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RESEARCH PAPER

A truncated MYB transcription factor from *Antirrhinum majus* regulates epidermal cell outgrowth

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Abstract

Plant *MYB* genes can be divided into subgroups on the basis of additional conserved regions of sequence. In some cases, genes within a subgroup share similarities in function, as well as sequence. The functions of three proteins in subgroup 9 have been described, with AmMYBMX regulating the differentiation of conical-papillate petal epidermal cells, PhMYB1 involved in extending the growth of these cells, and AmMYBML1 involved in differentiation of several petal epidermal cell types. Here, the isolation of a gene encoding a new member of MYB subgroup 9, AmMYBML3 (*Antirrhinum majus* MYB MIXTA-LIKE 3) is described, which contains the defining regions of conserved sequence but is lacking the majority of the C-terminus, including the amphipathic α-helix presumed necessary for transcriptional activation. *AmMYBML3* is expressed in all aerial organs, but its expression is restricted to outgrowing epidermal cells, including trichomes, stigmatic papillae, and petal conical-papillate cells. Ectopic expression of *AmMYBML3* in tobacco results in the formation of conical-papillate cells in the usually flat carpel epidermis. These data suggest that this protein is capable of altering epidermal development, thus resulting in cellular outgrowth, despite the missing C-terminus, and may act in conjunction with other transcriptional activators to enhance cellular outgrowth from the epidermis of all aerial organs.

Key words: AmMYBML3, *Antirrhinum*, cellular differentiation, conical-papillate cell, development, epidermis, MYB, transcription factor, trichome.

Introduction

Members of the MYB family of transcription factors contain between one and four repeats of a DNA-binding domain containing about 53 amino acids (Stracke *et al*., 2001). Each repeat encodes three α-helices, with the second and third forming a helix–turn–helix conformation to intercalate into the major groove of the target DNA (Frampton *et al*., 1989; Ogata *et al*., 1994). Once bound to DNA, a MYB protein may function to activate transcription through its C-terminus or through interaction with another protein such as a bHLH transcription factor (Goff *et al*., 1992). Most plant MYB proteins contain just two of the three repeats and are thus referred to as R2R3-MYB factors (Jin and Martin, 1999). In *Arabidopsis thaliana* some 126 R2R3 MYB genes have been identified. The plant MYB transcription factors have been divided into 42 subgroups (plus seven outliers) based on conserved sequence motifs (Kranz *et al*., 2000; Stracke *et al*., 2001; Jiang *et al*., 2004). In some cases it appears that MYB proteins within a subgroup have similar functions, although insufficient proteins have been characterized to date to allow predictions of protein function based on sequence.

Subgroup 9 (or G03, according to different authorities) of the plant MYB transcription factor family contains the MIXTA-LIKE MYBs, defined by a conserved sequence motif downstream of the DNA-binding domain (Kranz *et al*., 1998; Stracke *et al*., 2001; Jiang *et al*., 2004). The functions of only three members of this group have been characterized. *AmMYBMX* (or *MIXTA*) of *Antirrhinum majus* is required for conical-papillate cell formation in the petal epidermis (Noda *et al*., 1994; Glover *et al*., 1998). *PhMYB1* from *Petunia hybrida* has also been shown to play a role in conical-papillate petal cell

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development (Avila *et al*., 1993; van Houwelingen *et al*., 1998). *AmMYBML1* of *A. majus* is involved in conical-papillate petal cell development, petal trichome differentiation, and the formation of the hinge of the ventral petal (Perez-Rodriguez *et al*., 2005). The three known members of subgroup 9 in *A. thaliana* are *AtMYB16*, *AtMYB17*, and *AtMYB106*. To date, mutant phenotypes have not been described for loss of function in any of these *Arabidopsis* genes.

The first member of subgroup 9 to be fully characterized was *AmMYBMX* from *Antirrhinum*. Through analysis of a loss-of-function mutation, caused by transposon insertion in the gene, it was shown that *AmMYBMX* is necessary for the development of conical-papillate cells on the adaxial surface of the petals (Noda *et al*., 1994). The petal cells of the adaxial surface of the *mixta* mutant are flat compared with the wild type. The shape of the wild-type conical-papillate cells optimizes light capture by the pigments, causing the petal to appear deeper in colour (Noda *et al*., 1994). There is evidence to suggest that this specialized shape of the adaxial petal epidermal cells increases the attractiveness of the flower to pollinating bees (Glover and Martin, 1998; Comba *et al*., 2000). *AmMYBMX* expression in *Antirrhinum* is limited to a narrow window of time late in development of the petal epidermis. *In situ* hybridization has confirmed that the expression of *AmMYBMX* is confined to adaxial petal epidermal cells (Glover *et al*., 1998).

The ectopic expression of the *AmMYBMX* gene in tobacco resulted in the formation of both multicellular trichomes and conical-papillate cells (Glover *et al*., 1998; Payne *et al*., 1999). These data indicate that these two specialized cell forms use parts of a common developmental programme and suggest that a second gene may be responsible for the formation of multicellular trichomes. Two further subgroup 9 genes were isolated from *Antirrhinum*, using an *AmMYBMX* probe on a petal cDNA library. One of these genes, *AmMYBML1* (*Antirrhinum majus MYB MIXTA LIKE 1*), has been shown to play roles in the development of three distinct specialized petal cell types. AmMYBML1 is involved in epidermal conical cell development in the ventral petal hinge region, in the elaboration of the mesophyll below this epidermis to generate the landing platform of the *Antirrhinum* flower, and in the development of trichomes in the throat of the flower (Perez-Rodriguez *et al*., 2005). The function of *AmMYBML2* (*Antirrhinum majus MYB MIXTA LIKE 2*) is still under analysis (Martin *et al*., 2002). These data indicate that a family of closely related *MYB* genes, which are likely to specify different cell forms with different functions through expression at different developmental stages, exists in *Antirrhinum*.

