GL3 Encodes a bHLH Protein That Regulates Trichome Development in Arabidopsis Through Interaction With GL1 and TTG1

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ABSTRACT

Arabidopsis trichome development and differentiation is a well-studied model for plant cell-fate determination and morphogenesis. Mutations in *TRANSPARENT TESTA GLABRA1* (*TTG1*) result in several pleiotropic defects including an almost complete lack of trichomes. The complex phenotype caused by *ttg1* mutations is suppressed by ectopic expression of the maize anthocyanin regulator R. Here it is demonstrated that the Arabidopsis trichome development locus *GLABRA3* (*GL3*) encodes an R homolog. GL3 and GLABRA1 (GL1) interact when overexpressed together in plants. Yeast two-hybrid assays indicate that GL3 participates in physical interactions with GL1, TTG1, and itself, but that GL1 and TTG1 do not interact. These data suggest a reiterated combinatorial model for the differential regulation of such diverse developmental pathways as trichome cell-fate determination, root hair spacing, and anthocyanin secondary metabolism.

ARABIDOPSIS trichome cell-fate determination is positively regulated by at least three regulatory genes, *TTG1* (Koornneef 1981), *GL1* (Herman and Marks 1989), and *GL3* (Koornneef *et al.* 1982). It has been known for several years that the epidermal-specific defects caused by the *ttg1* mutation of Arabidopsis can be suppressed by overexpression of the maize basic helix-loop-helix (bHLH) protein R (Lloyd *et al.* 1992). In plants homozygous for the strong allele, *ttg1-1* (Larkin *et al.* 1999), these epidermis-specific defects include absence of trichomes, anthocyanin pigments, and seed coat mucilage (Koornneef 1981) and position-independent spacing of root hairs (Galway *et al.* 1994). It has been shown that R interacts with the MYB-class transcription factors C1 (Cone *et al.* 1986) and GL1 (Oppenheimer *et al.* 1991) when co-overexpressed in Arabidopsis (Lloyd *et al.* 1992; Larkin *et al.* 1994) and that R and GL1 interact when both proteins are expressed *in vitro* (Szymanski *et al.* 1998). R also interacts with PAP1, an Arabidopsis R2R3 MYB-like anthocyanin regulator (J. Borevitz, R. Dixon, C. Lamb and Y. Xia, personal communication) in plants and yeast two-hybrid assays (C. T. Payne and A. M. Lloyd, data not shown). Extensive work in maize has shown that the MYB-like anthocyanin regulators C1 and PL must interact with R or its homolog B to activate the pigment pathway (Cone *et al.* 1986, 1993; Paz-Ares *et al.* 1987; Ludwig *et al.* 1989; Goff *et al.* 1992). The observations that the *ttg1-1* mutation was suppressed by R and that this bHLH transcription factor could interact with endogenous Arabidopsis MYB-like proteins offered compelling but indirect evidence that in Arabidopsis one or more R homologs performed regulatory functions in TTG1-dependent developmental pathways. Until now, no *bona fide* Arabidopsis R homolog involved in the regulation of any of these pathways has been defined.

TTG1 was reported to be a WD repeat-containing protein [conserved repeating units usually ending in Trp-Asp (Neer *et al.* 1994)] without MYC homology (Walker *et al.* 1999), indicating that either the R overexpression results were artifactual or that one or more R homologs were downstream of TTG1 and had yet to be identified. Experiments in *Petunia hybrida* indicated that floral anthocyanin production required a WD repeat-containing protein, AN11 (de Vetten *et al.* 1997), and the MYB- and MYC-like transcription factors AN2 and JAF13, respectively (Quattrocchio *et al.* 1998). All three classes of regulators are probably required for anthocyanin production in other flowering plants as well. In fact, at least one R homolog is crucial to the regulation of anthocyanin production in every flowering plant where the pathway has been genetically dissected, with the exception of Arabidopsis. It was reasoned that at the very least Arabidopsis must also possess an R homolog regulating anthocyanin synthesis. Furthermore, because the WD-40 repeat protein TTG1 and another MYB-like transcription factor, GL1, were required for trichome differentiation, it seemed likely that an R homolog also regulated that pathway.

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Two Arabidopsis R-homologous genes have been described, *ATMYC1* (Urao *et al.* 1996) and *RAP1* or *RD22BP1* (Abe *et al.* 1997; de Pater *et al.* 1997). *ATMYC1* was cloned by homology to *DELILA*, an *Antirrhinum majus* bHLH anthocyanin regulatory gene (Goodrich *et al.* 1992). The function of ATMYC1 is unknown, but overexpression experiments conducted in our lab indicate that it neither suppresses the *ttg1-1* mutation nor upregulates any of the TTG1-dependent pathways in a wild-type background (F. Zhang and A. M. Lloyd, unpublished results). No increase in anthocyanin synthesis was observed when ATMYC1 was overexpressed in tobacco (C. T. Payne and A. M. Lloyd, unpublished results). *RAP1/RD22BP1* was independently cloned by two groups on the basis of its ability to bind a MYC recognition site. It is induced by dehydration and abscisic acid and may interact with ATMYB2 to activate transcription of dehydration response genes. Neither of these genes maps to positions of known trichome or anthocyanin mutations.

An Arabidopsis expressed sequence tag (EST), 146D23T7, which was highly similar to *R* and *DELILA*, was identified by the Arabidopsis genome effort. This EST was used to screen a phage library of Arabidopsis genomic DNA, which resulted in the cloning of a fragment of a second bHLH gene also highly similar to *R* and *DELILA* (F. Zhang and A. M. Lloyd, unpublished results) and present in the database as MYC6. Although other Arabidopsis bHLH sequences are also present in the database, the extensive homology between these two genes and the known anthocyanin regulatory loci from maize and Antirrhinum caused us to focus efforts on 146D23T7 and MYC6, the latter identified here as GL3.

In contrast to the *ttg1-1* and *gl1-1* mutants, the *gl3-1* mutant is not devoid of trichomes (Koornneef *et al.* 1982); *gl3-1* plants produce fewer trichomes and these are not fully developed (Figure 1, A and B; Figure 4). The nuclei of *gl3-1* trichomes typically undergo a total of three rather than the normal four rounds of endoreduplication, the final cell size is decreased, and these parameters appear to be correlated with the reduced branching observed in this mutant (Hülskamp *et al.* 1994).

Here we describe experiments that identify the trichome development regulatory factor encoded by the *GL3* gene as a bHLH protein with significant homology to R. It is furthermore demonstrated that the GL3 protein can interact synergistically with GL1 to promote trichome development in plants. Finally, complementary two-hybrid data showing interactions in yeast between GL3 and other known regulators of trichome development are presented and a regulatory model supported by these data is described. In this model, TTG1 regulates the several distinct developmental pathways defined by *ttg1* mutations by influencing the activity of one or more bHLH factors including GL3.

MATERIALS AND METHODS

Plant growth conditions: *Arabidopsis thaliana* was grown in Premier ProMix BX (Hummerts, St. Louis) at 22° under continuous fluorescent illumination. Plants were fertilized weekly with a dilute solution of Peters 20-20-20 all-purpose plant food.

