Heterologous myb genes distinct from GL1 enhance trichome production when overexpressed in Nicotiana tabacum

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Accepted 20 November 1998; published on WWW 20 January 1999

SUMMARY

Myb-class transcription factors implicated in cell shape regulation were overexpressed in Arabidopsis thaliana and Nicotiana tabacum in an attempt to assess the extent to which cellular differentiation programs might be shared between these distantly related plants. GLABROUS 1, a myb gene required for trichome development in Arabidopsis, did not alter the trichome phenotype of the tobacco plants in which it was overexpressed. MIXTA, which in Antirrhinum majus is reported to regulate certain aspects of floral papillae development, did not complement the glabrous 1 mutant of Arabidopsis. However, 35S:MIXTA transformants of N. tabacum displayed various developmental abnormalities, most strikingly production of supernumerary trichomes on cotyledons, leaves and stems.

In addition, floral papillae were converted to multicellular trichomes. CotMYBA, a myb gene which is expressed in Gossypium hirsutum ovules and has some homology to MIXTA, was also overexpressed in the two species. A similar but distinct syndrome of abnormalities, including the production of cotyledonary trichomes, was observed in 35S:CotMYBA tobacco transformants. However, CotMYBA did not alter trichome production in Arabidopsis. These results suggest that the trichomes of Arabidopsis and Nicotiana are merely analogous structures, and that the myb genes regulating their differentiation are specific and separate.

Key words: CotMYBA, MIXTA, Trichomes, Nicotiana tabacum

INTRODUCTION

Trichomes are specialized structures which originate and project from the above-ground epidermal tissues of most plants. A vast array of morphologies occur in addition to the hair-like shape suggested by the term. Trichomes may be composed of one or more cells, may be unbranched or branched, and may or may not possess glands which accumulate or secrete alkaloids like nicotine, terpenoids such as menthol and camphor, or other compounds repellent or toxic to phytophagous insects (Johnson, 1975; Levin, 1973; Rodriguez et al., 1984). Different trichome types may occur on the same plant of a given species, with type and spacing determined by the plant organ or organ surface (ie, adaxial vs. abaxial, leaf vs. stem) from which the trichome extends, and the developmental phase of the plant at the time of organ initiation.

The trichome is a useful cell type for studying regulation of cell differentiation in plants. It is morphologically distinct from surrounding epidermal cells, and its genesis and development from this population are readily observed due to its accessibility to visual inspection and its relatively large size. In the laboratory, trichomes are non-essential and thus can be genetically manipulated without a reduction in plant viability. A number of mutants lacking or producing fewer, aberrant, or mis-positioned trichomes have been isolated from Arabidopsis, and the order of action in the differentiation pathway of the genes so identified has been deduced by epistatic analysis (Hülskamp et al., 1994).

Wild-type Arabidopsis leaf trichomes are unicellular, typically have three branches, and lack glands. Progression through the stages of leaf trichome morphogenesis in this species is correlated with overall cell enlargement and endoreduplicative DNA increase. Three rounds of endomitosis occur initially, concomitant with extension growth from the epidermal surface. A fourth round of endomitosis occurs after primary branching of the developing trichome in a proximodistal orientation to the leaf primordium surface has begun. Subsequently the distal branch itself branches, and the nucleus migrates to the branching point from its previous position below it. Trichome cells are regularly spaced in the mature Arabidopsis leaf. Initiation and maturation of trichomes proceeds in a generally basipetal direction over the leaf primordium, but additional trichomes may be initiated between mature trichome cells as organ growth continues (Hülskamp et al., 1994).

Mutations at either of two loci, TRANSPARENT TESTA GLABRA 1 (TTG1) or GLABROUS 1 (GLI), result in the virtual absence of leaf trichomes from A. thaliana. TTG1 is thought to have roles in trichome precursor selection, trichome initiation, and in a nearest-neighbor inhibition of initiation in surrounding protodermal cells. Mutations at this locus have...
pleiotropic effects: in addition to the absence of trichomes, homozygous mutants fail to produce anthocyanin pigments and seed coat mucilage (Koornneef, 1981), and, conversely, produce supernumerary root hairs on root epidermal cells that are normally hairless (Galway et al., 1994). Previously, it was found that overexpression of the maize R gene (a myc-homologous transcription factor which regulates anthocyanin synthesis in that species) under control of the CaMV 35S promoter could suppress the various phenotypes associated with the ttg1-1 mutant. In wild-type Arabidopsis overexpression of R leads to production of extra trichomes on leaves and stems, and in some transformants to trichomes on organs which normally lack them, such as petals, stamens and pistils (Lloyd et al., 1992). TGT1 has recently been cloned and the gene is reported to encode a protein with WD-40 repeats and no myc homology (Walker et al., 1997). Presumably, TGT1 regulates or acts in concert with the activity of a myc-class transcription factor or factors. We have recently identified two endogenous Arabidopsis myc-class genes. When constitutively expressed in transgenic plants both suppress to varying extents the trichome and other defects of the ttg1-1 mutation, albeit less dramatically than R (Lloyd et al., unpublished).

Both GLI and TTG1 are necessary for normal Arabidopsis trichome initiation. The GLI gene was cloned by T-DNA tagging (Marks and Feldmann, 1989). It encodes a putative transcription factor with myb-type DNA binding and C-terminal acidic activation domains (Oppenheimer et al., 1991). By itself GLI is not sufficient to initiate trichomes in Arabidopsis. Overexpression from a 35S:GLI construct does not suppress the ttg1-1 mutation, but it does lead to the production of an occasional supernumerary trichome on the cotyledons of wild-type transformants. In fact, a reduction in leaf trichome number is observed, and this paradoxical result has been attributed to a squelching phenomenon (Larkin et al., 1994) akin to that described by Ptashne and Gann (1990).