Here, the isolation and characterization of a fourth *Antirrhinum* gene from MYB subgroup 9 is described. *AmMYBML3* (*Antirrhinum majus MYB MIXTA LIKE 3*) differs from the three genes previously described in lacking a putative activation domain in its extremely reduced C-terminus. Semi-quantitative RT-PCR indicates that the gene is expressed in all aerial organs of the plant, most strongly in young flowers, while promoter–GUS analysis and *in situ* hybridization indicate that this expression is limited to outgrowing epidermal cells (trichomes, stigmatic papillae, and petal conical-papillate cells) in these organs. Ectopic expression of *AmMYBML3* in tobacco results in the conversion of the usually flattened carpel epidermal cells to a conical-papillate form, suggesting that AmMYBML3 may play a weak but active role in the outgrowth of specialized epidermal cells of *Antirrhinum*.

Materials and methods

Gene isolation

An *Antirrhinum* genomic library in bacteriophage λEMBL4 was screened at very high stringency using a full-length cDNA of *AmMYBML1* as a probe. Five independent plaques hybridized with the probe. One contained a genomic clone of *AmMYBML1*, one hybridized less strongly to the probe and was not analysed further, while three contained the same previously unknown *MYB* gene, which was named *AmMYBML3*. Following restriction digestion of phage DNA containing this unknown gene, the restriction fragments which bound *AmMYBML1* probe on a Southern blot were isolated from a gel, ligated into pCR2.1, and sequenced.

To obtain a cDNA clone, RNA was extracted from young *Antirrhinum* shoots using lithium chloride precipitation (Glover *et al*., 1998). First-strand cDNA was synthesized using the Amersham Pharmacia Biotech First Strand cDNA synthesis kit and a B26 primer for 3′ RACE (Frohman *et al*., 1988). The *AmMYBML3* cDNA was amplified using a gene-specific 5′ primer designed to the genomic sequence and a 3′ RACE primer which binds the adaptor on cDNA ends. The PCR product was cloned into pCR2.1 and sequenced.

Semi-quantitative RT-PCR

RNA was extracted from different plant organs as described above, treated with DNase I to remove contaminating genomic DNA, and cDNA pools synthesized from each sample. Ubiquitin primers were used to establish the linear phase of the PCR (25 cycles) and to ensure the use of equal amounts of cDNA from each tissue. Ubiquitin primers generate multiple bands in *Antirrhinum*, as they anneal to multiple repeats within the ubiquitin genes. However, the bands are consistent between samples. PCR products generated using *AmMYBML3*-specific primers were separated on an agarose gel.

In situ *hybridization*

In situ hybridizations were performed by applying digoxigenin-labelled antisense *AmMYBMML3* RNA probes to 8 μm sections cut from wax-embedded samples (Hofer *et al*., 1997). Samples were counterstained with 0.1% (w/v) Calcofluor and viewed under epi-fluorescent illumination at low magnification. A sense RNA probe was used as a control.

Promoter::GUS construct

Following sequencing of bacteriophage fragments containing the *AmMYBML3* genomic clone, primers were designed to amplify the

complete promoter sequence (1665 bp) with *Bam*HI and *Xba*I restriction sites on the end. This PCR product was ligated into a *Bam*HI/*Xba*I-digested pGREEN-GUS binary vector (Hellens *et al*., 2000) to make construct pTRY27.

Construct for ectopic expression in tobacco

The sense construct pTRY30 for ectopic expression of *AmMYBML3* in tobacco was created by excising an 813 bp *Xho*I fragment containing the whole *AmMYBML3* cDNA from pCR2.1 and inserting it into the *Sal*I site of a modified pBIN19 (Bevan, 1984) containing the double CaMV 35S promoter and CaMV poly(A) terminator cassette from pJIT60 (Guerineau and Mullineaux, 1993).

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*. (1985). RNA was extracted as described above and transgene expression confirmed by northern analysis, using the method of Martin *et al*. (1985).

GUS assays

Tissue samples were immersed in GUS staining solution (100 µl 40 mg ml^{-1} X-Gluc in 8 ml staining buffer [100 mM NaH_2PO_4, 10 mM Na_2EDTA, 0.5 mM K ferrocyanide (MW 422.4), 0.5 mM K ferricyanide (MW 329.3), 0.1% Triton X-100, adjusted to pH 7.0 with NaOH)] and incubated at 37 °C for 3 h. Samples were destained with 70% ethanol and incubated at 37 °C for 30 min, twice. The samples were then transferred to a microscope slide and examined using a dissecting and light microscope.

Scanning electron microscopy

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

Results

Analysis of AmMYBML3 *sequence*

The *AmMYBML3* genomic clone was 7263 bp in length. The clone consisted of promoter sequence, three exons, two introns, and some 3′ sequence. The first two exons of *AmMYBML3* are identical in length to those of all other *Antirrhinum* and *Arabidopsis MIXTA-LIKE* genes. However, the sizes of the remaining exon and the two introns differ from those of other genes.