Arabidopsis strains: The *gl3-1*, *gl3-2*, and *ttg1-1* mutations are in the Landsberg *erecta* (L*er*) ecotype. With the exception of *gl3-2*, all strains are from the Ohio State Arabidopsis Biological Resource Center. The *gl3-2* mutant was a gift of Martin Hülskamp and is an EMS-induced mutation in the L*er* background.

Sequencing of *GL3* mutant alleles: PCR products spanning the entire *gl3-1* allele were sequenced in both directions and the mutation in codon 378 was identified. To verify the sequence, sequences of PCR products spanning the mutation were generated from three independent amplifications, each from a separate DNA preparation derived from different plants. Wild-type L*er* sequences, spanning the region containing the *gl3-1* mutation, from two independently generated PCR products, each from different template DNA preparations, agreed in both cases with the GenBank sequence for the MYC6 R homolog. PCR products from the *gl3-2* mutation were sequenced until the mutation at codon 46 was identified. This region was sequenced in both directions from PCR products from two independent DNA preps from different plants to verify the sequence. It is possible that *gl3-2* contains additional mutations later in the coding region.

Two-hybrid β-galactosidase lift assays: All two-hybrid analysis was done in the yeast strain Y190 (Durfee *et al.* 1993). β-Galactosidase assays were performed according to a protocol provided with the Matchmaker system (CLONTECH, Palo Alto, CA) using X-gal as a substrate. Observations of β-galactosidase activity (visual inspection of filter for development of the blue pigment) were made at 15-min intervals during the first hour of incubation at 30°, and then at hourly intervals up to 10 hr. Activation was arbitrarily designated strong (distinctly blue within the first hour) or moderate (faintly blue within the first 3 hr and distinctly blue at 10 hr). All results were reproducible.

Plasmid constructs: *pD2LX, GL3 genomic complementation plasmid:* The genomic *GL3* coding sequence plus ~1 kb of sequence 5′ and 3′ of the gene was amplified by PCR from the P1 clone MYC6 (AB006707), using the primers 5D25 (5′-GGGGGATCCGATCACTCAAATAGTAATAAGACTG-3′) and 3D23 (5′-GGGGGATCCCTGATCGCACACTTTGTGTG-3′) and the product cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) as a *Bam*HI fragment to create pD2L-2. The pD2L-2 insert was subcloned as a *Bam*HI fragment into the *Bgl*II site of the T-DNA vector pAL47 (Lloyd and Davis 1994) to create pD2LX.

pEZF1, GL3 antisense plasmid: A partial clone of *GL3* was isolated by homology to *146D23T7* from an Arabidopsis genomic library. A *Sal*I fragment of this was subcloned into pBluescript II KS+ to create pZF1. The primers M13R (5′-AGCGGATAACAATTTCACACAGGA-3′) and Fun102/SalI (5′-CGCGCGTCGACCAACTAAGTAGTCTTCAACAGATCCA-3′) were used to amplify a 1.8-kb PCR product from this template and it was cloned as an *Eco*RI-*Sal*I fragment into pLBJ22 digested with *Eco*RI and *Xho*I in the antisense orientation. pLBJ22 is a derivative of pKYLX71 created as follows: The preexisting *Eco*RI site of pKYLX71 (Schardl *et al.* 1987) was filled and reclosed to create the plasmid pLBJ17. The self-complementary oligo, 5′-CGGAATTCCG-3′, was ligated into the filled, unique *Xba*I site of pLBJ17. This replaces the unique *Xba*I site with a unique *Eco*RI site in the CaMV 35S expression cassette of pKYLX71.

pD22, GL3 plant overexpression plasmid: The P1 clone MYC6 was used as a template for PCR amplification of the GL3 gene.

The oligonucleotides Ros102/EcoRI (5′-GGGGGAATTCGC CATGGCTACCGGACAAAACAGAACAAC-3′) and Fun102/ SalI (5′-CGCGCGTCGACCAACTAAGTAGTCTTCAACAGA TCCA-3′) were used as primers, introducing *Eco*RI and *Sal*I sites, respectively. This product was cloned into the corresponding sites of pBSKS+ to create pDRAT2. The insert in pDRAT2 was sequenced and subcloned into pLBJ21 digested with *Eco*RI and *Xho*I as an *Eco*RI-*Sal*I fragment. Note that *GL3* contains an internal *Sal*I site. pLBJ21 is a derivative of pKYLX71 created as follows: The self-complementary oligo, 5′-CGGAATTCCG-3′, was ligated into the filled, unique *Hin*dIII site of pLBJ17. This replaces the unique *Hin*dIII with a unique *Eco*RI site in the CaMV 35S expression cassette of pKYLX71.

pGL3A and -B, GL3 full-length two-hybrid constructs: The *GL3* cDNA was amplified by reverse transcriptase (RT)-PCR from total RNA prepared from an Arabidopsis line overexpressing GL3 from the pD22 construct, using the primers DRA25 (5′-GGGAGCTCGAATTCGCCATGGCTACCGGACAAAAC AGA-3′)and DRA23 (5′-G GTCTAGAGGATCCTCAACAGAT CCATGCAACCC-3′). This product was cloned into pBluescript II KS+ as a *Sac*I-*Xba*I fragment to create pD2CD-7. After sequencing, the pD2CD-7 insert was subcloned as an *Eco*RI-*Bam*HI fragment into the corresponding sites of pGAD424 and pAS2-1 (both from CLONTECH) to create pGL3A and -B, respectively.

pGL3NTA and -B, GL3 400 amino acid, amino end, two-hybrid plasmids: A 5′ fragment of the *GL3* cDNA encoding the first 400 amino acids of the protein was amplified from the pD2CD-7 template using the DRA25 (see pGL3A and -B) and D2NT (5′-GGGGYCGACTCATCCTGATGATGATGACGATGAT-3′) primers and cloned into pBluescript II KS+ as a *Sac*I-*Sal*I fragment to create pD2NT. After sequencing, this insert was subcloned into pGAD424 or pAS2-1 as an *Eco*RI-*Sal*I fragment to create pGL3NTA and -B, respectively.

pGL396A and -B, GL3 96 amino acid, amino truncation, two-hybrid plasmids: The fragment encoding the carboxy-terminal 541 amino acids of GL3 was amplified from pD2CD-7 using the primers D2096 (5′-GGGTCGACCCATGGAATTCGCCG GATCTCAAGTCACC-3′) and DRA23 and cloned as a *Sal*I-*Xba*I fragment into pBluescript II KS− to create pD296. After sequencing, this insert was subcloned into pGAD424 and pAS2-1 as an *Eco*RI-*Bam*HI fragment to create pGL396A and -B, respectively.

