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Control of *GL2* expression in *Arabidopsis* leaves and trichomes

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SUMMARY

More than twenty genes are required for the correct initiation, spacing, and morphogenesis of trichomes in *Arabidopsis*. The initial selection of trichome precursors requires the activity of both the *GLABROUS1* (*GL1*) and *TRANSPARENT TESTA GLABROUS* (*TTG*) genes. The *GLABRA2* (*GL2*) gene is required for subsequent phases of trichome morphogenesis such as cell expansion, branching, and maturation of the trichome cell wall. Previous studies have shown that *GL2* is a member of the homeodomain class of transcription factors. Here we report a detailed analysis of *GL2* expression in the shoot using anti-GL2 antibodies and the GUS reporter gene fused to the *GL2* promoter. The *GL2* expression profile in the shoot is complex, and involves spatial and temporal variation in developing leaves and trichomes. Two separate promoter domains that are expressed in trichomes were identified. *GL2*, like *GL1*, is expressed in developing trichomes and in cells surrounding trichomes during early stages of trichome development. Unlike *GL1*, *GL2* expression persists in mature trichomes. It was found that while *GL1* and *TTG* were not required for the initiation of *GL2* expression in the non-trichome cells, the presence of a functional *GL1* or *TTG* gene was able to increase *GL2* expression in these cells compared to *ttg gl1* plants. The hypothesis that *GL1* regulates aspects of *GL2* expression is consistent with epistatic analysis of *gl1* and *gl2* and the expression patterns of *GL1* and *GL2*. In support of this hypothesis, it was found that ectopic expression of *GL1* in the presence of ectopic expression of the maize *R* gene, which can bypass the requirement for *TTG*, can ectopically activate *GL2* transcription.

Key words: Trichome, *GLABRA2*, Cell fate, Homeodomain protein, *Arabidopsis*

INTRODUCTION

Trichomes are specialized cell types that are present on the surface of nearly all land plants (Johnson, 1975). However, the morphology and pattern of trichomes vary greatly depending on the species. In *Arabidopsis*, leaf trichomes are unicellular and stellate. The process of trichome development is complex and involves genes that regulate their spacing, density, and morphology. Over twenty mutations that affect various aspects of trichome development have been identified (reviewed by Marks, 1997). The analysis of these genes will aid in understanding the molecular mechanisms that control cell fate and differentiation in plants.

The *GLABROUS1* (*GL1*) and *TRANSPARENT TESTA GLABRA* (*TTG*) genes are required for trichome initiation; the surface of *gl1* and *ttg* mutant leaves are glabrous. Mutations in *TTG* also affect anthocyanin synthesis, integument development (Koornneef, 1981), and root hair patterning (Galway et al., 1994). The *GL1* gene has been cloned, and shown to encode a putative *myb*-class transcription factor (Oppenheimer et al., 1991). *GL1* is expressed in fields of cells containing initiating trichomes, and commitment to the trichome cell fate is accompanied by an initial rise in *GL1* expression in the differentiated cell (Larkin et al., 1993). Genetic experiments showed that both *GL1* and *TTG* activity are required for trichome initiation, but the ectopic expression of *GL1* with the cauliflower mosaic virus 35S promoter (35S) does not lead to increased trichome formation nor does it bypass the requirement for *TTG* (Larkin et al., 1994). The maize *R* gene can supply an additional required activity because plants that ectopically express both *GL1* and *R* initiate ectopic trichomes (Larkin et al., 1994). Although the *R* gene can compliment all aspects of the *ttg* mutation (Galway et al., 1994; Lloyd et al., 1992), the *TTG* and *R* genes do not share significant deduced amino acid similarity (A. Walker and J. Gray, personal communication). Therefore the precise relationship between *TTG* and *R* is not known. Nonetheless, the available evidence is consistent with a strict requirement for both *GL1* and *TTG* for trichome initiation (Larkin et al., 1994).

Once an epidermal precursor is specified to enter the trichome pathway, an elaborate morphogenetic transformation occurs (Fig. 1). In wild-type trichome development, a focus of cell expansion centered on the external face of an epidermal precursor cell appears and develops into an elongating stalk. Additional foci of cell expansion are positioned on the growing stalk and produce branches (Hülskamp et al., 1994; Marks, 1994). After trichome expansion ceases the cell wall thickens and numerous papillae form on the outer surface of the trichome.

The *GLABRA2* (*GL2*) gene is required for normal trichome morphogenesis (Koornneef et al., 1982; Rerie et al., 1994; Marks and Esch, 1994). On *gl2* plants, expansion of trichomes is aberrant. Most *gl2* trichomes are either enlarged abortive epidermal cells that expand in the plane of the leaf or unbranched spikes.The walls of *gl2* trichomes do not appear to thicken or acquire papillae. The morphological defects in *gl2* trichome cell expansion and cell wall maturation suggest that *GL2*-dependent activity is required throughout trichome development.

Genetically, the *GL2* gene is downstream from *GL1* and *TTG*, as *gl2 gl1* and *gl2 ttg* double mutants lack trichomes (Hülskamp et al., 1994). Similar to *ttg* plants, *gl2* plants lack seed coat mucilage and form ectopic root hairs (Koornneef, 1981; Masucci et al., 1996). In roots, *GL2* is expressed in files of cells that become atrichoblasts, which suggests that *GL2* limits hair formation in the root (Masucci et al., 1996). The level of *GL2* expression in *ttg* roots is reduced, suggesting that *TTG* has a role in up-regulating *GL2* expression (Di Cristina et al., 1996). A key question centers on the mechanism by which *GL2* integrates positional information to regulate aspects of cell fate and differentiation in both roots and shoots.

GL2 has been sequenced and shown to have sequence similarity to homeodomain proteins (Rerie et al., 1994). Homeodomain proteins are transcription factors that often function to coordinate the expression of several target genes during diverse developmental processes (reviewed by Kessel and Gruss, 1990; Gehring et al., 1994). In *Arabidopsis*, there are known to be at least fifteen homeodomain proteins (Rerie et al., 1994; Lincoln et al., 1994; Ruberti et al., 1991; Carabelli et al., 1993; Schena and Davis, 1992; Quaedvlieg et al., 1995; Reiser et al., 1995; Lu et al., 1996). *GL2* is most closely related to the HD-Zip group that contains a homeodomain located toward the N terminus followed by an amphipathic alpha helical domain (Kerstetter et al., 1994). The alpha helical region of *GL2* is capable of interacting with itself in vitro (Di Cristina et al., 1996). C-terminal to the helical domain, *GL2* encodes an additional 527 amino acid residues. This region does not contain any recognizable motifs; however, other genes have been identified that show significant similarity to the C-terminal domain of *GL2*. For example, *Arabidopsis Thaliana Meristem Layer 1* (*ATML1*) and *GL2* are 37% identical throughout their sequence and 60% identical in the homeodomain (Lu et al., 1996). *GL2* also shares C-terminal sequence similarity to homeodomain genes from *Phalenopsis* (Nadeau et al., 1996), *Helianthus* (Valle et al., 1996), and to an uncharacterized *Panicum* sequence (Taniguchi et al., 1994).

To better understand the role of *GL2* during trichome development, and the mechanisms by which the *GL2* gene is regulated, we examined *GL2* expression throughout leaf development. GL2 protein levels and *GL2* promoter activity were analysed in developing leaves and trichomes. Variations in the level and location of *GL2* expression were detected in the subepidermal cells and the trichomes of developing leaves. Analysis of the *GL2* promoter indicates that two separate regions contribute to the observed leaf and trichome expression pattern. Evidence for the involvement of *GL1* in the activation of the *GL2* promoter in vivo is presented.

