Development 125, 3497-3508 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV3884

Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor

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Accepted 17 June; published on WWW 6 August 1998

SUMMARY

A MYB-related transcription factor (MIXTA) that controls development of conical cell form is expressed only in the inner epidermis of *Antirrhinum* petals. Expression of this gene throughout transgenic tobacco plants leads to excess numbers of multicellular trichomes on leaves and floral organs as well as the novel production of conical cells on leaves. These data indicate that conical cells and trichomes are produced by a common developmental pathway. The timing of *MIXTA* expression suggests that the choice between the cell types depends on the competence for cell division at the time at which the controlling gene is expressed. Duplication of genes and their association with different *cis*-regulatory regions may therefore result in the specification of novel plant cell types.

Key words: MIXTA, Trichome, *Nicotianum tabacum*, Cell differentiation, Transcription factor

INTRODUCTION

Plants are considered to be anatomically simple and composed of relatively few morphologically distinct cell types (Goldberg, 1988). The understanding of epidermal cell determination is greatest for trichomes (hairs) in *Arabidopsis*. These consist of a single epidermal cell which branches towards its apex. Specification of trichome development is governed by the activity of at least three genes, *GLABROUS1 (GL1)*, *TRANSPARENT TESTA GLABRA (TTG)* and *TRIPTYCHON (TRY)* (Hülskamp et al., 1994). While *GL1* and *TTG* control the commitment to trichome formation, the *TRY* gene product functions after cell commitment but before cell morphogenesis to limit the number of cells responding to the initial signal. A semidominant, quantitative trait locus, *REDUCED TRICHOME NUMBER (RTN)*, also influences trichome density and may affect the time period during which trichomes are initiated (Larkin et al., 1996). The activities of *TTG/GL1* and *TRY* are also thought to signal to adjacent epidermal cells to inhibit their development as trichomes (Larkin et al., 1994, 1996, 1997). Trichome formation is under somewhat different control on the adaxial (upper) and abaxial (lower) surfaces of the *Arabidopsis* leaf, because the cells of these leaf surfaces differ in their sensitivity to the trichome inducer gibberellic acid (Chien and Sussex, 1996; Telfer et al., 1997; Silverstone et al., 1997). Additional factors distinguish trichome formation on adaxial and abaxial leaf surfaces: trichomes form on only the adaxial surface of the first two rosette leaves of *Arabidopsis*, and trichome formation is selectively suppressed on the adaxial surface of cauline leaves, which is a developmental feature linked to floral induction (Telfer et al., 1997).

Trichomes of most other flowering plants include multicellular hairs, and little is known of the developmental genetics governing their specification. In tobacco, both simple and glandular multicellular trichomes are found. Glandular trichomes can be further subdivided into two distinct types: those with long stalks that are believed to be the site of diterpenoid production and secretion, and those with short stalks which exude nicotine and which may serve as hydathodes (Akers et al., 1978; Severson et al., 1985; Meyberg et al., 1991; Nielsen et al., 1991). Mutations affecting the morphogenesis and density of the long-stalked glandular trichomes have been described by Johnson et al. (1988). Those affecting their morphogenesis do not affect the formation of the shorter hydathodes, implying that discrete developmental programmes control the formation of these two types of glandular trichome (Delon and Schiltz, 1979; Roberts et al., 1981; Burk et al., 1982).

Thus there is circumstantial evidence that different types of trichome are specified by independent developmental programmes within a single plant species, and consequently the different trichome types in different species may be specified by distinct developmental programmes. Ectopic expression of the maize basic helix-loop-helix (bHLH) transcription factor LC (which regulates anthocyanin pigment production in maize) results in ectopic trichome production in *Arabidopsis*, indicating a role for an unidentified bHLH protein in trichome specification (Lloyd et al., 1992). However, *LC* has no effect on trichome formation in species with multicellular

trichomes such as tobacco or tomato or, indeed, in maize (Lloyd et al., 1992; Mooney et al., 1995), emphasising the differences in the specification processes for multicellular and unicellular types of trichome (Esau, 1977).

Stomata, which constitute the other predominant specialised cell type in shoot epidermis, form from an epidermal initial cell termed the stomatal meristemoid (Bünning, 1956). A series of divisions of the meristemoid provide the guard mother cell flanked by adjacent flat epidermal cells, termed subsidiary cells. The guard cells and pore develop from the guard mother cell. Stomata are spaced evenly over the leaf epidermis although they may be absent from regions over veins (Smith and Watt, 1986; Sylvester et al., 1996) and from some regions defined by underlying mesophyll cells (Rasmussen, 1986; Croxdale et al., 1992). The positioning of new stomatal meristemoids away from the stoma may contribute to patterning. In *Arabidopsis* two genes, *TOO MANY MOUTHS* (*TMM*) and *R*-558, affect stomatal density and may control recruitment of cells to a stomatal lineage. In *Arabidopsis* meristemoids form only after trichome initiation (Yang and Sack, 1995; Larkin et al., 1996, 1997).

Amongst epidermal cells one other clear and localised specialisation is the development of conical shape on the inner epidermis of flower petals; a morphological change distinct enough for it to be used as a marker for petal identity (Simon et al., 1994; Goodrich et al., 1997). The occurrence of specialised conical cells on the petals of 80% of flowering plant species has led to the suggestion that they might serve to attract pollinators by enhancing the amount of light reaching the pigments inside the cells (Kay et al., 1981), and by providing petals with a velvet sheen because of the reflective and refractive properties of the cells (Noda et al., 1994; Gorton and Vogelmann, 1996). An alternative explanation is that the conical cells in petals provide tactile clues to pollinators which can learn to recognise those species-specific cell types associated with successful foraging (Kevan and Lane, 1985).

The formation of conical cells in petals of *Antirrhinum majus* requires the activity of the *MIXTA* gene; in its absence, petal epidermal cells do not develop their conical form (Noda et al., 1994). *MIXTA* encodes a MYB-related transcription factor, and a similar function has been assigned to one particular MYB protein from *Petunia*, PhMYB1 (Avila et al., 1993; Noda et al., 1994; van Houwelingen et al., 1998), which shows very high structural similarity to MIXTA.

Conical cells are restricted to the inner epidermis of petals in most flowering plants and the more familiar types of specialised epidermal cells such as trichomes and stomata are absent from this tissue layer. *MIXTA* (and its functional homologue *PhMYB1*) is the only gene assigned a role in conical cell development.

We are interested in the specification of epidermal cells and, in particular, the role that *MIXTA* plays in controlling conical cell shape. We have investigated this role by ectopic expression of the *MIXTA* gene in transgenic tobacco, showing that it is sufficient to promote conical cell formation in some cells.

Ectopic expression of *MIXTA* also promotes trichome differentiation, showing that multicellular trichomes and conical cells share part of a common developmental pathway. Evidence from transgenic lines suggests that the decision between these alternative developmental routes depends on the time of initiation of cellular outgrowth relative to the overall development of the epidermal tissue. Consequently, while *MIXTA* normally controls the development only of conical cells in petals, other, very similar, MYB-related transcription factors probably promote trichome formation in other epidermal tissues. Commitment to conical cell/trichome formation is also mutually exclusive with commitment of epidermal cells to form stomata. Therefore these transcription factors can influence cellular patterning within the shoot epidermis, through promotion of trichome/conical cell formation and inhibition of stomatal development.