pGL3211A and -B, GL3 211 amino acid, amino truncation, two-hybrid plasmids: The fragment encoding the carboxy-terminal 426 amino acids of GL3 was amplified from pD2CD-7 using the primers D2211 (5′-GGGTCGACCCATGGAATTCCCGTA CGCTACAATATTACC-3′) and DRA23 and cloned as a *Sal*I-*Xba*I fragment into pBluescript II KS− to create pD2211. After sequencing, this insert was subcloned into pGAD424 and pAS2-1 as an *Eco*RI-*Bam*HI fragment to create pGL3211A and -B, respectively.

pGL3CTA and -B, GL3 400 amino acid, amino terminal truncation, two-hybrid plasmids: A 3′ fragment of *GL3* encoding the carboxy-terminal 237 amino acids of the protein was amplified by PCR from pD2CD-7 using the primers D2CT (5′-GGG TCGACCCATGGAATTCACCGCCACGGTCACGGC-3′) and DRA23 and cloned into pBluescript II KS+ as a *Sal*I-*Xba*I fragment to create pD2CT. After sequencing, the insert was subcloned into pGAD424 or pAS2-1 as an *Eco*RI-*Sal*I fragment.

pGL1A and -B, GL1 full-length two-hybrid plasmids: RT-PCR was employed to amplify a fragment encoding the entire 216 amino acid protein of GL1 from ecotype Col-O using the primers GL1A (5′-GGGGGGGGGAATTCATGAGAATAAGGA GAAGAGATG-3′) and GL1C (5′-GGGGGGGGCTGCAGATT AAACTAAAGGCAGTATC-3′). The product was cloned into pBluescript II SK− as an *Eco*RI-*Pst*I fragment to create pSRV2 and sequenced. This insert was subsequently cloned into pGAD424 and pGBT9 (CLONTECH) as an *Eco*RI-*Pst*I fragment to create pGL1A and -B, respectively.

pGL1NTA and -B, GL1 myb domain two-hybrid plasmids: RT-PCR was employed to amplify the 5′ fragment encoding the 121 amino-terminal residues of GL1 from ecotype Col-O using the primers GL1A (5′-GGGGGGGGGAATTCATGAGAATAA GGAGAAGAGATG-3′) and GL1B (5′-GGGGGGGGCTGCAG TTAATCTCCGACGAGTTTTTTGCTG-3′). The product was first cloned into pBluescript II SK− as an *Eco*RI-*Pst*I fragment to create pSRV1 and sequenced. This insert was subsequently cloned into pGAD424 and pGBT9 (CLONTECH) as an *Eco*RI-*Pst*I fragment to create pGL1NTA and -B, respectively.

pTTG1A and -B, TTG1 full-length two-hybrid plasmids: Full-length *TTG1* was amplified from genomic Wassilewskija (WS) wild-type DNA (the coding region contains no introns) using the primers WD40X5 (5′-GGGAATTTCGCCATGGATAATT CAGCTCCAGATTC-3′) and WD40X3 (5′-GGTCTAGACTC GACTCATTAGAATCTAGGCCTAGCAA-3′) and cloned as an *Eco*RI-*Sal*I fragment into pBluescript II KS+ to create pWS10. After sequencing, the pWS10 insert was subcloned into pGAD424 and pAS2-1 as an *Eco*RI-*Sal*I fragment to create pTTG1A and -B, respectively.

pTTG1MA and -B, TTG1 25 amino acid, carboxy-terminal truncation, two-hybrid plasmids: A truncated version of *TTG1* was amplified from pWS10 using the primers WD40X5 and TTG1M1 (5′-GGTCTAGAGTCGACCAACTAATTAATCTCCGAACC AGC-3′) and cloned into pBluescript II KS− as an *Eco*RI-*Xba*I fragment to create pMTTG. After sequencing, the insert was subcloned into pGAD424 and pAS2-1 as an *Eco*RI-*Sal*I fragment to create pTTG1MA and -B, respectively.

Plant transformation: Binary constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 containing pMP90 (Koncz and Schell 1986) by electroporation. Arabidopsis was transformed by vacuum infiltration essentially as described in Bechtold *et al.* (1993).

Trichome counts: Trichome numbers were counted for each of the first four fully expanded leaves on soil-grown seedlings using an Olympus dissecting scope. Leaves were numbered in order of appearance except the first two leaves, which emerge at the same time and are considered equivalent. The trichome branching phenotype in Table 1 refers to the number of processes rather than branching events; thus 1 is an unbranched spike, 2 is two-branched, etc.

Scanning electron microscopy: Plant materials were fixed overnight in 2% glutaraldehyde and 0.1 M cacodylate and then taken through an alcohol dehydration series, once in 35, 50, 65, 75, 85, and 95% ethanol, and twice in 100% ethanol, for at least 2 hr per step. Specimens were critical-point dried in a Tousimis Samdri-790 and sputter coated with a gold-palladium alloy using a Ladd instrument. Specimens were visualized with a Phillips 515 scanning electron microscope and photographed with Polaroid film.

RT-PCR experiments: Two-week-old Arabidopsis seedlings grown on germination medium, 1 × Murashige and Skoog salts (Sigma), 10 g/liter sucrose, 1 × Gamborg's B5 vitamins, and 8 g/liter tissue culture agar (Carolina Biological), under continuous fluorescent light at 22°, were frozen in liquid nitrogen. Total RNA was prepared using Trizol reagent (GIBCO BRL, Gaithersburg, MD) and the procedural modifications recommended by the manufacturer for plant tissue. RNA was quantified spectrophotometrically. A total of 2 μg of each sample was treated with amplification grade DNase (GIBCO BRL) as per the manufacturer's instructions. Oligo(dT) was used to prime reverse transcription by Superscript II (GIBCO BRL) from 1 μg DNase-treated RNA template. Parallel PCR reactions using each reverse transcription reaction (5 of 20 μl) as template were set up as follows in a total volume of 100

μl: 10 μl 10 × PCR buffer, 3 μl 50 mM $MgCl_2$, 5 μl 10 μM 5′ primer, 5 μl 10 μM 3′ primer, and 1 μl (= 5 units) Taq polymerase (GIBCO BRL). Control primers, APTC5 (5′-GAAATGGCGACTGAAGATGTG-3′) and APTC3 (5′-CTCC TTTCCCTTAAGCTCTG-3′), amplify a fragment of the Arabidopsis adenine phosphoribosyl transferase (APRT) message. APRT has been shown to be constitutively expressed at low levels in all cells (Moffatt *et al.* 1994) and was previously used as a control by Cowling *et al.* (1998) in quantitative PCR experiments. APRT-PCR reactions were subjected to 25 rounds of amplification. A fragment of the GL3 cDNA was amplified using the primers GL3RT5 (5′-ATGGCTACCGGAC AAAACAG-3′) and GL3RT3 (5′-CCTTCACCAATGTTGAAG ACG-3′), which are homologous to sequences from the first and second coding exons of the gene, respectively. The GL3 reactions were subjected to 45 rounds of amplification. Cycling parameters for both reactions were as follows: 1 min at 95°, 30 sec at 55°, and 1 min at 72°. A total of 10 μl samples of each reaction for a given first-strand template preparation were loaded together on a 1.4% agarose gel for electrophoresis.