MATERIALS AND METHODS

GUS histochemistry and microscopy

Plants were processed for GUS staining as described by Larkin et al. (1993). For sectioning of GUS-stained material, specifically staged plants were embedded in Tissue Tek OCT medium, and allowed to equilibrate for 4 hours at room temperature. Samples were cryo-sectioned in a Jung-Reichert Frigocut 2800 cryostat. Sections were transferred to poly-L-lysine coated slides and mounted using Aqueosmount for microscopy. For microscopic observations, samples were viewed using a Nikon SMU-Z stereoscope or a Nikon Diaphot-200 inverted microscope with DIC optics. Images were collected on color slides. Scanned images were labelled and compiled in figures using Adobe Photoshop (Adobe Systems Inc.) and Powerpoint$^{4.0}$ (Microsoft Corp.). For SEM analysis, soil grown plants were frozen in liquid nitrogen, mounted onto the cold stage of a Philips 500 scanning electron microscope and images were collected (Ahlstrand, 1996).

GUS enzyme assays and construction of mutant lines for analysis

GUS activity was analysed quantitatively essentially as described by Gallagher (1992). Seedlings were germinated on MS plates with 10% sucrose and the cotyledons and leaves were separated from the hypocotyl at the four leaf stage. Plant samples were frozen in liquid nitrogen an homogenized with a plastic pestle in a microfuge tube. Additional grinding was done in the presence of 200 μl of GUS extraction buffer containing 50 mM $NaPO_4$, 1 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 10 mM DTT. The homogenized sample was centrifuged for 10 minutes, and 5 μl of the supernatant was assayed for GUS activity in 195 μl extraction buffer containing 20% methanol and 1 mM 4-MUG (4-methyl-umbelliferyl glucuronide). The reaction was terminated by the addition of 800 μl of 0.2 M Na_2CO_3. MU (4-methyl-umbelliferone) flouresence was measured with a fluorometer (Hoefer Model TKO 100).

The gl1-1 null and ttg-1 alleles were used to analyze *GL2*::GUS expression. To quantitate GUS activity in *gl1, ttg, gl1 ttg,* 35S::*R-GR ttg,* and 35S::*R-GR ttg gl1* lines a cross was made between *gl1* GL2::GUS and *ttg* 35S::*R-GR*. F_1 progeny from that cross were allowed to self, creating F_2 segregating populations. For each genotype, at least 18 individual plants were analyzed for GUS activity. Plants that lacked significant GUS activity presumably did not contain the *GL2*::GUS reporter and were discarded from the analysis. F_2 seed was germinated on MS plates. *ttg* plants were scored based on the absence of anthocyanin production in the hypocotyl. Both *TTG*/n.d. and *ttg* plants were tranferred to MS plates containing 10 μM dexamethasone. 35S::*R-GR* is a dominant marker scored by a reduction of root hairs and the presence of increased trichomes in the presence of *GL1*. 35S::*R-GR gl1* plants had reduced root hairs and did not initiate increased trichomes on the leaf surface. In order to isolate the *ttg gl1* double mutant, F_2 *TTG*/n.d. *gl1* plants were identified as glabrous plants with normal seed coat muscilage. These F_2s were allowed to self, and F_3 seed was analyzed for segregation of *ttg* and the presence of the *GL2*::GUS transgene. One line was identified as homozygous for *gl1* and *GL2*::GUS, and heterozygous for *TTG*. This line was used to quantitate GUS activity in the *ttg gl1* and in *gl1 TTG*/n.d. plants. Similar results were obained with two other lines that were heterozygous for *GL2*::GUS and *TTG* and homozygous *gl1*. *GL2*::GUS quantitation of *ttg GL1*/n.d. was conducted on an F_3 line that was homozygous *ttg*, and segregating for *GL2*::GUS and *gl1*; heterozygosity at *GL1* was confirmed by crossing 8 F_3 plants with a *gl1-1* homozygote and scoring the F_1s for *ttg* and *gl1*.

Co-immunoprecipitations

The amino acid sequence of the *c-myc* epitope tag (MSEQKLISEEDL; Evan et al., 1985) was clone into the *Nco*I site at the 5′ end of the *GL1* full length cDNA using a synthesized

oligonucleotide. This new clone was moved as a cassette into an *Nco*I/*Eco*RI cleavage of the pSputk expression vector (Stratagene) downstream of the Sp6 promoter and the Kozak initiation site. This same epitope was cloned in a similar manner to the 5′ end of the *GL1 myb* domain cDNA (aa 1-126). These epitope fusions were cloned into the pSputk vector. The full length *R* cDNA (Ludwig et al., 1989) was also cloned into pSputk for in vitro expression.

In vitro transcriptions and translations were performed together using the TnT coupled reticulocyte lysate system (Promega). Translations were performed using [^{35}S]methionine and subsequently quantitated using TCA precipitation onto filter paper to ensure equal loading and molar ratios. Immunoprecipitations were performed with anti-c-myc antibody Ab-1 (Oncogene Science) and with protein A/G+ agarose beads (Oncogene Science) as suggested by the manufacturer. This involved clearing the lysate by incubation of the translation reactions with mouse normal anti-serum and beads for 1 hour on ice followed by low speed centrifugation. The cleared lysates were then incubated with the Ab-1 antibody at a 1:500 dilution and the beads at 4°C overnight with gentle shaking. The bead/antibody/protein complexes were then pelleted and washed 4 times with cold PBST buffer (1×PBS, pH 7.4, with 1% Triton X-100). Co-precipitations were treated identically except that the proteins were mixed at an equal molar ratio and allowed to sit at room temperature for 30 minutes prior to the clearing step.

Immunocytochemistry

Antibodies for immunocytochemistry were generated against a 21 amino acid peptide [SNGAHVQSIANLSKGQDRGNS] corresponding to residues 594-614 in the deduced amino acid sequence of GL2 (BioSynthesis Inc.). Antibodies were affinity purified using the peptide as an antigen. *Arabidopsis* seedlings were fixed overnight in buffered 4% paraformaldehyde at 4°C. Samples were dehydrated in an ethanol series, and infiltrated with Histoclear. Once equilibrated in 100% Histoclear, samples were infiltrated with Paraplast over a period of 4 days. Samples were mounted into blocks, and sectioned at 11 μm using a Reichert Jung microtome. Sections were dewaxed with Histoclear, and rehydrated in a graded ethanol series. Samples were detected using the Vectastain Elite Kit (Vector Laboratories) with minor modifications. Endogenous peroxidases were blocked in 0.3% hydrogen peroxide in methanol. After three washes in phosphate-buffered saline, samples were subjected to proteolysis with 20 μg/ml proteinase K (Sigma) for 20 minutes. Proteinase K digestion was stopped by incubation in 2 mg/ml glycine. Primary anti-GL2 antibodies were used at a 1:17,000 dilution along with 1:1,000 normal goat serum (Sigma). After incubation with a biotinylated goat secondary antibody and an avidin/biotin horseradish peroxidase complex, samples were detected with 10 mg/ml 3,3′-diaminobenzidine hydrochloride in 25 mM Tris-HCl, pH 7.5, 0.03% hydrogen peroxide, 0.04% nickel chloride. Staining was monitored under a stereomicroscope, and samples were dehydrated and mounted with Permount prior to microscopy.

Promoter deletion construction

A 2.1 kb *Hin*dIII/*Nhe*I fragment from the 5′-upstream region of the *GL2* gene was cloned into pUC 118 using standard molecular techniques (Ausubel et al., 1995) to form p*GL2*pro. A *Hin*dIII/*Bam*HI fragment containing the *GL2* promoter was cloned as a transcriptional fusion to GUS using the pBI101.1 plasmid (Jefferson, 1987) to form p*GL2*::GUS. The ΔRI and ΔRV constructs was generated by digestion of p*GL2*pro with *Eco*RI and *Eco*RV, filling in the protruding ends with T4 DNA polymerase (NEB), and digestion with *Bam*HI. These fragments were cloned into *Hin*dIII/*Bam*HI digested pBI101.1 vector in which the *Hin*dIII overhang had been filled in with T4 DNA polymerase (NEB). All of the other deletion constructs shown in Fig. 5 were made by double digesting the p*GL2*pro plasmid with the enzymes indicated, filling in the protruding ends with T4 DNA polymerase (NEB), and religating the DNA molecule. The truncated *GL2* promoter fragments were then cloned into pBI101.1 as *Hin*dIII/*Bam*HI fragments. The *GL1*::GUS promoter corresponds to the GGE4 promoter GUS fusion described by Larkin et al. (1993).

