Ectopic Expression of the Arabidopsis AtMYB23 Gene Induces Differentiation of Trichome Cells

Victor Kirik,[*],[†] **Arp Schnittger,**[‡] **Volodymyr Radchuk,**[*] **Klaus Adler,**[*]
Martin Hülskamp,[†] **and Helmut Bäumlein**[*],[1]

[*]*Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, 06466
Gatersleben, Germany;* [†]*Botanisches Institut, Universität Köln, Gyrhofstrasse 15,
50923 Köln, Germany; and* [‡]*Entwicklungsgenetik, ZMBP, Universität Tübingen,
Auf der Morgenstelle 1, 72076 Tübingen, Germany*

The control of epidermal cell fate is a complex molecular process and requires the regulatory activity of different transcription factors. Here, we describe the isolation of a member of the *Arabidopsis* MYB transcription factor family, *AtMYB23*, that is involved in trichome development. Expression of the *AtMYB23* gene under the control of the viral *CaMV 35S* promoter causes the development of ectopic trichomes. The formation of ectopic trichomes depends on *TRANSPARENT TESTA GLABRA1* but not on *GLABRA1*. The absence of the negative regulator TRIPTYCHON leads to branching of the ectopic trichomes on cotyledons and the formation of ectopic trichomes in the leaf subepidermal cell layer. The *CaMV 35S* promoter-controlled expression of *AtMYB23* can partially rescue the *glabra1* mutant phenotype. Together, the presented data indicate that the *AtMYB23* gene has partially overlapping functions with *GLABRA1* in controlling the initiation of trichome development. © 2001 Academic Press

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INTRODUCTION

The leaf epidermis is a derivative of the outer layer (L1) of the shoot meristem. During growth of the leaf primordia, the epidermis is maintained as a single cell layer and epidermal cells differentiate into characteristic cell types, including pavement cells, stomata, and trichomes. Studies of trichome initiation and development in *Arabidopsis* are facilitated by the large cell size, distinct form, and dispensability of trichomes under laboratory conditions.

The first detectable change in the development of the trichome precursor cell is the cessation of cell division concomitant with lateral expansion. The nuclear DNA undergoes three rounds of endoreduplication reaching a level of 16C (Hülskamp *et al.,* 1994). The committed cell then expands perpendicularly to the leaf surface. Branch formation coincides with the fourth round of endoreduplication (Hülskamp *et al.,* 1994). The mature trichome reaches a size of 300–500 μm. At the time of initiation, neighboring trichome precursor cells are separated by about

four epidermal cells (Hülskamp *et al.,* 1994; Larkin *et al.,* 1996). A lateral inhibition activity that prevents neighboring cells from adopting trichome fate has been suggested to be involved in the selection of the trichome precursor cell (Larkin *et al.,* 1994; Schnittger *et al.,* 1999).

Genes that regulate trichome initiation and spacing include *GLABRA1* (*GL1*), *TRANSPARENT TESTA GLABRA1* (*TTG1*), *GLABRA3* (*GL3*) (Koornneef, 1981; Koornneef *et al.,* 1982), and *TRIPTYCHON* (*TRY*) (Hülskamp *et al.,* 1994).

In the epidermis of the *try* mutant, two to four adjacent epidermal cells can adopt trichome cell fate and form clusters. Trichome nuclei in the *try* mutant exhibit increased DNA content level, reaching 64C (Hülskamp *et al.,* 1994). Thus, TRY acts as a negative regulator involved in the lateral inhibition of cells neighboring a trichome precursor cell and in the repression of the DNA endoreduplication in the developing trichome.

Mutations in *GL1* and *TTG1* result in the loss of trichomes, suggesting that they act as positive regulators of trichome initiation. TTG1 also plays a role in lateral inhibition since some weak *ttg* alleles produce trichome clusters (Larkin *et al.,* 1994). In addition, *ttg1* mutant plants

do not produce anthocyanin and seed coat mucilage, but have an increased number of root hairs (Koornneef, 1981). The *TTG* gene codes for a protein containing four conserved WD-40 repeats which are thought to be involved in protein–protein interactions (Walker *et al.,* 1999). The pleiotropic nature of the *ttg* phenotype suggests an interaction of TTG protein with components of several regulatory pathways.

A null mutation in the *GL1* gene results in the absence of trichomes, but has no other visible phenotypes. Cloning of the *GL1* gene revealed its structural similarity to transcription factors of the MYB gene family (Oppenheimer *et al.,* 1991). The *GL1* gene is expressed in the epidermis of young leaf primordia, and the transcript accumulates in developing trichomes. Constitutive expression of the *GL1* gene under the *CaMV 35S* promoter can rescue the *gl1* phenotype and leads to the formation of ectopic trichomes on cotyledons (Oppenheimer *et al.,* 1991; Larkin *et al.,* 1994). Rare trichomes are observed in the *gl1-1*-null mutant, suggesting that additional factors with partially overlapping function are involved in the induction of trichome development.

The *GL3* gene acts as a positive regulator of both the trichome initiation and growth. In the *gl3* mutant, trichomes are initiated with reduced frequency and show a lower level of DNA endoreduplication (Koornneef *et al.,* 1982; Hülskamp *et al.,* 1994; Szymanski *et al.,* 2000). Recent cloning of *GL3* revealed that it encodes a basic helix–loop–helix (bHLH) transcription factor that is similar to the regulators of the anthocyanin biosynthesis *DELILA* from *Antirrhinum* and *R* from maize. Ectopic expression of *GL3* can rescue the *ttg1* mutant phenotype, suggesting that *GL3* acts downstream of *TTG1* (Payne *et al.,* 2000).

Genetic analysis indicates that *TTG1* and *GL1* are upstream regulators of *GL2*. The *GL2* gene encodes a homeobox protein that is required for trichome cell expansion, branching, and maturation (Rerie *et al.,* 1994; Masucci *et al.,* 1996). Expression of the *GL2* gene is strongly reduced in *gl1* and *ttg1* mutants (Szymanski *et al.,* 1998), suggesting that *GL1* and *TTG* regulate *GL2* expression in trichomes. The activity of TTG1 is probably mediated by GL3, which directly interacts with GL1 (Szymanski *et al.,* 2000). Whereas the *GL1* expression is trichome-specific, the *gl2* mutation is pleiotropic and affects the development of seed coat, trichomes, and root hairs (Koornneef, 1981; Masucci *et al.,* 1996), suggesting that additional factors expressed in these tissues regulate *GL2* expression.

The *WEREWOLF* (*WER*) gene regulates *GL2* expression in the root and hypocotyl. (Lee *et al.,* 1999). *WER* encodes a MYB-related protein with high similarity to *GL1* and *At-MYB23*, and these three putative transcription factors define a distinct subgroup in the *Arabidopsis* MYB protein family (Kranz *et al.,* 1998).

Here, we show that the *35S CaMV* promoter-controlled ectopic expression of the *AtMYB23* can induce ectopic trichome formation.