RESULTS

The bHLH gene contained on MYC6 is *GLABRA 3*: The MYC6 P1 clone (genomic sequence AB006707) contains an *R*-homologous gene and is part of a contig that maps 16–18 Mb from the top of chromosome 5 near restriction fragment length polymorphism marker m423. The putative protein encoded by this clone contains 637 amino acids and is 52% homologous throughout its length to R from maize, a monocot. It is 55% homologous to DELILA (Goodrich *et al.* 1992), an R-homologous anthocyanin regulator from Antirrhinum, a dicot like Arabidopsis. The *GL3* locus was reported to map to the same general area of chromosome 5 as MYC6 (Koornneef *et al.* 1983; Larkin *et al.* 1998). It was reasoned that the *GL3* locus might be the *R*-like gene on MYC6, and PCR-generated fragments were used to sequence the MYC6 *R* homolog contained in wild-type and *gl3-1* mutant plants. A mutation was identified in *gl3-1* that converts codon 378, CAG, to a stop codon, TAG, which would delete the entire bHLH region of the encoded protein. A mutation identified in *gl3-2* converts codon 46, TGG, to a stop codon, TGA, which would delete ~93% of the encoded protein.

To verify that the mutation in the MYC6 homolog was responsible for the *gl3-1* mutant phenotype, the mutant was complemented by transformation with a MYC6 genomic fragment containing the *R*-homologous gene under the control of its native regulatory elements. A PCR fragment was generated that contained the entire coding region and ~1 kb of flanking genomic DNA both upstream and downstream of the putative start and stop codons. This fragment was subcloned into an *A. tumefaciens* binary vector to create pD2LX. The *gl3-1* allele is in the Landsberg *erecta* (L*er*) ecotype, which has fewer trichomes than most "wild-type" strains (Larkin *et al.* 1996). Figure 1, A–C, shows untransformed L*er* and *gl3-1* plants as well as the complemented mutant. In some cases the complemented mutants had more than wild-type trichome numbers and in some cases less (Table 1); this is likely due to differences in the expression level of the *GL3* transgene caused by position effects or T-DNA copy number variation. Table 1 shows the results of trichome counts for leaves one to four from seedlings of L*er*, L*er gl3-1*, and five L*er gl3-1* pD2LX-transformed lines. It should be noted that numbers of trichomes as well as the proportion of three-branched trichomes are increased in the transformants, approximating wild type. The association of a premature stop codon with the *gl3-1* and *gl3-2* phenotypes and the ability of the MYC6 genomic fragment to complement the *gl3-1* mutant indicate that *GL3* is the *R*-homologous gene from the MYC6 P1 clone.

Antisense GL3 expression: Further evidence in support of a role for the MYC6/*GL3* locus in trichome development is provided by antisense expression experiments. An ~1800-bp PCR fragment of the genomic region starting just after the stop codon was placed in reverse orientation under the control of the constitutive CaMV 35S promoter, and the resulting construct, pEZF1, was transformed into wild-type ecotype WS. *GL3* antisense expression caused the plants to produce altered proportions of two- and three-branched trichomes, approximating the *gl3-1* mutant branching phenotype (Figure 1, D and E; Table 1) but it had only a moderate effect at best on reducing trichome numbers. This phenotype is dominant and segregates with the T-DNA when the transformants are either selfed or outcrossed. Three out of 20 *GL3* antisense transformants examined show this altered trichome branching phenotype. It is interesting that the *gl3-2* mutation has the same effect of increasing the proportion of two-branched trichomes but has only a modest affect on total trichome number (Table 1).

GL3 overexpression increases trichome differentiation: A *GL3* genomic PCR fragment without the flanking regulatory regions was cloned in the sense orientation under the control of the CaMV 35S promoter to create pD22. Figure 1F shows a WS wild-type plant overexpressing GL3 from this construct (compare to Figure 1D). The increased trichome phenotype seen in these plants is very similar to that seen in plants overexpressing R (Lloyd *et al.* 1992).

GL3 overexpression suppresses *ttg1-1*: Figure 1K shows that *ttg1-1* plants overexpressing GL3 from this construct produce trichomes. GL3 overexpression also restores anthocyanin and seed coat mucilage production in *ttg1-1* (data not shown). In contrast to R, when overexpressed, GL3 is not a strong suppressor of the trichome defect, and in some cases transformed plants are as hairless as the *ttg1-1* mutant but low levels of anthocyanins are restored (data not shown).

The fact that overexpression of GL3 produces an abundance of trichomes in the presence of a full-length TTG1 protein, but not in plants without, indicates that