MATERIALS AND METHODS

Plant transformation

A construct of the *MIXTA* cDNA, expressed from the CaMV 35S promoter (with duplicated enhancer sequences) and linked to the CaMV Terminator of pJIT60 (Guerineau and Mullineaux, 1993; Fig. 1A) was cut with *Kpn*I and *Xho*I and inserted between the *Kpn*I and *Sal*I sites of the binary plasmid pBIN19 (Bevan, 1984) to produce the construct pJAM986. As a control to test that the morphogenetic effects of MIXTA operated through transcriptional regulation in tobacco, a modified construct of the *MIXTA* cDNA encoding a truncated protein product was built (*MIXTA*-T) using a *Taq*I fragment (from the *Taq*I site in the polylinker of pBLUESCRIPT which lay at the 5′ end of the cDNA to an intragenic *Taq*I site at position 503 in the cDNA) cloned into the *Acc*I site of pJIT60 to give construct pJAM1177; Fig. 1A. Transformation of *Agrobacterium tumefaciens* was by electroporation (Mattanovich et al., 1989). Tobacco (*Nicotiana tabacum* var. Samsun) was transformed by a modified version of the leaf disk method (Horsch et al., 1985; Martin et al., 1989). Tobacco was used because transformation of *Antirrhinum* is not currently routine; the close relationship between *Antirrhinum* and tobacco meant that tobacco was a reliable host for the analysis of *Antirrhinum* gene function (Martin et al., 1989; Mooney et al., 1995; Tamagnone et al., 1998) and because use of a heterologous host overcame possible complicating sense-suppression by the transgene.

RNA extraction and analysis

RNA was extracted from different organs of *Antirrhinum* and tobacco and RNA gels were run and blotted as described previously (Martin et al.,1985). DNA fragments from the following cDNA clones were labelled by random priming with [^{32}P]dCTP; the *MIXTA* cDNA clone in pBLUESCRIPT (pJAM980), the *PhMYB1* cDNA clone in pBLUESCRIPT (Avila et al., 1993); the *AmMYBML1* cDNA clone in pBLUESCRIPT (pJAM1257), *CYCLIN D3b* and *HISTONE H4* cDNA clones from *Antirrhinum majus* in pBLUESCRIPT (Forbert et al., 1996).

Scanning electron microscopy

Plant tissue was examined under a CamScan mark IV scanning electron microscope with a Hexland cryostage. Cell counts were undertaken from photographs at low magnification (×100).

Sectioning and light microscopy

Tissue was fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). It was postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate and embedded in LR White resin. Sections (0.5 μm) were stained with 0.5% toluidine blue in 0.5% borax and examined with a Nikon Microphot SA microscope.

Analysis of pollen by staining with aniline blue

Stylar tissue of self-pollinated wild-type flowers and non-dehiscent anther heads of transgenic tobacco were fixed in FAA (1:8:1 formalin, 80% ethanol, glacial acetic acid), and stained in 0.1% aniline blue in

0.1 M tribasic potassium phosphate. The slides were examined using dark-field microscopy, illuminated with ultraviolet light at 356 nm, with a Nikon Microphot SA microscope.

In situ hybridisation

RNA in situ hybridization was performed on sections of *Antirrhinum* inflorescences and buds at different developmental stages. The tissue was fixed in formaldehyde and embedded in wax (Jackson et al., 1991). Digoxigenin labelling and in situ hybridisation (Bradley et al., 1993) used a *MIXTA* probe made from plasmid pJAM1155 containing an internal fragment of *MIXTA* cDNA between nucleotides 1 and 503 subcloned into pBLUESCRIPT SK$^+$. The *CYCLIN D3b* vectors were supplied by J. Doonan (Forbert et al., 1996).

Identification of c-DNA clones encoding MIXTA-related proteins

A cDNA library was prepared (from RNA from petals of buds of a range of sizes from a *mixta* mutant line), in λgt10 according to the manufacturer's instructions (Amersham plc UK). Plaques (100,000) from the unamplified library were screened with the DNA fragment from the truncated *MIXTA-T* clone, pJAM1155. Filters were washed at moderate stringency (2× SSC, 0.5% SDS, 65°C) and two showed considerably stronger hybridisation to the probe than the others, reflecting sequence similarity that was confirmed by sequencing. These clones represented a partial and a full length version of a *MIXTA*-related gene, *AmMYBML1*. The sequence of the full length cDNA has been submitted to the EMBL database under accession number AJ006292.

RESULTS

Expression of *MIXTA* in tobacco

MIXTA is necessary for the formation of conical cells in petals of *Antirrhinum majus* (Noda et al., 1994). To test whether *MIXTA* expression is sufficient to promote conical cell formation, the *MIXTA* cDNA was placed under control of the 35S promoter from Cauliflower Mosaic Virus (CaMV) to give high level expression in transgenic tobacco (Fig. 1A). Nine primary transformants expressing *MIXTA* were produced. RNA gel blot analysis revealed that they expressed *MIXTA* at varying levels in mature leaves (Fig. 1C).

Effect of *MIXTA* in tobacco leaves

Scanning electron microscopy showed that compared to epidermal cells of untransformed lines the cells of the leaf epidermis of the transgenic lines were modified in shape. Almost every epidermal cell had a central outgrowth of the outer wall, causing the cells to resemble the conical cells of the petal epidermis (compare Fig. 2A and C). In some cells the outgrowth protruded a considerable distance from the epidermal surface and a cell wall was formed between the parental cell and the cell of the extension (Fig. 2C and E). In some examples, a secondary outgrowth subsequently formed in the parental cell, usually close to the base of the initial outgrowth (Fig. 2E). *MIXTA* is not expressed in leaves of *Antirrhinum* (Noda et al., 1994) nor is the homologous gene in tobacco expressed in leaves, because RNA gel blots probed with the *PhMYB1* gene from the very closely related species *Petunia hybrida* (which has the homologous function to *MIXTA*; van Houwelingen et al., 1998), showed an equivalent transcript in tobacco flowers but not in leaves (Fig. 1B). Ectopic expression of *MIXTA* in tobacco leaves demonstrated that *MIXTA* is not only necessary but also sufficient to direct the morphological transition to form conical cells (in the leaf epidermis).

Effect of *MIXTA* expression on the epidermal cells of floral organs

The inner epidermis of the petal limb was modified in the transgenic lines (Fig. 2D). Appreciable numbers of multicellular trichomes developed among the normal conical cells. These trichomes were of both the simple and the long-stalked glandular type found normally in tobacco. Since trichomes are never observed on the inner epidermis of the

