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Trichome Development in *Arabidopsis thaliana*. II. Isolation and Complementation of the *GLABROUS1* Gene

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We are using the formation of trichomes in *Arabidopsis thaliana* as a model system to study gene expression during cellular differentiation. To initiate the molecular characterization of this system, we tagged and isolated a gene that is specifically required for the development of the specialized trichome cell. We confirmed the identity of this gene, *GLABROUS1* (*GL1*), by complementation. These results demonstrate that a crucial gene in a plant developmental pathway can be successfully identified by complementation.

INTRODUCTION

Trichomes are the hairs that are present on the surface of the leaves and stems of many plants (Esau, 1965). In *Arabidopsis thaliana*, these trichomes are large, single cells that differentiate from individual protodermal cells in the developing epidermis of the leaves and stems (Rollins and Banerjee, 1975). We are using the process of trichome formation in *Arabidopsis* as a relatively simple model system to study the complex mechanisms that control gene expression during cellular differentiation. When compared with other cell types, the development of trichomes is an attractive model system for two reasons (Haughn and Somerville, 1988). First, the process can be easily monitored because it occurs on the accessible outer surface of the plant. Second, morphological mutants can be readily isolated and characterized because trichomes are not essential for the survival of the plant.

The accompanying paper (Marks and Feldmann, 1989) describes a new trichome mutant of *Arabidopsis* that was isolated from a population of plants that had been transformed by a seed transformation procedure (Feldmann and Marks, 1987; Feldmann et al., 1989). Several different genetic analyses showed that a T-DNA insert was tightly linked to this new trichome mutation. The mutant plants exhibited a unique phenotype in that they had normal leaf trichomes, but no stem trichomes. Crosses revealed that the new mutation is allelic to the previously described *glabrous1* (*gl1*) mutation, which is characterized by the absence of both leaf and stem trichomes (Koornneef, Dellaert, and van der Veen, 1982). The new allele was designated *gl1-43*. To isolate the junction between the T-DNA and flanking plant DNA, a genomic library was constructed with DNA from *gl1-43* and screened with a T-DNA probe. Restriction mapping and DNA gel blot hybridization identified one clone that contained sequences that flank the left border of the T-DNA insertion site.

In the experiments described here, we used the left border clone as a probe to isolate an uninterrupted sequence from a genomic library of wild-type *Arabidopsis* and subcloned it into a binary vector suitable for *Agrobacterium*-mediated transformation. The transformed shoots that regenerated from *gl1* root tissue exhibited normal leaf and stem trichomes. An analysis of the progeny from one of the transformed plants showed that the presence of trichomes cosegregated with the kanamycin resistance gene on the T-DNA. These results confirm that we have isolated *GLABROUS1* (*GL1*), a gene with an unknown product that is required for a specific type of cellular differentiation. This work also demonstrates that T-DNA tagging and complementation can be used successfully to isolate and identify a crucial gene in a plant developmental pathway.

RESULTS

Isolation of a DNA Sequence Tightly Linked to the *GL1* Locus

The accompanying paper reports the cloning of a DNA sequence that flanks a T-DNA insert that is tightly linked to the *GL1* locus. This 2.8-kb clone, pBS43LG, was used as a probe to isolate the uninterrupted *GL1* sequence from a wild-type *Arabidopsis* genomic library. When the library was screened with this probe, a hybridizing phage clone, designated NT2, was identified. After this clone was taken through three rounds of plaque purification, DNA was