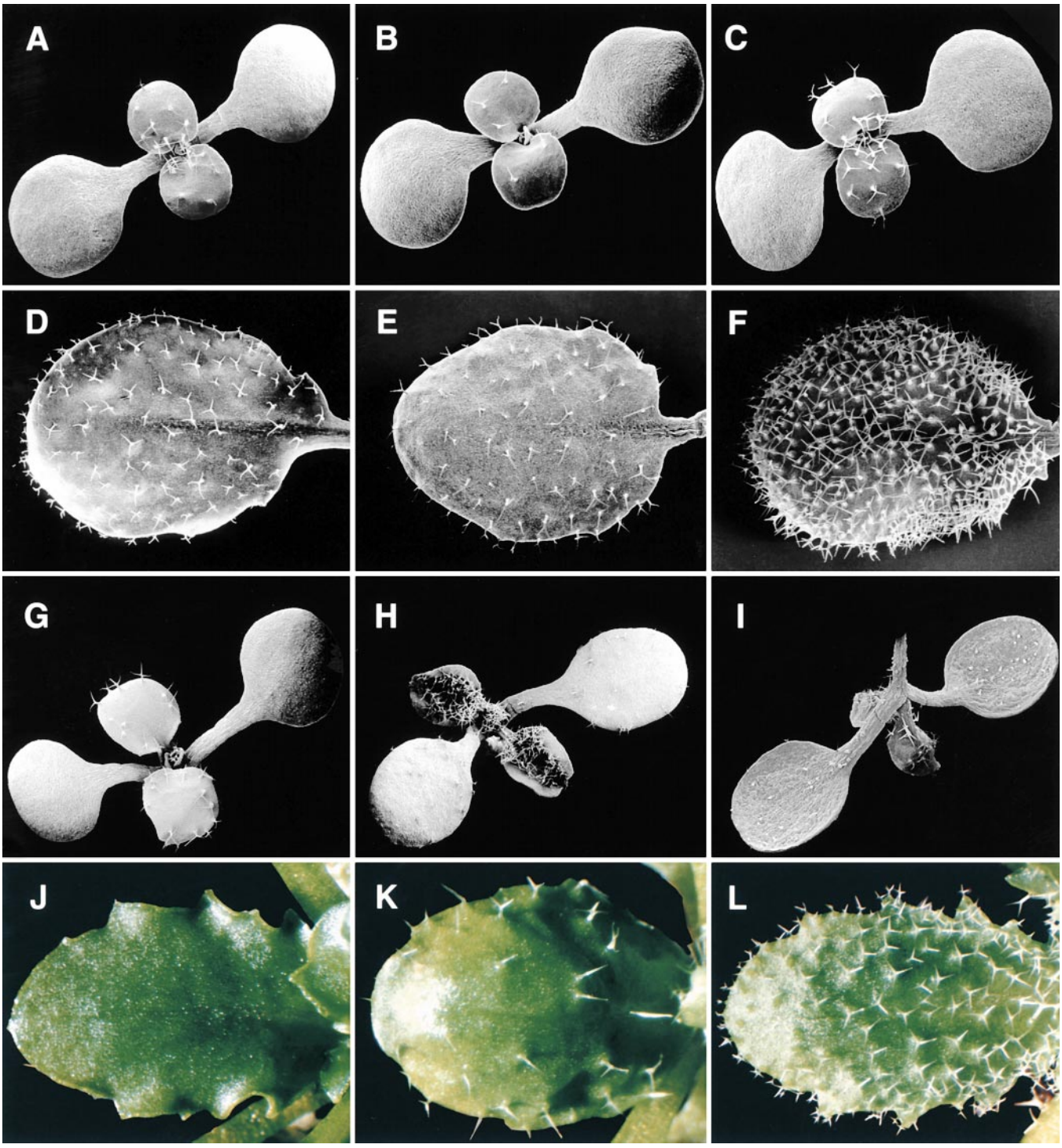

FIGURE 1.—Wild-type, mutant, and transgenic Arabidopsis phenotypes. (A–I) Scanning electron micrographs (SEMs). (A–C) 10-day-old seedlings. (A) Wild-type L*er*. (B) *gl3-1* mutant in L*er* background. (C) *gl3-1* mutant complemented with MYC6 genomic fragment. (D–F) SEMs of fourth true leaves. (D) Wild-type WS. (E) Antisensed *GL3* in WS background. (F) GL3 overexpressed in WS. (G–I) 10-day-old seedlings. (G) WS overexpressing GL1. (H) WS in which both GL1 and GL3 are overexpressed, adaxial view. (I) Same as H, but abaxial view. Note the presence of trichomes on both sides of the cotyledons and on the hypocotyl in H and I. (J–L) Fourth true leaves of transgenic plants produced in the *ttg1-1* background. (J) GL1 overexpressed in the *ttg1-1* mutant. (K) GL3 overexpressed in *ttg1-1* mutant. (L) Cross in which both GL1 and GL3 are overexpressed in the *ttg-1* mutant background.

TABLE 1

Leaf trichome phenotypes for wild-type, mutant, and transgenic Arabidopsis lines

Genotype	Leaf number	Branching phenotype				Total no. of trichomes
		1	2	3	4	
Ler wild type	1,2		0.1 ± 0.31	7.8 ± 2.5	3.9 ± 2.0	11.8 ± 2.0
n = 15	3		0.27 ± 0.59	25.5 ± 5.7	17.8 ± 5.4	43.7 ± 5.9
	4		0.2 ± 0.56	31.9 ± 5.6	23.7 ± 6.6	55.8 ± 6.3
gl3-1	1,2	0.59 ± 1.0	4.2 ± 1.9	0.32 ± 0.64		5.1 ± 2.1
n = 11	3	4.5 ± 1.8	14.6 ± 2.9	0.45 ± 0.52		19.5 ± 3.3
	4	9.3 ± 4.1	19 ± 5.0	0.18 ± 0.60		28.5 ± 8.3
gl3-2	1,2	0.45 ± 0.6	10.3 ± 4.0	1.9 ± 1.7		12.2 ± 4.9
n = 10	3	5.4 ± 3.1	28.3 ± 8.1	3.5 ± 1.4		37.2 ± 10
	4	7.6 ± 4.8	35.3 ± 10	2.0 ± 1.7		44.9 ± 14
gl3-1XLer wt	1,2	0.07 ± 0.23	0.71 ± 0.91	12.6 ± 3.2	0.36 ± 0.50	13.7 ± 2.9
n = 7	3		2.0 ± 1.5	39 ± 7.5	4.0 ± 3.5	45 ± 9.3
	4		1.6 ± 1.1	56.7 ± 11	5.1 ± 4.2	63.4 ± 11
1/14/99—3A	1,2		0.63 ± 0.92	9.9 ± 2.5	0.63 ± 0.74	11.1 ± 2.0
n = 4	3			26 ± 3.7	4 ± 0.82	30 ± 3.9
	4		1.0 ± 1.2	41.8 ± 10	5.3 ± 4.7	48 ± 14
1/14/99—6A	1,2		0.20 ± 0.41	9.6 ± 2.3	2.6 ± 2.0	12.4 ± 2.1
n = 10	3		0.20 ± 0.63	19.3 ± 4.7	11.5 ± 6.8	31 ± 4.2
	4			28.6 ± 5.9	12.6 ± 9.9	41.2 ± 8.1
1/14/99—6B	1,2	0.13 ± 0.34	0.50 ± 0.93	10.4 ± 1.5	0.38 ± 0.52	11.3 ± 1.6
n = 4	3			25 ± 2.4	2.5 ± 2.6	27.5 ± 4.9
	4			35.8 ± 2.9	3 ± 1.6	38.8 ± 3.9
1/14/99—4A	1,2		0.38 ± 0.52	8.0 ± 2.1	4.1 ± 3.2	12.5 ± 3.3
n = 4	3			17.8 ± 6.7	13.3 ± 9.7	31 ± 5.6
	4		1.0 ± 2.0	31.8 ± 16	11.8 ± 12	44.5 ± 7.2
12/21/98—8A	1,2		0.30 ± 0.67	8.9 ± 2.1	6.3 ± 1.3	15.6 ± 2.7
n = 5	3		0.40 ± 0.89	14.8 ± 2.7	21 ± 6.2	36 ± 7.9
	4			20.6 ± 5.3	23.3 ± 5.0	43.8 ± 8.9
WS wt	1,2		8.7 ± 3.9	33.6 ± 5.7		42.3 ± 6.3
n = 5	3		16.4 ± 4.4	92.8 ± 11		109 ± 11
	4		20.8 ± 4.3	106 ± 6.8		127 ± 9.8
4/15/98—17A	1,2		29.8 ± 6.0	11.7 ± 6.3		41.5 ± 12
n = 5	3		59.8 ± 10	36.8 ± 12		96.6 ± 11
	4		80.2 ± 14	38 ± 14		118 ± 19
4/15/98—17B	1,2		29.8 ± 3.9	11.7 ± 2.7		41.5 ± 5.1
n = 5	3		62.8 ± 9.6	39 ± 8.1		101 ± 13
	4		75 ± 12	38.4 ± 9.9		113 ± 22
4/15/98—17F	1,2		28.1 ± 9.1	15.9 ± 6.3		44 ± 8.1
n = 5	3		43.6 ± 19	26.3 ± 12		68.7 ± 30
	4		65.2 ± 13	36.8 ± 8.5		102 ± 17

Because trichome phenotypes on the first two leaves are indistinguishable, data were pooled. Consequently, *n* for leaf 1,2 is twice the *n* given for the number of plants. Branch phenotype refers to the numbers of processes on the trichome. The number 1 refers to an unbranched spike. The number 2 is a two-branched trichome, etc. 1/14/99—3A, 6A, 6B, 4A, and 12/21/98—8A are *gl3-1* transformed with the *GL3* genomic clone, pD2LX. 4/15/98—17A, 17B, and 17F are WS wild type transformed with the GL3 antisense clone, pEZF1. *n*, number of individual plants counted for each strain.