Little is known about the molecular basis of trichome differentiation in other species, although the inheritance of indumentum characters correlated with pest resistance has been studied in some crop plants such as cotton (Stephens and Lee, 1961; Wannamaker, 1957), soybean (Broersma et al., 1972), and tomato (Gentile et al., 1969; Goffreda et al., 1990). In his 1954 monograph, Goodspeed recognized five basic morphological classes of trichomes variously distributed within the genus Nicotiana, and assessed their transmission to F1 interspecific hybrids and naturally occurring amphidiploids. Such experiments are difficult to interpret, but differences in the inheritance of trichome types, especially in regard to organ distribution and degree of structural elaboration, were documented. Such differences are likely to be indicative of separate regulatory mechanisms for the various trichome classes.

Noda et al. (1994) have cloned the gene encoding another myb-class transcription factor, MIXTA, from a Tam4-tagged allele of Antirrhinum majus. MIXTA appears to be a positive regulator of a directional cell wall synthesis which results in the conical-papillate shape of epidermal cells on wild-type adaxial petal surfaces. This cell shape is thought to affect the optical properties of the petal epidermis such that incident light passes through the pigmented cell before it is reflected, and thus perceived intensity of pigmentation is enhanced. Epidermal cells of mixta mutant petals are flat by comparison, but retain other characters normally associated with the conical cell type (pentagonal symmetry, striations), indicating that the role of the MIXTA gene in this differentiation pathway is specific and late. Recently, Glover et al. (1998) have published a characterization of the MIXTA overexpression phenotype in Nicotiana tabacum. The data presented herein are in good agreement with their results which indicate that the transgene can also regulate trichome differentiation in tobacco.

Suppression of the pleiotropic Arabidopsis ttg1-1 mutant by overexpression of the R gene from maize, a monocot, dramatically demonstrated the potential utility of heterologous gene expression as a tool for manipulating and studying the regulation of cellular differentiation processes in plants. Such an approach necessarily assumes that the regulatory mechanisms of similar pathways and developmental programs have been at least partly conserved over evolutionary time. At the outset of the present study we hypothesized that this conservation would be reflected by heterologous functioning of known epidermal cell shape regulators. Herein we describe an unsuccessful attempt to replace the trichome-initiating function of the Arabidopsis GLI gene by overexpression of MIXTA, a myb-class cell shape regulator from another dicotyledonous plant, Antirrhinum, and CotMYBA, a closely related myb-class gene from cotton. We further describe the MIXTA overexpression phenotype in Nicotiana tabacum, which, in contrast to our results with Arabidopsis, includes the production of supernumerary trichomes. Overexpression of CotMYBA in Nicotiana also activates trichome initiation, additionally amplifying a distinct class of multicellular trichomes. Since overexpression of either GLI or R has no effect on trichome initiation in Nicotiana tabacum, our data indicate that the trichomes of Arabidopsis and Nicotiana are not homologous structures and that their differentiation is controlled by distinct regulatory genes. Furthermore, the development of papillate cells and certain classes of trichomes in Nicotiana appears to be controlled by the same regulatory pathway while separate myb-elements can differentially regulate the cell-fate decision to produce separate, partially overlapping subsets of trichomes.

MATERIALS AND METHODS

Plant strains and growth conditions

Arabidopsis thaliana. pMXCD, pCMAX41 and pLBJ4 constructs were used to transform the wild-type WS ecotype, the ttg1-1 mutant in Landsberg erecta outcrossed to WS one time, and the gl1-1 mutant of ecotype Columbia. The R gene was previously overexpressed in the wild-type WS, wild-type RLD, and in the Landsberg erecta ttg1-1 mutant outcrossed to either WS or RLD one time (Lloyd et al., 1992). Plants were potted in Sunshine Mix no. 1 (Premier Horticulture Inc.) and grown under continuous fluorescent illumination at 22°C.

Nicotiana tabacum. All constructs were used to transform tobacco introduction TiT347 ‘Praecox’ pMXCD was also transformed into strain NC744. Both were supplied by the US Tobacco Germplasm Collection. Previously, R and C1 overexpression phenotypes were also evaluated in the ‘Xanthi’ cultivar. Potting mix consisted of a 2:1:1 mixture of Bacto, Sunshine no. 1, and vermiculite. Plants were grown in a warm greenhouse with supplemental fluorescent light to achieve 16-hour days.
**Vector construction**

pJB34. The oligonucleotide GL1-C (5'-GGGGGGGGCTGCAGA-TTAACTAAGGGCAGCTACTC-3') was used to prime reverse transcription of the GL1 cDNA from Col-0 RNA. The cDNA was amplified by PCR using the primers GL1-C and GL1-A (5'-GGGGGGGGGATCATGAGAATTAGAGAAGAGATG-3') and then cloned into pBluescript II SK+ (Stratagene) as an EcoRI-PstI fragment to create pSRV2. The ends of the insert from pSRV2 were sequenced and subsequently cloned into the binary plant vector pKYLX71 (Scharld et al., 1987) as a HindIII-XbaI fragment. The g11-1 mutation has been complemented by transformation of this construct into Arabidopsis (Lloyd et al., unpublished).

pMXCD. The MIXTA cDNA was cloned by ligating a HindIII-SacI restriction fragment from the pUC-18-derived plasmid pJAM980 (Glover et al., 1998) into pKYLX71.