MATERIALS AND METHODS

Arabidopsis Strains and Plant Growth

Arabidopsis plants were grown at 22°C with 6000 lux of white light for 16 h a day. The mutant lines *ttg1-1*, *gl1-1*-null allele, *gl3*, and *try-EM1* were described previously (Koornneef, 1981; Koornneef *et al.,* 1982; Hülskamp *et al.,* 1994). The *GL1::GUS* transgenic line contains a *GL1* promoter fragment driving *GUS* expression specifically in trichomes and stipules (pGGE4; Larkin *et al.,* 1993). The *GL2::GUS* line contains a 2.1-kb fragment from the 5′-upstream region of the *Gl2* gene (Szymanski *et al.,* 1998). The *try/try 35S::AtMYB23* plants were selected by their strong cluster phenotype among the kanamycin-resistant F$_2$ progeny from the cross between *35S::AtMYB23* line and *try* mutant. This phenotype was persistent in all kanamycin-resistant F3 plants. The *try/try 35S::AtMYB23 GL1::GUS* plants were obtained from a cross between *try/try 35S::AtMYB23* line and *try/try GL1::GUS* line (Schnittger *et al.,* 1998). From the cross between *gl1-1* and *35S::AtMYB23*, 100% of the F$_2$ progeny grown on kanamycin had trichomes on cotyledons and leaves. *gl1/gl1 35S::AtMYB23* lines were obtained in the kanamycin-resistant population of the F$_2$ plants that displayed a new trichome phenotype with respect to trichome branching, clustering, and density (see Results). From the three selected F$_2$ plants, 100% of the kanamycin-resistant F$_3$ progeny displayed reduced trichome density and trichome clusters. Twenty-five percent of the F$_3$ progeny grown without selective antibiotic represented *gl1-1* phenotype. To verify the genotype of these plants, three of them were crossed with the *gl1-1* mutant. About 50% of the examined 140 F$_1$ plants develop trichomes on the cotyledons and reduced number of the irregularly branched trichomes on the leaves. Another 50% of the F$_1$ plants did not have trichomes either on their cotyledons or on the leaves.

3′ and 5′ RACE and RT-PCR

Total RNA was extracted as described by Heim *et al.* (1993). Poly (A)$^+$ RNA was isolated from total RNA by using the Oligo (dT)-Cellulose (Pharmacia) according to Sambrook *et al.* (1989). Synthesis of cDNA and PCR amplification were performed as in Frohman *et al.* (1989). For the 3′ end amplification of transcribed *MYB* gene family members, degenerated primers derived from the conservative regions of MYB proteins were used (Kirik *et al.,* 1998a). To obtain the full-length cDNA of *AtMYB23*, 3′ and 5′ RACE techniques using unidirectional primers were used (Frohman *et al.,* 1989). Poly(A)$^+$ RNA (150 ng) isolated from the leaves of *Arabidopsis* ecotype Columbia was used for reverse transcription with T1r1 primer (5′-TAC GTC AGT TGG TGT TGC GTG GAC-3′) (in 5′ RACE) or with UAP primer from GIBCO BRL (in 3′ RACE). In the 5′ RACE experiment, primers T1r2 (5′-TCT TGA TGA GAG GAG GAC GTT GTG-3′) and T1r3 (5′-TAT AAG GGC CAT AGA CGG TGG AG-3′) were used in combination with the Anchor primer (GIBCO BRL) for the PCR. For 3′ RACE, the combination of primers T1f1 (5′-GGT CTC GGA GAT CAT TCA ACT G-3′) and T1f2 (5′-TGT AGA GTC TCC ACC GTC TAT G-3′) with the UAP were used to amplify the *AtMYB23* cDNA fragment.

For RT-PCR, 100 ng of poly(A)$^+$ RNA isolated from the different *Arabidopsis* (ecotype Columbia) organs were used in reverse transcription with the oligo(dT) primer. To amplify the *AtMYB23* cDNA, primers T1-s1 (5′-TCG GGA TCA AGA AAA GTG AAG-3′) and T1-as1 (5′-TAC TTT TTA GAA GCT AAT CAT C-3′) were used; for amplifying the *GL1* cDNA, GL1-s1 (5′-ATG AGA ATA AGG AGA AGA GAT GA-3′) and GL1-as1 (5′-CTA GAA GCA

AAA TTC ATC ATT AC-3′) primers were used. Minimal number of PCR cycles, which resulted in visible in UV light bands in agarose gel, were selected, and amplification was assumed to be close to exponential. The data were controlled with two independent and by partial sequencing of the obtained PCR products.

Vector Constructs and Plant Transformation

To construct the *35S::AtMYB23* transcriptional fusion, a full-length cDNA of *AtMYB23* was amplified by RT-PCR [from 0.2 µg of poly(A)$^+$ mRNA isolated from leaves of the Columbia ecotype] using proofreading *Pfu* polymerase (Stratagene) and the primer oligonucleotides, including the *Xba*I endonuclease recognition site. Sequencing was performed to select an error-free cDNA fragment. The *Xba*I fragment was isolated from the selected plasmid (pDL13) and recloned into binary vector pBinAR (Höffgens and Willmitzer, 1990). The orientation of the cDNA was determined by restriction analysis. Plasmids harboring the *AtMYB23* in sense and antisense orientations were transformed into *Agrobacterium tumefaciens.* In addition, the construct with the partial *AtMYB23* coding region was made as follows: the *Bgl*II/*Bam*HI 0.4-kb fragment coding for the C-terminal part of the AtMYB23 protein, excluding the MYB domain, was isolated from the pDL13 and recloned into *Bam*HI site of pBinAR (Höffgens and Willmitzer, 1990).

To generate the *AtMYB23::GUS* construct, 2032-bp and 1926-bp promoter fragments as well as 1516 bp of 3′ gene fragment following cDNA coding region were PCR amplified with *Pfu* DNA polymerase. Error-free fragments were fused to the β-glucuronidase gene in pGUS1 (Peleman *et al.,* 1989) and the complete reporter construct was recloned into the binary vector pBin19. The constructs were used for the *Agrobacterium*-mediated transformation of *Arabidopsis,* using the vacuum infiltration technique (Bechtold *et al.,* 1993). Transgenic T1 plants were selected by plating on media containing kanamycin (50 µg/ml).

Microscopy and Cytophotometry

Histochemical analysis of plants containing the GUS reporter gene was performed as described previously (Vroemen *et al.,* 1996). 4′6-Diamidino-2-phenylindole (DAPI) and cytophotometry were done as previously described (Hülskamp *et al.,* 1994).

For scanning electron microscopy, plant material was fixed by slow-speed freezing as described previously (Adler *et al.,* 1996). Images were processed by using Adobe Photoshop 3.0, Fotofonish 3.0, Word 6.0, and Aldus Feehand 7.0 software.