The cDNA of *AmMYBML3* was isolated using RT-PCR and the sequence deposited in GenBank (accession number AY661654). Alignment of this cDNA sequence with the sequences of the other subgroup 9 genes indicated a high level of sequence conservation in the 5′ region, but a highly divergent 3′ sequence. Similarly, comparison of the proteins indicates a highly conserved N-terminus but a divergent C-terminus. The conservation of the N-terminus of MYB proteins is consistent with most other R2R3 MYBs and it can be seen that between previously identified *MIXTA-LIKE* genes and *AmMYBML3* there is a very high level of conservation in the first 170 amino acids (aa) of the protein products (Fig. 1). In these first 170 aa, on the few occasions when the residue at a position differs between two members of the subgroup, it is usually the case that both amino acids are functionally similar.

The C-terminus, however, varies quite significantly between the proteins (Fig. 1) and it is possible that this may be indicative of different protein–protein interactions, thus resulting in different regulatory effects. AmMYBML3 is much shorter than the other subgroup 9 proteins, being only 219 aa in length compared with others which are over 300 aa. This reduction in size is due to the absence of the majority of the C-terminus of the other proteins, although the final 26 aa of AmMYBML3 do show conservation with those of AmMYBMX, AmMYBML1, AtMYB16, and AtMYB106. This unusual reduction in protein length was confirmed both by 3′ RACE (which amplified the same cDNA from three separate plants) and by careful analysis of the 3′ region of the genomic clone, which contained no further open reading frames suggestive of a second stop codon. Noda *et al*. (1994) suggested that AmMYBMX was a transcriptional activator because of the identification of a potential amphipathic α-helix, but the C-terminus of AmMYBML3 does not contain an apparent amphipathic α-helix. This may have consequences for its ability to cause transactivation.

Production of a phylogenetic tree of subgroup 9 proteins indicates that AmMYBML3 is most similar to AtMYB16 and AtMYB106 of *Arabidopsis* (Fig. 2). This suggests that at least one gene of this group existed prior to the divergence of *Arabidopsis* and *Antirrhinum*, and perhaps significantly earlier. AmMYBML2 also clusters with these proteins, and may represent an ancestral form of AmMYBML3, prior to the loss of the C-terminus.

Expression analysis of AmMYBML3

Semi-quantitative RT-PCR was performed on a range of different organs. *AmMYBML3* transcript was found in all organs tested; floral buds, petals, sepals, stamens, carpels, inflorescence stems, leaves, hypocotyls, and the vegetative stem (Fig. 3). The lowest expression was seen in the carpel, but no difficulty was found in amplifying *AmMYBML3* from any organ tested.

Analysis of AmMYBML3 *promoter::GUS fusions in transgenic tobacco*

An *AmMYBML3* 1665 bp promoter fragment was isolated from the genomic clone and the sequence deposited in GenBank (accession number DQ228865). This fragment was used to drive GUS expression in transgenic tobacco plants. GUS expression was found in all aerial organs of the plant, but was mainly restricted to outgrowing epidermal cells (Fig. 4). This included the glandular trichomes found

