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Allele-Specific Interactions Between *ttg* and *gl1* During Trichome Development in *Arabidopsis thaliana*

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ABSTRACT

Trichome development in *Arabidopsis thaliana* is a well-characterized model for the study of plant cell differentiation. Two genes that play an essential role in the initiation of trichome development are *GL1* and *TTG.* Mutations in either gene prevent the initiation of most trichomes. The *GL1* gene encodes a *myb*-related transcription factor. Mutations in *TTG* are pleiotropic, affecting anthocyanins, root hairs, and seed coat mucilage in addition to trichomes. Six *ttg* alleles were examined and shown to form a hypomorphic series. The severity of all aspects of the *ttg* phenotype varied in parallel in this allelic series. The weakest allele, *ttg-10*, causes frequent clusters of adjacent trichomes, suggesting a role for *TTG* in inhibiting neighboring cells from choosing the trichome fate. This allele results from a mutation in the 5′-untranslated region of *ttg* and creates an out-of-frame upstream AUG codon. The *ttg-10* allele shows several unusual genetic interactions with the weak hypomorphic *gl1-2* allele, including intergenic noncomplementation and a synthetic glabrous phenotype. These interactions are specific for the *gl1-2* allele. The implication of these results for current models of trichome development is discussed.

CELL fate specification is a process of fundamental importance during development. Although much work has been done in plants on differentiation at the tissue level, relatively few systems are available in plants for the study of the differentiation of individual cell types. The development of *Arabidopsis thaliana* leaf hairs (trichomes) is a well-established model for the study of plant cell fate and differentiation (Larkin *et al.* 1997; Hülskamp and Schnittger 1998). Arabidopsis trichomes are highly specialized single cells that develop in the plant epidermis. These cells undergo a dramatic program of polarized cell expansion, ultimately elaborating several aerial branches. Trichomes are one of several possible epidermal cell fates; other cell types present in the epidermis include unspecialized epidermal cells, stomatal guard cells, and root hairs. In nature, trichomes may provide protection against insect herbivores (Mauricio and Rausher 1997), but under laboratory conditions trichomes are nonessential. This has allowed the identification of mutations in more than twenty genes involved in trichome development (Marks 1997). The accessibility of trichomes to genetic analysis makes this an excellent system for determining the cascade of developmental events involved in the differentiation of a single cell type.

Current evidence suggests that trichome precursors are selected from a field of developmentally equivalent cells by a mechanism similar to that involved in the development of Drosophila sensory bristles (Artavanis-Tsakonas *et al.* 1995) and Caenorhabditis vulval development (Sternberg and Horvitz 1984). Developing trichomes are distributed in a minimum-distance spacing pattern more regular than would be expected by chance, indicating the existence of an underlying mechanism controlling the patterning of trichome precursors (Hülskamp *et al.* 1994; Larkin *et al.* 1996). Cell lineage has been ruled out as a basis for this pattern (Larkin *et al.* 1996), and current models invoke lateral inhibition of neighboring cells to explain the distribution of these cells (Larkin *et al.* 1996), although the mechanistic basis remains unknown. Two genes, *GLABRA1* (*GL1*) and *TRANSPARENT TESTA GLABRA* (*TTG*), are known to play a central role in the trichome cell fate decision based on the virtually complete absence of trichomes on loss-of-function mutants of either gene. *GL1* encodes a protein containing a MYB DNA-binding domain and is expressed most highly very early during trichome development, consistent with its proposed role as a central regulator of the cell fate decision (Oppenheimer *et al.* 1991; Larkin *et al.* 1993). In addition, when *GL1* is expressed ectopically along with the maize *R* gene, which encodes a basic-helix-loop-helix (bHLH) transcription factor, trichomes are produced on epidermal surfaces that are normally glabrous (Larkin *et al.* 1994a). These observations suggest that the GL1 protein, probably in conjunction with an endoge-

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nous R-like protein, is a central regulator of the trichome cell fate decision. Mutations in *gl1* do not appear to affect any processes other than trichome development.

In contrast to *GL1*, *TTG* is required for a striking diversity of processes, as was first noted by Koornneef (1981). In addition to lacking trichomes, *ttg* mutants are defective in the synthesis of anthocyanin pigments, production of seed coat mucilage, and root hair development (Koornneef 1981; Galway *et al.* 1994). The role of *TTG* in root hair development is particularly noteworthy, because *TTG* is required for development of the nonhair cells in the root epidermis. Thus, *ttg* mutants produce extra root hairs.

Two observations suggest that *TTG* plays a role in the spacing of trichome precursors in the epidermis (Larkin *et al.* 1994a). First, some hypomorphic alleles of *TTG* produce frequent clusters of partially developed adjacent trichomes. Clusters of adjacent trichomes are rarely observed on wild-type (WT) leaves. Second, *ttg* heterozygotes containing a *35SGL1* constitutive overexpression construct also produce a high frequency of trichome clusters. One explanation for these observations is that a process downstream of *TTG* may be involved in lateral inhibition of cells neighboring a trichome precursor, preventing them from developing as trichomes. A candidate for a gene functioning in this pathway is the *TRIPTYCHON* (*TRY*) gene; leaves of *try* mutants produce numerous trichome clusters (Hülskamp *et al.* 1994).

Several parallels exist between the regulation of Arabidopsis trichome development and the regulation of anthocyanin biosynthetic enzymes in various plant species, in addition to the involvement of *TTG* in both processes in Arabidopsis. In maize, two gene families have been identified that encode transcription factors required for the expression of anthocyanin biosynthetic enzymes. The *R/B* family encodes bHLH proteins (Chandler *et al.* 1989; Ludwig *et al.* 1989), while the *C1/Pl* family encodes Myb proteins related to *GL1* (Cone *et al.* 1986, 1993; Paz-Ares *et al.* 1987; Grotewold *et al.* 1994). Members of these two gene families have also been identified as regulators of anthocyanin biosynthetic genes in several other plant species (reviewed in Mol *et al.* 1998). In maize, members of these two regulatory protein families cooperate to induce anthocyanin biosynthesis and appear to interact physically (Klein *et al.* 1989; Goff *et al.* 1992). The mode of action of Myb and bHLH proteins in the regulation of anthocyanin biosynthesis thus provides a model for understanding their role in trichome development.

The maize *R* gene can bypass the need for *TTG* in all *TTG*-dependent processes when it is expressed in Arabidopsis *ttg* mutants (Lloyd *et al.* 1992). This observation initially led to suggestions that *TTG* might encode an R homologue. Recently, however, *TTG* has been cloned and shown to encode a protein of 341 amino acid residues containing four WD repeats (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results). The petunia anthocyanin regulatory gene *an11* also encodes a WD-repeat protein closely related to *TTG* (de Vetten *et al.* 1997; A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results). Apparent homologues of the *TTG/an11* genes exist in humans, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae.* The WD repeat is a structural motif found in proteins regulating transcription, signal transduction, vesicular trafficking, RNA processing, and cytoskeletal assembly (Neer *et al.* 1994). Many WD-repeat proteins are known to physically interact with other proteins, and this domain is thought to function as a protein interaction domain.

Previous work has suggested that *GL1* and *TTG* act at essentially the same point in the trichome pathway (Larkin *et al.* 1994a). Several of the experiments used to draw this conclusion, however, were performed using the maize *R* gene as a surrogate for *TTG* function and may reflect the function of an endogenous *R*-like gene acting downstream of *TTG*, rather than *TTG* itself. In this work, we describe allele-specific genetic interactions between *ttg* and *gl1* mutations that suggest that the functions of these two genes are tightly intertwined.

MATERIALS AND METHODS

Growth conditions, plant strains, and genetic methods: Plants were grown under continuous illumination by 40-W Sylvania Cool White fluorescent bulbs (~100 μE m^2 s^{-1}) at 21° in a mixture of a peat-based potting medium and vermiculite and watered from below. Plants were fertilized with a modified Hoagland's solution (Epstein 1972) initially and again when the plants began to bolt.