RESULTS

Isolation of the AtMYB23 Gene

Using degenerate primers derived from conservative regions of MYB-related proteins, several *Arabidopsis MYB*-genes (*AtMYB13*, *AtMYB44*, *AtMYB77*) have been identified as putative downstream genes of the *FUS3* gene, an essential regulator of seed maturation (Luer βen *et al.,* 1998; Kirik *et al.,* 1998a,b). In addition to these genes, we have isolated the cDNA corresponding to the *AtMYB23* gene (accession no. Z68158). This gene exhibits high similarity to the key regulator of trichome development, GL1, and was chosen for further analysis.

Southern hybridization with the gene-specific 3′ end of the *AtMYB23* cDNA detected signals consistent with a single gene copy in the *Arabidopsis* genome (data not shown). To determine the position of the *AtMYB23* gene, 29 recombinant inbred lines produced from a cross between Columbia and Landsberg ecotypes (Lister and Dean, 1993) were tested for segregation against 94 RFLP markers. The *AtMYB23* was localized on chromosome V close to the RFLP marker m247. This gene position has been confirmed by the sequence of the P1 clone MPO12 that contains the *AtMYB23* gene (*Arabidopsis* Genome Initiative).

In order to study the putative function of the *AtMYB23* gene, the full-length cDNA was isolated by using 3′ and 5′ RACE (Frohman *et al.,* 1989). The cDNA clone *AtMYB23* shows high similarity both to the *GL1* and the *WER* genes, especially within the DNA binding domain (see Lee and Schiefelbein, 1999). The sequence similarities also extend into the 5′- and 3′-nontranslated regions of the three genes. A characteristic feature of the *AtMYB23* cDNA is the presence of a long TC microsatellite (76 nucleotides with 2 mismatches) in the 5′-untranslated leader region. Interestingly, the *GL1* gene also includes a stretch of TC nucleotides (16 nucleotides with 1 mismatch) followed by a stretch of 16 CA nucleotides preceding the protein coding sequence. A shorter and interrupted TC stretch (in total 16 nucleotides) is also present in the leader of the *WER* gene. The high structural conservation of the AtMYB23, GL1, and WER (see dendrogram in Kranz *et al.,* 1998) suggests their close evolutionary relationship and indicates putative common functional features in controlling epidermal cell differentiation.

Expression of the AtMYB23 Gene Promoter GUS Construct

The *AtMYB23* gene transcript could not be detected by conventional Northern analysis, probably due to the low abundance of the message. Therefore, we applied RT-PCR to compare the organ-specific expression of both genes. As shown in Fig. 1 the *GL1* transcript can be detected in leaves, stems, and flowers, but in contrast to *AtMYB23,* no expression was detectable in seed and root tissues. The data were confirmed with two independent RNA samples and by partial sequencing of the obtained PCR products. Thus, whereas *GL1* mRNA is not found in organs lacking trichome cells, *AtMYB23* mRNA could be detected in all analyzed plant organs.

To study the *AtMYB23* gene promoter activity at histological and cellular levels, the gene promoter was transcriptionally fused to the β-glucuronidase reporter gene. Two fragments of 2.0 and 1.9 kb of 5′ flanking sequence were analyzed. The 2.0-kb fragment contains 85 bp of the TC microsatellite sequence of the 5′-untranslated leader, which is absent in the 1.9-kb fragment. An additional construct includes both the 2.0-kb 5′ flanking sequence and a 1.5-kb sequence downstream of the polyadenylation site. We did not observe any difference in the reporter gene
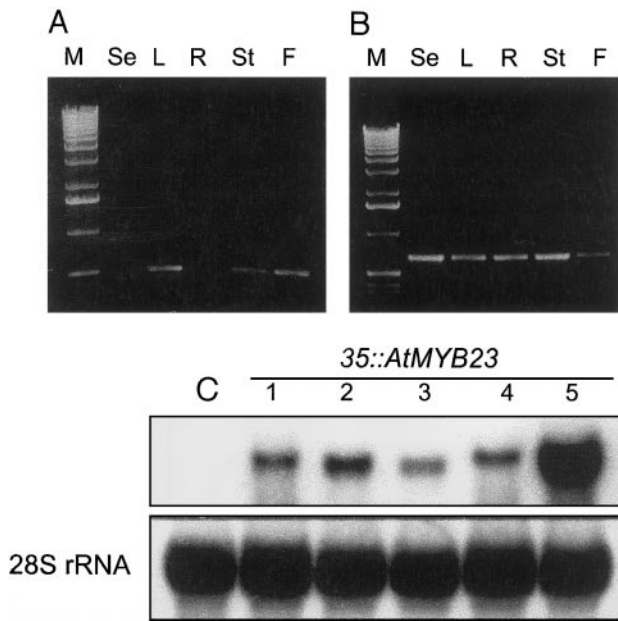
FIG. 1. (Top) *GL1* and *AtMYB23* expression in various organs: Se, seeds; L, leaves; R, roots; S, stems; F, flowers. Lane M contains a 1-kb DNA molecular weight marker (GIBCO/BRL). (A) RT-PCR amplification of *GL1*. (B) RT-PCR amplification of *AtMYB23*. (Bottom) Overexpression of *AtMYB23* under the control of the *CaMV 35S* promoter in different transgenic lines. Total RNA (10 μg per lane) from rosette leaves was hybridized with a gene-specific fragment. Lane C, Wild-type control. Lanes 1–5, Independent transgenic lines. RNA in lane 5 was isolated from a *35S::AtMYB23* plant with reduced trichome density. 28S rRNA was probed as a loading control.

expression in transgenic *Arabidopsis* plants transformed with these constructs (see below). Thus, in contrast to the *GL1* gene, which requires enhancer sequences about 1 kb downstream of the transcribed sequence for trichome-specific expression (Larkin *et al.,* 1993), the *AtMYB23* gene expression most likely does not depend on 3' regulatory sequences.

Strong *GUS* expression was observed in trichomes on leaves and stems (Figs. 2A–2C). In agreement with RT-PCR data, GUS activity was detected in the cell division and differentiation zone of the root (Figs. 2D and 2E) and at low level in the seed coat (not shown). Weak expression was also detected at the base of flowers and siliques (not shown). The *AtMYB23::GUS* activity was detected in developing leaf primordia (Fig. 2C) with the highest level in developing trichomes. A similar pattern has been described for the expression of *GL1* with high levels of activity in cells committed to the trichome cell fate (Larkin *et al.,* 1993). In contrast to *GL1*, the activity of the *AtMYB23::GUS* construct did not decline in the fully developed trichomes of older leaves (Fig. 2B).