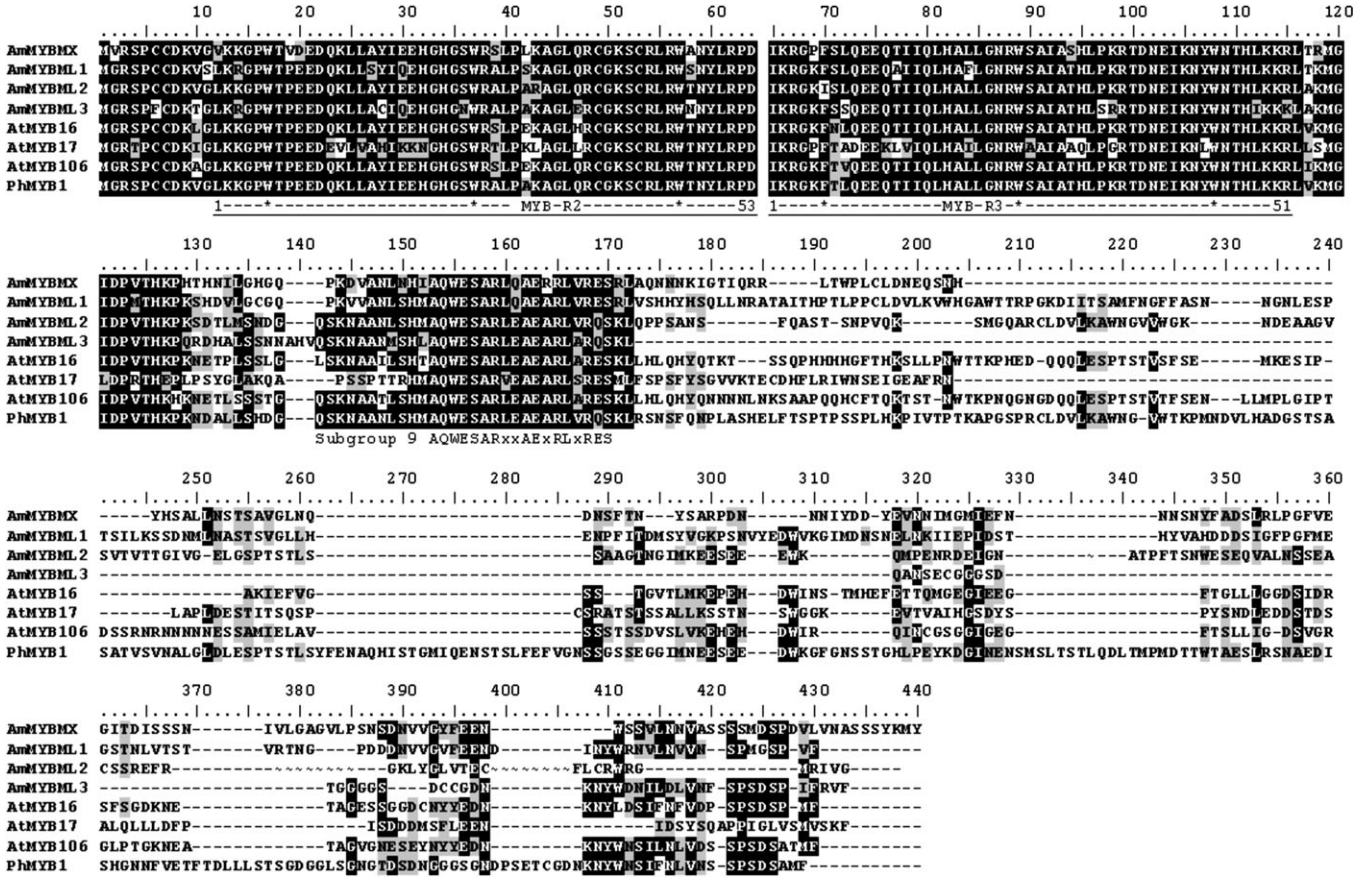

Fig. 1. Alignment of amino acid sequences of AtMYB16, AtMYB17, AtMYB106, AmMYBMX, AmMYBML1, AmMYBML2, AmMYBML3, and PhMYB1. This alignment was created using Clustal W software. It is clear that a substantial region of the C-terminus is missing from AmMYBML3, but that the final 26 amino acids of AmMYBML3 share some conservation with those of the other seven proteins.

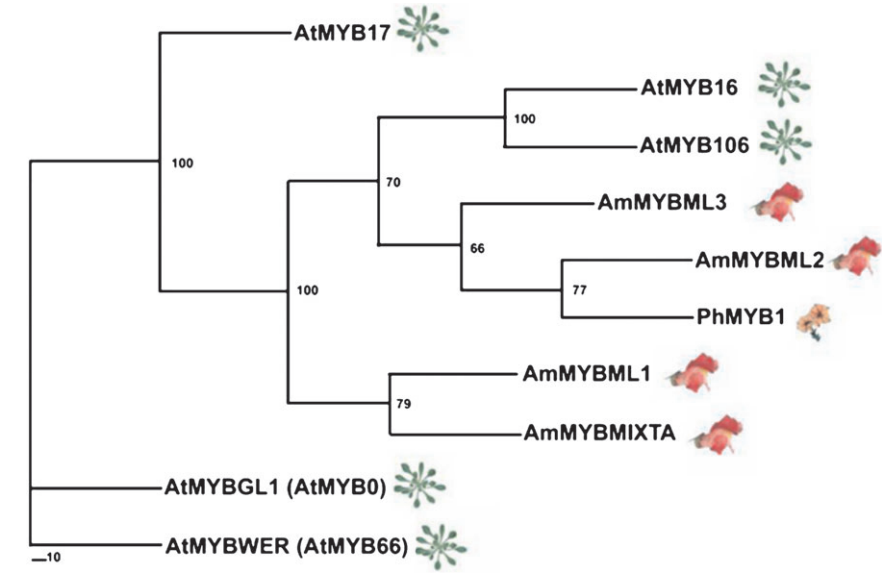

Fig. 2. Phylogenetic tree showing the similarity in sequence between the subgroup 9 *MYB* genes, including *AmMYBML3*.

over the vegetative surfaces and petals of the plant, and also the conical papillate cells of the petal and the stigmatic papillae. Stigmatic staining was particularly strong.

In contrast, *AmMYBML3* is expressed in leaf trichomes during their active development, but the transcript disappears once the trichomes are fully mature. In the leaves,

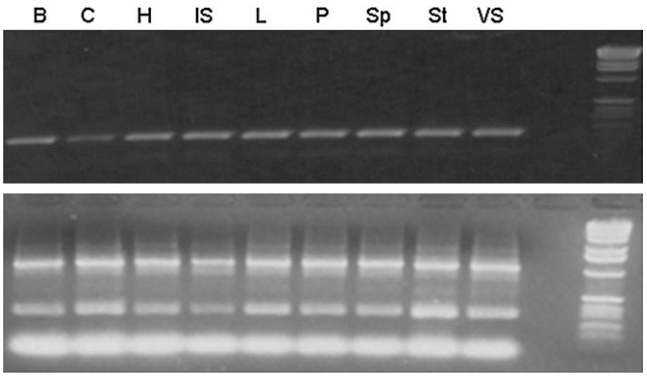

Fig. 3. Semi-quantitative RT-PCR showing *AmMYBML3* expression (top) and *ubiquitin* expression as a control (bottom). B, floral bud; C, carpels; H, hypocotyls; IS, inflorescence stems; L, leaves; P, petals; Sp, sepals; St, stamens; VS, vegetative stems. The multiple bands in the *ubiquitin* control are usual, and are consistent between samples and reactions.