TTG1 is necessary for full GL3 function. That R does not require functional TTG1 further suggests that there is a fundamental difference between the regulatory roles performed and the functional interactions participated in by the R and GL3 proteins.

Overexpressed GL3 and GL1 interact in plants: Arabidopsis overexpressing GL1 were crossed to GL3 overexpressers to assess the interaction between these proteins in plants. GL1 overexpression leads to a reduction in the number of trichomes in a wild-type background

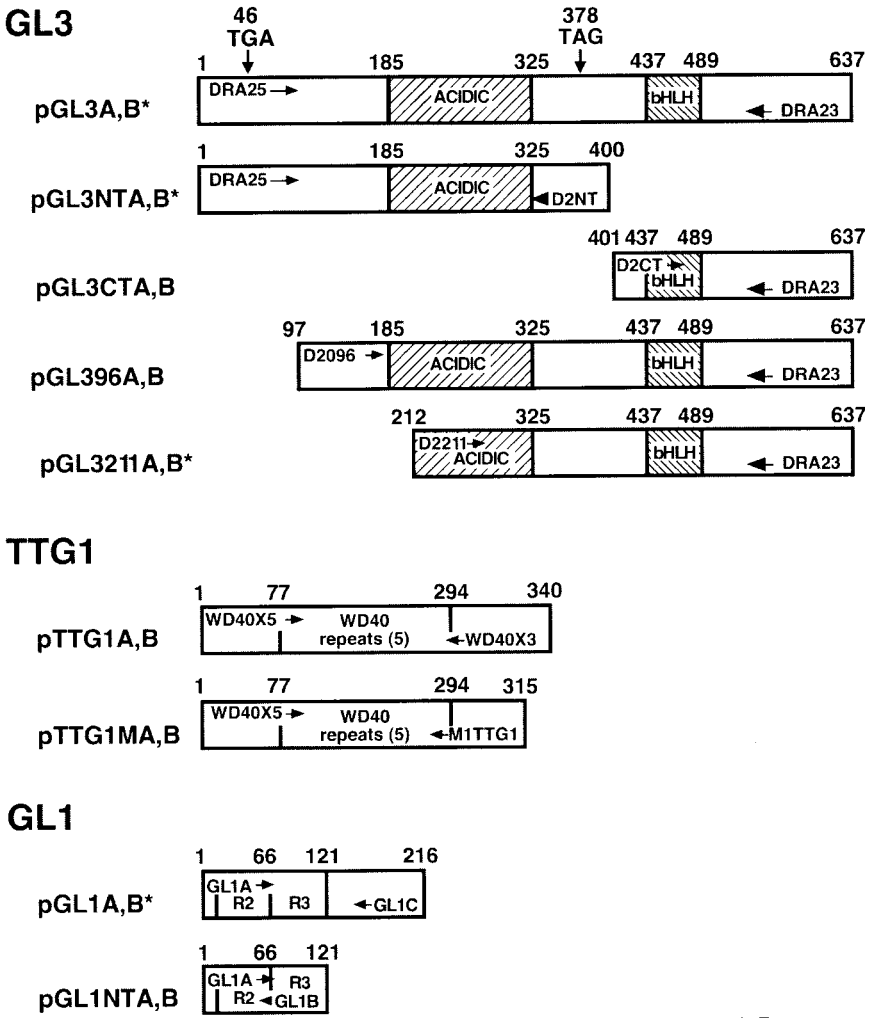

FIGURE 2.—Fusion constructs used in GL3 two-hybrid analyses. Constructs suffixed with "A" are fusions to the GAL4 AD in pGAD424; those suffixed with "B" are fusions to the GAL4 DBD in pAS2-1 or pGBT-9. Proteins are represented by bars; motifs or domains within proteins are delimited by vertical lines. Diagonal fill is used to denote the acidic and bHLH domains of GL3 and its derivative fragments. Numbering above bars indicates amino acid position within wild-type sequence. Oligonucleotides used as primers for PCR-based cloning (see MATERIALS AND METHODS) are shown in the top left and bottom right corners of bars, with arrows to indicate the direction of synthesis. The *gl3-1* and *gl3-2* mutant codons 378 and 46, respectively, are identified above the bar representing the full-length GL3 protein for reference. An asterisk beside a DBD fusion construct name indicates that the construct in question does not require an interaction partner to activate reporter gene transcription.

(Figure 1G; OPPENHEIMER *et al.* 1991; LARKIN *et al.* 1994) and to occasional unbranched trichomes on cotyledon edges but no ectopic trichomes on the hypocotyl. Plants overexpressing R do not produce trichomes on cotyledons or the hypocotyl. Plants overexpressing both R and GL1 produce many trichomes on cotyledon and hypocotyl surfaces and more trichomes on true leaves than plants overexpressing R alone (LARKIN *et al.* 1994). These findings indicate a synergistic interaction between these two genes that causes shoot epidermal cells to assume a trichome cell fate. A similar pattern of ectopic and supernumerary trichome initiation was observed in plants overexpressing GL3 and GL1 together (Figure 1, H and I). In addition, the effect of overexpressing both GL3 and GL1 in a *ttg1-1* mutant background was examined. As discussed above, GL3 overexpression suppresses the *ttg1-1* mutation, but this does not give a wild-type complement of trichomes (Figure 1K). Overexpression of GL1 does not suppress the *ttg1-1* mutation (LARKIN *et al.* 1994; Figure 1J). When F_1 hybrid plants overexpressing GL1 and GL3 in a *ttg1-1* background were produced, trichome numbers and distribution resembling wild type are achieved (Figure 1L). This finding indicates that GL1 and GL3 can interact synergistically in Arabidopsis plants, even in the absence of TTG1. However, the large numbers of trichomes produced by wild-type plants overexpressing both are not seen, suggesting that TTG1 is required for full function of the putative GL3:GL1 complex.

GL3 interacts with other trichome regulators in yeast: Prior to complementation of the *gl3-1* mutant, two-hybrid analysis in yeast (FIELDS and SONG 1989) suggested a role for the MYC6 gene product in trichome regulation. A construct in which the *GL3* cDNA was fused to the GAL4 activation domain (AD), pGL3A, was unable to activate transcription of reporter genes alone. When cotransformed with the GAL4 DNA binding domain (DBD) fusions to two known regulators of trichome initiation and morphogenesis, the MYB repeat domain of GL1 (pGL1B) or full-length TTG1 (pTTG1B), reporter genes were strongly activated (Figure 2 and Table 2), indicating physical interactions between GL3 and both GL1 and TTG1.