To check a putative upstream or downstream relation

between *GL1* and *AtMYB23*, we tested the *AtMYB23* gene promoter activity in the *gl1-1*-null mutant background. Like in wild type, the expression of the *AtMYB23::GUS* construct was detected in primordia of developing leaves (not shown). The expression of the *AtMYB23::GUS* construct was detected in the infrequently occurring trichomes of the *gl1-1*-null mutant (Fig. 2F). Together, these data suggest that the *AtMYB23* gene promoter activity does not depend on GL1 activity.
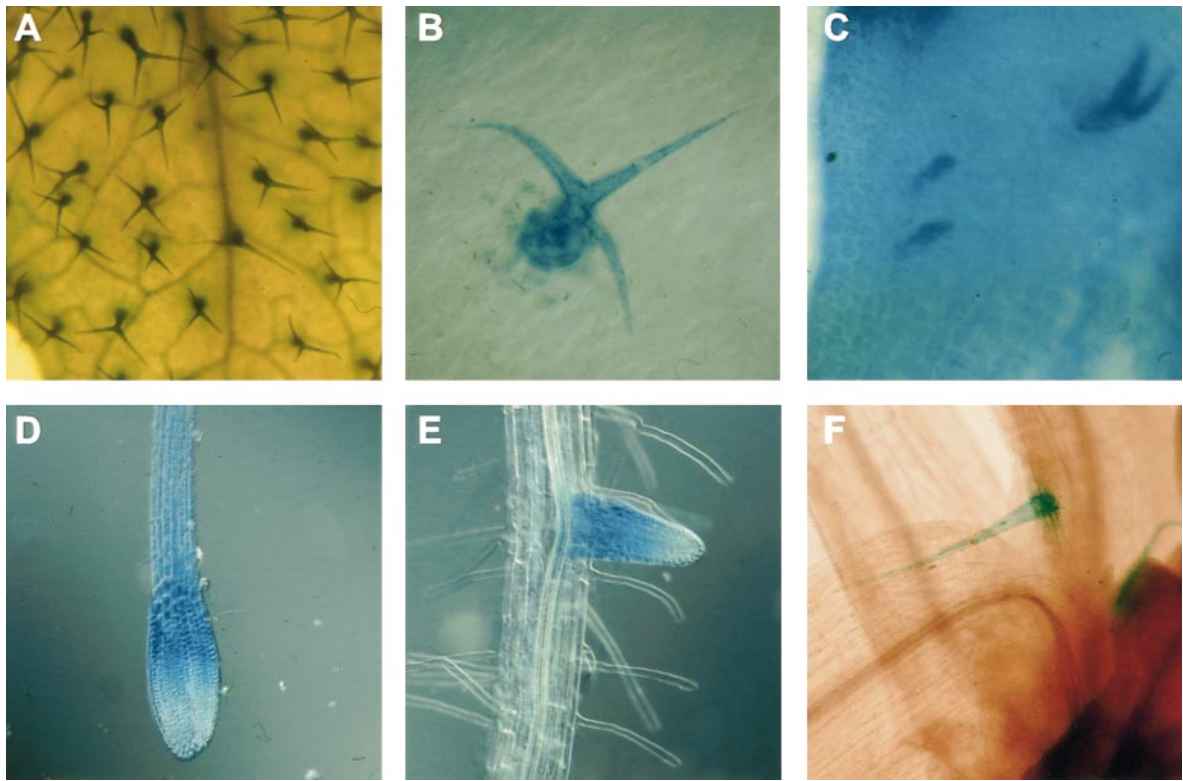
Constitutive Expression of the AtMYB23 Gene Causes Ectopic Trichome Formation

In order to provide further insights into the function of the *AtMYB23* gene, transgenic plants were generated in which the *AtMYB23* gene was expressed in the sense and antisense orientations from the *CaMV 35S* promoter. To prevent a potential cross reaction of the antisense RNA with other *MYB*-like gene transcripts, the highly conserved cDNA region corresponding to the MYB DNA-binding domain was excluded from the antisense construct. Among 34 independent transgenic lines with this antisense construct, no abnormal phenotype was observed.

In contrast, overexpression of *AtMYB23* results in an induction of ectopic trichomes. Many unbranched trichomes are produced on the adaxial surface of the cotyledons as well as on the abaxial surface of the first leaves in *35S::AtMYB23* plants (Figs. 3A, 4B, and 4C). A similar appearance of ectopic trichomes was reported for plants that express the *GL1* gene under the control of the *CaMV 35S* promoter (*35S::GL1*) (Larkin *et al.,* 1994). Whereas only a few trichomes develop on the cotyledon surface of *35S::GL1* plants, a dense trichome pattern was observed in *35S::AtMYB23* plants (Figs. 4B and 4C). Additionally, the cell shape of the hypocotyl epidermal cells is also changed in *35S::AtMYB23* plants. In comparison to wild type, the cells are shorter and have roundish or conical surfaces (Figs. 3F, 3G, 4D, and 4E).

Whereas most *35S::AtMYB23* plants have unchanged trichome density and distribution on the leaves, some transgenic lines have less trichomes than wild type. Several *35S::AtMYB23* lines exhibit reduced trichome number, both on the stem and leaf surface and are, in some cases, nearly glabrous in comparison to wild type (Figs. 3B and 3C). The trichome distribution in these transgenic lines is similar to the *GL1* gene overexpression phenotype with trichomes present at leaf margins only (Larkin *et al.,* 1994). Interestingly, the transgenic lines that show reduced trichome number exhibit the highest expression level of the transgene (Fig. 1).

To check whether ectopic trichomes in *35S::AtMYB23* plants share the molecular pathway of wild-type trichome development, we analyzed the expression of genes active during normal trichome development in ectopic trichomes. A *GL1* promoter *GUS* fusion construct was used as a marker for trichome initiation and early development, and a *GL2* promoter fusion construct was analyzed as a marker

FIG. 2. Expression of the *AtMYB23* gene promoter GUS fusion in *Arabidopsis*. (A) Leaf. (B) Old trichome on fully expanded leaf. (C) Developing trichome on young leaf. (D) Root tips. (E) Lateral root. (F) *AtMYB23* gene promoter activity in the infrequently occurring trichomes of the *gl1-1*-null mutant.

for both early and late trichome development. As shown in Figs. 3H–3K, both *GL1* and *GL2* promoters are active in the ectopic trichomes of *35S::AtMYB23* plants. Consistent with the *GL1* promoter activity in wild-type trichomes, we observed a transient expression of the corresponding reporter construct in young trichomes (Figs. 3H and 3I) with declining expression during their development (Fig. 3J). In contrast, the *GL2* promoter was constitutively active in the ectopic trichomes (Fig. 3K).

These results demonstrate the ability of the AtMYB23 gene product to modify the differentiation of epidermis cells and initiate trichome development. The development of ectopic trichomes is restricted to certain organs. No trichomes were observed, for example, on the abaxial surface of cotyledons or on petals, suggesting that only some epidermal cells are competent to respond to the AtMYB23 gene product activity and produce trichome cells.

Genetic Relation of the AtMYB23 Gene to Other Regulators of Trichome Development

To examine genetic interactions of *AtMYB23* with other genes required for trichome development, we crossed *35S::AtMYB23* plants with *gl3*, *ttg1*, and *try* mutant lines.

Trichomes on the leaves of *gl3/gl3 35S::AtMYB23* plants showed the *gl3* mutant phenotype (data not shown). The cotyledons of the *gl3/gl3 35S::AtMYB23* bear ectopic trichomes typical for the *35S::AtMYB23* phenotype.