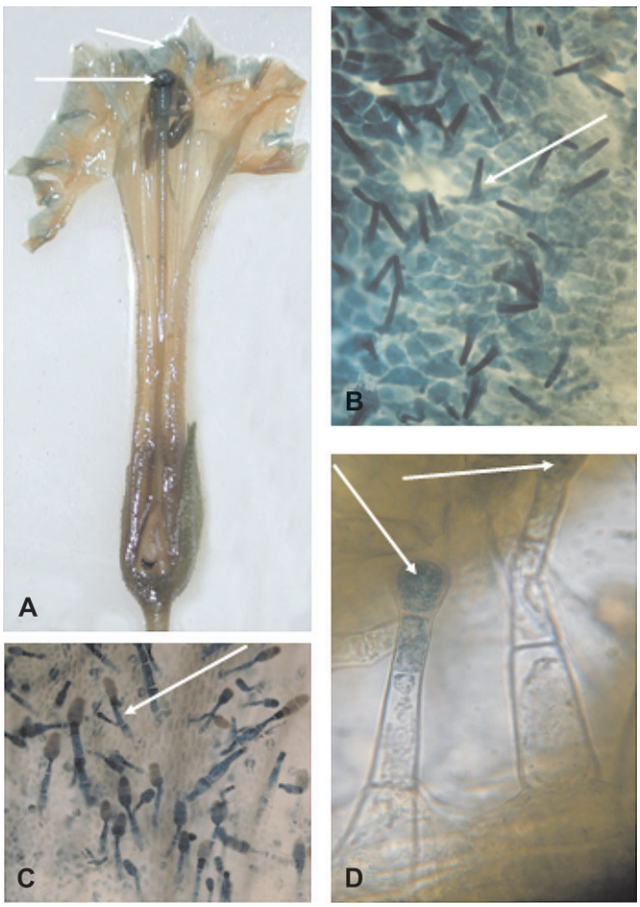

Fig. 4. Expression of *AmMYBML3::GUS* in tobacco. (A) Cross-section of flower, showing expression in the conical-papillate cells around the edge of the petals and in the stigmatic tissue. (B) Stigmatic tissue, showing expression in papillae. (C) Petal epidermis, showing expression in trichomes. (D) Leaf trichome expression. Arrows point to strong GUS expression.

trichome development is at different stages in different places, with the youngest trichomes at the proximal end still developing while the trichomes at the distal end are fully mature (Hülskamp *et al*., 1994; Turner *et al*., 2000). GUS expression in leaf trichomes could still be visualized towards the proximal end of the leaf, but was less clear in trichomes of the distal end. This suggests that *AmMYBML3* is involved in the development of the trichomes, but once they are fully developed its expression ceases.

To confirm that these results were not artefacts, the GUS vector without a promoter was transformed into tobacco but no GUS expression was detected in transformed plants. A 35S promoter–β-galactosidase-driven construct was used to provide a positive control for GUS staining.

In situ *hybridization*

To confirm the results of the promoter::GUS analysis, which identified particularly strong expression in stigmatic papillae, *in situ* hybridization was conducted using an *AmMYBML3* probe with sections of stigmatic tissue. Expression was found in the papillae themselves, but also in several cell layers beneath this surface layer (Fig. 5A). This broader distribution of *AmMYBML3* expression may account for the strong staining observed with GUS. This was the only indication of *AmMYBML3* expression in any non-epidermal tissue.

Phenotype of Nicotiana tabacum *plants ectopically expressing* AmMYBML3

A construct (pTRY30) containing the *AmMYBML3* cDNA under the control of two copies of the CaMV 35S promoter in pBin19 was used for transformation of tobacco. Expression of *AmMYBML3* in regenerated tobacco plants was confirmed by northern analysis. Detectable levels of *AmMYBML3* expression in leaves were identified in 13 out of 16 lines and these 13 were used in phenotypic analysis.

The phenotype of the *AmMYBML3* transformed plants was extremely similar to that of wild-type tobacco. There was no observable difference in roots, leaves, stems, sepals, petals, or stamens at any stage of development, whether in epidermal development or internal structure (determined by freeze fractures). The aerial parts of the plant appeared to have wild-type proportions of both types of trichomes, stomata, and pavement cells. The leaves appeared wild type in terms of colour, size, and trichome numbers on adaxial and abaxial surfaces, unlike the leaves of tobacco ectopically expressing *AmMYBMX*, which were reduced in size, paler in colour, and had rolled-in leaf margins (Glover *et al*., 1998). Essentially, the transgenic tobacco expressing *AmMYBML3* appeared wild type for all characteristics except for the shape of the epidermal cells of the carpel tissue, surrounding the ovary, which were transformed from irregularly shaped flat epidermal cells to more regularly shaped conical-papillate cells

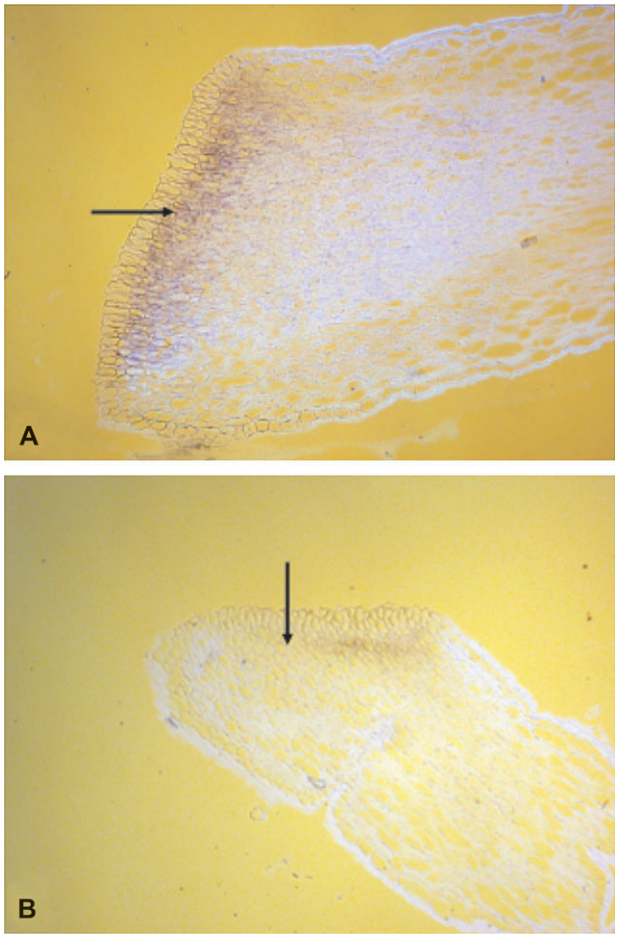

Fig. 5. *In situ* hybridization of *AmMYBML3* in *Antirrhinum* stigmatic tissue. (A) Mature stigma, with extensive staining visible in stigmatic papillae and several cell layers below the epidermis. (B) Sense control, with much more limited hybridization in stigmatic tissues. Arrows point to the region where the hybridization is stronger with the antisense probe.