Fragments of the *GL3* cDNA were subcloned as fusions to the GAL4 AD and tested for interaction with the GL1 and TTG1 constructs. The amino-terminal two-

TABLE 2

Yeast two-hybrid interactions for trichome regulators based on β-galactosidase lift assay results

Activation domain fusion constructs	DNA binding domain constructs				
	pGL1B	pGL1NTB	pTTG1B	pTTG1MB	pGL3CTB
pGL3A	++	++	++	−	+
pGL3NTA	++	++	++	−	−
pGL3CTA	−	−	−	−	+
pGL396A	−	−	++	−	+
pGL3211A	−	−	++	−	+
pGL1NTA	−	−	−	−	−
pTTG1A	−	−	−	−	−

Constructs and yeast strains are as in MATERIALS AND METHODS and Figure 3. ++, strong β-galactosidase activity was detected in 1 hr or less. +, activity was faint within the first 3 hr and dark blue within 10 hr. −, no activity could be detected in >10 hr.

thirds of the protein (pGL3NTA) was sufficient for both interactions to occur, but amino-terminal truncations of 96 (pGL396A) and 211 (pGL3211) amino acids from GL3 abolished the interaction with the MYB domain-containing GL1 construct. The interaction with TTG1 was not affected by these truncations, indicating that the two trichome regulators bind at different sites on the GL3 protein or that GL1 binding is relatively more sensitive to conformational changes resulting from the truncations. A DBD fusion to TTG1 lacking 25 amino acid residues from its carboxy terminus, pTTG1MB, which recapitulates the *ttg1-1* mutation, failed to interact with any of the GL3 fusions. This result agrees well with our interpretation of the plant overexpression data: GL3 exerts its regulatory effects on the trichome development pathway through interaction with TTG1, and TTG1 requires GL3, and possibly other bHLH class regulators, to activate the trichome pathway.

The GL3 amino-terminal truncations differed in their ability to independently activate reporter gene transcription when fused to the GAL4 DBD. Curiously, the pGL396B construct, from which relatively less of the amino terminus was removed, failed to activate, whereas pGL3211B activated strongly. GOFF *et al.* (1992) did not observe solo activation in yeast by B (a functional duplicate of R) when fused to the GAL4 DBD, which they attributed to an absolute requirement for heterodimerization with the MYB-class transcription factor C1. However, these investigators did note that B contains an acidic domain that might have transcriptional activation properties. LUDWIG *et al.* (1989) also described the existence of an acidic domain within R. While our two-hybrid results do not fine map a GL3 activation domain that functions in yeast, they confirm its existence and are consistent with a placement comparable to those suggested by previous authors on the basis of alignments and sequence acidity.

A GAL4 DBD fusion construct containing approximately the carboxy-terminal third of GL3 (pGL3CTB) did not activate reporter gene transcription alone or when cotransformed with GL1 or TTG1 AD fusion constructs. This fragment contains a bHLH dimerization and DNA binding motif and was sufficient to mediate self-interaction in yeast when cotransformation of AD and DBD fusions were performed. We also could find no indication that either TTG1 or GL1 form homodimers or that TTG1 and GL1 interact with each other.

RT-PCR of GL3: To observe the effects of mutant background on the expression of the GL3 transcript, semiquantitative RT-PCR was performed. APRT (MOFFATT *et al.* 1994; COWLING *et al.* 1998) was used as a control. As can be seen in Figure 3, both APRT and GL3 are easily detected in all lines regardless of muta-

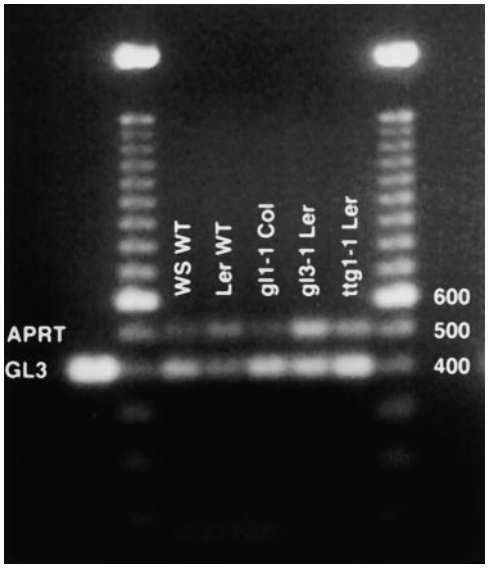

FIGURE 3.—RT-PCR of GL3. The position of the GL3 and APRT products is shown at the left. The left lane is a control reaction using the GL3 cDNA as a template. Next and last lanes are 100-bp size markers. The next five lanes show RT-PCR products from the genotypes listed above the reaction products. On the far right is shown the position of the 400-, 500-, and 600-bp marker bands.

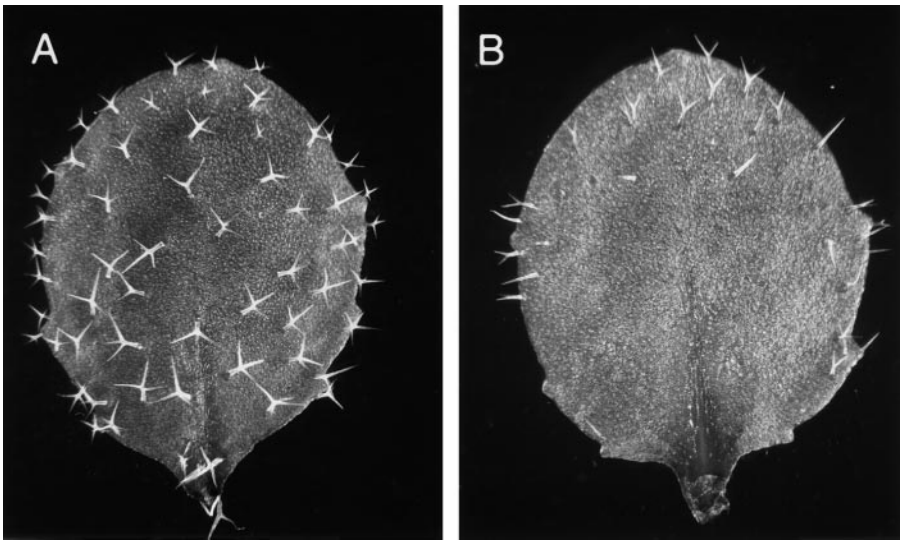

FIGURE 4.—Fourth leaf of wild-type and *gl3-1* mutant Arabidopsis. (A) L*er* wild-type leaf showing the regular spacing and mostly three-branched trichomes. (B) L*er gl3-1* mutant leaf showing aberrant trichome patterning around the edge of the leaf and the mostly two-branched phenotype.

tion. Thus GL1, GL3, and TTG1 do not appear to be required for the transcription of GL3.