In *ttg/ttg 35S::AtMYB23* plants, trichomes are absent both on the leaves and on the cotyledons. Therefore, *AtMYB23* requires the function of *TTG* to induce trichome development. This result also suggests that *TTG* activity is normally present in cotyledons and expression of the *MYB* genes *GL1* or *AtMYB23* is limiting for trichome formation on this organ.

In *try/try 35S::AtMYB23* plants, ectopic trichomes on the cotyledons are branching (Fig. 5A). Since *try/try 35S::GL1* plants develop trichomes in the subepidermal layer (Schnittger *et al.,* 1998; Szymanski and Marks, 1998) the structure of the mesophyll cell layer of *try/try 35S::AtMYB23* plants was examined. Interestingly, we could also observe the development of branched trichomes in the subepidermal layer. Occasionally, these subepidermal trichomes cause an epidermal bulge and even penetrate the epidermis (Figs. 5B–5D). Thus, similar to the *GL1* gene, overexpression of *AtMYB23* and an alteration of *TRY* activity can override normal positional control of trichome cell fate.
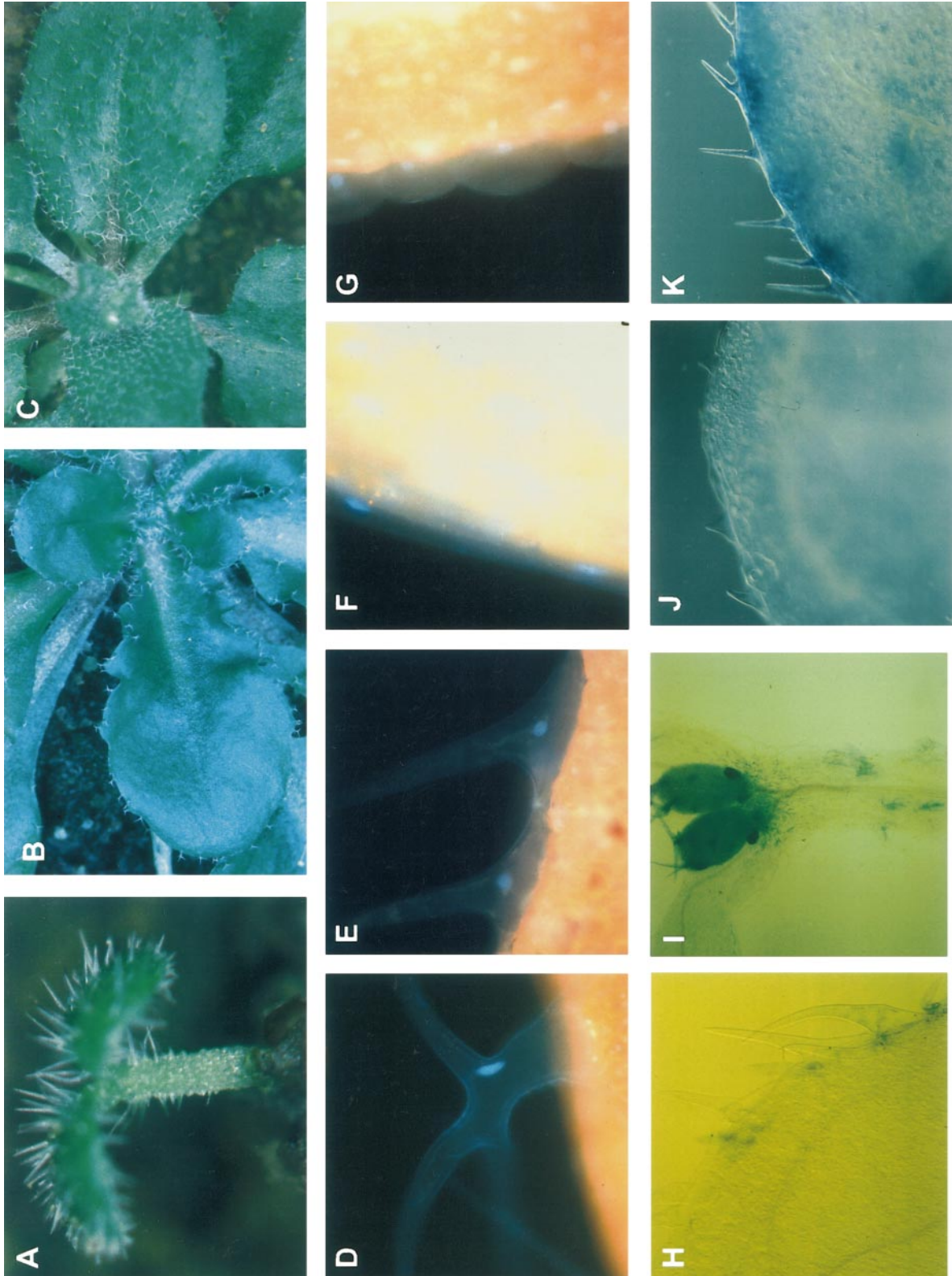
FIG. 3. Ectopic trichomes in 35S::AtMYB23 plants. (A) 35S::AtMYB23 seedling. (B) 35S::AtMYB23 seedling. (C) Wild-type rosette leaves. (D) 35S::AtMYB23 rosette leaves. (E) DAPI staining of the ectopic trichomes on the cotyledon of an 35S::AtMYB23 seedling. (F) DAPI staining of a wild-type leaf trichome. (E) DAPI staining of the ectopic trichomes on the cotyledon of an 35S::AtMYB23 seedling. (F) DAPI staining of wild-type hypocotyl epidermal cells. (G) DAPI staining of 35S::AtMYB23 hypocotyl epidermal cells. (H–K) Expression of trichome-specific molecular markers in 35S::AtMYB23 ectopic trichomes. (H) GL1 promoter::GUS expression in ectopic trichomes on the cotyledons of 3 DAG (days after germination) seedlings. (I) GL1 promoter::GUS expression in hypocotyl conical cells. (J) GL1 promoter::GUS expression in ectopic trichomes on the cotyledons of 6 DAG seedlings. (K) GL2 promoter::GUS expression in ectopic trichomes on the cotyledons of 6 DAG seedlings.
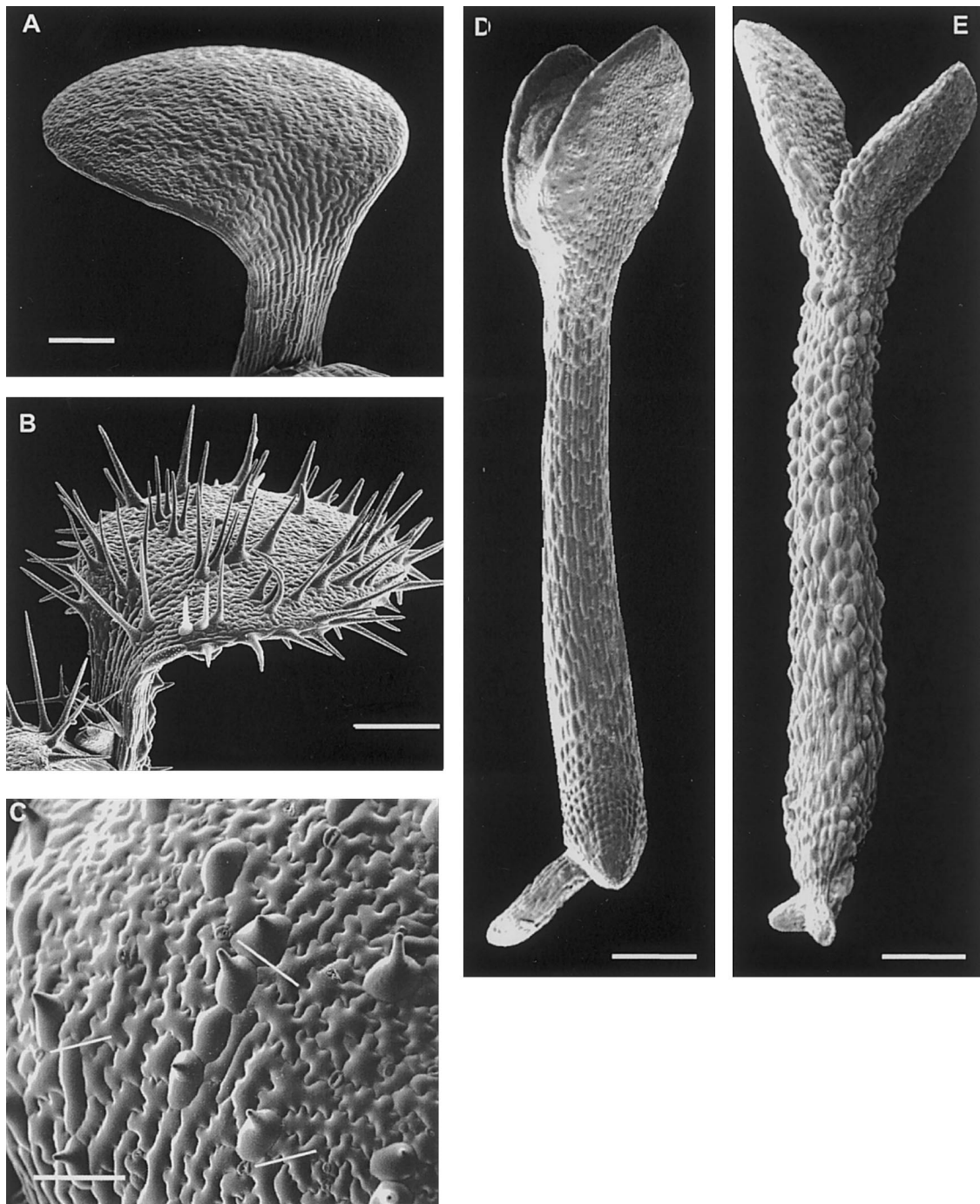
FIG. 4. Scanning electron microscopy of the seedling epidermis. (A) Wild-type cotyledons. Bar is 300 μm. (B, C) *35S::AtMYB23* cotyledons. Bars are 300 μm and 150 μm, respectively. Note the absence of socket cells at trichome base and stomata cells developed adjacent to the trichome (indicated with arrow). (D) Wild-type seedling. Bar is 250 μm. (E) *35S::AtMYB23* seedling. Bar is 250 μm.