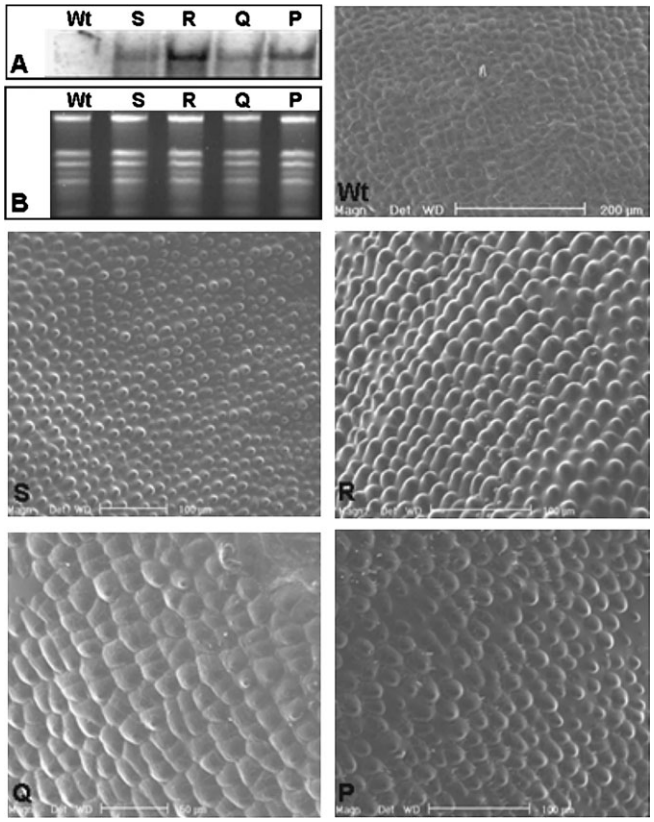

Fig. 6. Phenotypic effect of ectopic *AmMYBML3* expression in tobacco. (A) Northern blot showing expression of *AmMYBML3* in wild-type control (left) and four transgenic tobacco lines (S, R, Q, and P, left to right). (B) Ethidium bromide staining of the same RNA samples to show equal loading. The remaining five panels show SEMs of carpel epidermis from the same five plants, labelled Wt, S, R, Q, and P.

(Fig. 6). This subtle phenotype resulting from *AmMYBML3* expression was only visible using SEM. Analysis of the carpel epidermis of many wild-type tobacco plants, for comparison with a number of transgenic lines, has never resulted in the identification of such cellular outgrowths, from which it is concluded that *AmMYBML3* expression is responsible for the phenotype observed here (Glover *et al*., 1998; Martin *et al*., 2002; Perez-Rodriguez *et al*., 2005).

The severity of this phenotype varied, with some plants having only a few of the epidermal cells converted to conical-papillate cells (e.g. plant Q, Fig. 6). The strongest phenotypes were shown by plants which had all carpel epidermal cells converted to the conical-papillate form (e.g. plant S, Fig. 6). No correlation was found between level of *AmMYBML3* expression (as measured from transcript abundance in northern blots) and severity of phenotype. As Fig. 6 shows, some plants with very high *AmMYBML3* expression levels (such as plant R) had only a gentle rounding of carpel epidermal cells. Some plants with lower expression levels, such as line Q, had outgrowth of only a few epidermal cells, while other plants with similar expression levels (such as S) had quite extreme outgrowth of all carpel epidermal cells.

Effect of ectopic AmMYBML3 *expression on plants ectopically expressing* AmMYBML1 *or* AmMYBMX

To investigate the possibility that AmMYBML3 acts as either an enhancer or repressor of the activity of related MYB transcription factors, crosses were made between the transgenic plants described above and plants ectopically expressing *AmMYBMX* (Glover *et al*., 1998) or *AmMYBML1* (Perez-Rodriguez *et al*., 2005). Progeny containing both transgenes were selected by PCR, and expression of both transgenes confirmed by northern analysis. Ectopic expression of *AmMYBMX* resulted in transformation of almost all aerial epidermal cells to the conical fate, with production of excess trichomes on many organs (Glover *et al*., 1998). Introduction of the *AmMYBML3* transgene into this background had no visible effect, either to enhance or to repress the phenotype.

Similarly, ectopic expression of *AmMYBML1* resulted in transformation of carpel epidermal cells to steep cones or trichomes (Perez-Rodriguez *et al*., 2005), but *AmMYBML3* had no effect on these plants, failing either to enhance or to reduce the carpel phenotype and resulting in no novel phenotype on any other organ (Fig. 7). The plants expressing both *AmMYBML3* and *AmMYBML1* were indistinguishable from those expressing *AmMYBML1* alone, both by eye and under the scanning electron microscope.