Dexamethasone induction

A cross was made between 35S::*GL1*/35S::*GL1 GL2*::GUS /n.d. and 35S::*R-GR*/n.d.*ttg*/*ttg* plants. In addition, ΔRI::GUS and ΔXH::GUS were crossed to 35S::GL1/n.d. 35S::R-GR/n.d. *ttg/ttg* plants. The F_1 progeny were germinated on MS plates with 10 μM dexamethasone, putative 35S::*GL1* 35S::*R-GR* plants were transferred to soil. Seed was collected from at least five plants. The genotype of these individual plants was confirmed by segregation data from the F_2 generation, and by PCR amplification of genomic DNA with primers specific to 35S::*GL1* and 35S::*R-GR*. F_2 progeny were analyzed histochemically for GUS activity after growth on MS plates containing 10 μM dexamethasone. For *GL2*::GUS, F_3 plants were scored as *ttg*, 35S::*GL1 ttg*, 35S::*R-GR ttg*, or 35S::*GL1* 35S::*R-GR ttg*. Floral explants from F_3 individuals were surface sterilized and incubated on MS plates with or without dexamethasone for 24 hours. Floral explants were then stained for GUS activity.

RESULTS

Stages of wild-type trichome development

To establish a framework for discussing trichome development, trichome development will first be divided into

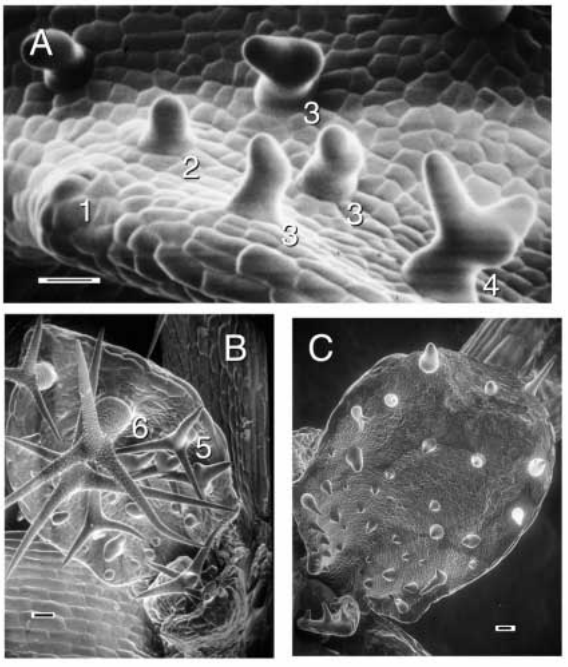

Fig. 1. Scanning electron micrographs demonstrating the different developmental stages of trichome morphogenesis. Numbers positioned at the lower right corner of each cell indicate the specific developmental stages. (A) Early stages of wild-type morphogenesis: stage 1, radial expansion of the trichome precursor in the plane of the leaf; stage 2, stalk emergence and expansion; stage 3, formation of branch structures; stage 4, expansion of the stalk and branches, which have blunt tips. (B) Late stages of wild-type trichome development: stage 5, continued expansion of the stalk and branches, which develop pointed tips; stage 6, mature trichome with papillate surface. (C) Late stages of trichome development in the *gl2* mutant. Bar, 10 μm.

several stages (Fig. 1). During the first stage, a committed cell begins to expand radially relative to its surrounding cells (stage 1; Fig. 1A), and the nucleus undergoes endoreduplication (Hülskamp et al., 1994). After the trichome precursor expands to two to three diameters greater than the surrounding cells, it emerges perpendicular to the epidermal plane and produces what will become the stalk of a trichome (stage 2; Fig. 1A). Next, the top of the growing cell develops secondary foci of cell expansion that will form the trichome branches (stage 3; Fig. 1A). Leaf trichomes typically produce two to four branches depending on the geographic race (Marks and Esch, 1994). After all branches have been initiated, the cell continues to expand via diffuse growth, such that it increases in diameter as well as in height. Initially, the growing branch tips are blunt (stage 4; Fig. 1A); but later, the tips become pointed (stage 5; Fig. 1B). After cell expansion ceases, the outer trichome surface develops numerous papillae (stage 6; Fig. 1B).

Expression pattern of the *GL2* gene

A 2.1 kb *Hin*dIII/*Nhe*I fragment from the 5′-untranslated region of the *GL2* gene was cloned as a transcriptional fusion to the β-glucuronidase gene (*GL2*::GUS), and used to transform wild-type *Arabidopsis* plants. The activity of the *GL2*::GUS fusion throughout leaf development is shown in Fig. 2. In the young leaf primordia and in developing leaves up to approximately 400 μm in length, *GL2*::GUS activity was detected in developing trichomes and surrounding epidermal cells. In leaves longer than 500 μm, most of the non-trichome *GL2*::GUS activity was restricted to the basal regions of the leaf (Fig. 2A). *GL2*-dependent staining also was detected in the petiole and in the midvein of developing leaves. Transverse sections of GUS-stained leaves that were less than 400 μm in length showed that the promoter was active in all cell layers in the developing leaf (Fig. 2B). Once the developing leaf reached approximately 600 μm, *GL2*::GUS activity was detected in the basal regions of the leaf where trichomes continue to initiate (Fig. 1C), but GUS activity was only observed in trichomes in the apical two thirds of the leaf (Fig. 2C,D). Early stage trichomes (stages 1-3) forming at the base of older leaves (greater than 600 μm in length) often showed higher *GL2*::GUS activity relative to surrounding adjacent epidermal cells that do not enter the trichome pathway (Fig. 2E). However, on younger leaves, the level *GL2*::GUS activity in stage 2 and stage 3 trichomes relative to neighboring epidermal and subepidermal cells was often similar (Fig. 2F). GUS activity persisted during stages 2 and 3, but after stage 3, the level of GUS activity was variable and similarly staged trichomes often displayed differential *GL2*::GUS activity (compare trichomes A and B in Fig. 2G). As a trichome reached its final size, the level of activity appeared to increase (Fig. 2G, trichome B) and in mature trichomes, *GL2*::GUS activity remained stable for the lifetime of the cell (Fig. 2H). The data are consistent with the hypothesis that *GL2* is expressed throughout trichome development.

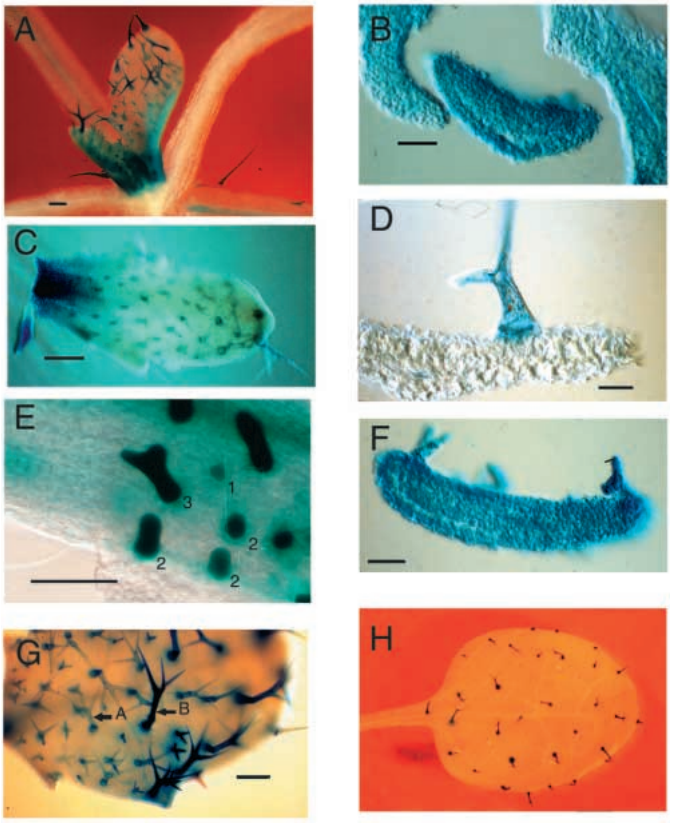

Fig. 2. Histochemical localization of the *GL2*::GUS transgene activity in developing wild-type leaves. (A) Developing leaves. (B) Transverse section through a developing leaf primordia. (C) Adaxial surface of a developing leaf. (D) Cross section through the apical third of a developing leaf. (E) Emerging trichomes at the base of a mature leaf. Trichomes are labeled as stage 1 to stage 3 as described in Fig. 1. (F) Transverse section through leaf base with developing trichomes. (G) Developing leaf showing trichomes at several developmental stages. Trichomes at similar developmental stages are labeled A and B and indicated with an arrow. (H) Mature leaf. Bars (A,C,E,G) 100 μm; (B,D,F) 50 μm.