Trichomes were counted on either of the first two postembryonic leaves, referred to as "first leaves" throughout this report. These leaves are developmentally equivalent and have a similar number of trichomes. To avoid miscounting inconspicuous abortive trichomes on the *ttg* mutants, trichomes were counted only if they clearly protruded from the surface of the leaf (~50 μm). In general, trichomes were counted on a single first leaf from 10 independent plants per pot. Root hairs were counted on plants grown on vertically placed agar plates, essentially as described by Galway *et al.* (1994). Anthocyanin phenotypes were assessed by visual inspection of seedlings grown on both agar plates and soil. Seed-coat mucilage phenotypes were determined by viewing seeds mounted on microscope slides in a dilute suspension of India ink (Rerie *et al.* 1994).

Trichome clustering phenotypes: Trichome clusters were defined as any instance where two or more trichomes were located immediately adjacent to each other, without intervening adjacent cells. This phenotype has been previously documented (Larkin *et al.* 1994a) and is readily scored in a dissecting microscope.

Plants homozygous for the *ttg-10* allele exhibit a distinctive phenotype, with numerous incompletely developed trichomes that often occur as clusters (Larkin *et al.* 1994a,b; Figure 1H).

TABLE 1

Origins of mutant alleles used in this study

Allele	Ecotype of isolation	Mutagen	Reference	Crosses to Col-0 before selfing[a]
ttg-1	L*er*	EMS	Koornneef (1981)	5
ttg-9	Col-0	EMS	Larkin *et al.* (1994a,b)	3
ttg-10	WS	EMS	Larkin *et al.* (1994a)	5
ttg-11	Col-0	EMS	Larkin *et al.* (1994a)	3
ttg-12	Col-0	EMS	This study	3
ttg-13	RLD	Fast neutrons	This study	3
gl1-1	Col-1?	Unknown	Oppenheimer *et al.* (1991)	WS[b]
gl1-2	Col-1	X rays	Esch *et al.* (1994)	1
try-JC	Col-0	EMS	This study	1

[a] Crosses done in this study prior to use in experiments.
[b] Crossed six times to WS before selfing. See materials and methods.

Many of these trichomes develop as unbranched spikes, which are very noticeable in the dissecting microscope. In discussion of genetic interactions between *ttg-10* and *gl1* alleles, the phenotype exhibited by *ttg-10* homozygotes in an otherwise WT background is referred to as "spiky."

Origins of mutant alleles: Unless otherwise noted, WT refers to the Columbia-0 (Col-0) genetic background throughout this paper. The origins and crossing history of the six *ttg* alleles used in this study are summarized in Table 1. Allelism of all *ttg* mutations was confirmed by examining both the F_1 and the F_2 of crosses between the new alleles and the *ttg-1* reference allele. Both the defective trichome phenotype and the yellow seed phenotype (caused by the absence of anthocyanin pigmentation) were used to confirm noncomplementation. Linkage of putative *ttg* alleles to the *ttg* locus was confirmed by examining the F_2 progeny of crosses to the reference allele. The origins of *ttg-1*, *ttg-9*, *ttg-10*, and *ttg-11* have been described previously (Larkin *et al.* 1994a). The *ttg-12* allele, isolated in the Col-0 genetic background, was obtained from A. Sessions (University of California, Berkeley, CA). The *ttg-13* allele was isolated in the Rschew (RLD) genetic background by D. Oppenheimer in the lab of M. D. Marks (University of Minnesota, St. Paul) in a screen of fast-neutron mutagenized seed purchased from Lehle Seeds (Round Rock, TX). Alleles isolated from a Col background (*ttg-9*, *ttg-11*, and *ttg-12*) were crossed to Col-0 three times before selfing and selecting *ttg* mutant plants. Alleles isolated from a background other than Col were crossed to Col-0 five times before selfing and selecting *ttg* mutant plants, with the exception of *ttg-13.* This allele was added late in the study and was crossed to Col-0 only three times prior to selfing. This reduced level of introgression is unlikely to influence the results, because first leaves of Col-0 and RLD WT plants have similar numbers of trichomes, and no aberrant trichome phenotypes have been observed to segregate from numerous crosses between these ecotypes. To ensure that all potential modifier loci had been eliminated by backcrossing, the WT siblings of each backcrossed *ttg* mutant were examined to confirm that the number of trichomes per leaf was similar to that of Col-0. Particular care was taken in backcrossing *ttg-1* plants to choose families that no longer segregated the *er* mutation derived from the Landsberg *erecta* (L*er*) background. L*er* carries an allele of the *RTN* locus that reduces the number of trichomes per leaf approximately threefold relative to Col-0, and *rtn* is tightly linked to *er* (Larkin *et al.* 1996).

The *gl1-1* allele used in this study was described previously (Oppenheimer *et al.* 1991). In the line used here, *gl1-1* had been introgressed into the Wassileskjia (WS) background by crossing six times before selfing was used in these studies. Crosses to WS were included as controls in some experiments and gave results essentially identical to results obtained with Col-0 (Table 4; data not shown). The *gl1-2* allele originated in the Col-1 background (Esch *et al.* 1994). The 35S*GL1* line used here has been described previously as 35S*GL1* 4-1 (Larkin *et al.* 1994a). This line was generated by Agrobacterium-mediated transformation of Col-0, and it is homozygous for the T-DNA insert. The *try-JC* allele was generated in Col-0 and was provided by J. Chien (University of California, Berkeley, CA). Allelism with *try* was confirmed both by failure to complement the recessive trichome clustering phenotype associated with the *try-82* allele (provided by M. Hülskamp, University of Tübingen, Tübingen, Germany) and by the absence of phenotypically WT plants in the subsequent F_2, demonstrating linkage of *try-JC* to the *try* locus. Of the three *try* alleles that we have examined, *try-JC* appears to have the strongest phenotype. The *gl2-p1* and *gl2-p2* alleles were obtained from M. D. Marks and originated with R. S. Poethig (University of Pennsylvania, Philadelphia). These alleles were generated in the Col-0 background and were not backcrossed before use in the experiments described here. The *gl3-1* reference allele in the L*er* background was obtained from M. D. Marks and was not backcrossed before use in the experiments described here.

Identification of *ttg-10 gl1-2* double mutants: The double mutant was initially identified in the F_2 of a cross between *ttg-10* and *gl1-2* as a completely glabrous plant that produced only completely glabrous plants with yellow seeds in the F_3. The *gl1-2* genotype of this plant was confirmed by PCR amplification of genomic DNA with the *gl1-2*-specific primers gl1-2R (5′GCAAAATTCATCATTACGAGTG3′) and gl1-2F (5′TCATCTCAGCAAAAAACTCG3′), using an annealing temperature of 57° with 3 mm $MgCl_2$ for 30 cycles, followed by analysis on a 2.5% agarose gel. These primers flank the *gl1-2* deletion (see results) and identify the *gl1-2* allele as a length polymorphism relative to WT in the amplified product.

Scanning electron microscopy: Samples fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid) were prepared for scanning electron microscopy by standard methods (Irish and Sussex 1990).