Discussion

The AmMYBML3 protein is a member of MYB subgroup 9 with a reduced C terminal region

The classification of *A. thaliana MYB* genes was based on conserved domains within the protein products. These groupings may sometimes provide information about gene function, as it has been shown that several genes that cluster together have conserved functions. These include *AtMYB91* (*AS1*) and *AmMYBPHAN*, which group together but fall outside the designated subgroups. Both negatively regulate *KNOX* (*KNOTTED*) expression in organ primordia (Byrne *et al*., 2000). *AtMYB0* (*GL1*) and *AtMYB66* (*WER*) of subgroup 15 (or G07) can functionally complement each other, but display different functions due to their different expression patterns (Lee and Schiefelbein, 2001). *Production of Anthocyanin Pigment 1* and *2* (*PAP1*/*AtMYB75* and *PAP2*/*AtMYB90*) of *Arabidopsis* and *Anthocyanin 2* (*An2*) of *Petunia* can be grouped into subgroup 6 (or N09) and have been shown to control anthocyanin biosynthesis (Quattrocchio *et al*., 1999; Borevitz *et al*., 2000).

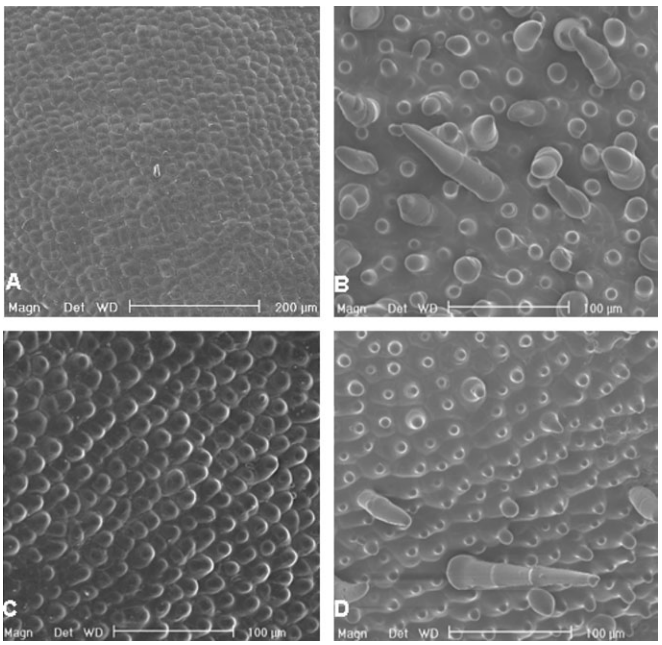

Fig. 7. Effect of combining ectopic expression of *AmMYBML1* and *AmMYBML3*. (A) Carpel epidermis of wild-type plant. (B) Carpel epidermis of plant ectopically expressing *AmMYBML1*. (C) Carpel epidermis of plant ectopically expressing *AmMYBML3*. (D) Carpel epidermis of plant ectopically expressing both transgenes.

Stracke *et al*. (2001) placed *AtMYB16*, *AtMYB17*, *AtMYB106*, and *AmMYBMX* into subgroup 9 based on a conserved domain (AQWESARxxAExRLxRES) in their protein products. Within these MYB proteins this domain is the next most highly conserved domain after the MYB repeats.

The genomic and cDNA sequences of *AmMYBML3* confirm that it is a member of subgroup 9. The defining conserved domain is present (aa 142–172, Fig. 1). The first two exons of the genomic clone are identical in size to those of the other *Antirrhinum* and *Arabidopsis* subgroup 9 genes. Phylogenetic trees based on amino acid sequence similarity confirm that AmMYBML3 is most similar to AmMYBML2 and to AtMYB16 and AtMYB106 (Fig. 2), indicating an ancestry before the divergence of the lineages which contain *Antirrhinum* and *Arabidopsis* (the Euasterids and the Eurosids, respectively). However, the C-terminal amphipathic α-helix, presumed to be necessary for transcriptional activation of target genes, is missing from the protein product. This suggests that the protein could function as a dominant negative, a suggestion used to explain the dominant negative C1-I allele of maize which negatively regulates anthocyanin production in kernels (Edwards *et al*., 2001; Singer *et al*., 1998; Paz-Ares *et al*., 1990). DNA-binding proteins with transcriptional activation domains usually initiate or enhance transcriptional initiation from specific promoters. However, DNA-binding proteins without transcriptional activation domains may function as competitors, binding promoter sequences without the ability to activate transcription. Thus the presence of such a dominant negative in a cell as well as a transcriptional activator may result in less mRNA of the target gene being produced than if only the transcriptional activator were present. Such systems may be used by plants to regulate the precise level of expression of transcription factor target genes. Moyano *et al*. (1996) showed that two MYB transcription factors of *A. majus* (AmMYB305 and AmMYB340) bound the promoters of the same genes encoding enzymes of flavonoid synthesis, but had differential transcriptional activation activity. As a result, variations in the expression levels of the two transcription factors with respect to each other could precisely regulate the level of target gene expression. A similar interaction has been postulated between AtMYB71 and AtMYB79, as AtMYB79 is very similar in sequence to AtMYB71, but with a truncated activation domain (Kranz *et al*., 1998).

While the conventional explanation for the function of a DNA-binding protein without a transcriptional activation

domain is that it functions as a dominant negative, it is also possible that such proteins can enhance transcriptional activation, through interactions with other transcription factors. By analogy with proteins in other MYB subgroups with conserved functions, it is likely that AmMYBML3 plays a role, either negative or positive, in the regulation of epidermal cell outgrowth.