Ectopic Expression of AtMYB23 Induces Trichome Development in the gl1 Mutant

High structural similarity between *GL1* and *AtMYB23* and similar overexpression phenotypes suggest that both gene products might share common functions. To address the functional relationship between these two genes, the *gl1-1*-null allele was crossed with the *35S::AtMYB23* line. It was found that 100% of the F$_2$ progeny seedlings selected on

kanamycin develop ectopic trichomes on the cotyledons and abaxial surface of the first leaves. The density of ectopic trichomes was not changed in comparison to *35S::AtMYB23*-expressing plants in the wild-type background. However, a new phenotype showing trichomes on stems and in reduced number on the adaxial side of leaves was observed (Figs. 5F and 5G) in *gl1/gl1 35S::AtMYB23* plants. These trichomes showed irregular branch numbers from zero (unbranched) to three branches and were often found in clusters (Fig. 5H). These data demonstrate that ectopic trichomes in the *35S::AtMYB23* plants do not require the *GL1* gene activity for their development. Moreover, the *AtMYB23* gene expressed under the control of the *CaMV 35S* promoter in the *gl1* mutant background is able to partially rescue the *gl1* phenotype.

DISCUSSION

AtMYB23, GL1, and WER Represent a Structurally Distinct Subgroup of Arabidopsis MYB Factors

The *AtMYB23* gene was isolated in a screen for *MYB*-related genes that are expressed during seed development (Kirik *et al.,* 1998a,b). The *Arabidopsis MYB* gene family encodes proteins with two trypthophan repeats (R2R3) and consists of more than 100 members that share high sequence similarity in the N-terminal MYB DNA-binding domain (Martin and Paz-Ares, 1997; Kranz *et al.,* 1998). The majority of MYB proteins show no obvious sequence similarity outside the MYB domain. However, short stretches of identical amino acids have been detected in the C-terminal part of some MYB proteins, allowing their classification into 22 subgroups (Kranz *et al.,* 1998). The AtMYB23 gene product was classified to subgroup 15 which includes WER (Lee *et al.,* 1999) and GL1. Members of this subgroup share a conserved motif of 19 amino acids in the putative transcription activation domain at the C-terminal end (Kranz *et al.,* 1998).

Mutations in *GL1* result in the absence of trichomes, indicating that the *GL1* gene is a key factor involved in the initiation of trichome development. The *wer* mutation affects root hair initiation and causes the development of ectopic root hairs. The high structural similarity of the AtMYB23, AtMYB66, and GL1 proteins (Lee *et al.,* 1999) implies their close evolutionary relationship and suggests that these factors regulate an overlapping set of target genes. Moreover, the conserved C-terminal part of the AtMYB23, WER, and GL1 proteins includes 27 amino acids that are predicted to be missing in the gl1-2 mutant protein (Esch *et al.,* 1994). The *gl1-2* mutation results in a reduced number of trichomes, and, in combination with the weak *ttg1-10* mutant, it acts as a dominant mutation. This suggests that the truncated gl1-2 protein acts as a repressor of trichome initiation (Larkin *et al.,* 1999). It was hypothesized that the region missing in the gl1-2 protein is part of the C-terminal transcription activation domain (Esch *et al.,* 1994).