To confirm the expression patterns of the *GL2*::GUS fusion observed in developing leaves, anti-GL2 antibodies were used to localize the endogenous GL2 protein. Wild-type and *gl2*-2 leaves were prepared for immunocytochemical staining using a polyclonal antibody directed against an epitope in the C terminus of GL2. *gl2*-2 served as a negative control because it contains a T-DNA insertion consisting of several tandemly linked T-DNA units in the coding sequence upstream from the deduced 20 amino acid GL2 peptide against which the anti-GL2 antibodies were generated (Rerie et al., 1994). The anti-GL2 staining results obtained were consistent and the results from a single experiment are shown in Fig. 3. Wild-type plants showed significantly greater staining than *gl2*-2 plants (compare Fig. 3A,B). Wild-type sections processed for immunocytochemistry without primary antibody or probed with pre-immune serum, as a control, did not display any significant staining (data not shown). Immunolocalization of GL2 protein in young wild-type leaves detected higher levels of GL2 protein in all cell layers compared to older leaves (compare Figs 3B and 3C). In the subepidermal cells of young leaves, the anti-GL2 signal was distributed throughout the cytoplasm with some nuclear staining (Fig. 3D); however, in trichomes at stage 4 (Fig. 3B,C) and at stage 3 (Fig. 3E) the signal was localized to the nucleus. Immunolocalization experiments with antibodies against GL1 did not reveal any significant cytoplasmic staining, and all GL1 signal was

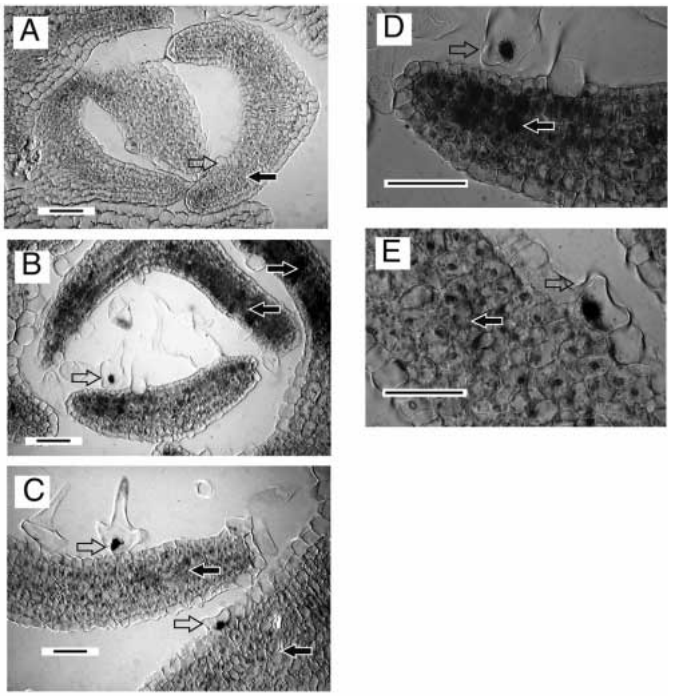

Fig. 3. Immunolocalization of GL2 protein in the developing leaves of wild-type and *gl2*-2 plants. (A) Transverse section through developing leaf of *gl2*-2 plants. (B) Transverse section of a wild-type leaf. (C) Transverse section through later stage wild-type leaves. Solid arrows indicate subepidermal cells, and open arrows indicate developing trichomes. (D) Higher magnification of trichome indicated in B. (E) Higher magnification of lower trichome indicated in panel C. Bars, (A,B,C,D,E) 100 μm.

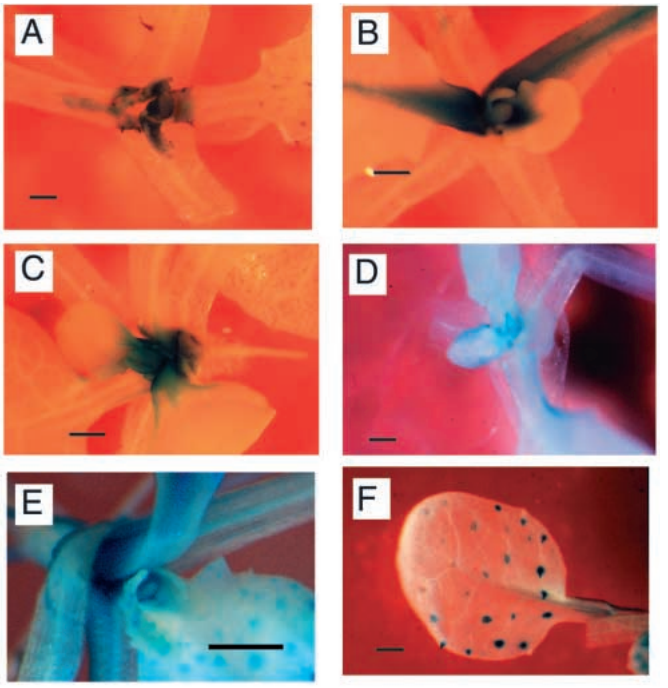

Fig. 4. Expression of *GL2*::GUS in different mutant backgrounds. Histochemical localization of *GL2*::GUS transgene activity in wild-type and mutant *Arabidopsis* plants. (A) Wild-type, (B) *gl1*, (C) *ttg*, (D) *gl1 ttg* and (E) *gl2* leaf primordia. (F) *gl2* mature leaf. Bar, 300 μm.

localized to the nucleus (D. Szymanski, unpublished results). Methylene blue staining of similar sections showed that the cytoplasm of developing trichomes was present and presumably accessible to the antibody reagent. The staining pattern of the anti-GL2 antibodies corroborate the results obtained using the GL2::GUS reporter construct. Furthermore, the partitioning of GL2 protein between the cytoplasm and nucleus revealed another potential level of control on GL2 activity.