Sequence analysis of mutant *ttg* alleles: Genomic DNA was extracted from 4-wk-old plants using the method of Dellaporta *et al.* (1983). The DNA was purified further by centrifugation on CsCl gradients (Walker *et al.* 1997). Using genomic DNA from mutants *ttg-10*, *ttg-11*, *ttg-12*, and their parental ecotypes (WS and Col) as templates, PCR products from the

region of the *TTG* gene were generated by amplification using a mixture of thermostable polymerases BioTaq (Bioline) and Pfu (Stratagene, La Jolla, CA) in the ratio of 10:1. Four pairs of oligonucleotide primer pairs were designed to give overlapping fragments of about 700 bp. PCR products, which covered the gene from 500 bp upstream from the start of translation to 400 bp into the intron sequence (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results), were subjected to electrophoresis on 1% agarose TAE gels (Sambrook *et al.* 1989) and extracted from the gels using a Qiaex II gel extraction kit (QIAGEN, Valencia, CA). The purified DNA was sequenced directly using both primers by an ABI (Columbia, MD) automated sequencer. Comparisons between mutant and parental WT sequences were carried out using the SeqEd program (ABI) to determine the position of the mutations.

Gel blot analysis of transcripts of *TTG* from mutants: Total RNA from floral buds of 4-wk-old soil-grown WS and mutant *ttg-10*, *ttg-11*, and *ttg-12* plants was extracted from 200 mg of tissue using Tripure (Boehringer Mannheim, Mannheim, Germany). RNA (10 μg) was subjected to electrophoresis on a 1.2% agarose denaturing formaldehyde gel and blotted onto Genescreen Plus (Dupont, Wilmington, DE) in accordance with the manufacturer's instructions. Double-stranded probes were prepared as in Walker *et al.* (1997) by random-primer extension using [α^{32}P]dATP (Amersham, Arlington Heights, IL). The *TTG* probe contained the entire coding region. Hybridization was carried out at 42° in 50% formamide for 16 hr. Blots were washed in 0.1× SSC, 2% SDS at 65° for 15 min. The filter was exposed to X-Ograph XB-200 film (X-Ograph Imaging Systems, Inc.) with two intensifying screens for 2 days at −80°. The blot was stripped by boiling in 0.1× SSC, 2% SDS for 5 min and checked by exposure to film before hybridization to an Arabidopsis β-tubulin cDNA probe (GenBank accession no. Z25960, 3′-untranslated region of the *TUB5* gene).

RESULTS

Genetic characterization of *ttg* alleles: Koornneef (1981) was the first to describe the pleiotropic phenotype of *ttg* mutants in detail. He examined eight independent alleles and concluded that the trichome, anthocyanin, and seed-coat aspects of the *ttg* phenotype were the result of mutations at a single locus. Since this description, however, the trichome clustering and root hair effects of *ttg* mutations have been described (Galway *et al.* 1994; Larkin *et al.* 1994a). While it is virtually certain that this phenotypic syndrome is caused by mutations in a single gene, it is possible that the *TTG* gene could have multiple functional domains. If this is the case, some mutations might affect different *TTG* functions to different extents. For these reasons, the genetic characterization of a series of *ttg* alleles was undertaken.

Six *ttg* alleles were characterized in a predominantly Col-0 genetic background (Figure 1; Table 2). All of these alleles were fully recessive to WT. All aspects of the *ttg* phenotype were found to vary in parallel, with *ttg-13* being the strongest allele and *ttg-10* the weakest. None of the *ttg* alleles resulted in visible anthocyanin pigmentation in the seed coat, but seedlings homozygous for the *ttg-10* allele produced some anthocyanin pigment on the cotyledons and the upper part of the hypocotyl. Seed coats of *ttg-10* mutants also produced mucilage. Seedlings homozygous for the *ttg-9* allele did not produce visible anthocyanins but did produce patchy seed-coat mucilage (J. C. Larkin, unpublished observations). None of the other alleles resulted in production of either anthocyanins in the seedling or seed-coat mucilage. As previously reported (Larkin *et al.* 1994a,b), weak *ttg* alleles caused clusters of adjacent trichomes at a fairly high frequency (Tables 2 and 3). Recently, *ttg-13* has been shown to be a null allele containing a deletion of at least 4 kb that includes the *TTG* gene (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results), consistent with *ttg-13* being the strongest allele. Despite the absence of any *TTG* coding sequences, a few trichomes and clusters of adjacent trichomes were occasionally found on *ttg-13* mutant leaves (Figure 1C). These observations demonstrate that *TTG* is not absolutely necessary for limited trichome development.

All combinations of *ttg* allele *trans*-heterozygotes were constructed (Table 3), and all combinations resulted in a trichome phenotype intermediate to that of the parental alleles. In particular, all alleles produced fewer trichomes when *in trans* to the *ttg-13* null than when they were homozygous. Therefore, these six *ttg* alleles formed a hypomorphic allelic series, with the order of strength of the alleles from strongest to weakest being *ttg-13* > *ttg-1* > *ttg-12* ≈ *ttg-11* > *ttg-9* > *ttg-10*. There was no intragenic complementation, and no evidence was found for the existence of multiple functional domains in *TTG*.

Genetic characterization of the *gl1-2* allele: The genetic behavior of the weak *gl1-2* allele also was characterized. This allele contains a 14-bp deletion that results in an in-frame stop codon at the point of the deletion (Esch *et al.* 1994). The mutation would result in a polypeptide missing 27 amino acids from its C terminus. *gl1-2* mutant plants produce very few trichomes on their first two leaves (Table 4). On subsequent leaves, the mutants have an increasing number of trichomes, but few trichomes develop near the midvein of the leaf (Figure 2A). The *gl1-1* allele is a null allele caused by a 6.5-kb deletion that completely removes the *GL1* coding region (Oppenheimer *et al.* 1991). The *gl1-2* allele is fully recessive to WT (Figure 2B; Table 4), and when placed *in trans* to the null allele, the phenotype is more severe than the phenotype of *gl1-2* homozygotes (Table 4). The *gl1-2* allele thus behaves as a typical hypomorph.

The *ttg-10* allele interacts with alleles of *gl1* and *try*: When mutations in two different genes fail to complement in a double heterozygote, this often indicates that the two genes act in a common pathway (Stearns and Botstein 1988; Simon *et al.* 1991). Intergenic noncomplementation has been used to identify new interacting mutations, but it also can be used to examine preexisting