### Ectopic Activity of AtMYB23 Can Replace GL1 in Controlling Trichome Initiation

To investigate the effect of the ectopic expression of *AtMYB23*, this gene was introduced into wild-type *Arabidopsis* under the control of the *CaMV 35S* promoter. Transgenic plants developed ectopic trichomes. In wild type *Arabidopsis,* trichomes develop on the adaxial surface of early rosette leaves and on the adaxial and abaxial surfaces of later rosette and cauline leaves, inflorescence, stem, and sepals. They are not found on cotyledons, hypocotyl, petals, and carpels. The distribution of the trichomes in *35S::AtMYB23* transgenic plants is similar to that reported in *35S::GL1* plants (Larkin *et al.,* 1994). In particular, *35S::AtMYB23* plants produce ectopic trichomes on the adaxial surface of the cotyledons. In contrast to *35S::GL1* plants, where only a few trichomes have been reported on the cotyledons, *35S::AtMYB23* cotyledons develop numerous trichomes, often occurring in clusters and strings. This suggests that lateral inhibition of trichome development is less pronounced in *35S::AtMYB23* ectopic trichomes. Moreover, ectopic trichomes on cotyledons and the abaxial surface of the first leaf pair are always unbranched, while, in the *35S::GL1* plants, branched trichomes were also observed (Larkin *et al.,* 1994). In *35S::AtMYB23* plants, branched trichomes were found only in the absence of the functional *TRY* gene product. Thus, the unbranched morphology of *35S::AtMYB23* ectopic trichomes can be explained by the suppression activity of TRY.

Transgenic plants with high expression of the *35S::AtMYB23* construct produced substantially fewer leaf trichomes than wild type. Larkin *et al.* (1994) also reported a reduced trichome number in the plants ectopically expressing the *GL1* gene. This phenomenon was suggested to be the result of "squelching," the titration of an interacting factor (Larkin *et al.,* 1994). A member of the bHLH protein family is a good candidate for such an interacting factor since overexpression of the maize bHLH R gene causes an increased number of trichomes in the *35S::GL1* plants (Lloyd *et al.,* 1994). Thus, the similar trichome-patterning phenotype of *35S::AtMYB23* plants and *35S::GL1* plants suggests that both MYB factors may interact with the same member of the bHLH family.

Ectopic trichomes in *35S::AtMYB23* plants are not branched, possess a nuclear DNA content of 16C (V.K., unpublished observations), and grow in clusters without support cells at the base of the trichome. This indicates that the *AtMYB23* gene alone is not sufficient to activate the entire gene expression program of wild-type trichome development. However, the activity of the *GL1* and *GL2* promoters in the ectopic trichomes demonstrates that ectopic expression of the *AtMYB23* gene can trigger the gene expression program of early trichome development.

To test whether *AtMYB23* can function downstream of *GL1* within the trichome formation pathway, we checked the *AtMYB23* promoter activity in the *gl1-1*-null mutant background. As shown in Fig. 2F, the GL1 function is not
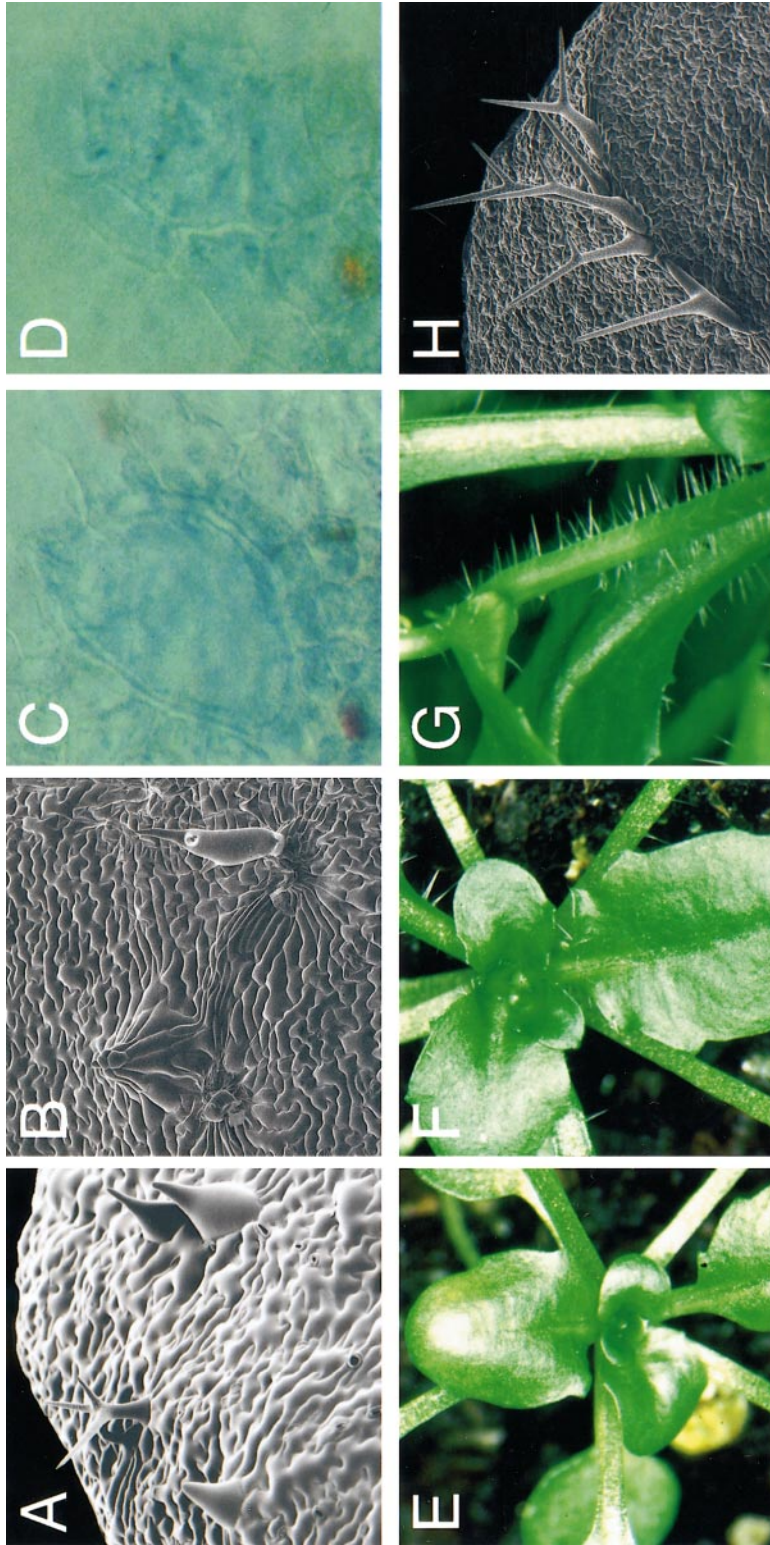
FIG. 5. Ectopic expression of AtMYB23 in different genetic backgrounds. (A) SEM of try/try 35S::AtMYB23 trichomes on a cotyledon. (B) SEM of subepidermal trichomes growing through the leaf epidermis of a try/try 35S::AtMYB23 plant. (C, D) DIC image of GUS-stained try/try 35S::AtMYB23 GL1::GUS plants, focused on the leaf ground tissue (C) and epidermis (D). (E) gl1 rosette leaves. (F) Rosette leaves of a gl1/gl1 35S::AtMYB23 plant. (G) Stem trichomes in a gl1/gl1 35S::AtMYB23 plant. (H) SEM of trichomes on leaves of a gl1/gl1 35S::AtMYB23 plant.

necessary for the normal activity of the *AtMYB23* gene promoter.