Dependence of *GL2*::GUS activity on genes that affect trichome development

Genetic analysis of the trichome developmental pathway showed that the *TTG* and *GL1* genes are required for trichome initiation. Epistatic analysis placed *GL2* downstream from *GL1* and *TTG* (reviewed by Marks, 1997; Hülskamp et al., 1994), which suggests that *GL2*::GUS expression may require *GL1* and/or *TTG*. In order to address this issue, the *GL2*::GUS construct was analyzed in *gl1*, *ttg*, and *gl1 ttg* backgrounds. For *gl1* and *ttg* mutants, the GUS staining patterns were observed in at least 5 shoots removed from transformed callus tissue, and were confirmed in at least three independent transformants grown from seed, as well as in F_2 and F_3 populations in which *gl1*, *ttg* and *GL2*::GUS were segregating. The transformant *gl1 GL2*::GUS D-1 was used as a parent in a cross with a *ttg* plant to analyze *GL2*::GUS in the double mutant. Results from representative plants are shown in Fig. 4. The leaf primordia and petiole staining observed in wild-type plants (Fig. 4A) was also observed in *gl1* (Fig. 4B). However in *gl1* there was a consistent reduction in *GL2* staining in the marginal and apical domains of developing leaves compared to wild-type. In contrast, *ttg* plants lacked significant *GL2*::GUS staining in the petiole of fully expanded leaves, and staining was restricted to the margin of developing leaves (Fig. 4C). However, the diffuse leaf primordia staining in *ttg* was similar to *gl1* and wild-type. In both *gl1* and *ttg*, staining was not detected in any epidermal cells in mature leaves. This suggests that the *GL2*::GUS expression in fully expanded leaves of wild-type plants is dependent upon the presence of trichomes induced by the activities of *GL1* and *TTG*; otherwise, a subset of epidermal cells on mature *gl1* and *ttg* leaves would accumulate GUS. The detection of significant *GL2*::GUS in both *gl1* and *ttg* suggested that either *GL1* or *TTG* was sufficient for *GL2* transcription in developing leaves. To address this possibility, the *GL2*::GUS reporter was crossed into a *gl1 ttg* background and analyzed for GUS staining. In the double mutant, *GL2*::GUS activity is faintly detected in the leaf primordia, and is restricted to the margins of developing leaves. In later leaves *GL2*::GUS activity was restricted to the margins of the leaf base and petiole (Fig. 4D). The *GL2*::GUS construct was also transformed into *gl2* plants. It was found that *GL2*::GUS expression in *gl2* shoots (Fig. 4E) and mature leaves (Fig. 4F) mirrored the expression in wild-type plants. Therefore *GL2* does not function as an essential factor that autoregulates its expression in the shoot and in developing trichomes.

Table 1. Activity of the *GL2*::GUS reporter construct in coytledons and leaves in wild-type, mutant, and 35S::*R-GR* containing *Arabidopsis* plants at the four leaf stage

Genotype[a]	N[b]	GUS activity (pmolMU/min·mg)[c]
GL1/n.d.[d]; TTG/n.d.[e]	17	2200±1100
gl1-1/gl1-1; TTG/n.d.[f]	14	280±130
GL1/n.d.; ttg/ttg[f]	10	250±100
gl1-1/gl1-1; ttg/ttg[f]	14	59±19
35S::R-GR; GL1/?; ttg/ttg[e]	15	4200±2200
35S::R-GR; gl1-1/gl1-1; ttg/ttg[e]	11	540±270

a: Plants analyzed for GUS activity are progeny from the cross gl1/gl1 GL2::GUS X ttg/ttg 35S::*R-GR*. F2 progeny were scored for *TTG* activity based on anthocyanin production in the hypocotyl and the presence of a seed coat muscilage. *GL1* activity was scored according to the presence of trichomes and *TTG* and/or 35S::*R-GR* activity. 35S::*R-GR* was scored by the presence of increased leaf trichomes and inhibition of root hair initiation during dexamethasone induction.

b: Number of plants analyzed for GUS activity. Plants that did not have any significant GUS activity presumably lacked the reporter transgene, and were discarded from the analysis. In the case of the F3 *ttg/ttg* GL1/gl1 family, the GUS activity of presumptive *ttg gl1* double mutants were significantly lower than *ttg* mutants and were discarded from the analysis.

c: Activity reported as mean ± standard deviation.

d: n.d.: Precise zygotic constitution at this locus is not determined.

e: Analyzed as F_2 individuals.

f: Analyzed as F_3 individuals.

In order to quantify the relative levels of *GL2* transcription, the total GUS activity of leaves and cotyledons at the four leaf stage was measured in vitro (Table 1). Plants that contained at least one wild-type copy of *GL1* and *TTG* had a mean GUS activity of 2200 pmol MU/minute·mg. *gl1* and *ttg* plants lacked trichomes and had reduced mean GUS activities of 280 and 250 pmol/minute·mg respectively. Consistent with the histochemcal staining, the *gl1 ttg* double mutant had a mean GUS activity of only 59 pmol MU/minute·mg. The ability of the maize *R* gene to complement *ttg* phenotypes was also reflected in its ability to rescue *GL2*::GUS transcription in the *ttg* background. 35S::*R-GR ttg* plants had a mean GUS activity of 4200 pmol MU/minute·mg. The ability of 35S::*R-GR* to activate *GL2* transcription in the absence of *GL1* was demonstrated in 35S::*R-GR gl1 ttg* plants that displayed a mean GUS activity of 540 pmol MU/minute·mg.

Deletion analysis of the *GL2* promoter

Previous molecular complementation experiments have shown that the presence of the upstream domain between the *Eco*RI and *Eco*RV sites is required for full *GL2* function in the leaf (Rerie et al., 1994). This result suggested that transcriptional control was a significant component of *GL2* function in wild-type plants. To address this issue, several nested and internal deletions of the *GL2* promoter were cloned as transcriptional fusions to GUS and analyzed for expression patterns in transformed plants. The physical map of the analyzed constructs and a summary of the observed expression patterns are shown in Fig. 5. Internal deletions from the *Hpa*I site at position −2007 (relative to the first translation start codon) to the *Xba*I site at position −880 (construct 3) or to the *Msc*I site at position −421 (construct 2) eliminated *GL2*::GUS trichome expression. However, faint staining in leaf primordia was detected with construct 3, suggesting that sequences between −880 and −421 may also play a role in regulating *GL2* shoot expression. The results with constructs 2 and 3 indicate that the deleted region contains important elements for shoot and trichome expression. Plants containing constructs with external deletions down to the *Eco*RV site at position −1373 (construct 4) exhibited both non-trichome and trichome staining in the shoot. If the region between the *Eco*RV site and *Msc*I of construct 4 are replaced with the region upstream of the *Eco*RV site (the region between the *Hin*dIII site at position −2149 and the *Eco*RV site; construct 6), shoot expression is maintained. This analysis indicates that sequences both upstream and downstream of the *Eco*RV site can mediate shoot and trichome expression. Both domains contained several sequence elements with similarity to well characterized binding sites for vertebrate (Biedenkapp et al., 1988) and plant (Grotewald et al. 1994; Sablowski et al., 1994) *myb* proteins.

Relationship between *GL1* and *GL2*

Both the *GL1* and *TTG* loci are required for trichome initiation, while *GL2* is required for the earliest morphogenetic events of trichome growth. The presence of *myb*-class binding sites in the *GL2* promoter provides additional circumstantial evidence for the involvement of *GL1* in *GL2* regulation. The spatial and temporal expression of the *GL1* and *GL2* genes also is consistent with the idea that *GL1* regulates *GL2* expression. A comparison of the staining patterns of representative *GL1*::GUS and *GL2*::GUS containing transgenic plants reveals the similarity between *GL1* and *GL2* expression profiles (Fig. 6). The *GL1* promoter was active in non trichome cells at the base of developing leaves, and in developing trichomes. *GL1*::GUS staining was not detectable in older trichomes at the tip of the first leaf pair (Fig. 6A) or in the trichomes of mature leaves (data not shown). The *GL2*::GUS staining pattern in developing leaves

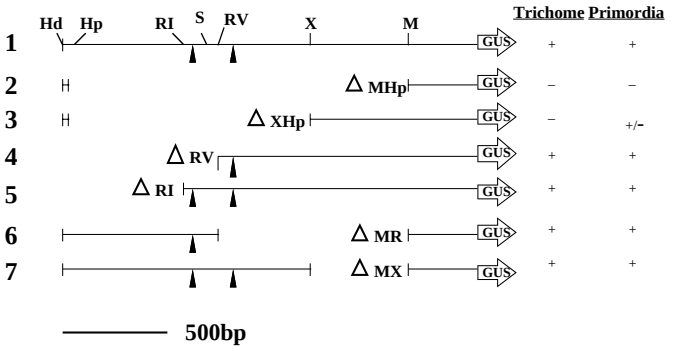

Fig. 5. Identification of trichome and leaf primordia specific promoter elements in the *GL2*::GUS reporter construct. Diagram of the restriction enzymes used to generate the different *GL2*::GUS promoter constructs used in this study. The full length construct is shown at the top, and deletion constructs are shown to scale below. Numbers are used to refer to each construct. The presence or absence of leaf primordia and trichome specific expression are indicated with a '+' or '–' for each construct. H, *Hin*dIII; Hp, *Hpa*I; RI, *Eco*RI; S, *Spe*I; RV, *Eco*RV; X, *Xba*I; M, *Mse*I. Arrows indicate the position of putative *myb* binding sites. Bar, 500 bp.

is similar, except staining persists in mature trichomes (Fig. 6B). Transverse sections through mature *GL1*::GUS (Fig. 6C) and *GL2*::GUS (Fig. 6D) leaves confirmed a difference in transcription between *GL1* and *GL2* in mature trichomes. As indicated above, *GL1* is not required for *GL2* expression in the shoot, but the overlapping expression pattern of *GL1* and *GL2* suggests that *GL1* could mediate *GL2* expression during some stages of trichome development.