pMKMY-1. Oligonucleotides MXM5 (5'-GGGAAGTTGAAATCTGTTAGATGCGAGTCTACTC-3') and MXM3 (5'-GGGGAGCTGCACCTAACGCTTCTTTAGATGAGTGTTCCACG-3') were used to amplify the first 356 nucleotides of MIXTA from pJAM980. MXM3 incorporates a stop codon after the arginine residue which terminates the R3 myb repeat. The PCR product was cloned into pBluescript II KS+ (Stratagene) as a SacI-HindIII fragment to create pMXM1-1. The pMXM1-1 insert was completely sequenced from M13 forward and reverse primers, and then subcloned into pKYLX71 (Scharld et al., 1987) as a SacI-HindIII fragment.

pCMAX41. The CotMYBA gene was amplified by PCR from genomic Gossypium hirsutum cv. Texas Marker-1 DNA using the primers COTMYBA-5' (5'-GGCGATTCCTCGGACCATGGAAGAAGT-3') and COTMYBA-3' (5'-GGGCTTGACTCTGACCTTTACGGATGATGAGTCTACTC-3'). After digestion with XhoI and XhoI, the PCR product was ligated into pBluescript II KS+ (Stratagene) to create pBSCMAX4. The ends of this insert were sequenced and then the insert was subcloned into pKYLX71 as an XhoI to XbaI restriction fragment.

pCMANX2. This overexpression construct contains an intronless version of CotMYBA amplified by RT-PCR from a pCMAX41-transformed TI1347 line using the COTMYBA-5' and -3' primers described above. The PCR product was cloned into pBluescript II KS+ (Stratagene) as an XbaI to XhoI fragment to create pCMP2. The insert was completely sequenced and compared to the GenBank cDNA accession to verify correct splicing. The cDNA insert from pCMP2 was then subcloned into pKYLX71 as an XhoI to XhoI fragment. TI1347 plants transformed with pCMANX2 displayed the same range of developmental abnormalities seen in pCMAX41 transformants (data not shown).

The construction of pAL144, 35S:R, and pAL71, 35S:CL, is described elsewhere (Lloyd et al., 1992).

**Plant transformation**

Binary constructs were introduced into Agrobacterium tumefaciens strain GV3101 containing pMP90 (Koncz and Schell, 1986) by electroporation. Arabidopsis was transformed using the protocol of Valvekens et al. (1988), except in the case of the pCMANX2 construct, which was vacuum infiltrated essentially as described by Bechtold et al. (1993). Nicotiana tabacum transformation was accomplished essentially as described by Horsch et al. (1985), except that explants were derived from hypocotyls of one-week-old seedlings. With the exception of pMMKY, a minimum of fifteen to twenty-five independent transformants were produced for each construct. Where overexpression phenotypes were not observed, transcription of the transgenes was verified for subsets of transformants using either northern blot analysis or RT-PCR.

**Scanning electron microscopy**

Plant materials were fixed overnight in 2% glutaraldehyde and 0.1 M cacodylate, then taken through an alcohol dehydration series, once in 25, 50, 75, 85, 95% and twice in 100% ethanol, for at least 2 hours per step. Specimens were critical point dried in a Tousimis Samdri-790 and sputter coated with a gold-palladium alloy using a Ladd instrument. Specimens were visualized with a Phillips 515 scanning electron microscope and photographed with Polaroid film.

**Plant sectioning and light microscopy**

Tissue samples were fixed and stained as described by Mauseth et al. (1984). Sections were photographed through an Olympus BX60 microscope with an Olympus PM-C35DX camera.

**Image processing**

Images for figures were scanned with a Sharp XJ325 High Resolution Color Scanner. Figures were constructed using Adobe Photoshop 4.0. Colors were corrected to match the living specimens and adjustments in brightness and contrast were made using this program. Images were printed with a FUJIX Pictography 3000 printer.

**RESULTS**

**Overexpression of MIXTA in Arabidopsis thaliana does not complement the g11-1 mutation**

Given the structural similarities between GL1 and MIXTA and their roles in the control of epidermal cell differentiation, we hypothesized that MIXTA might be able to substitute for the function of the Arabidopsis trichome regulator. The MIXTA cDNA was placed under the transcriptional control of the CaMV 35S promoter in a binary vector and used to transform by means of Agrobacterium tumefaciens wild-type Arabidopsis and the g11-1 and ttg1-1 mutants. No differences in trichome production or other epidermal characters could be discerned. Transcription of the transgene was verified by northern blot analysis (data not shown).

Since the MIXTA gene was isolated from Antirrhinum majus based on its effects on floral papillation, we also examined the petals of pMXCD transformants. Scanning electron micrographs of petal epidermis (data not shown) revealed no differences between 35S:MIXTA and untransformed plants of any of the three genotypes. No published transformation protocols for Antirrhinum exist, and the reciprocal experiment, overexpression of GL1 in that species, was not attempted.

**Mixta overexpression in Nicotiana tabacum results in supernumerary trichome production**

Like Antirrhinum majus and unlike Arabidopsis thaliana, Nicotiana tabacum produces flowers pigmented by anthocyanins. Concurrent with the Arabidopsis experiment, we transformed N. tabacum with pMXCD to evaluate the effects of MIXTA overexpression on floral pigmentation. Noda et al. (1994) have previously shown that in Antirrhinum the gene exerts its effects on floral phenotype by altering epidermal cell shape and thus perception of pigmentation rather than quantity of pigment. One floral phenotype of 35S:MIXTA transformants is shown in Fig. 1D. Several other transformants produced mottled pink flowers different from the one shown. The adaxial floral epidermis of the transformants was examined by scanning electron microscopy. The SEM shown in Fig. 1H is from the same transformant that produced the small flower in Fig. 1D. Remarkably, many of the papillae have elongated to produce what appear to be multicellular, uniseriate trichomes. These results are similar to those presented by Glover et al., (1998). For comparison, both a flower and floral epidermis
from untransformed TI1347 are shown in Fig. 1A,E, respectively. The red flower in Fig. 1B is from a plant overexpressing the maize R gene. Note that R overexpression up-regulates anthocyanin production in flowers but does not alter floral papillation (Fig. 1F).