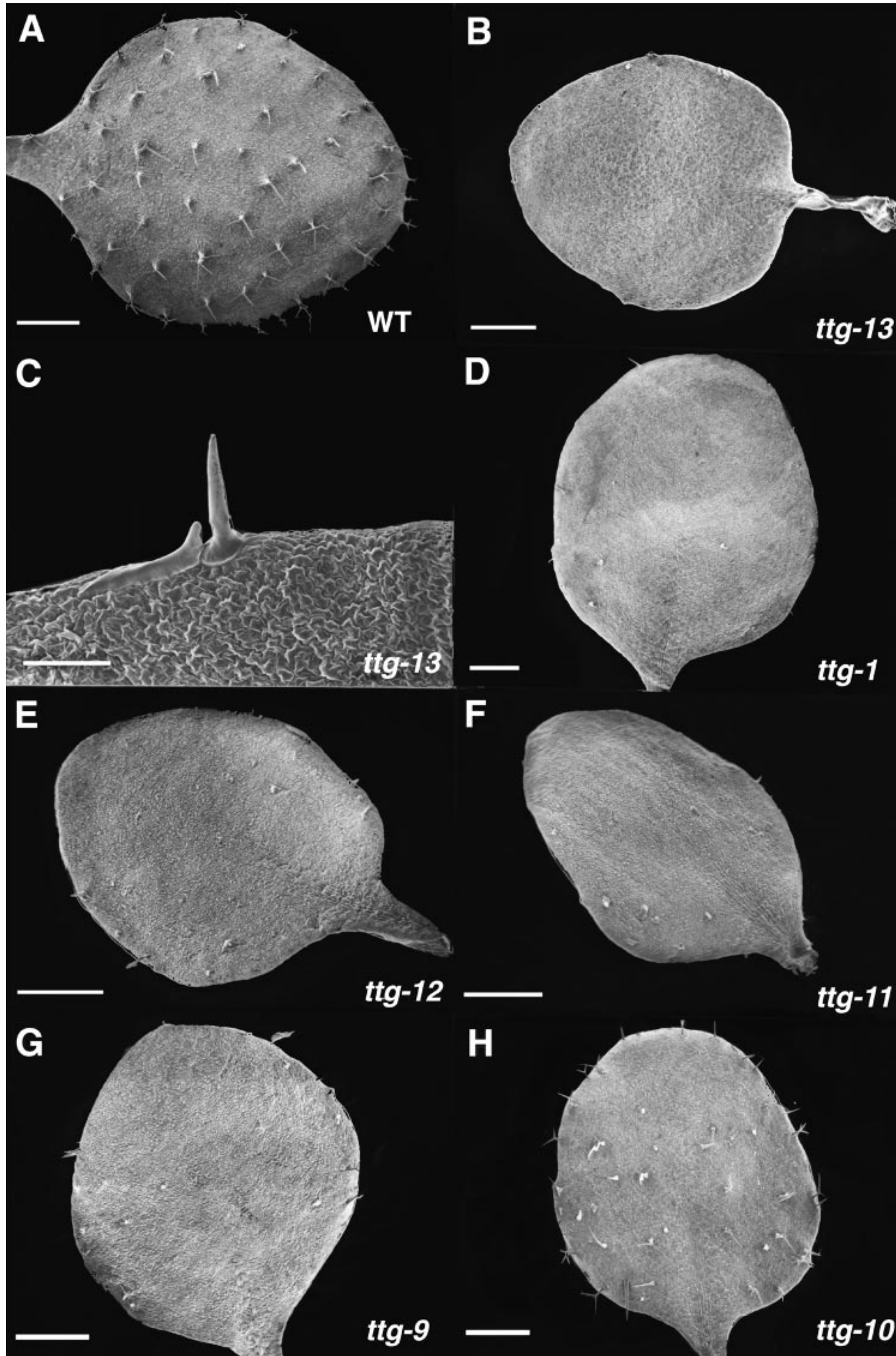

Figure 1.—Phenotypes of first leaves of strains carrying the indicated *ttg* alleles. (A) Col-0 WT. (B) *ttg-13.* (C) Trichomes on *ttg-13* leaf. (D) *ttg-1.* (E) *ttg-12.* (F) *ttg-11.* (G) *ttg-9.* (H) *ttg-10.* Bars in A, B, D, E, F, G, and H, 500 μm. Bar in C, 100 μm.

mutations for genetic interactions (Theisen *et al.* 1994). To investigate the relationship between *ttg* and other mutations in the trichome pathway, the weak *ttg-10* allele was crossed with alleles of various genes involved in trichome development (*gl1-2*, *gl2-p1*, *gl2-p2*, *gl3-1*, and *try-JC*), and the F_1 progeny (*ttg-10*/+ *m*/+, where *m* is another trichome mutation) were examined for altered trichome phenotypes. Plants heterozygous for *ttg-10* and either *gl2-p1*, *gl2-p2*, or *gl3-1* were indistinguishable from *ttg-10* single heterozygotes (J. Larkin, unpublished observations). In contrast, plants doubly heterozygous for *ttg-10* and either *try-JC* or *gl1-2* showed an increase in the number of trichomes present in clusters (Figure 2C; Tables 5 and 6).

A high frequency of trichome clusters also was observed in the F_1 of crosses of several other *ttg* alleles with *gl1-2* (Table 6), indicating that this clustering interaction was not dependent on a particular allele of *ttg.*

TABLE 2

Detailed phenotypes of *ttg* alleles

Allele	No. of trichomes[a]	Seedling			Seed coat mucilage[e]
		% in clusters [b]	Anthocyanin[c]	% root hairs[d]	
WT	30.9 ± 6.1	0.0	+	39.6 ± 3.9	+
ttg-13	0.8 ± 1.5	5.3[f]	−	85.6 ± 3.9	−
ttg-1	2.4 ± 1.7	0.0	−	84.4 ± 2.6	−
ttg-12	4.6 ± 3.5	9.7	−	77.6 ± 4.6	−
ttg-11	5.0 ± 3.3	8.6	−	76.4 ± 6.7	−
ttg-9	8.1 ± 3.1	7.7	−	61.6 ± 3.9	+/−[g]
ttg-10	25.8 ± 5.8	27.9	+/−	53.2 ± 1.8	+

[a] Number of trichomes on the adaxial surface of first leaves (mean ± SD).
[b] Mean percentage of trichomes adjacent to another trichome.
[c] Visible anthocyanins on 10-day-old seedlings.
[d] Percentage of root epidermal cells that are root hairs (mean ± SD).
[e] Exclusion of ink particles from vicinity of hydrated cells by mucilage "halo."
[f] This 5.3% of trichomes in clusters on *ttg-13* represents a single cluster of two trichomes.
[g] Three of 10 seeds had a partial halo because of the presence of patches of mucilage.

In contrast, the high frequency of trichome clusters on *ttg*/+ *gl1-2*/+ plants was specific for the *gl1-2* allele. When each of the six *ttg* alleles was crossed with the *gl1-1* null allele, less than one-fourth as many trichomes were produced in clusters as were seen on *ttg*/+ *gl1-2*/+ plants (Table 6). In fact, fewer trichomes in clusters were produced on *ttg*/+ *gl1-1*/+ plants than were produced on any of the *ttg* allele heterozygote controls (Table 6).

The frequency of trichome clusters on leaves of *ttg*/+ controls (Table 6) was somewhat higher than was reported previously (Larkin *et al.* 1994a). This difference may be due to the use of a Col-0 genetic background, because in the previously reported experiments the *ttg-1* allele was in an L*er* background. *ttg-1* homozygotes produced trichomes at a higher frequency in a Col-0 background than in an L*er* background (J. Larkin, unpublished observations). This suggests that a genetic modifier present in the Col-0 background can increase the frequency of trichome initiation on leaves of *ttg-1* mutants. The L*er* background contains an *rtn* allele that results in a reduced number of trichomes relative to Col-0 (Larkin *et al.* 1996). It is possible that the *rtn* allele from Col-0 also modifies the frequency of clustering in *ttg* heterozygotes. However, we cannot rule out the possibility that this difference is due to other modifiers that differ between the two genetic backgrounds.

Allele-specific interactions between *gl1* and *ttg-10*: Further evidence of an interaction between *gl1-2* and *ttg-10* was obtained from the segregation ratio of trichome phenotypes in the self-pollinated F_2 of the double heterozygotes. As noted above, the first two leaves of *gl1-2* plants produce few, if any, trichomes. The first

TABLE 3

Trichome counts and percentage clustered trichomes on *ttg* allele *trans*-heterozygotes

	ttg-13	*ttg-1*	*ttg-12*	*ttg-11*	*ttg-9*	*ttg-10*
ttg-13	0.2 ± 0.5 0.0%	1.7 ± 1.6 0.0%	2.4 ± 1.2 0.0%	2.5 ± 1.6 4.0%	4.1 ± 2.1 4.8%	8.8 ± 3.8 14.3%
ttg-1		0.7 ± 0.8 0.0%	1.5 ± 1.8 0.0%	1.4 ± 1.5 0.0%	6.1 ± 3.0 16.4%	6.9 ± 2.6 8.0%
ttg-12			2.0 ± 1.6 0.0%	3.7 ± 2.3 10.8%	6.0 ± 2.1 13.3%	6.3 ± 2.5 5.3%
ttg-11				2.7 ± 2.0 0.0%	1.8 ± 1.3 0.0%	5.1 ± 2.2 2.6%
ttg-9					9.0 ± 2.1 14.1%	14.6 ± 1.7 23.0%
ttg-10						15.6 ± 4.1 23.6%

Data are reported as number of trichomes on the adaxial surface of first leaves (mean ± SD) and mean percentage of trichomes adjacent to another trichome.