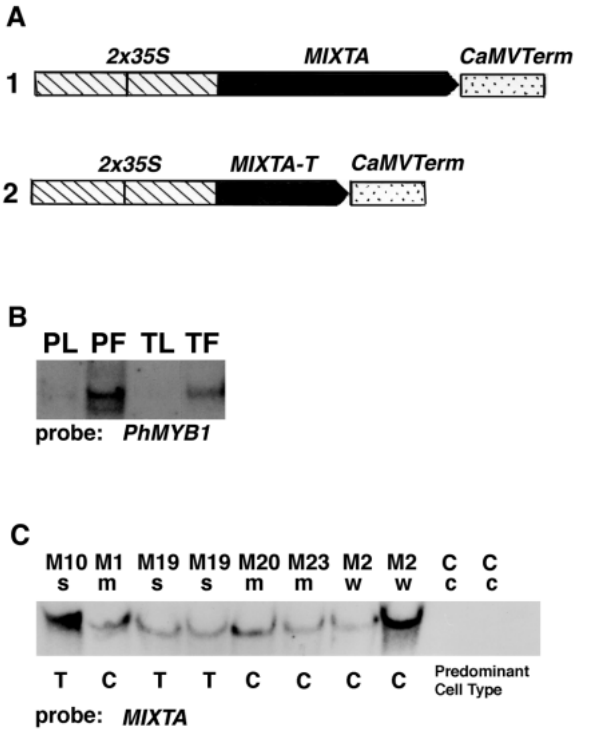

Fig. 1. Gene constructs for ectopic expression of *MIXTA* and the resulting expression levels in transformed tobacco lines.
(A) Structure of the gene constructs used to express; (1) the *MIXTA* cDNA and, (2) the truncated *MIXTA-T* cDNA in transgenic tobacco. Gene expression in each case was driven by the 35S promoter from CaMV and termination was ensured by the addition of the CaMV terminator sequence at the 3′ end of the MIXTA cDNA sequence. (B) RNA gel blot of 10 μg total RNA from *Petunia* leaves (PL) and *Petunia* flowers (PF) and 2 μg poly(A)+ RNA from tobacco leaves (TL) and tobacco flowers (TF) probed with the cDNA encoding PhMYB1 (the functionally homologous protein to MIXTA from *Petunia*). (C) Expression of *MIXTA* in transgenic tobacco lines. An RNA gel blot loaded with 15 μg of total RNA from tobacco leaves was probed with the *MIXTA* cDNA. Samples from independent transformants M10, M1, M19, M20, M23 and M2 together with RNA from control leaves and control flowers (C) were used. Two lines of the primary transformant M19 were used (which had been derived from cuttings of the original plant). Two lines of M2 were used which had been obtained as separate progeny from seeds from the primary transformant back-crossed to wild-type tobacco. The strength of the overall plant phenotype is indicated below the line number: s, strong; m, medium; and w, weak; c indicates control. Below the gel the predominance of either conical cells (C) or trichomes (T) on the epidermal surfaces of the transformed lines is indicated.

petal limb in untransformed tobacco, this morphological change was directed by *MIXTA*.

This novel effect of *MIXTA* was observed even more clearly in the epidermal layer of the upper part of the carpel of transgenic lines. These cells are normally flat without significant trichome development (Fig. 2G). In transgenic lines abundant single and long-stalked multicellular trichomes were formed in this tissue (Fig. 2H). Outgrowths could also be observed on the individual cells of the trichomes. These secondary outgrowths were always restricted to one per cell, unless division separated the outgrowth to form a branch (Fig. 2E,F,H). The outgrowths on the cells of the ectopic trichomes were randomly orientated with respect to the outgrowths on other cells within the same trichome.

Effect of *MIXTA* on trichome formation in leaves

Multicellular trichomes are normally present on both adaxial and abaxial surfaces of tobacco leaves. In some of the transgenic lines considerably more trichomes than usual were formed on both leaf surfaces. As seen in carpels, these trichomes were often branched, with outgrowths on the composite cells (Fig. 2F), whereas trichomes of tobacco normally branch only very occasionally, at the base (Roberts et al., 1981). Counts of cell types on the adaxial leaf surface of six independent lines (with two replicates for two lines) (Table 1) showed that the lines could be separated into those showing predominantly conical cells on their leaf epidermis (lines M1, M2, M20 and M23) and lines that also showed a very significant increase in the number of multicellular trichomes (lines M10 and M19). Stomatal density was

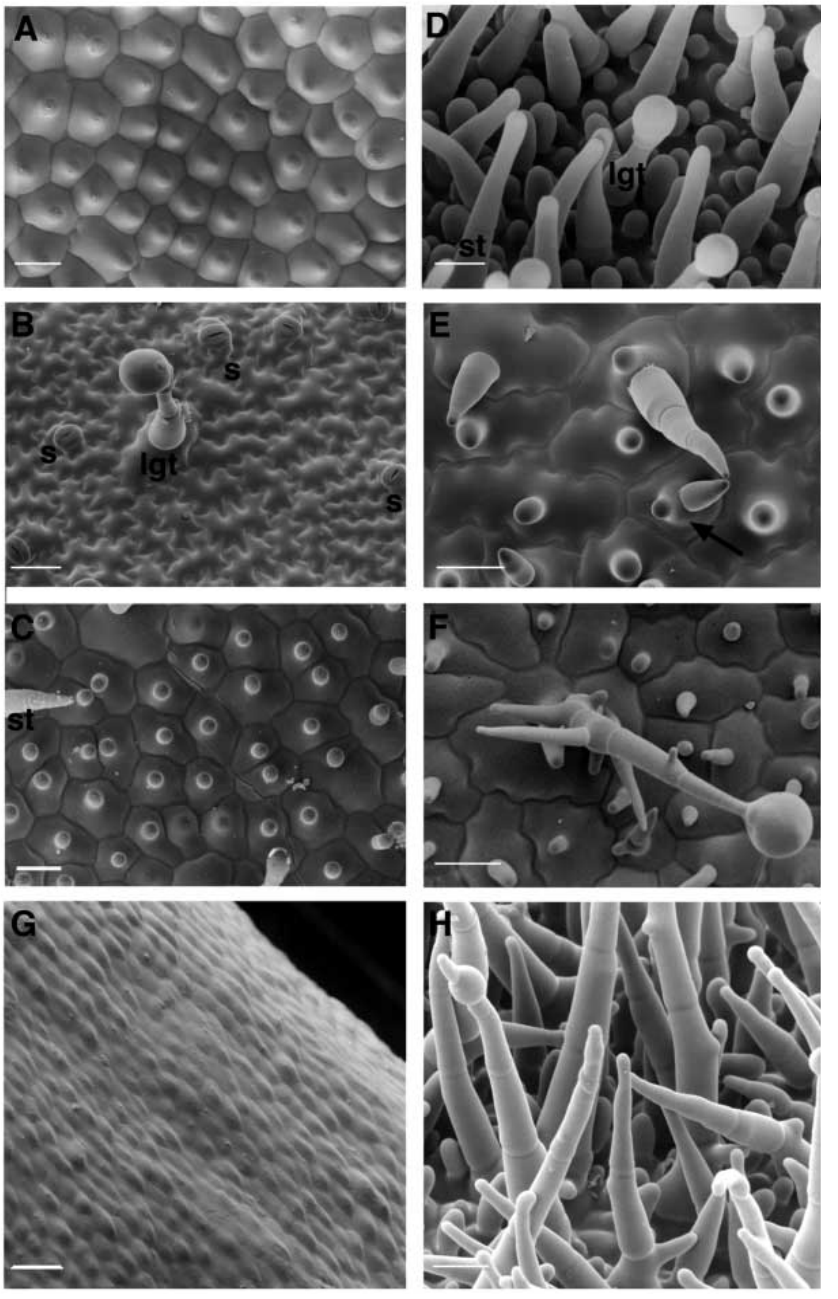

Fig. 2. Appearance of epidermal cells expressing *MIXTA*. (A) Scanning electron micrograph (SEM) of inner epidermis of a control tobacco petal limb. All cells are conical. (B) SEM of adaxial epidermis of a control tobacco leaf. The large flat epidermal cells predominate but stomata (s) and a long stalked glandular trichome (lgt) are also visible. (C) SEM of adaxial leaf epidermis of a tobacco line transformed with *MIXTA*. Each cell shows a single, central outgrowth of the outer, cuticularised wall. St, simple trichome. (D) SEM of inner epidermal cells of tobacco petal transformed with *MIXTA*. Many of the conical cells have developed into trichomes, and both simple (st) and long-stalked, glandular trichomes (lgt) are clearly visible. These are never seen in inner epidermal tissue of untransformed tobacco petals. (E) SEM of cellular outgrowths on the adaxial leaf surface of a *MIXTA* transgenic line. Once a cell plate has separated the outgrowth from the parental cell, a second outgrowth may arise close to the base of the first outgrowth (arrow). (F) SEM showing outgrowths on a long-stalked glandular trichome on the leaf of a *MIXTA*-transformed line. Usually tobacco trichomes branch only very occasionally at the base. The branches promoted by *MIXTA* arise as single outgrowths on the individual cells of the trichome. The orientation of the outgrowths is random relative to each other. (G) SEM of epidermis of upper part of carpel of control tobacco. The cells are smooth. (H) SEM of epidermis of upper part of carpel of *MIXTA*-transformed tobacco. A forest of trichomes have formed including both simple and glandular multicellular trichomes. Outgrowths have developed on the component cells of the trichome including the cells of the glandular heads. Scale bars represent 30 μm.