A basic model of the control of *GL2* is one in which *GL1* directly activates *GL2* transcription. Previous experiments have shown that over expression of the *GL1* gene using the 35S promoter results in a reduction in the number of leaf trichomes and the production of a few ectopic trichomes on the cotyledons (Larkin et al., 1994). However, over expression of the maize *R* gene with 35S::*GL1* provided an additional activity such that there was a massive increase in the initiation of both ectopic and leaf trichomes (Larkin et al., 1994).

To examine the relationship between *GL1, R* and the *GL2* promoter activity in vivo, plants that ectopically express *GL1* and an inducible form of the maize *R* gene were analyzed for ectopic *GL2* promoter activity. In *Arabidopsis*, the 35S::*GL1* and 35S::*R* transgene combination causes seedling lethality (Larkin et al., 1994). However, 35S::*GL1* plants that contain an inducible form of the *R* gene, that includes an N-terminal fusion to the vertebrate glucocorticoid receptor (35S::*R-GR*; Lloyd et al., 1994), are viable in the absence of the glucocorticoid analog dexamethasone. Plant viability is sensitive to *TTG* gene dosage; the most vigorous 35S::*GL1*, 35S::*R-GR* plants are homozygous for the *ttg* mutation. Inducible *R* activity has allowed us to monitor the effects of ectopic *GL1* and *R* expression on the activity of the *GL2*::GUS transgene. Fig. 7 shows that in a *ttg* background (Fig. 7A) neither the 35S::*GL1* (Fig. 7B) nor the 35S::*R-GR* (Fig. 7C) transgenes alone ectopically activate the *GL2*::GUS reporter in cotyledons and roots. Increased *GL2*::GUS staining in the midvein and margin of 35S::*GL1* plants in wild-type or *ttg*/*ttg* background was often observed. Strong ectopic expression from the *GL2* promoter was observed in five lines that ectopically expressed both *GL1* and *R-GR* in the presence of dexamethasone (Fig. 7D). Induced plants that ectopically expressed *GL1* and *R-GR* displayed widespread ectopic trichome initiation on their cotyledons, hypocotyls and leaves.

The ability of dexamethasone to induce ectopic expression of the *GL2* promoter was tested using inflorescence explants from plants harboring the *GL2*::GUS reporter. Inflorescence explants from soil grown *ttg*/*ttg*, 35S::*GL1*; *ttg*/*ttg*, 35S::*R-GR*; *ttg*/*ttg* and 35S::*GL1*; 35S::*R-GR*; *ttg*/*ttg* plants were subcultured on MS plates with (induced) or without (uninduced) 1 μM dexamethasone. After 24 hours, the explants were analyzed for GUS activity (Fig. 7E-L). In wild-type plants, *GL2*::GUS is active in the outer cell layer of the seed coat and stained seeds are faintly visible through a cleared silique (Sattler and Marks, unpublished data). In the *ttg* background, wild-type *GL2*::GUS expression in developing seeds (data not shown) is absent (Fig. 7E,F), and ectopic expression of *GL1* cannot rescue this activity (Fig. 7G,H). The 35S::*R-GR* construct can rescue *GL2*::GUS expression in developing seeds in a dexamethasone-independent manner, but does not give rise to ectopic *GL2*::GUS activation in the siliques (Fig. 7I,J). Strong dexamethasone-dependent activation in siliques was only observed in plants that ectopically expressed *GL1* and *R-GR* in the presence of dexamethasone. 35S::*GL1* 35S::*R-GR* activation of *GL2*::GUS did not require adoption of the trichome cell fate, because differentiated epidermal cells of siliques ectopically expressed *GL2*::GUS. To rule out the possibility that the ectopic activation of the *GL2* promoter in the 35S::*GL1* and 35S::*R-GR* background represents a non-specific transcriptional activation, two additional *GL2* reporter constructs were analyzed in that genetic background. The ΔXD construct, which lacks the *GL2* promoter regions required for detectable trichome expression, was not ectopically activated by the 35S::*GL1* 35S::R-GR transgenes (Fig. 8A). The ΔRI construct, which contains both putative shoot transcription domains, was ectopically activated in the presence of ectopic *GL1* and *R-GR* (Fig. 8B). These results indicate that *GL1* and *R* require the region between the *Xba*I and *Eco*RI sites (Fig. 5) for the ectopic *GL2* activation.

Because both *GL1* and *R* are required to ectopically activate *GL2*, it is possible that *GL1* and *R* function as a complex. This possibility has been examined through the use of in vitro co-immunoprecipitation experiments (Fig. 9). In order to provide a strong antigen, a construct containing the *GL1* gene with a *myc* epitope as an N-terminal fusion was generated. This same epitope fusion was made to a truncated version of the GL1 protein that expressed only the N-terminal 122 amino acids, which contains the entire *myb* homologous domain. These two protein fusions, as well as the full length untagged *R* protein, were cloned into Sp6 expression vectors for the in vitro production of mRNA and subsequent reticulocyte translations in the presence of [^{35}S]methionine. Fig. 9A shows an autoradiograph of the SDS-PAGE gel of the translated myc-GL1 (lane 1), myc-GL1 myb (lane 2), unprogrammed lysate (lane 3), and R protein (lane 4). Two high molecular mass bands are observed in the R translation reactions; the lower molecular mass band may be due to the spurious initiation or termination of R translation reaction (lane 4). Following incubation of each translation reaction with the anti-myc antibodies, the antibody bound fraction was purified with sepharose G+/A beads. The resulting complexes were further separated by SDS-PAGE (Fig. 9B). Only the GL1 proteins expressing the *myc* epitope were precipitated (Fig 9B, lanes 1,2). It is important to note that while R is a *myc* homologous protein, it does not have any homology to the *myc* epitope tag and is not precipitated by the anti-myc antibodies (Fig. 9B, lane 3).

The translated proteins were incubated together prior to immunoprecipitation. Under these conditions it was found that R only immunoprecipitated in the presence of full-length GL1 (Fig. 9C, lane 1). The immunoprecipitated R protein corresponds to the high molecular mass form of R that is observed in Fig. 9A lane 4. The nature of the smaller products in Fig. 8A lane 4 are unknown; however, the fact that only the larger product was precipitated indicates that the interaction between GL1 and R is specific. In addition, since the *myb* domain of GL1 could not efficiently precipitate R it indicates that something in the carboxy terminus of GL1 is also necessary for a stable interaction and that the *myc* epitope itself is not the source of the interaction.