Genetic interactions between the ectopically expressed *AtMYB23* gene with different trichome mutants provided additional evidence supporting a partial functional redundancy between AtMYB23 and GL1. The *ttg/ttg 35S::AtMYB23* plants did not produce trichomes on cotyledons or leaves. Thus, ectopic expression of *AtMYB23* cannot bypass the lack of TTG activity. *GL1* and *TTG* were postulated to act in a nonlinear pathway, where the activity of both genes is necessary for trichome development via the regulation of *GL2* (Larkin *et al.,* 1994; Szymanski *et al.,* 1998). The expression of *GL2* is induced in *35S::AtMYB23* ectopic trichomes, suggesting that *AtMYB23* is an activator of *GL2*. *GL1* expression is also activated in ectopic *35S::AtMYB23* induced trichomes, but trichome development does occur in the absence of *GL1*. These results show that *AtMYB23* can replace *GL1* activity necessary for the initiation of trichome development.

Although ectopically expressed *AtMYB23* can rescue the glabrous phenotype of the *gl1* mutant, it is only a partial restoration with respect to the frequency of trichome initiation, pattern formation, and trichome morphogenesis. Trichomes that develop on the leaves of *gl1/gl1 35S::AtMYB23* plants are reduced in number compared to wild type, are not regularly branched, and often grow in clusters.

Another similarity between the *GL1* and *AtMYB23* gene functions was observed in *try/try 35S::AtMYB23* plants. The cotyledon trichomes in these plants occasionally branch and trichomes also develop in the subepidermal mesophyll cell layer of leaves. A similar phenotype was reported previously for the *try/try 35S::GL1* plants (Schnittger *et al.,* 1998; Szymanski and Marks, 1998).

A further argument in support of a partial functional overlap between *AtMYB23* and *GL1* is their corresponding expression patterns. The *AtMYB23::GUS* construct is expressed in a pattern that is similar to that of the *GL1::GUS* construct. Both gene constructs are expressed in the epidermis of the developing leaf primordia with rapidly increasing expression in cells upon their commitment to the trichome developmental pathway (Fig. 3; Larkin *et al.,* 1993).

In the *gl1-1*-null mutant allele, which suffered a deletion of the complete gene coding sequence (Oppenheimer *et al.,* 1994), trichomes occasionally develop at leaf margins (Koornneef *et al.,* 1982), suggesting that another gene can substitute the function of *GL1*. Moreover, in the absence of *GL1*, an inducible form of the *R* gene (Lloyd *et al.,* 1994) could activate the expression of *GL2* (Szymanski *et al.,* 1998) and weak *GL2* promoter activity restricted to the margins of developing leaves was still observed in the *gl1-1* mutant and even in the *gl1-1 ttg* double mutant (Szymanski *et al.,* 1998). Together, these results strongly suggest the existence of additional factors required for trichome development. Our data suggest that *AtMYB23* can have a redundant function with *GL1*, which is manifested in trichome formation at the leaf margins in the *gl1-1* mutant.

Other Possible Functions of AtMYB23

In contrast to *GL1*, the activity of the *AtMYB23::GUS* construct persists in mature trichomes. Another transcription factor necessary for trichome development, GL2, has a similar expression pattern. Since the promoter region, essential for the correct *GL2* expression, has several putative MYB-binding sites, and the *gl1* mutation results in reduced expression of *GL2*, it was suggested that GL1 functions as a direct regulator of *GL2* expression (Szymanski *et al.,* 1998). However, the expression of *GL1* is down-regulated in mature trichomes, whereas the *GL2* gene is expressed at a high level through the entire trichome development. Therefore, it was suggested that additional MYB factors could regulate the expression of *GL2* in mature trichomes (Szymanski *et al.,* 1998). We propose that *AtMYB23* may function in maintaining the *GL2* gene expression level in mature trichomes. Late activity of the *GL2* gene may be required for the proper trichome maturation since the cell wall of the *gl2* mutant trichomes remains thin and lacks papillae.

The activity of the *AtMYB23::GUS* construct also overlaps with that of the *GL2* gene in seed coat and in roots. In addition to the trichome phenotype, plants without a functional *GL2* gene exhibit a hairy root phenotype due to ectopic hair development in atrichoblast cell files, and they fail to produce seed coat mucilage (Koornneef, 1981; Masucci *et al.,* 1996). Another mutation that affects trichome initiation and mucilage production in the seed coat and causes the development of ectopic root hairs is *ttg*. Ectopic expression of the *R* gene is able to rescue all aspects of the *ttg* mutant (Lloyd *et al.,* 1992). Since GL1 can interact *in vitro* with R and ectopic expression of *GL1* and *R* results in ectopic induction of the *GL2* promoter, it has been suggested that the *GL2* promoter is a target for an interacting complex between a bHLH protein and MYB protein GL1 (Szymanski *et al.,* 1998). However, ectopic expression of *R* can complement the *GL1*-independent phenotype of *ttg* plants, suggesting that interaction of the bHLH protein is not limited to GL1.

Expression of *WER* (*AtMYB66*) is restricted to the atrichoblast cell files of the root and to the files of hypocotyl epidermal cells located outside single cortical cell (Lee *et al.,* 1999). WER was shown to interact with a bHLH protein and to activate the expression of *GL2* in these cell files in root and hypocotyl (Lee *et al.,* 1999). Since the expression of *AtMYB23::GUS* construct overlaps with the *WER* expression in the root but is not restricted to the atrichoblast cell files, it is conceivable that *AtMYB23* may maintain a basic level of *GL2* expression in both trichoblast and atrichoblast cell files. *WER* and *CAPRICE* (*CPC*), another MYB-related gene apparently lacking a transcriptional activation domain (Wada *et al.,* 1997), might enhance and suppress this basic level of *GL2*.

Among the three closely related *MYB* genes *GL1*, *WER*, and *AtMYB23*, the expression in the seed coat is specific to *AtMYB23*. Considering the related roles of these three genes in the regulation of *GL2* expression, *AtMYB23* may

be specifically required to regulate the expression of *GL2* in the seed coat. No obvious seed coat mucilage phenotype has been observed in our antisense lines. However, we cannot exclude that the *35S CaMV* promoter does not provide an appropriate strength or timing of the expression in the seed coat.

Expression during trichome development, the ectopic expression phenotype, the genetic interaction with *TRY*, and the rescue of *gl1* mutant provide an indication for the partial functional redundancy of *GL1* and *AtMYB23* genes in the control of epidermal cell fate. The partial replacement of a gene function by a related gene product might be an important evolutionary principle to back up high-fidelity regulatory functions (Cooke *et al.,* 1997).

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