Table 1. Cell counts from adaxial leaf surface of independent tobacco lines expressing *MIXTA*

	% cell type on adaxial leaf surface				
Plant no.	Flat epidermal cells	Conical cells	Simple/glandular longstalked trichomes	Hydathodes	Stomata
M19-1	13.0	40.1	46.9	0.0	0.0
M19-2	17.4	47.3	34.9	0.4	0.0
M10	8.0	42.9	47.9	1.2	0.0
M23	19.2	68.2	10.6	1.6	0.4
M20	29.5	53.8	15.6	1.2	0.0
M1	29.0	66.7	2.7	1.1	0.5
M2-1	22.4	70.1	6.8	0.0	0.7
M2-2	30.1	66.9	1.5	0.8	0.8
Control	93.6	0.0	0.6	1.2	4.6

M19-1 and M19-2 were two individuals generated from cuttings. M2-1 and M2-2 were two individuals segregating in the progeny of a primary transformant backcrossed to wild-type tobacco. An area of 100 mm^2 was counted in each case.

significantly reduced in all transgenic lines, suggesting that commitment to forming conical cells and multicellular trichomes may be a developmental route mutually exclusive to commitment to develop the stomatal meristemoid and stomata.

Cell counts (Table 2) of both the adaxial and the abaxial surfaces showed that on the adaxial surface of control leaves 7% of epidermal cells formed stomata, 1.5% formed long multicellular trichomes (simple and long-stalked glandular trichomes combined) and 0.8% formed the short glandular hydathode type of trichome. On the abaxial leaf surface the proportion of epidermal cells giving rise to long multicellular trichomes was approximately the same at 1.1%, the frequency of hydathodes was reduced to 0.4% and the proportion of stomata was increased to 14.8%.

In the transgenic line exhibiting predominantly cellular outgrowths (M2) there was an increase in the number of long multicellular trichomes on both the adaxial and abaxial epidermis (to 8.8% and 11.7% respectively). However there was little effect (or even a decrease) on the density of hydathodes, showing that *MIXTA* did not promote formation of this type of trichome. There was a significant reduction in stomatal frequency, which was particularly marked on the adaxial epidermis. This confirmed the view that commitment to form either stomata or conical cells are mutually exclusive decisions. If a cell commits initially to form a stoma, it and its clonally derived descendants appear to be unable to form an outgrowth later. This idea was supported by examination of the cells around the stomata in the transformed lines. The subsidiary cells around the guard cells, which are derived from the meristemoid, were always free from outgrowths even when all the cells beyond the stomatal complex showed epidermal outgrowths (Fig. 3A). The effect of ectopic *MIXTA* expression on stomatal complex formation was reduced on the abaxial leaf epidermis compared to the adaxial epidermis. This difference was reflected in a significantly higher proportion of cells without outgrowths on the abaxial epidermis. These unaffected epidermal cells were always clustered around the stomata. It appears that the signal specifying stomatal formation was stronger on the abaxial leaf epidermis while the signal for cellular outgrowth and trichome formation was relatively stronger on the adaxial leaf surface of the same plant.

The same trends were shown by the epidermal cells of the line producing predominantly trichomes (M19). Here, there was a very significant increase in the frequency of long multicellular trichomes (to 58.7% on the adaxial surface and 45.9% on the abaxial surface), including increases in the density of long-stalked glandular trichomes. There was no effect on hydathode density on either surface in this line. Stomata were completely absent from the adaxial leaf epidermis, and were reduced in density (to 8.3%) on the abaxial epidermis (Fig. 3B).

Effects of *MIXTA* on non-epidermal cells

Despite the very dramatic changes in epidermal cell form promoted by *MIXTA*, sectioning and light microscopic examination of leaves, petals, stamens and carpels revealed that the non-epidermal cell layers of all these organs were unaffected (Fig. 4). The internal wall of the ovary, opposite the placental

Table 2. Cell counts from abaxial and adaxial leaf surfaces of control leaves, a line (M2) producing predominantly conical cells and a line (M19) producing predominantly trichomes

	Flat epidermal cells	Conical cells	Simple trichomes	Glandular trichomes	Hydathodes	Stomata
Adaxial surface				% cell type		
Control	90.7±5.8	0.0	0.6±0.8	0.9±0.2	0.8±0.2	7.0±6.4
M2	24.8±6.3	65.3±8.8	8.1±4.3	0.7±0.4	1.0±0.2	0.1±0.1
M19	6.6±4.6	34.0±8.2	56.3±8.4	2.4±0.7	1.0±0.9	0.0
Abaxial surface						
Control	83.6±3.2	0.0	0.4±0.3	0.7±0.4	0.4±0.1	14.8±3.2
M2	51.4±2.6	27.9±4.0	10.1±2.6	1.6±0.6	0.2±0.1	8.9±0.8
M19	24.5±8.4	20.8±10.8	44.3±6.9	1.6±0.9	0.5±0.2	8.3±1.5

Mean values plus or minus standard errors are given.

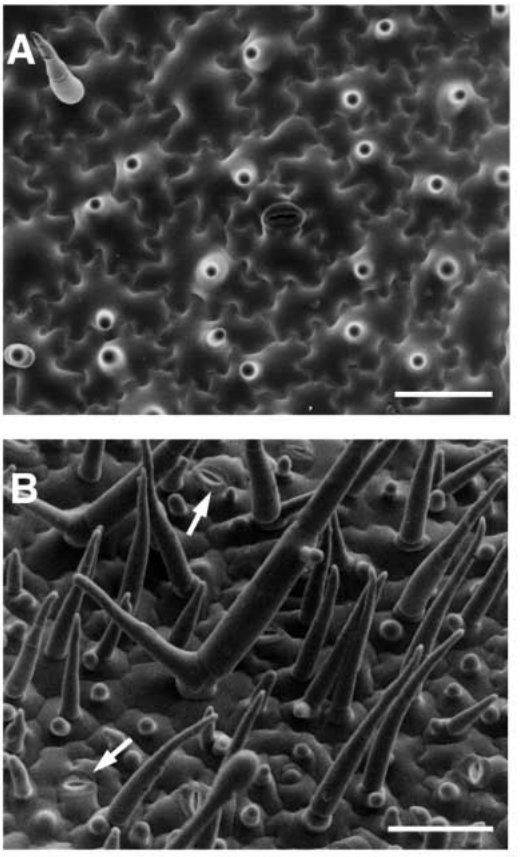

Fig. 3. Interaction between formation of stomata and cellular outgrowths/trichomes. (A) SEM of adaxial leaf surface from a transgenic line (M2) showing predominantly cellular outgrowths. Although stomatal frequency was dramatically decreased in this plant, where stomata formed, the subsidiary cells surrounding them were free from outgrowths. (B) The abaxial leaf surface of a transgenic line making predominantly trichomes (M19), produced fewer stomata than control plants. However, where the stomata did form the subsidiary cells were free from cellular outgrowths or trichomes (arrowed). Scale bars represent 75 μm.