TABLE 4

Genetic characterization of the *gl1-2* allele

Genotype	No. of trichomes[a]	% in clusters[b]
WT	37.9 ± 4.6	0.0
gl1-2	4.6 ± 3.3	0.0
gl1-1	0.0 ± 0.0	0.0
WT/*gl1-2*	35.6 ± 3.7	4.5
WS WT/*gl1-2*	42.9 ± 7.1	3.5
WT/*gl1-1*	38.3 ± 14.6	1.7
gl1-2/gl1-1	0.9 ± 1.1	0.0

[a] Number of trichomes on the adaxial surface of first leaves (mean ± SD).

[b] Mean percentage of trichomes adjacent to another trichome.

leaf phenotype of *gl1-2* will be referred to hereafter as "glabrous." Plants carrying the *ttg-10* mutation produce numerous clusters of trichomes on the first leaves. These trichomes generally are unbranched spikes or have a reduced number of branches. This distinctive phenotype will be referred to hereafter as "spiky."

The most obvious expectation for the F_2 trichome phenotypes of self-pollinated *ttg-10/+ gl1-2/+* plants is that the glabrous *gl1-2* phenotype would be epistatic to the spiky *ttg-10* phenotype. The predicted segregation ratio in this case would be 9 WT:3 spiky:4 glabrous. Unexpectedly, a significant deficit of spiky plants was noted in this cross (Table 7). The observed phenotypes were consistent, with a 9 WT:1 spiky:6 glabrous ratio (Table 7). This ratio could be explained if plants of the genotype *ttg-10/ttg-10 gl1-2/+* were also glabrous, in addition to *+/+ gl1-2/gl1-2* single mutants and *ttg-10/*

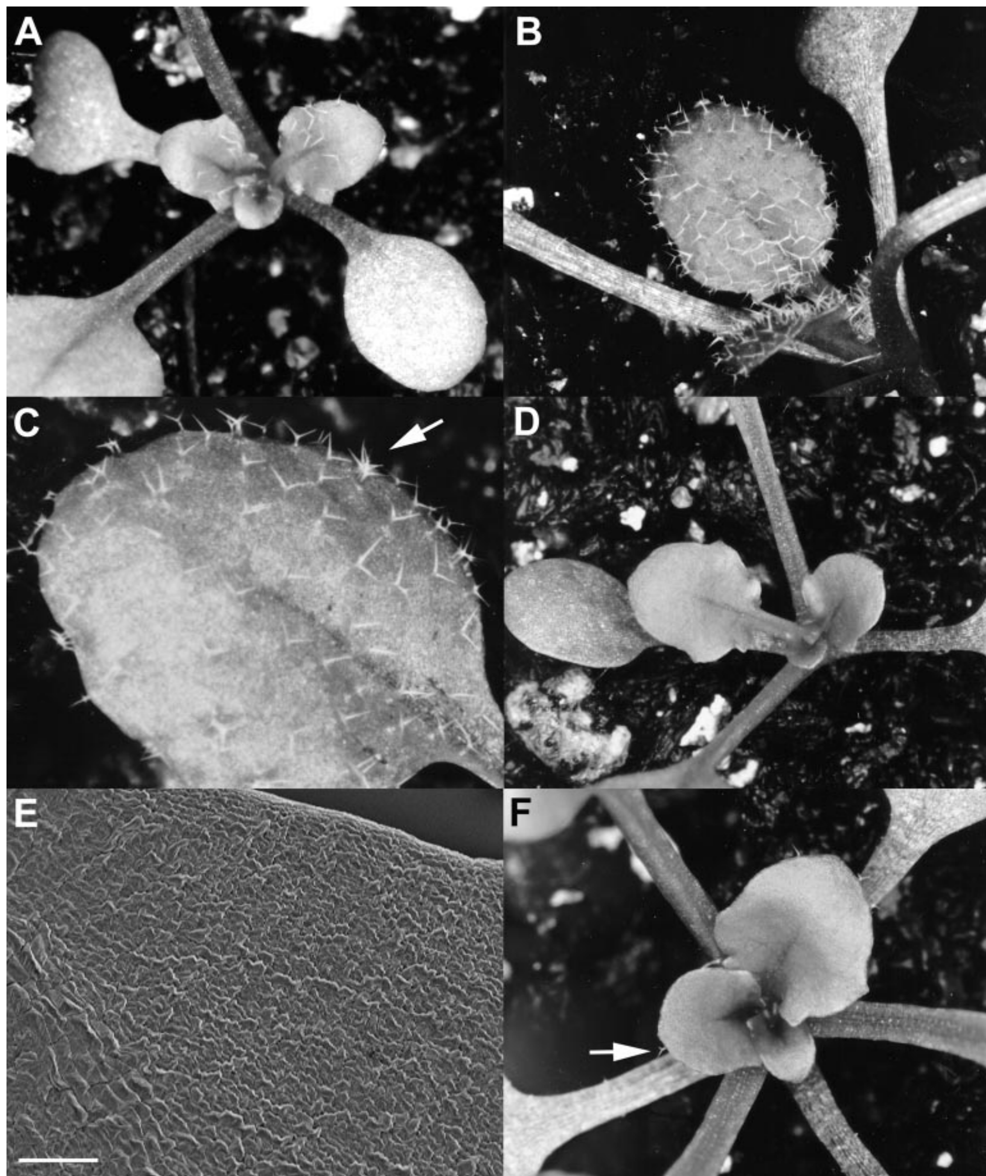

Figure 2.—Genetic interactions between *ttg-10* and *gl1-2*. (A) *gl1-2*. (B) *gl1-2/+*. (C) *ttg-10/+ gl1-2/+*. Arrow indicates a trichome cluster. (D) *ttg-10/ttg-10 gl1-2/+*. (E) Scanning electron micrograph of *ttg-10/ttg-10 gl1-2/+* leaf. Bar, 50 μm. (F) *ttg-10/+ gl1-2*. Arrow indicates a trichome.

TABLE 5

Interactions between *ttg* and *try* alleles

ttg allele	Cross for F_1	
	× WT	× *try-JC*
WT	35.3 ± 5.8 0.6%	27.4 ± 4.4 1.5%
ttg-13	NT[a]	34.6 ± 5.2 29.2%
ttg-1	34.7 ± 7.0 7.8%	32.9 ± 7.7 32.4%
ttg-10	38.2 ± 7.2 9.8%	34.5 ± 4.2 28.4%

Data reported are the number of trichomes on the adaxial surface of first leaves (mean ± SD) and the mean percentage of trichomes adjacent to another trichome. The data in Tables 5 and 6 were collected in the same experiment and are directly comparable.

[a] Not tested.

ttg-10 gl1-2/gl1-2 double mutants. In other words, the *gl1-2* allele appeared to be dominant in *ttg-10* homozygotes.

To test this hypothesis, 15 glabrous F_2 plants were allowed to self, and the resulting F_3 families were examined for trichome phenotype and anthocyanin production (Table 8). Seven of these F_2 plants produced brown seeds, indicating that these plants had been either +/+ or +/*ttg-10.* As expected in both models, all of the F_3 plants derived from these seeds were glabrous, confirming that the F_2 parent had been *gl1-2/gl1-2.* Eight of the F_2 plants produced yellow seeds, indicating that they were homozygous for *ttg-10.* Two of these families produced only glabrous plants in the F_3, suggesting that they were *ttg-10/ttg-10 gl1-2/gl1-2* double homozygotes. The *gl1-2* homozygous genotype of these two families was confirmed by PCR using primers flanking the *gl1-2* deletion. The remaining six F_2 plants that produced yellow seed segregated 1 spiky:3 glabrous in the F_3 (Table 8). This is the expected ratio if the F_2 parent is homozygous for *ttg-10* and heterozygous for *gl1-2*, and if *gl1-2* is dominant when *ttg-10* is homozygous.