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isolated and characterized by restriction enzyme analysis (Figure 1). To identify the region that corresponded to the site of the T-DNA insertion into the *gl1-43* mutant, a DNA gel blot hybridization analysis was performed. Fragments from two regions of the pBS43LG border clone were used as probes and hybridized to a filter containing restricted NT2 DNA cleaved with ScaI (data not shown). One probe was a 1-kb SalI-AccI fragment from the left side of the border clone and the other probe was a 0.8-kb ScaI-EcoRI fragment from the right side of the border clone directly adjacent to the T-DNA insertion site. The SalI-AccI probe only hybridized to the sequences immediately to the left side of the ScaI site located 4.2 kb from the left end of the NT2 clone. The ScaI-EcoRI fragment only hybridized to the sequences immediately to the right of the same ScaI site. These results demonstrate that the second ScaI site from the left end of the NT2 clone corresponds to the ScaI site of the pBS43LG probe. The restriction map in Figure 1 shows that the NT2 clone has fragments of 4.2 kb and 9.5 kb flanking the site of the T-DNA insertion into the *gl1-43* mutant. Because the NT2 genomic clone contained large regions of sequence flanking both sides of the *gl1-43* T-DNA insertion site, it was used as a source of subclones for subsequent attempts to identify the *GL1* gene by complementation.

Regeneration of Wild-Type and *gl1* Root Explants

Before an *Agrobacterium*-mediated transformation protocol could be used for complementation experiments, it was necessary to determine whether the expression of the trichome phenotype was affected by the process of tissue culture. Shoots were regenerated from root explants taken from wild-type and from *gl1* mutant seedlings in the Columbia and Landsberg backgrounds using modifications of the protocol described by Valvekens, Van Montagu, and Van Lijsebettens (1988). The shoots regenerated from the *gl1* Columbia and the *gl1* Landsberg root explants lacked leaf and stem trichomes (Figure 2A), the expected mutant phenotype. In contrast, the shoots regenerated from the wild-type Columbia and Landsberg root explants had normal leaf and stem trichomes (Figure 2B). Interestingly, there were discernible differences in the extent and in the time course of the regeneration response that were apparently a reflection of the genotypic variation in the root explants. For example, the Columbia root explants (both wild-type and *gl1*) yielded several more shoots per explant than the Landsberg root explants (data not shown). Also, shoot primordia were visible on the *gl1* Columbia root explants at least a week before they became visible on the wild-type Columbia root explants.

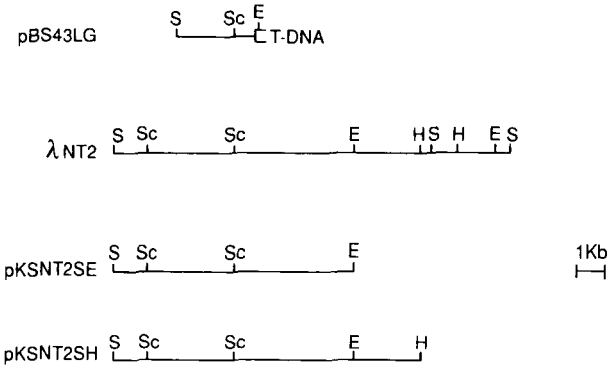

Figure 1. Restriction Maps of the Probe, Genomic Clone, and Subclones Used To Isolate the *GL1* Gene.

The construct pBS43LG was used as a probe to isolate the NT2 genomic clone from a wild-type EMBL3 library. Two overlapping fragments from the NT2 clone, both including the T-DNA insertion site of the probe, were then subcloned into pBluescript KS+ to give pKSNT2SE and pKSNT2SH. Restriction sites are: S, SalI; E, EcoRI; H, HindIII; Sc, ScaI.

Complementation of the *gl1* Allele

For the initial transformation experiments, *gl1* Columbia and *gl1* Landsberg root explants were cocultivated with *Agrobacterium* strain LBA4404 bearing the constructs pBINNT2SE and pBINNT2SH. As a negative control, *gl1* root explants were also transformed with *Agrobacterium* carrying pBI121, a plasmid derived from the binary vector pBIN19 that contains the β-glucuronidase (GUS) gene driven by the constitutive cauliflower mosaic virus 35S promoter (Jefferson, Kavanagh, and Bevan, 1987). Transformed calli were selected on shoot-inducing medium containing 50 mg/L kanamycin. After 10 days, small green calli were visible on the roots. Most of the calli derived from the *gl1* Columbia root explants produced shoots after 4 to 6 weeks on shoot-inducing medium, but the calli from the *gl1* Landsberg root explants became necrotic and died during this time.