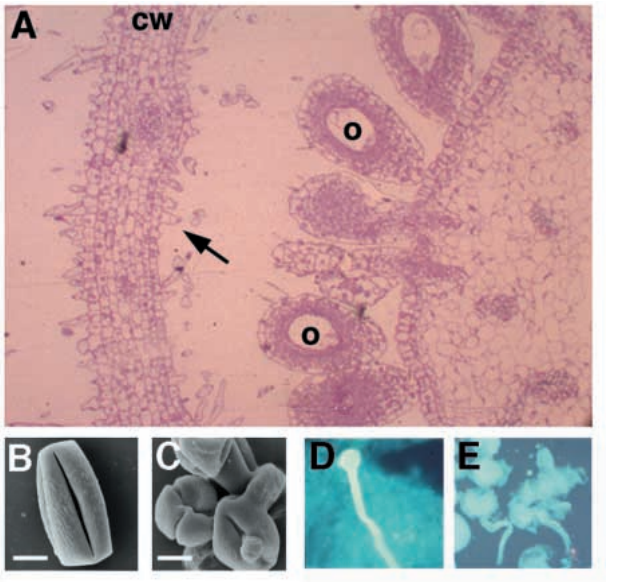

Fig. 4. Effects of *MIXTA* expression on cells of carpel and pollen. (A) Longitudinal section through carpel of *MIXTA*-transformed tobacco. Abundant conical cells and trichomes (arrowed) have formed on both the outer epidermis of the carpel and the inner epidermis of the carpel wall (CW). No outgrowths of the cells of the developing ovules (o), on the placental epidermis nor in the mesophyll/parenchyma cells were ever observed. (B) SEM of pollen grain from untransformed tobacco. (C) SEM of pollen from anther of *MIXTA*-expressing tobacco transformant. The pollen grains are distorted with cellular outgrowths. (D) Aniline blue staining of a germinated control pollen grain and tube on a tobacco stigma. The fluorescence indicates the presence of callose. (E) Aniline blue staining of pollen from anther of *MIXTA*-expressing tobacco. No callose is present in the tubular outgrowths. Scale bar represents 10 μm.

tissue, developed protrusions in the same way as the epidermal layers (Fig. 4A). However this response may be attributed to the epidermal derivation of this tissue (Esau, 1977). The ectopic production of conical cells and multicellular trichomes in epidermal layers was clear, while there was no corresponding change in the form of mesophyll cells.

A modification of the pollen grains was observed in the transgenic lines. A significant proportion of the pollen grains from the transgenic lines developed protrusions when still within the anthers. The cellular extensions observed were thicker than pollen tubes, and the texture of the exine was maintained (Fig. 4C). It appears that *MIXTA* can promote cellular outgrowths in pollen as well as in epidermal cells. Such outgrowths could represent premature pollen tube development. Pollen tubes growing from control pollen on a control stigma stained positive for callose (Fig. 4D), but equivalent staining was not observed from the outgrowths from the pollen in the anthers of the transgenic lines (Fig. 4E). Germination in vitro confirmed that the pollen grains from the transgenic lines could germinate and form pollen tubes, despite their aberrant morphologies. Although this would be predicted for 50% of the pollen because the *MIXTA* gene was hemizygous in the parental lines examined, some of the pollen grains with pre-existing cellular outgrowths were observed to germinate and develop normal pollen tubes.

Reciprocal crosses between *MIXTA* lines and untransformed control plants confirmed that there was no reduction in pollen viability as a result of *MIXTA* expression as measured by the inheritance of kanamycin resistance. Nevertheless, the transgenic lines were effectively male sterile because the anthers failed to dehisce. This was probably the result of morphological changes in the stomium because this region did not break and the anthers did not dehisce in the transgenic plants (compare Beals and Goldberg, 1997).

Lack of effects of *MIXTA* in tobacco roots

Despite the plethora of morphological changes observed in the shoot epidermal cells and pollen, light microscopy revealed no changes in the cellular morphology of roots in transgenic seedlings, either in non-hair cells or in root hair number or form.

Phenotype of tobacco plants ectopically expressing *MIXTA*

The transgenic plants were shorter and slower growing with a

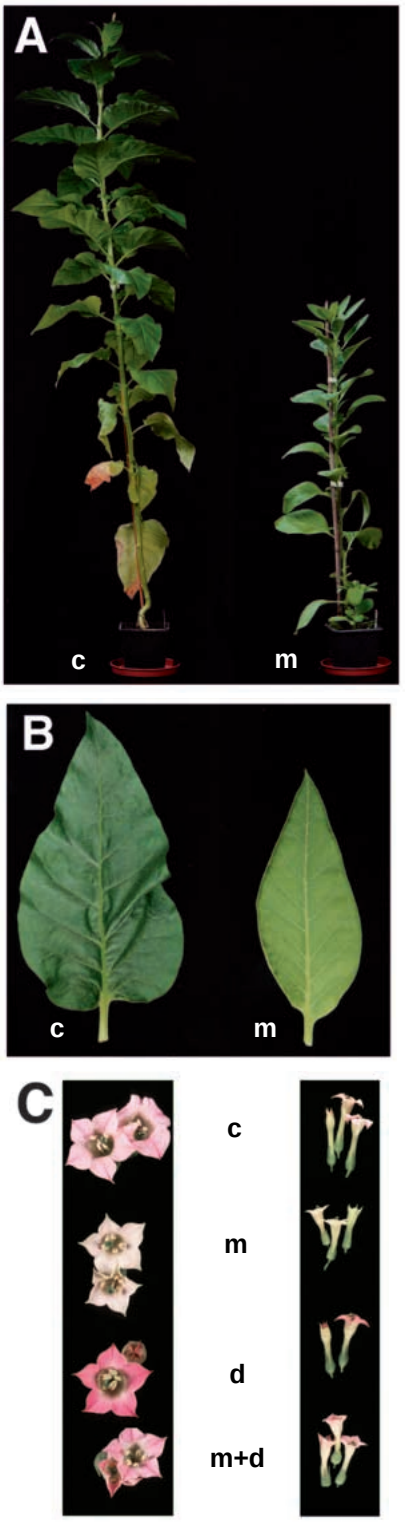

Fig. 5. Effects of ectopic *MIXTA* expression on the whole plant phenotype of tobacco. (A) Tobacco plants expressing *MIXTA* (m) showed reduced stature with paler, more spoon-shaped leaves compared to controls (c). (B) Comparison of a control leaf (c) to a leaf from a transgenic line expressing *MIXTA* (m). The leaves from the transgenic lines were paler with a more rounded shape with rolled-in leaf margins. (C) Effect of *MIXTA* on floral form and anthocyanin production in petals. Flowers from control plants were pink (c) whereas flowers from plants expressing *MIXTA* were very palely pigmented or acyanic (m). *MIXTA* expression also modified perianth form, presumably as a result of limitations on epidermal tissue expansion. The flowers were cornet-shaped as opposed to trumpet-shaped. Ectopic expression of *DELILA* in tobacco augmented pigment production (d; Mooney et al., 1995). A cross between a line expressing *MIXTA* and one expressing *DELILA* resulted in progeny (m+d) with restored levels of pigmentation but with the cornet-shaped perianth remaining.