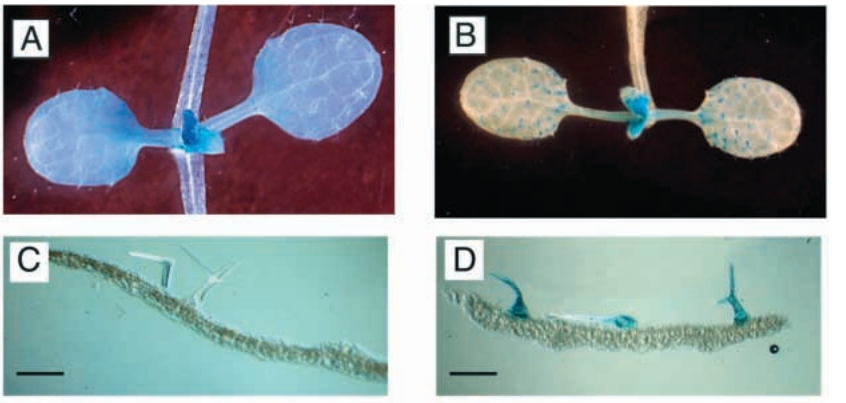

Fig. 6. Histochemical analysis of *GL1* and *GL2* promoters fused to the GUS reporter. (A) Staining pattern in wild-type plants containing the *GL1*::GUS transgene. (B) Staining pattern in wild-type plants containing the *GL2*::GUS transgene. (C) Transverse section through a 600 μm *GL1*::GUS leaf. (D) Transverse section through a 600 μm *GL2*::GUS leaf. Bar, (C,D) 100 μm.

DISCUSSION

Profile of *GL2* expression throughout leaf development

In *Arabidopsis* the *TTG* and *GL1* genes are required for trichome initiation. *GL2* function does not appear to be required for initiation since *gl2* leaves have approximately normal numbers of trichomes. However *gl2* trichome morphology is variable, and shows defects from stage 2 to stage 6. The expression of *GL2* reflects a complex requirement for its activity during leaf development, and includes spatial and temporal regulation in developing leaves and within trichomes (Figs 2, 3). In leaf primordia, the *GL2* gene is expressed in all cell layers. The apparently uniform level of expression in the epidermis of leaf primordia is low relative to that observed during some stages of trichome development. It is possible that this expression pattern reflects a permissive level of *GL2* required for normal progression of the trichome morphogenetic program. The functional significance of the diffuse subepidermal primordia and petiole expression pattern is not known because the expression precedes trichome initiation and occurs in cell types that do not normally enter the trichome pathway. Because the number of cell layers and leaf morphology of developing *gl2* leaves are not obviously different from wild-type, the non-trichome function of *GL2* in developing leaves is unknown. GL2 protein appears to show cell-type specific subcellular localization in developing leaves, and is localized in the nucleus in developing trichomes (Fig. 2). The partitioning of GL2 within the cell may play a functional role in regulating *GL2* activity.

Consistent with the early requirement for *GL2* for accurate initial outgrowth of the trichome stalk, significant *GL2*::GUS activity was detected in developing trichomes. The apparent lack of this early *GL2* activity in plants homozygous for strong mutant alleles results in a loss of trichome outgrowths. Some of the trichome outgrowths on *gl2* plants expand somewhat normally, but have fewer branches and lack papillae. Therefore, the detection of GL2 protein from stages 2 through 5, and the expression of *GL2*::GUS during stage 6 is consistent with *GL2* regulating multiple aspects of trichome morphogenesis.

Molecular analysis of *GL2* promoter function

The definition of the promoter elements that activate trichome gene expression is an important step in understanding the relationship between *GL2* transcription and its regulation. Leaf primordia, leaf base, and trichome specific expression

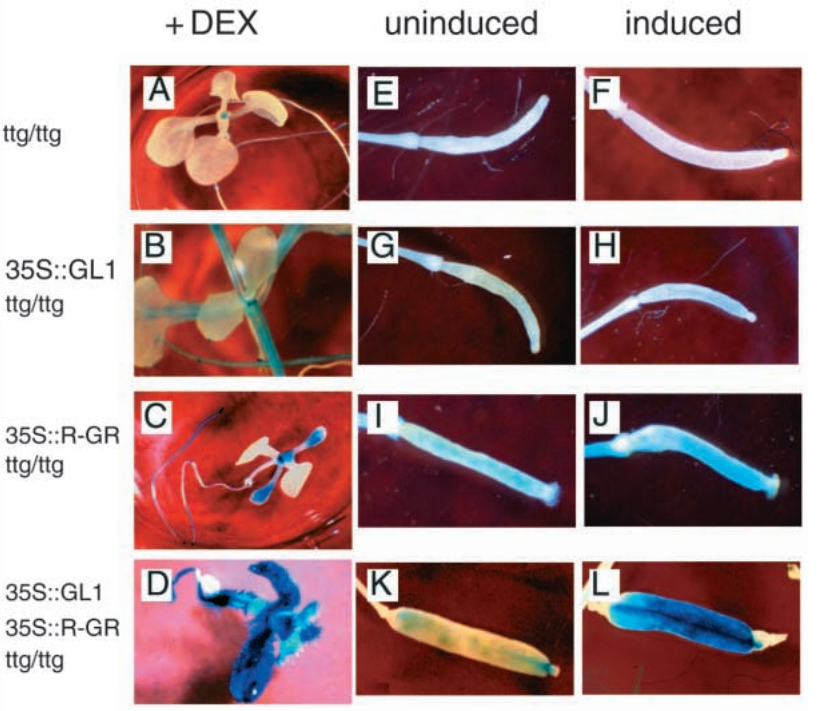

Fig. 7. Ectopic activation of *GL2*::GUS is dependent on ectopic expression of both *GL1* and R. (A-D) Seedlings from four classes of F_3 individuals from the cross 35S::*R-GR*, *ttg*/*ttg* × 35S::*GL1*, *GL2*::GUS were germinated on dexamethasone-containing plates and analyzed for ectopic *GL2*::GUS expression. All individuals were homozygous *ttg*/*ttg* plants and contained the *GL2*::GUS transgene. (A) *ttg*. (B) 35S::*GL1*. (C) 35S::*R-GR*. (D) 35S::*GL1*, 35S::*R-GR*. (E-L) Dexamethasone induction of ectopic *GL2*::GUS activity was tested in inflorescence explants from each genotype subcultured in the absence or presence of dexamethasone: (E,F) *ttg*, (G,H) 35S::*GL1*, (I,J) 35S::*R-GR*, (K,L) 35S::*GL1*, 35S::*R-GR*. The genotypes of the individual plants are shown to the left and the treatments are shown above each plant.

were not clearly separable activities and could be detected in two separate promoter domains flanking the *Eco*RV site at −1373 (Fig. 5). Either domain is sufficient for leaf and trichome expression. Inspection of the DNA sequences flanking the *Eco*RV site in the *GL2* promoter revealed two sequence elements with similarity to vertebrate *myb*-class transcription factor binding sites. The orientation of both elements is inverted with respect to the directionality of the *GL2* coding sequence. The more 5′ putative *myb*-binding site element (pMBS1: GAC**TAACGG**TAAG) matches the consensus vertebrate myb-binding site in six of six positions (shown in bold; Biedenkapp et al., 1988). The proximal sequence element (pMBS2: TAC**TAAC**A**G**TATA) conforms to the vertebrate *myb* consensus in five of six positions (shown in bold). The sequence similarity between pMBS1 and pMBS2 extends beyond the *myb* consensus; they are identical in nine of ten positions. It is possible these binding sites are composite elements recognized by more than one factor and/or trichome-specific *myb*-binding sites that have additional sequence constraints outside of the *myb*-core sequence. The *Eco*RI to *Eco*RV domain also contains potentially significant sequences with identity in five of six positions to the core recognition sequence CC[T/A]ACC that is bound by the maize P gene (Grotewald et al., 1994) and myb305 from *Antirrhinum* (Sablowski et al., 1994). Previous molecular complementation experiments have shown that the presence of the upstream domain between the *Eco*RI and *Eco*RV sites is required for full *GL2* function in the leaf (Rerie et al., 1994). Removal of this region resulted in trichomes with fewer branches. Therefore, the level of expression conferred by the two leaf expression domains appears to be important for normal trichome development, and the presence of multiple putative *myb*-binding sites reveals the possibility of complex combinatorial control of the *GL2* promoter. The same two regions also mediate *GL2*::GUS expression in the outer seed coat (Sattler and Marks, unpublished data). The possibility that shared promoter elements control *GL2* expression in seeds, shoots and trichomes will be addressed in future experiments.