To confirm that *ttg-10/ttg-10 gl1-2/+* plants were glabrous, *ttg-10* mutant plants were crossed to the *ttg-10 gl1-2* double mutant. The resulting F_1 plants were completely glabrous; no trichomes were produced even on subsequent leaves (Figure 2D). No evidence of trichome initiation was detected by scanning electron microscopy (Figure 2E). This phenotype is even more severe than that of *gl1-2* homozyotes, all of which produce at least some trichomes on the fourth and subsequent leaves (10 out of 10 plants).

Plants of the genotype *ttg-10/+ gl1-2/gl1-2* were also constructed. These plants had a phenotype stronger than that of *gl1-2* homozygotes; 4 of 10 plants had no trichomes on any of the first seven leaves, and no plant had more than two trichomes per leaf on any of the first seven leaves (Figure 2F). This result indicates that *ttg-10* has a dominant effect on trichome initiation in plants that are homozygous for *gl1-2.*

In contrast to the *gl1-2* allele, the *gl1-1* null allele is fully recessive in a *ttg-10* homozygous mutant background. The expected 9 WT:3 spiky:4 glabrous ratio was observed in the F_2 of a cross between *ttg-10* and *gl1-1* (Table 7). Analysis of F_3 families confirmed that 18 of 22 spiky F_2 plants segregated 3 spiky:1 glabrous in the

TABLE 6

Interactions between *ttg* and *gl1* alleles

ttg allele	Cross for F_1			
	× WT	× *gl1-1*	× *gl1-2*	× 35S*GL1*
WT	35.3 ± 5.8 0.6%	23.4 ± 6.0 0.0%	28.9 ± 5.7 1.0%	17.7 ± 2.5 1.1%
ttg-13	NT[a]	28.1 ± 6.0 5.7%	NT	40.1 ± 9.1 33.2%
ttg-1	34.7 ± 7.0 7.8%	30.1 ± 5.6 3.9%	35.0 ± 6.6 26.0%	43.2 ± 10.6 40.3%
ttg-12	34.3 ± 7.2 14.8%	26.9 ± 4.4 4.2%	26.4 ± 6.7 22.6%	NT
ttg-11	37.4 ± 5.3 14.2%	24.6 ± 3.8 8.1%	40.2 ± 8.3 28.3%	NT
ttg-9	37.0 ± 6.4 15.4%	29.3 ± 3.3 7.3%	39.7 ± 7.4 25.9%	36.9 ± 7.6 38.5%
ttg-10	38.2 ± 7.2 9.8%	NT	50.6 ± 12.8 28.4%	NT

Data reported as number of trichomes on the adaxial surface of first leaves (mean ± SD) and mean percentage of trichomes adjacent to another trichome.

[a] Not tested.

TABLE 7

F_2 analysis of crosses between *ttg-10* and *gl1* alleles

Cross	F_2 Phenotype			Null hypothesis	*P*
	WT	Spiky	Glabrous		
ttg-10 × +	21	8	0	3:1	>0.5
gl1-2 × +	59	0	22	3:1	>0.5
ttg-10 × *gl1-2*	157	18	85	9:3:4	<0.005[a]
				9:1:6	>0.1
ttg-10 × *gl1-1*	121	39	59	9:3:4	>0.5
				9:6:1	<0.005[a]

Trichome phenotypes were scored on the first two true leaves of seedlings from a self-pollinated F_2 of the indicated cross.

[a] Null hypothesis rejected.

F_3. The remainder of these families segregated only spiky plants in the F_3. Eleven glabrous F_2 plants produced only glabrous progeny. These results are exactly as expected if *gl1-1* is acting as a simple recessive mutation that is epistatic to *ttg-10* in these crosses.

The interaction between 35S*GL1* and *ttg* is not allele-specific: When *GL1* is expressed in a WT background from the constitutive cauliflower mosaic virus 35S promoter (35S*GL1*), the number of trichomes per leaf is reduced (Larkin *et al.* 1994a; Table 7). In contrast, plants heterozygous for *ttg-1* and carrying the 35S*GL1* construct also produce a high frequency of trichome clusters, as reported previously (Larkin *et al.* 1994a). The allele specificity of this interaction was tested in this study. A high frequency of trichome clusters was found on leaves of *ttg*/+ 35S*GL1*/+ plants for all three of the *ttg* alleles tested, including the *ttg-13* null allele (Table 7). Thus this interaction is not specific for any single *ttg* allele.

Molecular analysis of *ttg* alleles: The recent isolation of the *TTG* gene (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results) allowed the nature of the mutations present in each of the *ttg* alleles to be determined. The sequences of the *ttg-10*, *ttg-11*, and *ttg-12* alleles were determined during this study (Figure 3, A and B). All three mutations were single nucleotide substitutions. The *ttg-10* mutation was a G to A transition 43 nucleotides upstream of the start codon. This muta-

TABLE 8

F_3 analysis of the *ttg-10* × *gl1-2* cross

F_3 family	Seed phenotype of F_2 parent[a]	F_3 phenotypes		*P* (for 1:3 ratio)
		Spiky	Glabrous	
1	Brown		All glabrous	ND[b]
2	Brown		All glabrous	ND
3	Brown		All glabrous	ND
4	Brown		All glabrous	ND
5	Brown		All glabrous	ND
6	Brown		All glabrous	ND
7	Brown		All glabrous	ND
8	Yellow	13	49	>0.5
9	Yellow	10	28	>0.5
10	Yellow	17	46	>0.5
11	Yellow	15	55	>0.5
12	Yellow	5	23	>0.5
13	Yellow	13	31	>0.5
14	Yellow	0	40	<0.005[c]
15	Yellow	0	10	<0.01[c]

Trichome phenotypes were scored on the first two true leaves of seedlings from a self-pollinated F_2 of the indicated cross. For families 1–7, a minimum of 30 F_3 plants were scored.

[a] Presence (brown seeds) or absence (yellow seeds) of anthocyanins in the seed coat.

[b] Not determined.

[c] Null hypothesis rejected.

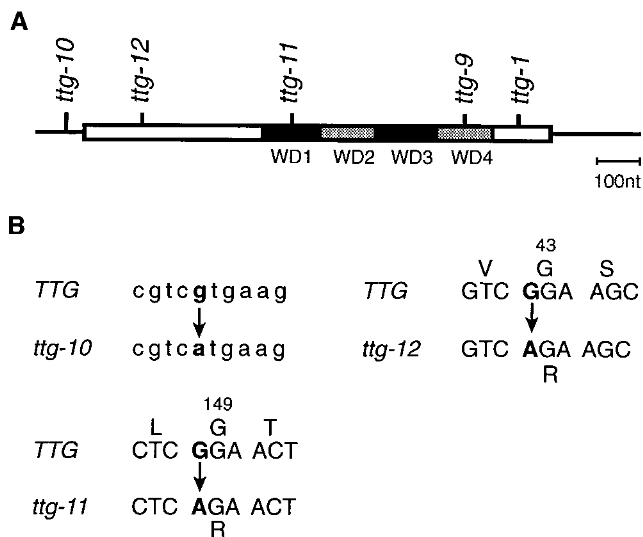

Figure 3.—DNA sequence changes in *ttg* alleles. (A) Schematic representation of the locations of five *ttg* mutations within the *TTG* mRNA. Noncoding sequences are indicated by lines, and coding sequences are indicated by boxes. WD repeats are indicated as solid or shaded boxes. The positions of *ttg-1* and *ttg-9* are from the results of A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray (unpublished results). The *ttg-13* allele is a deletion of the entire coding region (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results). (B) DNA sequence alterations in *ttg-10*, *ttg-11*, and *ttg-12.* Noncoding sequences are in lowercase, protein coding sequences are in uppercase, and the bases affected by the mutation are in boldface. For coding regions, the single-letter amino acid code is given, and the residue changed by the mutation is numbered.

tion was within the 109-bp untranslated leader and results in an out-of-frame upstream AUG codon in the *ttg* mRNA. Translation from this upstream AUG codon would produce a peptide 77 amino acids long. This new open reading frame was followed by two stop codons. The *ttg-11* mutation was a G to A transition, causing a Gly to Arg change in amino acid 149, in the first WD repeat. The *ttg-12* mutation was a G to A transition, causing a Gly to Arg change in amino acid 43, upstream of the first WD repeat. As described (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results), the *ttg-13* mutation is a deletion of greater than 4 kb encompassing the entire locus, the *ttg-1* mutation creates a stop codon 25 amino acids from the C terminus of the WT protein, and the *ttg-9* mutation causes a Ser to Phe change in the fourth WD repeat (Figure 3A). Transcript levels of the three *ttg* alleles sequenced in this study were examined by RNA blotting with a *TTG* probe (Figure 4). Steady state levels of RNA were essentially normal in the *ttg-11* and *ttg-12* alleles, while the level of mRNA was much lower in the *ttg-10* allele.