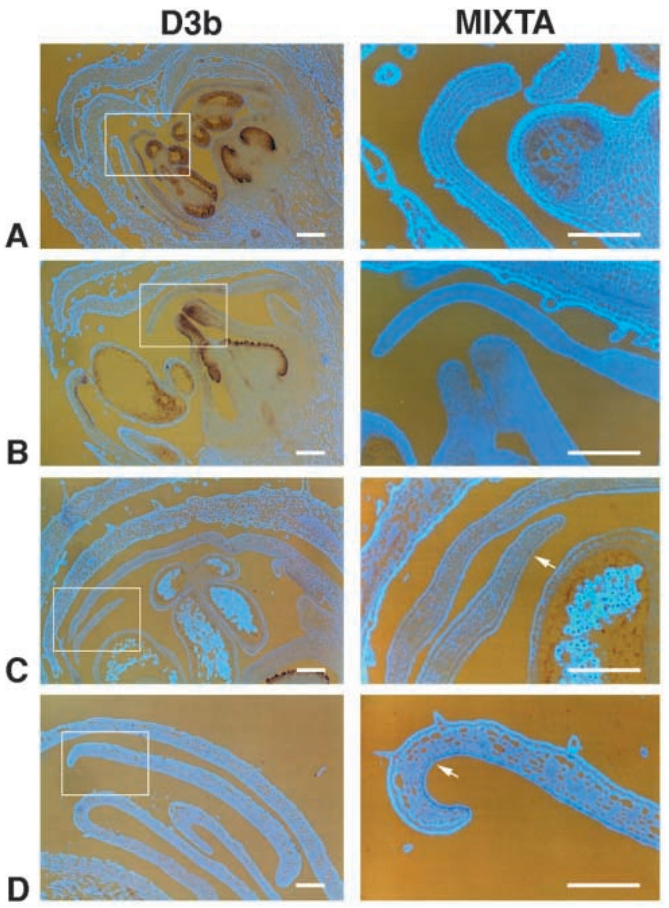

Fig. 6. Expression of *MIXTA* during *Antirrhinum* flower development. In situ hybridization of RNA probes to sections of *Antirrhinum* inflorescences; (A-D) Sections from buds of increasing size on an inflorescence. Serial sections were probed with antisense RNA probes to the cDNA encoding CYCLIN D3b from *Antirrhinum* (Forbert et al., 1996) and with an antisense probe to *MIXTA*. Viewed under bright-field illumination, the signal appears purple/brown on a light blue tissue background. *CYCLIN D3b* transcript was detected predominantly in the younger buds (stages in A and B). Most expression was seen in the ovules and anthers reflecting the high rates of cell division involved in gamete production. Expression was also observed in petals at early stages (A,B) and in a small patch at the base of the petal in slightly older buds (C). No expression was detected at the stage shown in D. The sections showing *MIXTA* transcript are at higher magnification than those for *CYCLIN D3b* expression. The areas of petal tissue on the *CYCLIN D3b* sections corresponding to the areas shown for *MIXTA* expression are indicated with a square. *MIXTA* was not expressed in the younger buds (A and B). Very low expression was just discernable on the inner epidermis of the petal limb at the stage shown in C (arrow). Strong expression of *MIXTA* limited to the single inner epidermal cell layer of the petal lobe was observed at the stage shown in D. Scale bars indicate 100 μm.

bushier appearance, reduced apical dominance and smaller rolled leaves (Fig. 5A,B). Transgenic flowers (m) had much paler corollas than controls (c; Fig. 5C) with very low levels of anthocyanin production. The flowers of transgenic lines were cornet-shaped rather than trumpet-shaped as in the untransformed control, as a result of the perianth opening less widely (Fig. 5C).

The reduction in anthocyanin levels in the transgenic tobacco lines was unexpected since *MIXTA* does not control anthocyanin production in *Antirrhinum* and affects floral pigmentation only indirectly through its control of petal cell shape (Noda et al., 1994). MYB-related transcription factors interact with bHLH factors in plants to control anthocyanin production, and the activity of bHLH factors may limit the rate of anthocyanin

accumulation in tobacco (Ludwig et al., 1989, Goff et al., 1992, Lloyd et al., 1992; Mooney et al., 1995). Consequently high levels of MIXTA protein production in transgenic tobacco flowers might reduce anthocyanin production by titrating out the bHLH-related factor (Ptashne, 1988). To test the extent to which the reduction in floral pigmentation and any other aspects of the *MIXTA*-promoted phenotype in tobacco could be attributed to such processes, we crossed two independent transgenic lines expressing *MIXTA* to a line of tobacco overexpressing the *DELILA* gene that encodes a bHLH-related transcription factor that is required for anthocyanin production in *Antirrhinum* (Goodrich et al., 1992, Mooney et al., 1995). This line showed elevated pigment production in the flowers (d in Fig. 5C; Mooney et al., 1995). The high level of the bHLH-factor restored pigmentation to the flowers in the progeny (m+d in Fig. 5C), although all the other characteristics of ectopic expression of *MIXTA*, including ectopic conical cell and trichome formation and the consequent modification of corolla shape (Fig. 5C) were retained. This confirmed that the effect of MIXTA on petal colour is likely to result from titrating out another transcription factor rather than being the direct consequence of MIXTA activity. The result also suggested that MIXTA might be able to interact with other bHLH proteins in the promotion of cellular outgrowth.

The C-terminal domain of MIXTA is required for its function

It is possible that the morphogenic consequences of overexpression of MIXTA in transgenic tobacco result from artificially high levels of protein in a heterologous host. To test whether the ability of MIXTA to determine epidermal cell fate was a consequence of the protein acting as a transcription factor, we transformed tobacco with a gene construct in which there was a deletion in the *MIXTA* cDNA so that the sequence encoding the potential activation domain at the C terminus of the MIXTA protein was lost (*MIXTA-T*; Fig. 1A). Transgenic lines expressing this construct were phenotypically normal except that their petals were paler than controls. This result confirmed that the C terminus of the MIXTA protein is necessary for its function and that the reduction in floral pigmentation associated with *MIXTA* expression is likely to be an indirect effect of the protein. The region of MYB-related transcription factors believed to interact with bHLH factors lies within the N-terminal DNA binding domain (Goff et al., 1992) which was present in the otherwise non-functional truncated MIXTA protein.

The importance of the timing of *MIXTA* expression in *Antirrhinum*

The results of ectopic expression of *MIXTA* in tobacco showed that the gene product can induce the formation of both conical cells and multicellular trichomes. However, in *Antirrhinum MIXTA* is necessary only for conical cell formation in petals and does not affect trichome formation in flowers, inflorescences or young vegetative tissues (Noda et al., 1994; B. J. Glover, unpublished results). We looked at the timing of *MIXTA* expression in *Antirrhinum* relative to the overall development of the petal tissue using a gene encoding a predictive marker for the cell cycle/cell division, *CYCLIN D3b* (Forbert et al., 1996). In situ hybridisation of RNA probes of *MIXTA* and *CYCLIN D3b* to serial sections of *Antirrhinum* inflorescences with buds of different ages (Fig. 6) revealed that *MIXTA* is expressed late in petal development after the end of the cell division phase, as indicated by loss of *CYCLIN D3b* gene expression in petal tissues prior to *MIXTA* expression. *MIXTA* expression was also limited to the inner epidermis of the petals (Fig. 6).

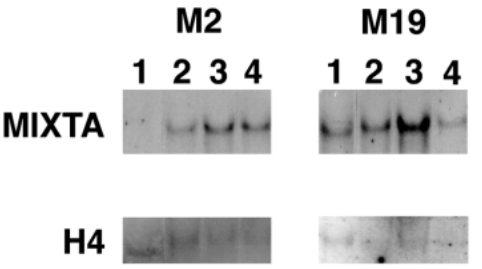

Fig. 7. Comparison of expression of *MIXTA* in developing leaves of transgenic lines producing predominantly conical cells or predominantly trichomes. The expression of *MIXTA* at four stages of leaf development is shown on RNA gel blots for the two transgenic lines. Lane 1, RNA from leaves less than 2 cm long; lane 2, RNA from leaves between 2 and 7 cm; lane 3, RNA from leaves between 7 and 10 cm; and lane 4, RNA from mature leaves. The expression of the genes encoding HISTONE H4 is shown below to mark the progression of cell division.

The importance of timing of *MIXTA* expression in tobacco

The specificity of *MIXTA* function in *Antirrhinum* might therefore arise as a consequence of its expression pattern. Clearly the formation of multicellular trichomes requires cell division subsequent to cell determination and an alteration in the plane of division from anticlinal to periclinal (Esau, 1977). In contrast, conical cell formation occurs only after the cessation of cell division in *Antirrhinum* petal epidermis (Noda et al., 1994). Since the predominant production of either trichomes or conical cells in the different transgenic lines was not correlated with the different levels of *MIXTA* expression in mature leaves (Fig. 1C), it seemed likely that the parameter determining whether trichomes or conical cells were formed was the timing of *MIXTA* expression relative to the progress of leaf development. Trichome formation might require early *MIXTA* expression to guarantee subsequent cell division, while later expression of *MIXTA*, after the completion of cell division, might result solely in the formation of conical cells.