Effects of MIXTA overexpression in tobacco were, however, visible prior to flowering. After transfer to soil, rooted shoots began to produce abnormal concave leaves with a spoon-like appearance. This leaf curvature was reversed in later leaves, which were often convex. Leaf blades also showed a characteristic variegation, dark green tissue associated with veins against pale green ground tissue, the latter becoming increasingly yellow as the leaves aged. The leaf epidermis in the vein-associated dark green areas possessed a sheen which was absent from the rough-looking epidermis of the lighter green areas. This variegation and its corresponding uneven pattern of reflectivity are seen in a close-up photograph of the adaxial leaf blade in Fig. 2B. To determine whether this variegation might be due to sub-epidermal cell shape changes, thick sections (25 μm) through adult leaves were sputter coated and examined by SEM, data not shown. No gross differences in sub-epidermal morphology or wall thickness judged to be sufficient to account for the variegation were seen (data not shown). Thinner sections (10 μm) from the same specimens were stained with safranin and counterstained with fast green, then examined by light microscopy. Adaxial epidermis with a pale, rough green appearance was primarily composed of papillate cells and trichomes, whereas dark green areas were composed of mostly rounded cells and occasional trichomes like those seen in the leaf epidermis of untransformed controls. This differential pattern of trichome occurrence and papillation was subsequently confirmed in SEMs of adaxial leaf epidermis (Fig. 2C-G). Leaves and petals are presumed to be homologous structures (Esau, 1977), so it is perhaps not surprising that MIXTA overexpression alters the epidermal characters of both, but our results are particularly interesting in that they suggest these alterations can affect perception of pigments with absorption spectra as diverse as anthocyanins and chlorophylls.

Tobacco plants transformed with the 35S:MIXTA construct were selfed and the resulting T2 seedlings examined by SEM. The cotyledons of N. tabacum are normally devoid of trichomes except at the very base of the petiole (Fig. 11), but in plants overexpressing MIXTA most of the cells of the cotyledonal epidermis have a papillate appearance, and many differentiate into glandular trichomes which are either unicellular or multicellular and uniseriate (Fig. 1L). Hypocotyls and true leaves also produce supernumerary trichomes. These same seedlings were fixed, sectioned, and stained for light microscopy as above. Longitudinal sections through untransformed TI1347 and 35S:MIXTA seedling cotyledons are compared in Fig. 3A and B, respectively. Note that the 35S:MIXTA cotyledon is smaller than the untransformed cotyledon; only the distal portion of the latter is shown. In general, cell expansion appears to be reduced in 35S:MIXTA cotyledons, perhaps due to premature cell wall thickening. The papillate epidermis (both adaxial and abaxial) is visible in Fig. 3B; associated debris is derived from trichomes projecting across the plane of the section. 35S:MIXTA root phenotypes have not been extensively characterized, but a pronounced tendency toward reduced elongation has been documented and increased cell wall thickening is suggested by Fig. 4B and D, as compared witho A and C respectively. This is in contrast to the findings of Glover et al. (1998), who note that they were unable to correlate a root phenotype with MIXTA overexpression.

When GL1 was overexpressed in Nicotiana tabacum the phenotype of transgenic plants was indistinguishable from wild type (compare Fig. 1C, G and K, with A, E, and I). Transcription of the 35S:GL1 transgene was confirmed by RT-PCR (data not shown). This result is further evidence that GL1 and MIXTA have distinct regulatory activities. In addition, R was not observed to affect cotyledonary or leaf trichome development (Fig. 1J).

The myb gene CotMYBA affects trichome differentiation when overexpressed in Nicotiana tabacum

A Blast search (Altschul et al., 1990) of the National Center for Biotechnology Information database using the complete Antirrhinum MIXTA cDNA sequence as a query, calls up a long
Myb genes enhance tobacco trichome production

A distinctive root phenotype is also associated with overexpression of CotMYBA in tobacco. As seen in Fig. 5E, 35S:CotMYBA seedling roots produce lollipop-like root hairs bordered by elongated, turgid-looking cells. The exaggerated appearance of these border cells is emphasized in Fig. 4E,F. In untransformed seedlings they are similarly discernible as a double layer of translucent cells at the cotyledonary margin, but lack the turgid appearance of the transformants. The angle of attachment of cotyledons to hypocotyl varies; some seedlings are Y-shaped as in Fig. 4B, with cotyledons remaining at less than 45° relative to the long axis of the hypocotyl, while others assume a normal perpendicular orientation. Interestingly, transformant cotyledon emergence from the seed coat is delayed relative to untransformed seedlings, whereas radicle emergence is not.

One of the most striking characteristics of tobacco seedlings overexpressing CotMYBA is “loss” of the cotyledonary petiole. The cotyledonary petioles of untransformed seedlings are narrower than the cotyledonary blade, and epidermal cells are elongate and occur in longitudinally aligned files (see Fig. 4G). In the most severe 35S:CotMYBA phenotypes (Fig. 4D,H) the cotyledonary petiole becomes a wider shoulder region in which tissue bulges out from the plane of the blade, epidermal cells have a rounded appearance, and cell files are difficult to recognize.

Like the plants overexpressing Mixta, 35S:CotMYBA plants produce supernumerary epidermal trichomes on cotyledons and other organs. In many of the transformants a preponderance of short-stalked, multicellular “jingle bell” trichomes (corresponding in Goodspeed’s classification scheme to E1, also referred to as hydathodes) like those shown in Fig. 4I, occur. Supernumerary trichomes of this type were not observed in plants overexpressing MIXTA. Whereas 35S:MIXTA cotyledonary trichomes in general appear to be regularly spaced, trichome initiation in 35S:CotMYBA transformant cotyledons looks more random, at least partly as a consequence of the aforementioned epidermal irregularities.