The shoots transformed with both of the pBIN19 constructs from the NT2 genomic clone developed normal leaf and stem trichomes (Figures 2D and 2E). The shoots transformed with the pBI121 plasmid had GUS activity when they were tested with a fluorometric assay. However, these shoots exhibited the *gl1* phenotype because they lacked leaf and stem trichomes (Figure 2C). When the transformation experiments were repeated using the pBINNT2SE and the pBI121 constructs in *Agrobacterium* strain C58, the same results were observed.

The transformed shoots were transferred to Magenta boxes to allow seed set. Seeds were collected from one of the plants that had been transformed with the pBINNT2SE construct and germinated on medium without kanamycin. Five of the progeny from this transformant lacked trichomes, whereas 28 displayed normal trichomes on the leaves and stems (Figure 2F). When the progeny

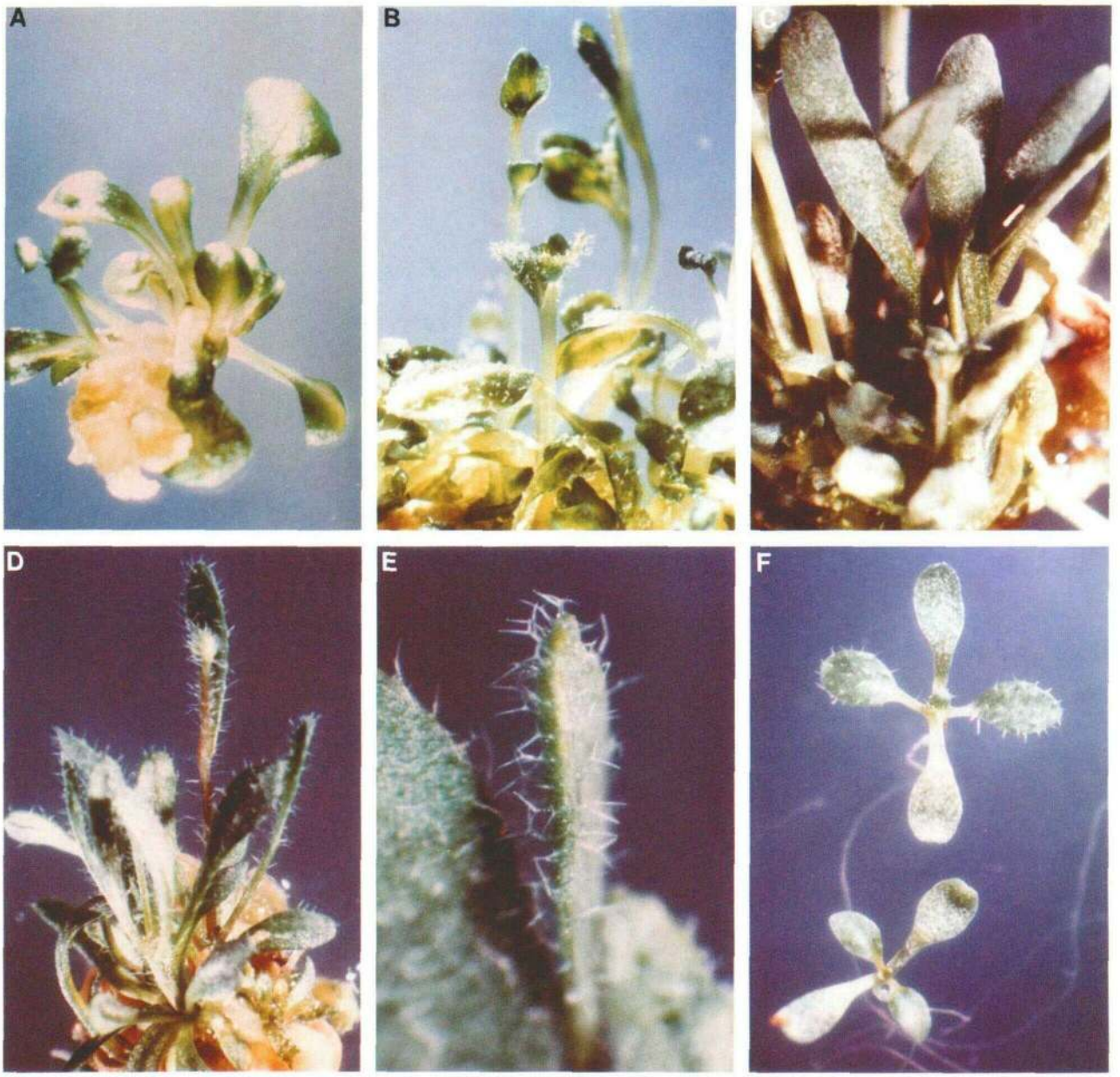

Figure 2. Complementation of the *gl1* Mutation of *Arabidopsis* (Columbia) with the pBINNT2SE Construct Containing the *GL1* Gene.

(A) Plantlets without leaf trichomes regenerating from a *gl1* root explant.
(B) Plantlets with leaf trichomes regenerating from a wild-type root explant.
(C) Plantlets without leaf trichomes regenerating from a *gl1* root explant transformed with the pBI121 construct that contains the GUS gene. The plantlets exhibit GUS activity.
(D) Plantlets with leaf trichomes regenerating from a *gl1* root explant transformed with the pBINNT2SE construct.
(E) Surface view of a leaf from a transformed *gl1* plantlet in **(D)** showing the branched trichomes characteristic of a wild-type leaf.
(F) Progeny from a *gl1* plant transformed with the pBINNT2SE construct segregating for leaf trichomes. One seedling has trichomes on the first pair of true leaves, whereas the other seedling completely lacks leaf trichomes. (The cotyledons, which are also visible, do not develop trichomes.)

were transferred to medium supplemented with 50 mg/L kanamycin, the plants with trichomes grew rapidly and set seed. In contrast, the five plants without trichomes grew very slowly and eventually became chlorotic.

DISCUSSION

