some conical cells in the *mixta* mutants (Fig. 4A,B), indicating that a gene other than *MIXTA* is sufficient for the development of conical cells in this restricted region of the corolla. In combination with the ability of *AmMYBML1* to direct the formation of conical cells, the localisation of its transcript to this region and the fact that promoter analysis confirmed the expression of *AmMYBML1* in conical cells of tobacco petals (Fig. 5C) strongly support the idea that AmMYBML1 is responsible for the differentiation of conical cells on the epidermis of the ventral petal hinge.

AmMYBML1 induces differential expansion of the mesophyll of the ventral petal, which reinforces the hinged landing platform for pollinating bees

In situ hybridisation also identified *AmMYBML1* expression in the adaxial mesophyll cells of the ventral petal hinge (Fig. 2E,F and Fig. 4C). Expression in mesophyll cells was not observed for *MIXTA* (Fig. 2G) and suggests a novel role for *AmMYBML1*. Scanning electron microscopy revealed that the mesophyll cells in this hinge region were expanded and tightly packed together, creating folds of tissue (Fig. 4D). Differential expansion of the mesophyll on the adaxial side may contribute to the overall form of the ventral petal which facilitates pollination. The folded region reinforces the corolla, which is important because the ventral petal functions as a landing platform for large pollinating bees. When *AmMYBML1* was expressed in tobacco under the control of the CaMV35S promoter, petal lobes were considerably thicker than controls, and thickening was correlated with increased expansion of mesophyll cells (Fig. 4G,H). Therefore the combination of expression of *AmMYBML1* in the hinge mesophyll and ability of AmMYBML1 to enhance mesophyll cell expansion in transgenic tobacco flowers leads us to conclude that AmMYBML1 is involved in expansion of the adaxial mesophyll cells of the ventral petal, helping to create the folds of the reinforced hinged landing platform. Interestingly, *MIXTA* was not as effective as *AmMYBML1* at promoting mesophyll thickening in transgenic tobacco, suggesting a further functional distinction between the two proteins.

DIV controls *AmMYBML1* expression and defines *AmMYBML1* function in cell specialisation during corolla development

We have been unable to identify a mutant of *AmMYBML1*, despite extensive reverse genetic screens. However, examination of the *div* mutant of *A. majus* supported our interpretation of the function of *AmMYBML1*. DIV promotes *AmMYBML1* expression significantly, although it does not affect expression of *MIXTA*. *div* mutants lack trichomes in the throat of their corolla tubes. This supports the idea that AmMYBML1 promotes trichome formation in the ventral petal tube directly. *div* mutants also lack conical cells in the region of the ventral petal epidermis around the hinge (Fig. 6H). As *MIXTA* expression is not affected by DIV, although DIV is expressed throughout the corolla in the early stages of development (Galego and Almeida, 2002), this observation supports a role for *AmMYBML1* in promoting conical cell formation in this restricted region. Finally, *div* mutants lack the folds of tissue that normally form at the hinge, supporting the view that *AmMYBML1* expression in the adaxial mesophyll in this region promotes the formation of the folds of petal tissue that reinforce the hinge and provide a robust landing platform for pollinating bees.

DIV determines ventral petal identity in *A. majus* (Almeida et al., 1997; Galego and Almeida, 2002) and promotes *AmMYBML1* expression in the ventral petal. Our data suggest that DIV is an activator of *AmMYBML1*. Certainly, a significant number of the specific events in ventral petal development appear to be realised through DIV activation of *AmMYBML1* expression.

The B-function floral homeotic genes, *DEF* and *GLO*, also contribute to the control of *AmMYBML1* expression, as

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evidenced by the reduction in transcript levels of the gene in flowers from plants homozygous for weak *def* alleles (Fig. 7E), and by the reduction of those cellular features promoted by *AmMYBML1* in the ventral petal of flowers of *def101* (Fig. 7A-D). Traditionally, DEF and GLO are defined as functioning in the determination of organ identity, and consequently it might be that their regulation of *AmMYBML1* expression is indirect. However the inhibition of *AmMYBML1* promoter activity by the combination of DEF and GLO in protoplast transfection assays (see Fig. S2 in the supplementary material) suggests that they may interact directly with the promoter. The absence of transcriptional activation of the *AmMYBML1* promoter by DIV/DEF/GLO in any combination suggests that there are additional components in the transcriptional activation complex. Potential candidates are the *SEPALLATA*-like genes and *SQUAMOSA* encoding MADS box transcription factors known to form ternary complexes with DEF and GLO, and to be essential for their transcriptional regulatory activity (Egea-Cortines et al., 1999; Gutierrez-Cortines and Davies, 2000).

Evolution of *AmMYBML1* function

Phylogenetic analysis suggests *AmMYBML1* to be the product of a relatively recent duplication of an ancestral subgroup 9 gene. The specialised function of *AmMYBML1* may have evolved at the same time as the zygomorphic floral structure of *Antirrhinum* and other Scrophulariacae. Its activity contributes to the adaptations of the *Antirrhinum* flower to specialised pollination by bees. Our data suggest that the divergence of developmental function between *MIXTA* and *AmMYBML1* is largely the result of differences in the expression patterns of the two genes although there are also subtle differences in the biochemical functions of the proteins they encode. Gene duplication and specialisation of the *AmMYBML1* expression pattern may have been the primary changes underpinning the evolution of the specialised cell types to provide some of the features of *Antirrhinum* flowers that adapt them to bee pollination. The AmMYBML1 protein directs the formation of three different specialised cell types in the ventral petal of *Antirrhinum*. The conical epidermal cells on the hinge epidermis may play a role in directing pollinators towards their nectar reward. The hinge structure created by the thickening and folding of the mesophyll acts as both a landing platform for bees and a deterrent to potential nectar robbers, as it requires an animal with the weight of a bumblebee to open the corolla at the hinge. The trichomes in the throat of the corolla tube, on the ventral petal, collect pollen from the surface of pollinating bees and redistribute it to the stigmatic surface for fertilisation. The coordination of development of these three different specialised cell forms results in the morphogenesis of a structurally complex petal, which is the function of this one transcription factor, AmMYBML1. The divergence of *AmMYBML1* function from that of *MIXTA* and other *R2R3 MYB* genes of subgroup 9 in *A. majus* illustrates one of the ways specialised floral forms have been achieved in plants.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/2/359/DC1

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