To date, only T2 plants with the mildest phenotypes (Fig. 4A) have survived transplantation to soil. This is not surprising given our observations of seedlings allowed to continue growth on nutrient medium without hormones for up to one month (Fig. 4J-L). The cotyledons of such seedlings may become extremely chlorotic, whereas cotyledons of untransformed seedlings of the same age maintained on the same medium remain green and healthy. Subsequent true leaves may or may not be green at emergence, but often pale as they age. Invariably they are distorted to some degree. In the most severely affected seedlings the epidermis coarsens and becomes increasingly disorganized as it ages, even to the point of being callus-like. Bulbous projections from the epidermis occur, and these are distinct from observed trichomes (compare Fig. 4I, with J and L). The “shoulder” regions of the cotyledons may become pronounced, flattened projections distinguishable from young first true leaves by their positions relative to the rest of the cotyledon (Fig. 4K).

A section through a one-month-old 35S:CotMYBA cotyledon is seen in Fig. 3D. Extreme variation in cell size and shape is characteristic. Large cells lack chloroplasts. (The overall appearance of tissue prior to fixation was chlorotic.) Whereas some tissue is collapsed and apparently necrotic, there are also isolated pockets of adventitious mitotic activity (Fig. 3F compare with Fig. 3C and E, untransformed).

SEM of tobacco seedlings overexpressing the CotMYBA transgene are shown in Fig. 4. Whole 10-day-old T2 seedling shoots seen in A-D demonstrate the variation in overall severity of the phenotype observed in progeny of independent transformants. In all cases the cotyledons are distorted in comparison to untransformed plants (see Fig. 1I), with many surface irregularities. Cotyledonary margins are wavy and list of myb-homologous plant genes. One of the highest scoring but presumably non-orthologous sequences (Clement, Payne, and Lloyd, unpublished) is a GenBank accession, CotMYBA (L04497), isolated from Gossypium hirsutum 3-day pre-anthesis ovules. Cotton fibers are trichomes which are initiated and begin elongation from the epidermis of the cotton ovule at about the time of anthesis (Basra and Malik, 1984). Cotton fiber-specific promoters have previously been shown to direct transcription of reporter genes in trichomes of N. tabacum (Dang et al., 1996), suggesting that the two trichome types share some ontogenetic similarities. To explore the possibility that CotMYBA is a positive regulator of trichome differentiation, we overexpressed the genomic sequence (see Materials and Methods) in tobacco.

SEMs of tobacco seedlings overexpressing the CotMYBA transgene are shown in Fig. 4. Whole 10-day-old T2 seedling shoots seen in A-D demonstrate the variation in overall severity of the phenotype observed in progeny of independent transformants. In all cases the cotyledons are distorted in comparison to untransformed plants (see Fig. 1I), with many surface irregularities. Cotyledonary margins are wavy and

![Fig. 2. Textural variation of Nicotiana tabacum adaxial leaf epidermis. (A) Photograph of untransformed adaxial leaf epidermis. The midvein is vertical. (B) 35S:MIXTA leaf. (C-G) SEMs of adaxial leaf epidermis. (C) Untransformed. (D) 35S:MIXTA, from region adjacent to vein, corresponding to reflective areas in (B). (E) 35S:MIXTA, from interveinal, rough-looking regions as seen in B. Scale bar, 0.1 mm. (F) Detail of non-papillate cells seen in D. (G) Detail of papillate epidermis as in E. Area shown in F and G is 1 mm².](image)
with bulbous tips. Similar structures are occasionally seen on untransformed TI1347 seedling roots, but on 35S:CotMYBA seedling roots they are much more numerous, and their frequency is directly correlated with severity of the phenotype of the aerial organs. A tendency toward disorganization and uncoordinated cell expansion has also been observed as 35S:CotMYBA roots age (data not shown).

*Arabidopsis thaliana* was also transformed with the pCMAX41 construct. Neither of the trichome mutants (ttg1-1, gl1-1) were suppressed. Initial experiments using the transformation protocol of Valvekens et al. (1988) resulted in a small number of regenerants which were stunted and produced aberrant flowers. Attempts to secure transgenic seed or correlate floral phenotypes with degree of papillation on petal epidermis were likewise unsuccessful.

In a recent experiment, vacuum infiltration was used to transform wild-type *Arabidopsis* with the pCMANX2 construct, which would allow us to evaluate transgenic cotyledon phenotypes even in the absence of fertile transgenics. Cotyledonary abnormalities somewhat comparable to those seen in 35S:CotMYBA tobacco seedlings were observed in approximately one quarter of the V1 transformants, but no cotyledonary trichomes were seen. Of 56 kanamycin-resistant V1 seedlings transplanted to soil, twenty-five survived to flowering. Survival on soil was inversely correlated with severity of the cotyledon phenotype. Among the survivors were six plants (derived from three different infiltration seed lots) which shared a syndrome of developmental abnormalities. These included small concave or convex rosette leaves (Fig. 6A) as well as floral defects like those seen in the earlier transformation experiment. In these plants first- and second-whorl floral organs fail to expand properly, or do so in an uncoordinated fashion. As a consequence, fourth- and to a lesser extent third-whorl organs are exposed prematurely (Fig. 6B). To date neither pollen dehiscence nor self-set siliques have been observed, although the plants appear to be fertile when pollinated with wild-type pollen. Further characterization of the 35S:CotMYBA *Arabidopsis* phenotype is planned for these out-crossed progeny of the surviving abnormal V1 seedlings once sufficient seed stocks have been accumulated.