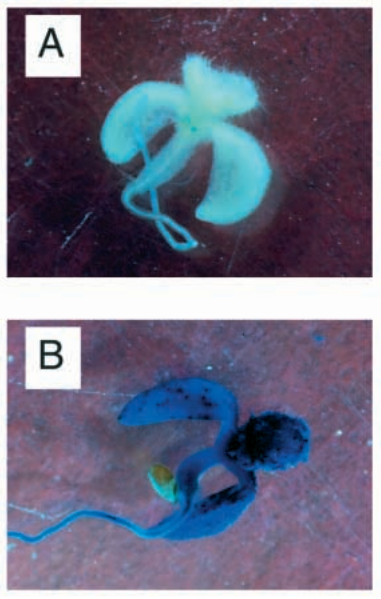

Fig. 8. 35S::*GL1* 35S::*R-GR* mediated ectopic activation of the *GL2* promoter is restricted to a region that contains trichome and shoot expression domains. The ΔXD and ΔRI constructs were analyzed in the 35S::*GL1* 35S::R-GR background under inducting conditions. (A) ΔXD, (B) ΔRI.

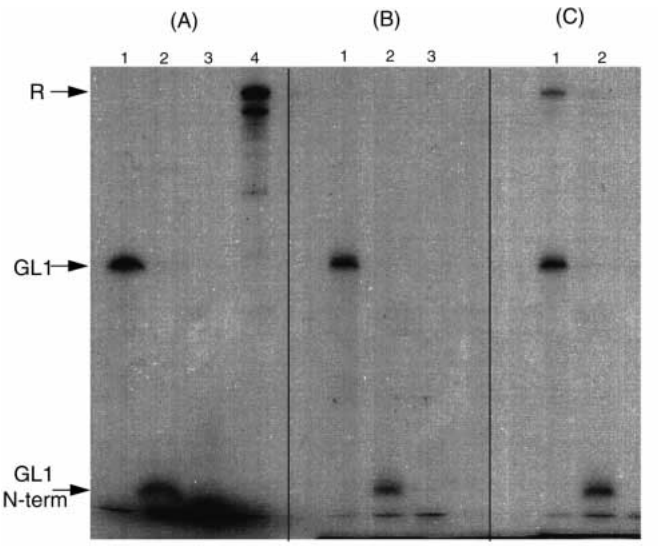

Fig. 9. Co-precipitation of R with tagged GL1. (A) Rabbit reticulocyte in vitro translations of the following in vitro translated mRNAs; (lane 1) *myc*-epitope tagged full length GL1, (lane 2) *myc*-epitope tagged amino terminal half of GL1, (lane 3) mock translation, (lane 4) full length *R.* (B) Anti-myc immunoprecipitations of the following translations; (lane 1) tagged GL1, (lane 2) tagged amino terminal half of GL1, and (lane 3) R. (C) Co-immunoprecipitations of the following translation mixes; (lane 1) full length tagged GL1 and R, (lane 2) and tagged amino half of GL1 and R.

Regulation of *GL2* shoot expression

There is extensive overlap in the shoot expression patterns of *GL1* and *GL2*. Expression of both *GL1* and *GL2* is detected throughout young leaf primordia and during early trichome development. Mutations in *GL1* are epistatic to those in *GL2* and the *GL2* promoter region contains potential GL1 binding sites. All of these findings suggest that *GL1* may directly regulate some aspects of *GL2* expression. It is also apparent that there are *GL1*-independent components to *GL2* expression in the shoot. In mature trichomes *GL2* transcription persists, while *GL1* is apparently downregulated. It is possible that additional *myb* genes that are expressed in trichomes regulate *GL2* (Li et al., 1996). In the absence of *GL1* and *TTG*, mean GUS activity in the shoot was 59 pmol MU/minute·mg. In plants containing either *TTG* or *GL1* mean GUS activity increased to approximately 250 pmol MU/minute·mg. This indicates either *TTG* or *GL1* is sufficient to independently increase *GL2* expression. The spatial pattern of *GL2* expression in the leaf primordia in *gl1 ttg* plants is similar to *gl1*, *ttg*, and wild-type plants (Fig. 4); the major difference is in the relative level of expression and/or its persistence as the leaf develops (Table 1). Since this expression pattern does not persist in developing *ttg gl1* leaves, the role of *GL1* and *TTG* appears to be to stabilize and activate *GL2* transcription in specific cells in the developing leaf. If the ttg-1 allele is a null, the reduced but detectable level of *GL2* transcription in *ttg gl1* mutants also indicates that an

additional unknown factor(s) participates in *GL2* transcription in developing leaves.

Ectopic *GL2* expression cannot be induced by the ectopic expression of *GL1* alone. In fact, 35S::*GL1* transgenic plants express high levels of GL1 protein (D. Szymanski, unpublished results), yet show a reduction in trichome number (Larkin et al., 1994) and apparently normal *GL2*::GUS expression in the developing shoot and trichomes (Fig. 7). The maize *R* gene provides an additional activity such that plants that overexpress *GL1* and *R* display widespread trichome initiation over much of their aerial surface and ectopic *GL2*::GUS transcription (Fig. 7). The presence of both ectopic *GL1* and *R* bypasses the requirement for *TTG* and presumably the normal trichome initiation control mechanisms that operate via wild-type levels of *GL1* and *TTG*. The detection of physical interaction between GL1 and R in co-immunoprecipitation experiments provides biochemical evidence that GL1 and R function as a complex. The interaction of GL1 with R is stable, specific, and occurs in the absence of DNA (Fig. 9). Therefore, it is possible that the association of GL1 and R, rather than the convergence of GL1 and R-dependent parallel pathways, is the mode of the activation of the *GL2* promoter. The ability of this complex to constitute a unique biochemical activity is unknown, since we have not been able to identify a high affinity DNA-binding site for GL1, R, or the GL1-R complex on the *GL2* promoter. Therefore it is not known if GL1 and R are part of a complex that directly or indirectly activates the *GL2* promoter or if additional factors are required for a stable interaction with DNA. The fact that *R* can activate *GL2* transcription in the absence of *GL1* and *TTG* activity (Table 1) suggests that if *R* is part of a complex that regulates *GL2*, it is capable of interacting with additional components of the complex, perhaps the putative *gl1-ttg*-independent factor. Since *R* can compliment *GL1*-independent phenotypes of *ttg* plants it is expected that *R* interactions are not limited to *GL1*.

The relationship between the maize *R* gene and *TTG* is not clear. The dominant effects observed by overexpressing *R* have not been observed with any other plant bHLH containing gene. Furthermore, recent results indicate that *TTG* is not a *R* homolog, and its deduced amino acid sequence encodes WD 40 repeats (A. Walker and J. Gray, personal communication). The presence of *R* could bypass the need for *TTG* is several ways. For example, the function of *TTG* could be to regulate the expression or the activity of a *myc* factor. In this case, *R* could function independently of *TTG*. Alternatively, both *GL1* and *TTG* may directly bind to the *GL2* promoter, either alone or as part of a larger complex, but the interaction between GL1 and R somehow eliminates the requirement for *TTG*. The characterization of *TTG* and the identification of components of DNA-protein complexes that bind to the *GL2* promoter should help to resolve this issue.

In conclusion, it has been found that both *TTG* and *GL1* can influence *GL2* expression in the shoot. Furthermore, the region of the *GL2* promoter shown to be important for shoot expression is also needed for *GL1-R* mediated ectopic *GL2* expression. However, many aspects of *GL2* expression remain to be understood. What is the identity of the *ttg-gl1*-independent factor that gives rise to leaf primordia expression? What factors control the specific and persistent *GL2* expression in mature trichomes? Finally, *GL2* expression is unaltered in the roots and seeds of *gl1* mutants. This indicates the presence of other factors that may participate in the regulation of *GL2*. The complexity of *GL2* regulation and its expression pattern reflects its function throughout trichome development and in the control of cell fate in the root and seed. Further studies of the mechanisms of *GL2* regulation will aid in the understanding of how complex and integrated gene networks control plant development.

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