The proof that a gene has been isolated through the use of gene tagging is the ability of the uninterrupted sequence to restore gene activity. In the accompanying paper (Marks and Feldmann, 1989), we showed that a new trichome mutation allelic to *gl1* was tightly linked to a T-DNA insert. The T-DNA "tag" was used to isolate the DNA flanking the insertion site. This flanking sequence was then used to isolate the uninterrupted sequence. In this paper, we showed that the uninterrupted sequence restored the ability of the *gl1* plants to produce trichomes.

The fact that the insertional mutation was allelic to *gl1* gave us flexibility in the source of tissue to be used for the complementation assay. The insertional mutant had normal leaf trichomes and was kanamycin-resistant. Because the binary vector pBIN19 carries the neomycin phosphotransferase gene that confers kanamycin resistance, we found it much more convenient to attempt to transform the known *gl1* mutants because they are naturally sensitive to kanamycin. The use of the *gl1* mutant also allowed the success of the experiment to be assessed at an earlier stage. Since the *gl1* mutants lack leaf trichomes, complementation was confirmed by the expression of branched leaf trichomes on the surface of the young leaves in the regenerating shoots. The *gl1* mutant in the Columbia background, which regenerated very efficiently under our tissue culture conditions, carries another mutation. This mutation, designated *an*, results in narrower leaves and is unlinked to *gl1*. The *an* phenotype served as a contamination control in the complementation experiments. The only plants in the laboratory that have the *an* mutation also have either the *dis1* mutation that results in distorted trichomes or the *gl1* mutation. All of the transformed plants in the complementation experiments expressed the *an* phenotype and normal trichomes. This confirms that the complementation results were valid and not due to wild-type seeds contaminating our *gl1* stocks.

Two overlapping constructs from the genomic clone successfully complemented the *gl1* mutation. These experiments allowed the *GL1* gene to be localized to an 8.3-kb SalI-EcoRI subclone. We are now in the process of transforming smaller fragments from this subclone into *Arabidopsis* to determine the precise location of the gene. We are also attempting to isolate a cDNA clone homologous to the region to the left of the T-DNA insertion, and we have begun to sequence the 8.3-kb subclone. These analyses should allow us to determine the sequence of the *GL1* gene.