**DISCUSSION**

We have shown that the independent overexpression of two myb-class transcription factors, MIXTA and CotMYBA, significantly perturb development of transgenic tobacco plants, engendering complex phenotypes which include the production of supernumerary trichomes. These same transgenes failed to alter the trichome phenotype of *Arabidopsis*. A known regulator of trichome initiation from *Arabidopsis*, GL1, likewise had no effect on the trichome complement of *Nicotiana tabacum* when overexpressed in that species. Previous experiments in which the myc-homologous *R* gene was overexpressed in the two species suggested that whereas the regulation of the anthocyanin pathway was conserved even between monocots and dicots and in both *Arabidopsis* and *N. tabacum*, the development of trichomes in these dicots was regulated differently. Present results corroborate this hypothesis by identifying two genes which can control trichome cell-fate in *Nicotiana* but not in *Arabidopsis*.

**Limited correlations between myb gene sequence homology and function are seen**

Fig. 7 shows an alignment of the myb DNA binding domains of the three plant myb-element transcription factors used in the
myb genes recently conducted in this lab (Clement, Payne and Lloyd, unpublished) revealed the existence of many previously unrecognized conserved motifs outside the myb DNA binding domain, some conserved between monocots and dicots. Since a majority of known plant myb genes have been cloned by DNA binding domain sequence homology (for example, Li and Parrish, 1995), gene function is often unknown, making the assignment of function to the motifs largely conjectural. Heterologous overexpression experiments such as those described herein, together with sequence analysis, may enable such assignments to be made with greater confidence, especially when the gene in question is derived from a species in which complementable or revertable mutants and transformation/regeneration technology is lacking.

A short N-terminal motif occurs in 31 of 50 R2R3 plant myb genes examined. Immediately C-terminal to the myb DNA binding domain, 20 genes examined encode a GIDPXXH amino acid sequence, as described in Avila et al. (1993); of these, 18 also encode the small amino-terminal motif, including MIXTA and CotMYBA. These motifs are boxed in Fig. 7. None of the flavonoid biosynthesis regulating mybs possess the GIDPXXH sequence, whereas it is present in all mybs directly implicated in plant cell shape regulation except GL1. A role in plant cell shape regulation for another myb from Arabidopsis, AtMYB305, has been inferred from experiments...
in which *Arabidopsis* genes were expressed under an inducible promoter in *Schizosaccharomyces pombe*, and isolated based on their apparent ability to cause cell division anomalies in the yeast (Xia et al., 1996). However, Moyano et al. (1996) have reported that its most similar counterpart in *Antirrhinum majus* is a regulator of phenylalanine ammonia lyase and other phenylpropanoid biosynthetic genes, suggesting that the result in *S. pombe* is due to a coincidence in gene product structure not correlated with gene function in plants.

*AmMYB308*, which also contains the GIDPXXH motif, may affect pallisade cell shape in *Antirrhinum* (Noda et al., 1994) and it is reported to inhibit lignin biosynthesis when overexpressed in tobacco (Tamagnone et al., 1998).

As previously mentioned (see Introduction), the morphogenesis of unicellular *Arabidopsis* trichomes is associated with endoreduplicative DNA increase. Melaragno et al. (1993) have suggested that once a plant cell undergoes a round of endoreduplication it becomes incompetent to undergo further cell division. Endoreduplication may thus constitute cellular commitment to a terminal differentiation program. Although no attempt to quantify DNA content has been made in the present study, the tobacco trichomes affected by overexpression of *MIXTA* and *CotMYBA* are frequently multicellular, which might imply that endoreduplication is unnecessary for *Nicotiana* trichome morphogenesis. If trichome genesis in the two species is as dissimilar as our experiments indicate, one would not expect to encounter homology between the implicated myb genes other than that which was minimally required for DNA binding domain function. Besides the common N-terminal motif, homology between *CotMYBA* and MIXTA outside the myb DNA binding domain is confined to an identically positioned GIDPXTH amino acid sequence, which suggests a role for the latter motif in cell shape regulation in tobacco.

**The phenotypes of tobacco plants overexpressing *MIXTA* or *CotMYBA* are distinct**

The most parsimonious explanation for our results would be that in *Nicotiana tabacum*, a myb-class transcription factor with extensive homology to MIXTA or CotMYBA regulates trichome differentiation, and that overexpression of the homologous genes from other species can substitute for or augment its activity to initiate ectopic trichome production. But such a facile interpretation fails to take into account the complexity of the observed phenotypes and the superficiality of their resemblance.

A hypothetical gene-for-gene relationship is a better fit for the *MIXTA* data than for *CotMYBA*, since the effects of its overexpression appear to be specific to the epidermis. As previously stated (see Results), overexpression of *MIXTA* in tobacco results in hyperpapillate leaf and petal epidermis, which in turn alters perception of plant pigments consistent with the reported function of the gene in *Antirrhinum*. However, in tobacco plants overexpressing *MIXTA* many of these papillate cells become what appear to be multicellular trichomes. One would imagine that natural selection by pollinators has optimized pigment perception in the *Antirrhinum* floral epidermis and thus the duration of the directional cell wall deposition signal presumably directed by *MIXTA*. In the *35S:MIXTA* transgenic tobacco plants an unregulated exogenous signal has been applied and some epidermal cells have demonstrated a prolonged capacity to respond. The multicellularity achieved by some of these trichomes may reflect a general cellular mechanism set to maintain the nuclear DNA/cytoplasmic volume ratio of cells within viable parameters.