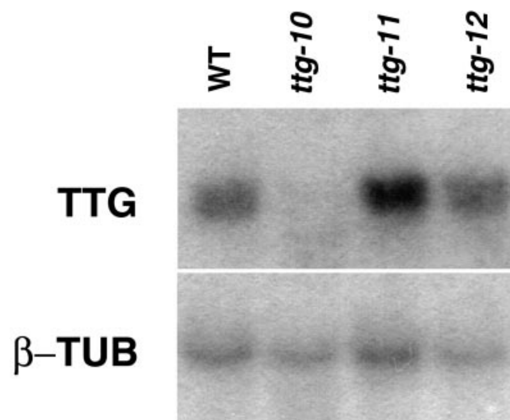

Figure 4.—Transcript levels of the *ttg-10*, *ttg-11*, and *ttg-12* homozygotes. RNA gel blots loaded with 10 μg of total RNA per lane were probed with the entire *TTG* coding region. To control for loading, the blot was stripped and probed with a β-tubulin cDNA.

DISCUSSION

TTG encodes a member of a novel subfamily of WD-repeat proteins that is widely conserved among eukaryotes, including humans (A. R. Walker, P. A. Davison, A. C. Bolognesi-Winfield, C. M. James, N. Srinivasan, T. L. Blundell, J. J. Esch, M. D. Marks and J. C. Gray, unpublished results). The only other member of this subfamily with a genetically identified function is the *AN11* gene of petunia (de Vetten *et al.* 1997). The Arabidopsis *TTG* gene acts as an important regulator in several diverse pathways, and understanding the role that *TTG* plays in these pathways should clarify our understanding of the role of related proteins in other organisms. However, the molecular basis of the pleiotropy of *ttg* mutations remains unclear. One possibility is that *TTG* encodes a protein with multiple independent functional domains. In this case, mutations affecting each function might be expected to cluster in different regions of the gene, as has been seen in other studies (Brennan *et al.* 1997). Alternatively, *TTG* may encode a protein with a single function that is needed by several pathways.

This study reports the genetic characterization of six *ttg* loss-of-function (LOF) alleles. The alleles include a null allele (*ttg-13*) and a weak allele (*ttg-10*) that reduces the *ttg* mRNA level without altering the protein sequence. Of the four mutations that would produce an altered protein, *ttg-12* was a missense mutation upstream of the first WD repeat, *ttg-11* was a missense mutation in the first WD repeat, *ttg-9* was a missense mutation in the fourth WD repeat, and *ttg-1* resulted in a C-terminal truncation of the protein downstream of the last WD repeat (Figure 3). Despite this diversity, all of these mutations affected all aspects of the *ttg* phenotype that were associated with the *ttg-13* null allele. Intercrosses among the alleles demonstrate that these six alleles form a simple allelic series of hypomorphs, with the strongest

alleles always severely affecting all aspects of the phenotype and the weakest alleles having moderate effects on all aspects of the phenotype. These results indicate that *TTG* probably does not encode a protein with independently mutable domains that are specific for subsets of the pathways in which it is involved.

The molecular basis of the *ttg-10* allele is particularly significant. Nonsense codons that interfere with translation of a message, including nonsense codons resulting from an introduced upstream reading frame, often result in reduced mRNA levels due to a decrease in mRNA stability (Jacobson and Peltz 1996). Nonsense-mediated mRNA decay also occurs in plants (van Hoof and Green 1996). Although the AUG codon created in the RNA by this mutation is a poor match to the consensus sequence for known plant start codons (Joshi *et al.* 1997), it seems likely that the reduction in mRNA levels seen in Figure 4 is due at least in part to nonsense-mediated mRNA decay. We cannot at present rule out the possibility that the reduced mRNA level is the result of a defect in transcription caused either by the mutation that we have identified or by another mutation located outside of the region that we have sequenced. Nevertheless, because the sequence of the *TTG* open reading frame is unaltered by the *ttg-10* mutation (Figure 3), it is clear that the phenotype of this allele is ultimately caused by a reduction in the level of TTG protein, not by an altered protein product.

Previous work had suggested that *TTG* and *GL1* act at the same stage in the initiation of trichome development (Larkin *et al.* 1994a). However, this work involved the heterologous *R* gene from maize. Additionally, both *R* and the Arabidopsis *GL1* gene were expressed from the strong constitutive 35S promoter from cauliflower mosaic virus. Constitutive overexpression of transcription factors can result in neomorphic phenotypes that may be hard to interpret. Additional evidence based on LOF mutations bearing on the relationship between *TTG* and *GL1* during trichome development would clearly be of value.

Several types of genetic interaction often indicate that two genes function in the same pathway (Huffaker *et al.* 1987). These include intergenic noncomplementation by mutations in different genes and synthetic phenotypes observed when a double mutant exhibits a phenotype not seen with either individual mutant. Such genetic interactions often occur between genes whose products interact (Hays *et al.* 1989; Akada *et al.* 1996). We have identified both of these types of interaction in combinations of LOF alleles of *ttg* and *gl1.* The increased trichome clustering in *ttg/+ gl1-2/+* double heterozygotes is an example of intergenic noncomplementation (Figure 2C; Table 7). In addition, *ttg-10 gl1-2* double mutant homozygotes have no trichomes, a synthetic phenotype that is more severe than the phenotype of either single mutant. Neither of these interactions is observed in crosses with the *gl1-1* null allele, demonstrating that both interactions are allele specific with respect to *gl1.* The noncomplementation interaction is not allele specific with respect to *ttg* (Table 7). This lack of allele specificity is not surprising, given that *ttg-10* most likely produces a WT protein, albeit at reduced levels. Similar synthetic phenotypes in double mutants and intergenic noncomplementation phenotypes have been seen in *clavata1* and *clavata3* mutants in Arabidopsis, although no allele specificity was observed in these interactions (Clark *et al.* 1995). The *clavata* genes have been proposed to function close together in the same pathway, and it is possible that their products interact.

Another aspect of the interaction between *ttg* and *gl1* shows a similar specificity for the *gl1-2* allele. Plants of the genotype *ttg-10/ttg-10 gl1-2/+* produce no trichomes (Figure 2, D and E), even though *ttg-10* plants have a readily detectable spiky phenotype and *gl1-2* by itself is fully recessive. The *gl1-1* null allele, which abrogates making of a protein product, is fully recessive in *ttg-10* homozygous plants. The dominance of *gl1-2* in *ttg-10* homozygotes must be due to the product of the *gl1-2* allele interfering with the function of the $GL1^+$ allele. This only occurs when the level of TTG product is limiting, however, suggesting that the GL1-2 protein product is competing with WT GL1 protein for access to some TTG-dependent process.