Our successful identification of the *GL1* gene by complementation should permit us to isolate other interesting genes that affect trichome differentiation. Besides *GL1*, there are five other distinct loci affecting trichome formation which have been characterized. The phenotypes of these mutants suggest that they contain blocks at different points in trichome differentiation. We are continuing to screen our transformed *Arabidopsis* population for trichome mutants and we have also initiated chromosome walks to some of the known loci.

The molecular characterization of *GL1* and the other trichome genes should provide information on the complex mechanisms that control the differentiation of trichome cells. An understanding of this model system may lead to a better understanding of the process by which other types of plant cells differentiate.

METHODS

Genetic Material

Seeds of *Arabidopsis thaliana* ecotype Landsberg *erecta* and of the *gl1* trichome mutant in the Landsberg *erecta* background were obtained from Dr. Maarten Koornneef (Agricultural University, Wageningen, The Netherlands). Seeds of *Arabidopsis thaliana* ecotype Columbia and of the *gl1* trichome mutant in the Columbia background were obtained from Dr. George Redei (University of Missouri, Columbia, MO).

Genomic Clones and Subclones

All procedures, unless indicated otherwise, were performed using standard protocols as described by Maniatis, Fritsch, and Sambrook (1982). The genomic library was constructed from partially digested Sau3A fragments of genomic DNA that had been isolated from wild-type *Arabidopsis* in the Wassilewskija background by the method of Weeks, Beerman, and Griffith (1986). These fragments were cloned into the bacteriophage vector EMBL3. The library was screened using the pBS43LG clone (Marks and Feldmann, 1989), labeled with ^{32}P by nick translation, as a probe. Two overlapping fragments from the NT2 genomic clone, both including the T-DNA insertion site, were subcloned into the pBluescript KS+ vector (Stratagene, Inc.) to generate the constructs pKSNT2SH and pKSNT2SE. pKSNT2SE contains an 8.3-kb SalI-EcoRI fragment from the NT2 clone. pKSNT2SH contains a 10.6-kb SalI-HindIII fragment that includes the same region as the pKSNT2SE subclone and an additional 2.3-kb region (Figure 1).

Agrobacterium Constructs

The *Arabidopsis* specific fragment in each of the subclones was ligated into the binary vector pBIN19 (Bevan, 1984) to generate

pBINNT2SE and pBINNT2SH. The constructs were then transferred by direct transformation (An et al., 1988) into a competent *Agrobacterium tumefaciens* strain harboring a helper Ti plasmid to supply the virulence functions necessary for DNA transfer from the bacterium to the plant. Two *Agrobacterium* strains were used: LBA4404 containing the plasmid pAL4404 (Hoekema et al., 1983), and C58 containing the plasmid pGV3850 (Zambryski et al., 1983).

Regeneration and Transformation of Root Explants

Root explants were transformed and regenerated using modifications of the method described by Valvekens et al. (1988). The callus-inducing medium was R3 medium (Christianson, 1979), and the shoot-inducing medium was .05/7 medium (Christianson and Warnick, 1983). All hormones were added to the media before it was autoclaved at 121°C for 15 min.

Seeds were sterilized in pure Clorox for 2 min, washed four times with sterile distilled water, rinsed with 95% ethanol, and dried in a laminar flow hood. They were germinated on a solidified medium that contained MS salts (Murashige and Skoog, 1962), 1% sucrose, and 0.8% Difco agar. Seedlings were grown for at least 3 weeks under fluorescent light (100 $\mu E/m^2/sec^2$) on a 12-hr light/dark cycle at 22°C in Magenta boxes on a solidified medium containing MS salts and vitamins, 3% sucrose, and 0.8% Difco agar. Root explants about 1 cm in length were taken from these in vitro seedlings and incubated on solid callus-inducing medium for 3 days before cocultivation for 48 hr with *Agrobacterium* cells grown overnight in 3 mL of YEP medium (An et al., 1988).

Measurement of β-Glucuronidase Activity

The fluorometric assay of Jefferson (1987) was used to measure GUS activity in plants that had been transformed with the pBI121 binary vector.

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