In the case of *CotMYBA*, the effects of overexpression in...
tobacco are global, implying that some fundamental cellular process is disrupted or exaggerated, one result being the production of supernumerary trichomes. These trichomes are of several distinct morphological classes, again indicating that the process regulated by CotMYBA is less specific. In severely affected 35S:CotMYBA transformants, non-trichome cells maintain the ability to elongate and expand long after normal cotyledon development has ceased. Pattern maintenance as well as pattern formation is defective. It may be that in cotton ovules CotMYBA positively regulates cell wall extensibility to allow for the considerable unidirectional fiber growth which occurs during development of the cotton boll. In Nicotiana tabacum, increased cell wall extensibility might trigger a certain subset of competent epidermal cells (perhaps those having conducive oriented cytoskeletal elements) to differentiate into trichomes.

In a preliminary characterization of tobacco plants overexpressing both MIXTA and CotMYBA, F1 progeny seedlings closely resemble seedlings overexpressing MIXTA alone (Payne and Lloyd, unpublished) indicating that the MIXTA transgene is epistatic to the CotMYBA transgene. MIXTA may function earlier than CotMYBA in cell morphogenesis or the expansion-promoting activities regulated by the two transgenes may in fact be antagonistic.

In Antirrhinum majus floral papillae, MIXTA is thought to regulate directional deposition of cell wall material (Noda et al., 1994). Random, excessive deposition of cell wall material coupled with an epidermal cell’s normal tendency to expand might result in the genesis of a trichome, since the integrity and shape of the wall facing outward would not be reinforced or constricted by the walls of surrounding cells. The supernumerary trichomes 35S:MIXTA plants produce are uniseriate vectors essentially perpendicular to the epidermis.

The intriguing possibility exists that both MIXTA and CotMYBA perturb cellular processes such that the physical properties of cell walls or cytoskeletal elements are changed, and that these physical manifestations in turn trigger the expression of trichome genes downstream of endogenous trichome initiation regulators, bypassing them. A possible role for physical constraints in determining organ differentiation has been demonstrated in Helianthus (Hernandez and Green, 1993).

The results of overexpression studies are potentially artifactual. Because the transgenes are constitutively transcribed from the strong CaMV 35S promoter, the results must be interpreted carefully. It is possible that the overexpression phenotypes of MIXTA and CotMYBA in tobacco are the result of non-productive interactions with gene promoters or endogenous regulatory proteins. The maize C1-I mutant allele of C1 is an endogenous dominant repressor of anthocyanin structural gene transcription. The C1-I gene product is truncated relative to C1, resulting in loss of the C-terminal amphipathic alpha helix, which functions in the full-length allele as an activator of transcription (Paz-Ares et al., 1990; Goff et al., 1992). C1-I retains the myb DNA binding domain, and apparently represses anthocyanin synthesis by competing with the full-length C1 gene product for binding to structural gene promoters. C1-I may also titrate out transcriptional activators by formation of non-functional heterodimers with C1 (Franken et al., 1994) or other regulatory proteins. When the Arabidopsis CAPRICE (CPC) gene is
overexpressed in wild-type A. thaliana, an overall reduction in trichomes and an increase in root hair production are seen. It has been proposed that CPC, which encodes a single R2-homologous myb motif and no obvious transcriptional activation domain, competes with GL1 in the regulation of the GLABROUS 2 gene since the phenotypes of gl2 mutants and CPC overexpressing wild-type plants are similar (Wada et al., 1997). It is worthwhile to note that like GL1, C1 does not alter the development of tobacco plants in which it is overexpressed (Lloyd, unpublished). MIXTA or CotMYBA might bind to heterologous negative regulatory elements in gene promoters or titrate regulatory proteins such that trichome structural gene transcription would be derepressed. Such a competition hypothesis was tested by overexpressing MIXTA from which the putative activation domain had been deleted.

A preliminary characterization of N. tabacum plants transformed with pMMKY, a construct encoding a MIXTA truncation from which everything C-terminal of the myb repeats is deleted, has revealed no developmental abnormalities, indicating that putative C-terminal transcriptional activation domains are necessary for production of the 35S:MIXTA phenotype (data not shown). This result suggests that the overexpression phenotype of the full-length MIXTA transgene is unlikely to be an artifact caused by titration of a MIXTA partner or nonproductive occupation of downstream gene promoters.

It is unlikely that MIXTA or CotMYBA alter the normal course or timing of overall plant development

Changes in epidermal characters such as trichome production have been correlated with phase change and the transition of the shoot from vegetative to inflorescence development programs. In maize, glossy-15 mutants result in the precocious acquisition of adult epidermal characteristics, including the elaboration of hairs (Moose and Sisco, 1994). Arabidopsis leaf trichome distribution changes gradually from exclusively adaxial (on early rosette leaves) to exclusively abaxial (on bracts) as the shoot develops (Telfer et al., 1997). Plants overexpressing R do not violate this trend, implying that in Arabidopsis competence to respond to trichome-specific differentiation regulators is governed by higher-order regulatory factors which are themselves modulated by such environmental stimuli as photoperiod. Increases in stem pubescence are correlated with inflorescence development in Antirrhinum majus (Bradley et al., 1996). None of the genes known to alter the timing of such developmental changes in trichome distribution (including genes involved in floral induction) are myb-class transcription factors. Time to flowering of plants overexpressing MIXTA is similar to that of untransformed plants. Slow growth of surviving 35S:COTMYBA T2 seedlings makes evaluation of flowering time problematical, but delays are likely due to pleiotropic effects of overexpression not associated with transition to flowering per se.