As noted above, the *gl1-2* mutation results in a protein with a C-terminal truncation removing 27 amino acids. The Myb DNA-binding domain of GL1 is located in the N-terminal region, and it was hypothesized that the region deleted in *gl1-2* was part of a C-terminal transcription activation domain (Esch *et al.* 1994). Consistent with this possibility, the C-terminal portion of GL1 can activate transcription in yeast (A. Lloyd, personal communication). In some instances, known transcription factors or proteins resembling transcription factors can act as dominant-negative repressors if they lack a transcription activation domain (Keegan *et al.* 1986; Benezra *et al.* 1990; Rieping *et al.* 1994). The recently discovered *CAPRICE* gene of Arabidopsis is particularly relevant (Wada *et al.* 1997). This gene encodes a protein containing a single MYB repeat related to GL1. There is no apparent transcription activation domain. Mutations in *caprice* reduce the number of root hairs. When *CAPRICE* is expressed constitutively, excess root hairs are present and trichomes are absent on the leaves. The root hair developmental pathway is also a *TTG*-dependent pathway in which *TTG* acts to suppress root hair development. It is possible that *CAPRICE* and *gl1-2*, both of which encode MYB-related proteins that lack an activation domain, interfere with trichome development by a similar mechanism.

Recent work on the regulation of anthocyanin pigment biosynthesis in petunia also provides some insight into the mechanisms that may underlie these interactions. In petunia, anthocyanin biosynthesis is regulated by the *TTG* homologue *an11*, the *GL1* homologue *an2*,

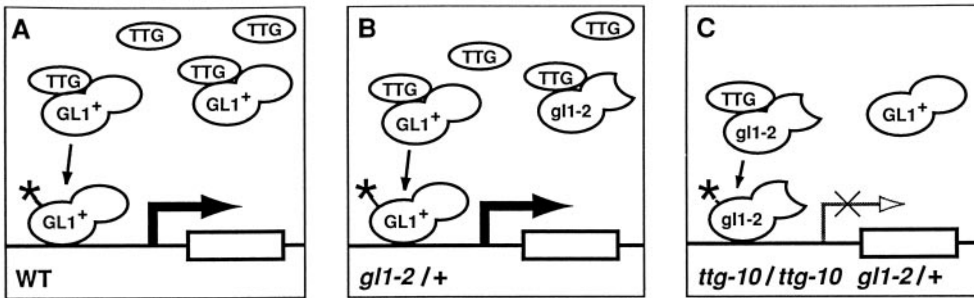

Figure 5.—Model of *TTG*-*GL1* interactions. (A) Wild type. TTG is present in excess. TTG activates $GL1^+$ in the cytoplasm, and the activated $GL1^+$ enters the nucleus and stimulates transcription of downstream genes. Activation is indicated by *. (B) *gl1-2/*+. TTG is present in excess. TTG activates both the $GL1^+$ and gl1-2 proteins, but sufficient $GL1^+$ is activated to promote downstream transcription. (C) *ttg-10/ttg-10 gl1-2/*+. TTG protein is limiting. $GL1^+$ and gl1-2 compete for activation by TTG, and insufficient $GL1^+$ is activated to promote downstream transcription. Additional competition could occur at downstream promoters.

and the *R*-related bHLH genes *an1* and *jaf13* (de Vetten *et al.* 1997; Quattrocchio *et al.* 1998). The regulation of Arabidopsis trichome development may thus closely resemble the regulation of petunia anthocyanin biosynthesis, with a WD-repeat protein, a MYB protein, and an as yet unidentified R-like bHLH protein. The AN11 protein appears to be located in the cytoplasm and may play a role in activating the AN2 MYB transcription factor (de Vetten *et al.* 1997).

A model consistent with our results is illustrated in Figure 5. *TTG* is assumed to encode a cytoplasmic protein that activates the GL1 protein outside of the nucleus, which then enters the nucleus and activates transcription of genes downstream in the trichome development pathway (Figure 5A). In plants heterozygous for the *gl1-2* mutant allele, there is an excess of TTG, and sufficient $GL1^+$ is activated to induce transcription of downstream genes (Figure 5B). In *ttg-10/ttg-10 gl1-2/*+ plants, the level of functional TTG protein produced is limiting, and $GL1^+$ and gl1-2 proteins compete for activation by TTG, resulting in reduced transcription of downstream genes. Although a direct interaction between TTG and GL1 is the simplest explanation, we cannot rule out the possibility that GL1 interacts with some factor downstream of TTG in the pathway. The increase in trichome clustering seen in *ttg-10/*+ *gl1-2/*+ plants can be explained if the lateral inhibition pathway is more sensitive than the trichome initiation pathway to reductions in TTG levels.

In Figure 5, TTG is shown activating GL1 alone for simplicity. However, it is likely that GL1 functions together with an R-like protein and that these proteins could be activated as a heterodimer. In support of this hypothesis, Szymanski *et al.* (1998) have shown that GL1 physically interacts with the maize R protein in *in vitro* translation reactions and that *R*-like genes have been identified in Arabidopsis (A. R. Walker, unpublished results; A. Lloyd, personal communication). The proposed activation of GL1 could occur through a variety of different mechanisms, including post-translational modifications such as phosphorylation, regulation of transport of GL1 into the nucleus, or assembly of GL1 into a complex with other proteins such as an R homologue. Because WD repeat proteins never have been found to act as enzymes, but are often involved in protein-protein interactions, TTG may act on GL1 in conjunction with other proteins. In previous work, the inability of constitutively-expressed *GL1* to bypass a *ttg* mutation was interpreted as evidence that *GL1* acts either at the same step or in parallel to *TTG.* The current model, placing *GL1* downstream of *TTG* but dependent on *TTG* for activation, is consistent with the previous data (Larkin *et al.* 1994a). The ability of *R* to bypass the requirement for *TTG* may be due to the use of a heterologous gene or due to the high level of expression from the 35S promoter used in these experiments. These issues cannot be resolved without the analysis of Arabidopsis *R* homologues that clearly function in trichome development.

The phenotype of 35S*GL1* plants remains difficult to explain. As described previously (Larkin *et al.* 1994a), WT plants carrying a 35S*GL1* transgene have a reduced number of trichomes (Table 6). One model proposed to explain this observation is that excess GL1 titrates some essential factor via a "squelching" process (Larkin *et al.* 1994a). Alternatively, excess GL1 that is not activated by TTG may act as a repressor. It is difficult, however, to reconcile these hypotheses with the phenotype of *ttg/*+ 35S*GL1* plants, which have an increased number of trichomes and a high frequency of trichome clusters (Larkin *et al.* 1994a; and Table 6). The observations presented here, demonstrating that *ttg/*+ *try/*+ double heterozygotes show increased trichome clustering, suggest that *TRY* acts at a point very close to *TTG* in the trichome differentiation pathway. One possible explanation is that *TRY* is under the direct control of *TTG. TRY* has been proposed to be an antagonist of *GL1* function (Schnittger *et al.* 1998). Perhaps TTG activates both GL1, a positive regulator of trichome development, and TRY, an inhibitor of trichome development. Such an arrangement could result in the extreme sensitivity to dosage of *TTG* that has been observed. It is also worth noting that dosage sensitivity can sometimes be a hallmark of direct protein-protein interactions (Meeks-Wagner and Hartwell 1986;

Burke *et al.* 1989), as we have proposed in this study for TTG and GL1.

The pathway for the differentiation of the trichome cell type has revealed a great deal of complexity. The present work suggests some hypotheses about biochemical interactions between components of the trichome development pathway. The recent isolation of *TTG* will allow these hypotheses to be tested in the near future. However, a complete understanding of the mechanisms behind this cell fate decision will require a detailed analysis of additional genes, particularly *TRY* and the hypothesized *R*-like genes.

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