Consequently, we compared the patterns of *MIXTA* expression in two transgenic tobacco lines that showed either predominantly conical cells or predominantly trichomes on their epidermis. Expression was compared in line M19 in which about half of the epidermal cells formed trichomes and in line M2 in which only about 9% of epidermal cells formed trichomes, but in which about half of the epidermal cells showed outgrowths of the outer wall. Transcript levels were measured at four stages of leaf development (Fig. 7). Leaves of less than 2 cm (in which cell division is very frequent) showed very low or undetectable levels of *MIXTA* transcript in line M2, but much higher levels in line M19 (Fig. 7). Expression of *MIXTA* peaked earlier during leaf development in line M19 than in line M2. This difference in timing of expression was also significant with respect to the timing of cell division as shown by the expression of the gene(s) encoding HISTONE H4 which served as a marker for cell division in tobacco (Fig. 7).

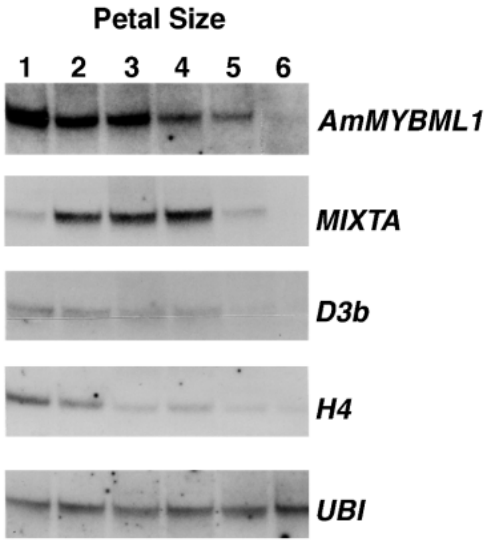

Fig. 8. RNA gel blots of expression of *MIXTA*-like *MYB* genes during petal development of *Antirrhinum*. Poly(A) +RNA (2 μg) was extracted from petals of increasing size: (1) 0-5 mm; (2) 5-10 mm;(3) 10-15 mm; (4) 15-20 mm; (5) 20-25 mm; (6) 25-30 mm long, separated on agarose gels blotted onto nitrocellulose and probed with ^{32}P-labelled DNA from the *AmMYBML1*, *MIXTA*, *CYCLIN D3b* (*D3b*) and *HISTONE H4* (*H4*) genes. As a loading control the blot was probed with a cDNA probe from the *UBIQUITIN* gene (*UBI*) of *Antirrhinum*.

Although the CaMV35S promoter is generally considered to be constitutive, integration of the transgenes at different chromosomal locations may give rise to variable expression patterns resulting from 'position effects' which could explain the differences in the time of expression of *MIXTA* in the transgenic lines. These results support the interpretation that conical cells and long-stalked multicellular trichomes share a common developmental pathway in tobacco, and that the decision determining cell development depends upon the timing of cellular outgrowth relative to the overall development of the tissue; probably to the progress of cell division in that tissue.

Control of trichome formation in *Antirrhinum*

Antirrhinum forms multicellular glandular trichomes in profusion on its outer petal epidermis and on the petal tube. Our results would predict that, in *Antirrhinum*, another *MYB* gene very similar to *MIXTA* is expressed early in petal development to promote trichome formation in flowers. This was confirmed by screening a cDNA library (prepared from RNA from petals of a mixta mutant line) at moderate stringency with a probe encoding the MIXTA DNA binding domain. Two cDNA clones encoding a protein, AmMYBML1, with very high homology to MIXTA were isolated. The DNA binding domain of AmMYBML1 is 87% identical to the MIXTA DNA binding domain and there is sequence similarity between the C-terminal domains of the two proteins including a region with potential to form amphipathic alpha-helix that might serve as a transcriptional activation domain (Noda et al., 1994). *AmMYBML1* is expressed predominantly early in petal development, with maximum transcript levels coinciding with the maximal expression of the genes encoding cell cycle markers CYCLIN D3b and HISTONE H4 whereas *MIXTA* gene transcript levels peak later (Fig. 8). *AmMYBML1* is, therefore, a good candidate for a factor that determines floral trichome development, because it is expressed in very young *Antirrhinum* petals when cell division is predominant.

DISCUSSION

Plant cells respond selectively to *MIXTA* to modify their shape

The ectopic expression of the *MIXTA* gene from *Antirrhinum majus* in tobacco leaves demonstrated that *MIXTA* is not only necessary for the formation of conical cells (Noda et al., 1994) but also sufficient to induce their formation. This might imply that *MIXTA* functions as a primary regulator, alone controlling the specification of conical cells. However the influence of *MIXTA* was not realised in all cell types, since no outgrowths of mesophyll cells or of root epidermal cells were ever observed even in those transgenic lines that showed very high frequencies of cellular outgrowths of their shoot epidermal cells. At present it is not possible to conclude whether other factors negatively regulate responses to *MIXTA* in mesophyll and root cells or whether there is a requirement for additional positive regulators that are active only in shoot epidermal cells and pollen grains. Analogy to the action of other plant MYB transcription factors suggests that there could be an obligate interaction with bHLH transcription factors, structurally similar to the *R*(*LC*) gene product of maize or the *DELILA* gene product of *Antirrhinum*, as has been suggested for the product of the *GL1 MYB* gene that controls trichome formation in *Arabidopsis* (Lloyd et al., 1992; Larkin et al., 1994). Circumstantial evidence for such an interaction is provided by the suppression of anthocyanin biosynthesis in the flowers of transgenic tobacco lines by high levels of MIXTA production. This suppression (although not the promotion of cellular outgrowths) can be reversed by concomitant overproduction of DELILA, suggesting that MIXTA titrates out the protein orthologous to DELILA in tobacco and implying the general possibility of physical interactions between MIXTA and bHLH proteins. If the specificity of MIXTA action is defined by the positive activity of a second interacting factor, our results would imply that such a factor is normally active in most shoot epidermal cells and in pollen grains.

MIXTA-promoted cellular outgrowths of epidermal cells were always limited to a single site on the cuticularised outer cell wall. Outgrowths arose on the component cells of the multicellular trichomes which are also cuticularised. In addition, the exine of pollen grains is cuticularised, even though these cells are not of epidermal origin. The function of *MIXTA* is, therefore, closely correlated to the production of cuticularised cell wall and the specificity of *MIXTA* activity may be associated with the production of this type of specialised cell wall.

MIXTA can direct the formation of both conical cells and long-stalked multicellular trichomes

MIXTA expression in transgenic tobacco promoted formation of long stalked multicellular trichomes as well as conical cells. This result links the developmental programmes specifying conical cells and multicellular trichomes. Our results do not

link all trichome types with conical cell formation, because the density of short glandular hairs (hydathodes) was unaffected or slightly reduced by ectopic expression of *MIXTA*, indicating that the hydathodes are formed by an independent developmental pathway. *MIXTA* had the most significant effect on the formation of simple, long- stalked trichomes, although it also increased the frequency of glandular, long-stalked trichomes, especially in flowers, suggesting that simple and glandular long-stalked trichomes of tobacco also share a common developmental pathway. The differentiation of gland cells may occur in only a proportion of the long-stalked trichomes. Alternatively, the simple trichomes may represent immature glandular trichomes as suggested by Cannon (1909). Supporting evidence for the idea that long-stalked glandular and simple trichomes use the same developmental route comes from microscopic inspection of the occasional branched trichomes in wild-type tobacco which may have one branch with a glandular tip and one with a simple tip (Roberts et al., 1981 and see also Fig. 2F).

We have found no effect of ectopic *MIXTA* expression on conical cell or trichome formation in *Arabidopsis* (B. J. Glover, C. Martin, H. Ichikawa and H. Jin, unpublished results). This would imply that neither conical cell differentiation in *Arabidopsis* petals, nor formation of unicellular trichomes are limited by the activity of a *MIXTA*-related gene. Significantly, although the *GL1* gene, which controls trichome formation in *Arabidopsis*, encodes a MYB-related protein, it is not particularly closely related to MIXTA in its overall primary structure nor in the sequence of its DNA-binding domain (Oppenheimer et al., 1991, Noda et al., 1994, Romero et al., 1998).