The leafy cotyledon (lec) mutants of A. thaliana bear a superficial resemblance to N. tabacum plants overexpressing MIXTA or CotMYBA, in that they produce cotyledonary trichomes (Meinke et al., 1994; West et al., 1994). However, the precocious germination phenotype characteristic of lec1-1 seeds is not seen in tobacco plants overexpressing either MIXTA or CotMYBA, and supernumerary trichomes are not confined to embryonic epidermis. A number of Nicotiana species produce cotyledonary trichomes as a matter of course, and a change in the pattern of expression of a trichome regulatory gene seems a simpler evolutionary explanation than homeotic conversion or heterochrony. Although the cotyledons of plants overexpressing CotMYBA are distorted, their progressive loss of photosynthetic capacity makes them decidedly unlike normal true leaves. Since radicle emergence is simultaneous, slight delays in cotyledonary escape from the seed coat relative to wild-type are probably due to these distortions rather than to abnormalities in GA or ABA metabolism or response, which one would expect to have an effect on germination.

Apparent contradictions between evolutionary distance and heterologous function of transgenes probably reflect parallel conservation of post-transcriptional regulatory mechanisms

Paradoxically, overexpression of the Antirrhinum majus MIXTA gene was not observed to perturb development of floral papillae in Arabidopsis thaliana, even though the latter genome contains a putative orthologue which is 91.3% similar through the myb DNA binding domain. A likely explanation might be that the two genes are insufficiently diverged to overcome post-transcriptional checks operating in A. thaliana. Sequence-specific DNA binding by the vertebrate MYB oncoprotein has been shown to be regulated by a variety of post-transcriptional means, including phosphorylation and redox state (Guehmann et al., 1992; Lüscher et al., 1990; Myrset et al., 1993). Capacity for interactions with other regulatory proteins may also be vital to myb gene function. In maize, the C1 (a myb) and B (one of several R orthologues in this species, a myc) gene products interact to regulate anthocyanin synthesis (Goff et al., 1992). Myb- and myc-class transcription factors also co-regulate anthocyanin synthesis in Petunia hybrida (Quattrocchio, 1994); recently, another anthocyanin regulatory locus, An 11, has been cloned (de V etten et al., 1997). It encodes a cytosolic protein with WD-40 repeats which acts upstream of the myb gene An 2. The TTG1 locus of Arabidopsis is reported to encode a WD-40 protein as well (Walker et al., 1997). Preliminary results of experiments conducted in this lab indicate that while overexpression of the maize R gene can strongly suppress the phenotype of the ttg1-1 mutant, two endogenous myc-class homologues do so more weakly. Overexpression of these same genes in a non-mutant background results in both excess trichome and anthocyanin production equivalent to wild-type plants overexpressing R, indicating that TTG1 influences the functioning of the endogenous myc gene products. The monocot gene may be sufficiently diverged to circumvent restraints imposed by TTG1 on its activity. MIXTA from A. majus may be too similar to its Arabidopsis orthologue to overcome equivalently similar regulatory mechanisms. It is interesting to note in this regard that overexpression of DELILA, the R orthologue from A. majus, results in less anthocyanin accumulation in tobacco than does overexpression of its monocot counterpart, and will not suppress the ttg1-1 mutant phenotype in A. thaliana (Mooney et al., 1995).

The term “trichome” has been used as a catch-all designation for plant epidermal protruberances that in fact vary widely in morphology and function (Mauseth, 1988). Arabidopsis trichomes are exclusively unicellular, and probably function as
physical and chemical barriers to insect feeding (Mauricio, 1998). Of the five distinct morphological classes of trichomes produced by *N. tabacum* (Goodspeed, 1954), two (types C and E) are glandular. Type D trichomes are defined by the presence of specialized stalk cells which may also accumulate secretory products. The fact that these do not occur together in every species of the genus *Nicotiana* implies that development of each type proceeds from the activity of specific and in some cases separate sets of regulatory genes, which would not preclude sharing of structural genes or trichome building mechanisms between types. *MIXTA* appears to exert its effects primarily on trichome types A and C. Overexpression of *CotMYBA* also promotes the development of Type E trichomes, which may indicate that it regulates or perturbs some growth process or fate-determining mechanism fundamental to all three. At least 23 myb gene sequences have already been cloned from *Arabidopsis* (Wang et al., 1997), and according to one estimate up to a hundred may be present in the genome (Martin and Paz-Ares, 1997). It has been suggested that proliferation of the myb regulatory gene family was correlated with the evolution in plants of specialized physiological functions (Martin and Paz-Ares, 1997). Indeed, the generalization can be made that those mybs to which functions have been assigned regulate late or downstream events in plant organ development, such as the epidermal characteristics of petals or leaves. Of course this bias in recovery might stem from the lethality of mutations in more fundamental developmental pathways. Given the large size of the myb gene family and observed regulatory association of these genes with downstream developmental events, it is perhaps not surprising that myb genes have been implicated in the differentiation of trichomes as different morphologically as those of *Arabidopsis* and *N. tabacum*. Our results imply that these myb genes (*GL1* vs. *MIXTA* or *CotMYBA*) are as distinct as the structures their activities give rise to. This is in sharp contrast to the well-documented conservation of regulatory genes which exists in flower development (Coen and Meyerowitz, 1991; Mandel et al., 1992). From a hierarchical standpoint this disparity is readily explained by the non-essential character of the trichome relative to the fundamental importance of the flower in the plant life-cycle. And since trichomes are simpler organs, one would suppose that fewer structural genes need be subjected to coordinate regulation in order to produce them.

We thank Dr Cathie Martin of the John Innes Institute for pJAM 980, Barbara Goettgens and John Mendenhall of the University of Texas Cell Research Institute for assistance with SEMs and image processing, and Dr Verne Sisson of the Oxford Tobacco Research Station for supplying *Nicotiana tabacum* germplasm. This work was partially supported by the Institute for Cellular and Molecular Biology, UT-Austin, and the Texas Higher Education Coordinating Board.

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