AmMYBML3 *is expressed in outgrowing cells of all aerial organs*

Semi-quantitative RT-PCR and promoter::GUS analysis both demonstrated that *AmMYBML3* is expressed in all aerial tissues. These data contrast with the expression pattern of *AmMYBMX*, which has been shown to be expressed only in the adaxial epidermal cells of *Antirrhinum* petals (Glover *et al*., 1998). In all aerial tissues of tobacco this expression was restricted to epidermal cells, and indeed to epidermal cells which had adopted a specialized cell fate through outgrowth. This expression of *AmMYBML3* in epidermal outgrowths, such as trichomes, conical-papillate petal cells, and stigmatic papillae, suggests that the protein product has a function in the development of these specialized cell types. However, this function is apparently less restricted than that of either *AmMYBMX* or *PhMYB1*, both of which are only involved in the development of petal conical-papillate cells (Noda *et al*., 1994; van Houwelingen *et al*., 1998).

Based on the reduced C-terminal domain of AmMYBML3 it is possible that the protein functions as either a negative or a positive regulator of transcription. If the protein acts as a negative regulator, *AmMYBML3* expression may be triggered early in tissues which do not have many outgrowing cells, but in tissues which have many outgrowths its expression could be late, so stopping continued outgrowth. In this case the role of AmMYBML3 may be to inhibit cellular expansion or division. Alternatively, if the protein functions as a weak activator of cell expansion it could be required in many cell types to enhance their outgrowth in response to other, stronger, transcriptional activators.

Ectopic expression of AmMYBML3 *causes the outgrowth of tobacco carpel epidermal cells*

Ectopic expression of *AmMYBML3* in tobacco caused the outgrowth of carpel epidermal cells, so that, in extreme cases, they resembled the conical-papillate cells normally found on the petal epidermis (Fig. 6). No effect was observed on any other organ. This phenotype was much more restricted than that obtained from ectopic expression of *AmMYBMX* or *AmMYBML1* in tobacco. Glover *et al.* (1998) showed that ectopic *AmMYBMX* expression resulted in the development of conical-papillate cells on all aerial epidermal surfaces, along with an increase in multicellular trichome density in many organs. Perez-Rodriguez *et al*. (2005) showed that ectopic *AmMYBML1* expression resulted in the development of conical-papillate cells and trichomes on carpels of transgenic tobacco. It is apparent from these data that AmMYBML3 is a less potent activator of cellular differentiation than the closely related proteins AmMYBMX and AmMYBML1. This may be a reflection of the reduction of the C-terminal domain of AmMYBML3.

Restriction of the phenotypic consequences of *AmMYBML3* expression to the carpel epidermis, the same tissue which responded to *AmMYBML1* expression, suggests that AmMYBML3 activity is limited by similar factors to those that limit AmMYBML1 (Perez-Rodriguez *et al*., 2005). Ectopic expression of *AmMYBML2* from the same constitutive promoter also results in cellular outgrowth restricted to a subset of the floral organs (Cathie Martin, personal communication). The restriction of the effects of overexpression of all three *MIXTA*-like genes implies that carpel tissue contains some other factor which allows the activity of these proteins to result in cellular outgrowth. Many MYB transcription factors are known to interact physically with bHLH transcription factors (Ramsey and Glover, 2005). For example, the *Arabidopsis* MYB GLABROUS1 interacts with the bHLH protein GL3 to induce trichome development (Payne *et al*., 2000). However, the subgroup 9 proteins do not contain the consensus motif necessary for physical interaction with a bHLH protein (Serna and Martin, 2006), making interaction of AmMYBML3 with a bHLH protein which is present only in carpel epidermis an unlikely scenario. An explanation involving the need for a partner transcription factor, perhaps of another family, is attractive because it would also account for the apparent ability of AmMYBML3, without a transcriptional activation domain, to cause cellular outgrowth. Physical interaction with a protein with such an activation domain would allow DNA binding by AmMYBML3 to result in transcriptional activation of target genes.

It is also apparent from these data that AmMYBML3 can function as a positive regulator of epidermal cell outgrowth, and does not appear, at least in a heterologous system, to regulate the growth of trichomes or other epidermal cells negatively. No reduction in trichome density, size, or cell number was observed in the transgenic tobacco plants. This suggests that the reduced C-terminus is sufficient either to activate low levels of transcription of genes involved in cellular outgrowth, or to interact with other transcriptional activators and to enhance their activity.

Any interaction between AmMYBML3 and other transcription factors does not seem to involve AmMYBMX or AmMYBML1. Combination of ectopic expression of pairs of these genes in transgenic tobacco plants resulted in phenotypes indistinguishable from that of the parent with the strongest phenotype (Fig. 7). AmMYBML3 was

unable to repress the activity of AmMYBMX or AmMYBML1, but also failed to enhance the phenotypes observed. It is possible that AmMYBML3 interacts with AmMYBML2, to which it is most similar in sequence. It is also possible that AmMYBML3 operates through interactions with less closely related proteins.

Conclusions

The truncated AmMYBML3 protein is sufficient to cause outgrowth of carpel epidermal cells in tobacco, but not to activate cellular differentiation in any other tissue. However, it is expressed in all organs of *Antirrhinum*, in a pattern restricted to outgrowing cells. These data suggest that AmMYBML3 functions as a very weak enhancer of the cellular differentiation induced by AmMYBMX in petal epidermal cells, and as a weak enhancer of cellular differentiation of other epidermal outgrowths regulated by other, as yet unknown, transcriptional activators. Likely candidates for the activation of trichome and stigmatic papillae outgrowth include the previously identified subgroup 9 protein AmMYBML2. Further investigation of this protein, along with analysis of the *Arabidopsis* members of the subgroup, should help to resolve the functions of this closely related set of transcriptional regulators.

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