Not all trichomes induced by *MIXTA* developed as full-sized, long-stalked trichomes. Some were defined only by one or two cell divisions (Fig. 2C,E). The formation of the first periclinal cell wall usually occurred when the cellular outgrowth reached a critical length (estimated to be about 25 μm). This suggested that the cellular outgrowth, itself, programmed the change in the orientation of epidermal cell division associated with trichome formation. The relationship between longitudinal cell expansion and the promotion of transverse cell division has previously been stressed in developing plant meristems (Lloyd and Barlow, 1982). The first stages of multicellular trichome formation might therefore follow as a direct consequence of the promotion of cellular outgrowth, provided the targeted epidermal cells are still competent to divide. However, more complex cellular differentiation programmes are probably induced following *MIXTA*-promoted outgrowth because a fraction of initiated trichomes then go on to form gland cells.

The specificity of *MIXTA* function resides in the pattern and timing of its expression

Given that our results show that *MIXTA* can induce both conical cell and trichome formation, the reason why *MIXTA* function is normally restricted to the control of conical cell formation should be addressed. In situ hybridisation analysis showed that the expression of the *MIXTA* gene in *Antirrhinum* petals is restricted to a specific cell layer (the inner epidermal layer) and to a specific time late in petal development when genes encoding predictive markers for cell division such as CYCLIN D3b are no longer expressed. This late expression of *MIXTA* may dictate its functional specificity. It has been observed in a number of species that conical or papillate cells normally arise very late in epidermal cell differentiation compared to trichomes which normally develop very early (Esau, 1977; Lersten and Curtis, 1992; Marysia, 1992; Sachs, 1996). Therefore, the timing of cellular outgrowth relative to overall tissue development, and specifically to the progress of cell division, may be a general developmental mechanism specifying different cell types. It is interesting that trichome development also occurs very early in *Arabidopsis* leaf development before the cessation of epidermal cell division, even though, in this case, the trichomes are unicellular (Hülskamp et al., 1994). In *Arabidopsis* the differentiation programme must involve a mechanism to suppress cell division, although DNA replication continues (Hülskamp et al., 1994). This may be an important feature distinguishing *Arabidopsis* trichome differentiation from that of multicellular trichomes and emphasises that different types of trichome are probably specified by discrete developmental programmes.

Our data on the timing of *MIXTA* expression in different transgenic lines predominantly producing either conical cells or trichomes, support the interpretation that cell differentiation is a function of the timing of cell outgrowth relative to the progress of tissue development, and especially relative to the progress of cell division.

MIXTA-related transcription factors probably control multicellular trichome formation

Multicellular trichome differentiation in *Antirrhinum* and tobacco is probably normally controlled by MYB-related transcription factors very similar in their structure to MIXTA, but with different patterns of expression. Trichomes are produced in abundance on *Antirrhinum* petals on the outer epidermal surface and on the corolla tube. *MIXTA* plays no part in the development of these trichomes as evidenced by the phenotype of null mutants. However, a MYB gene product (AmMYBML1) showing 63% overall structural identity to MIXTA (and 87% identity within the DNA binding domain) is expressed very early in *Antirrhinum* petal development. This gene is a good candidate for controlling multicellular trichome differentiation. Comparisons between other plant MYB-proteins with similar levels of structural similarity to those between AmMYBML1 and MIXTA have demonstrated them to have analogous biochemical functions (Tamagnone et al., 1998, van Houwelingen et al., 1998, Quattrocchio et al., 1998). A similar assignment of discrete developmental roles by functionally equivalent transcription factors has been described in *Drosophila* embryogenesis for the paired (*prd*) and gooseberry (*gsb*) genes (Li and Noll, 1994). Normally the products of these genes serve quite distinct functions both in terms of their control of morphogenesis and their control of specific target genes. Experimental induction of *gsb* expression early in embryogenesis causes an effect which mimics the role of *prd* early in development. Induction of *prd* later in development mimics the role of *gsb*. The conclusion from these studies was that gene duplication and subsequent alterations in the expression patterns of the individual genes allowed the acquisition of new and discrete functions by these related regulatory genes. Clearly, a similar pattern of events could well have allowed the diversification of *MIXTA*-related gene function in controlling epidermal cell differentiation. These data support the view that modification of *cis*-acting regulatory

sequences may be very significant in the development of regulatory systems controlling morphogenesis (Jacob, 1977).

Cellular outgrowth is mutually exclusive with commitment to form the stomatal meristemoid

Both the production of cellular outgrowths and the increase in trichome density inhibited the formation of stomata in the leaves of the transgenic tobacco lines. This inhibition was most marked on the adaxial leaf epidermis where stomata were virtually eliminated following ectopic expression of *MIXTA*. On the abaxial leaf epidermis stomatal density was reduced by about 50%. These data show that specification of a cellular outgrowth or trichome is mutually exclusive to specification of a stoma. The cells around the stomata in transgenic lines were always free from outgrowths or trichomes, indicating that the choice between these alternative developmental fates must occur at the point of initiation of the stomatal meristemoid which later subdivides to form the guard mother cells and the subsidiary cells. The decision to form either a trichome or a stomatal complex must contribute to the patterning of the leaf epidermis although we have no evidence for cell to cell signalling of the type proposed to laterally inhibit adjacent cells forming trichomes in *Arabidopsis* (Larkin et al., 1994, 1996, 1997).

There was a difference in the degree of inhibition of stomatal production on the adaxial compared to abaxial leaf surfaces in the lines ectopically expressing *MIXTA*. Significantly more stomata are normally formed on the abaxial leaf surface and it is possible that the predominance of stomatal formation over cellular outgrowth/trichome formation in the transgenic lines was due to a relatively stronger developmental signal to form stomata on the abaxial epidermis. Alternatively, stomata may start to develop earlier on the abaxial epidermis, ensuring that a proportion of epidermal cells commit early to stomatal production and are removed from the pool of cells available to form trichomes. A similar increase in the time available for stomatal development has been proposed to account for the increase in stomatal density observed in tmm mutants of *Arabidopsis* (Yang and Sack, 1995).

Ectopic expression of *MIXTA* therefore resulted in three dramatic phenotypic modifications to transgenic tobacco plants; increased trichome production, reduced stomatal density and, because of limitations on epidermal cell expansion, leaf inrolling at the margins (Fig. 5B). All these traits are also seen in plants adapted to dry and/or salty environmental conditions (Oppenheimer, 1960, Esau, 1977, Fahn, 1985, Weigler and Winter, 1991), where reduced stomatal density reduces water loss through transpiration, and increased trichome density is thought, principally, to reduce leaf temperature through increasing light reflectance (Ehleringer, 1984). Indeed, transgenic tobacco plants ectopically expressing *MIXTA* resemble, in their most extreme forms, plants adapted to hot, dry conditions (Fig. 5A). Our results suggest that these relatively complex morphological adaptations could be achieved through selection of simple changes in the expression patterns and/or levels of a single gene. In this way, new developmental functions may arise from changes in the context in which genes with homologous biochemical functions operate.

We thank Steve MacKay, Kim Findlay and Pilar Cubas for technical advice. We are grateful to Mark Mooney and Enrico Coen for the gift of the tobacco plants expressing 35S-*DELILA*. We thank Javier Paz-Ares for discussion and the gift of *PhMYB1*, David Hanke, David Hopwood and Keith Roberts for comments on the manuscript, and Theresa Warr and Anne Williams for preparation of the manuscript. B. J. G. acknowledges the support of a BBSRC PMB2 studentship during the course of this research. M. P. R. acknowledges the support of the EU Biotech programme (BIOT93/0101) and the BBSRC (CAD Initiative).

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