DISCUSSION

Sequence analysis indicates that the *gl3-1* mutant may produce a truncated protein lacking a bHLH domain but maintain those domains that two-hybrid data suggest mediate interactions with GL1 and TTG1. Thus the *gl3-1* allele might encode a partially functional GL3 protein able to participate in the R-suppressible developmental pathways defined by the *ttg1-1* mutation. The mutant protein would lack both of the R-homologous nuclear localization signals (NLSs) inferred for wild-type GL3 (based on SHIEH *et al.* 1993), although its reduced size might enable entry into the nucleus in the absence of these signals. GOFF *et al.* (1992) showed that a maize B protein construct from which the bHLH domain and M (middle) and C (carboxy-terminal) NLSs had been deleted could still activate reporter genes, albeit to a reduced extent. In their studies of *Ds* insertion and excision alleles of *R-sc*, LIU *et al.* (1998) found that partial truncations in Helix 2 of the bHLH region had more severe effects on the activation properties of mutant proteins than complete loss of the domain. The bHLH domain, a well-conserved bifunctional motif mediating DNA binding and homo- and heterodimeric protein-protein interactions (ATCHLEY and FITCH 1997), is unlikely to be dispensable. In yeast the GL3 bHLH has been observed to dimerize with itself and with R and 146D23T7 (not shown), indicating that the motif is functional. In other systems heterodimerization of bHLH proteins has profound regulatory consequences (BENEZRA *et al.* 1990; GARRELL and MODOLELL 1990; CAMPUZANO and MODOLELL 1992) and this is likely to be true in plants as well.

We have recently identified the lesion in the *gl3-2* allele as a G to A transition, which replaces the tryptophan codon TGG with a stop, TGA, at codon 46. If translated, the mutant transcript would yield a polypeptide of only 45 amino acids. Such a severely truncated protein is unlikely to have any significant regulatory function. The trichome phenotypes of the *gl3-1* and *gl3-2* mutants are very distinct. Both mutations result in a shift from three and four branched trichomes to mostly two branched (Table 1). However, the *gl3-1* mutation results in a more severe reduction in trichome number than *gl3-2*. This is despite the fact that the respective lesions would imply that the *gl3-2* mutation is more likely to be a null. The finding that the *gl3-1* truncation gives a more severe reduction in trichome number may imply that this partial protein can interfere with the trichome initiation machinery. A model for interference by *gl3-1* may be consistent with the findings for certain *R* alleles noted above (LIU *et al.* 1998) and with the phenotype of plants overexpressing GL1. Figure 1G shows the trichome pattern of GL1 overexpression with trichomes produced around the edge of the leaf and absent from the center of the blade consistent with earlier findings (LARKIN *et al.* 1994). Figure 4 shows a typical fourth leaf of L*er* wild type and L*er gl3-1*. As can be seen, the mutant also tends to produce trichomes around the edge of the blade and none in the center, reminiscent of the GL1 overexpression pattern. Perhaps this is a similar interference phenomenon that is absent in the null *gl3-2* mutant. *gl3-2* does not result in altered trichome patterning on the leaf blade (Table 1). However, F_1 hybrids between L*er* wild type and *gl3-1* do not show alteration in the number or placement of trichomes that might argue against interference by *gl3-1*.

Is GL3 redundant? The phenotypes of the *gl3* mutations are trichome specific and the mutants produce significant numbers of trichomes. No obvious seed coat or anthocyanin defects have been observed as might have been predicted from the overexpression and *ttg1* suppression experiments. This result could be readily

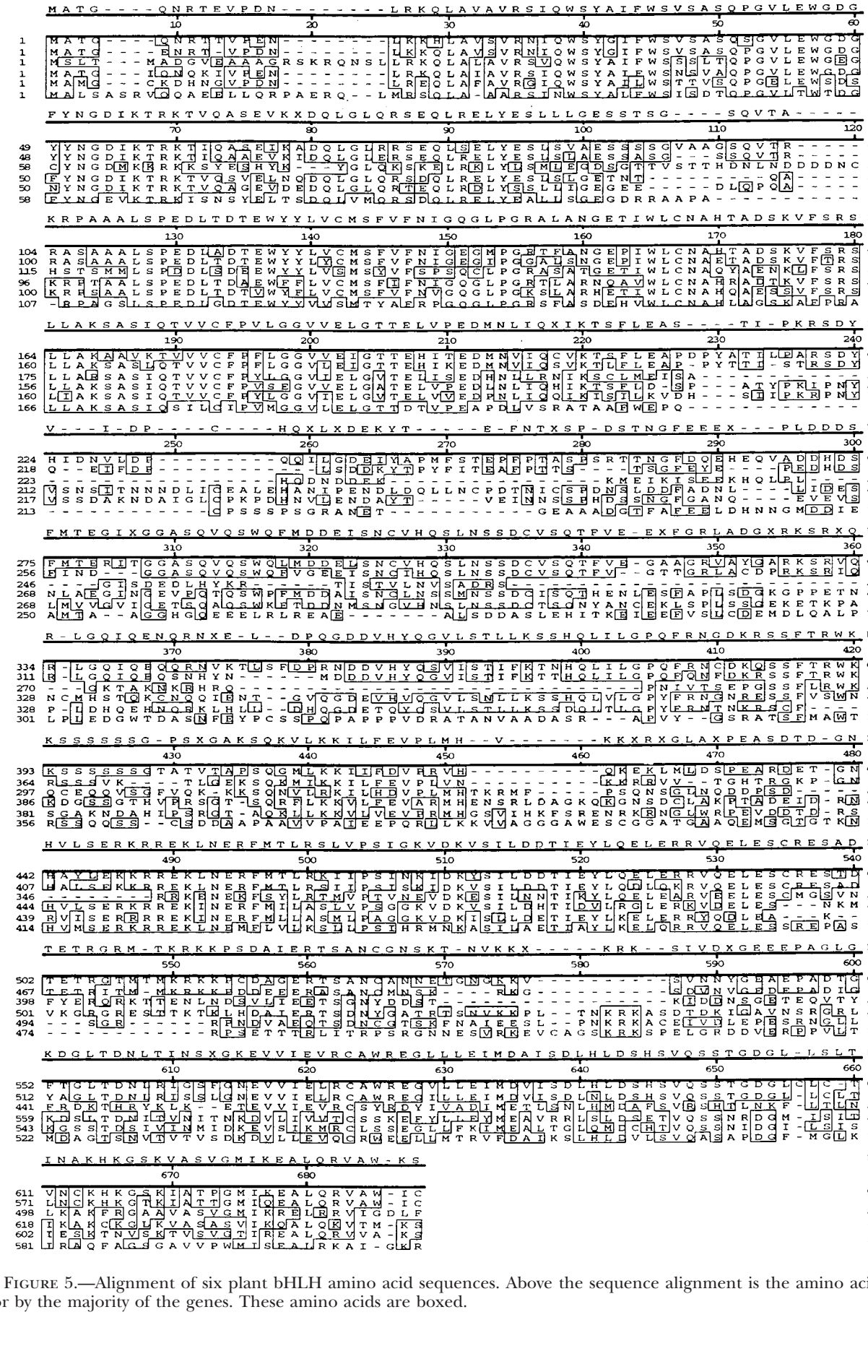

FIGURE 5.—Alignment of six plant bHLH amino acid sequences. Above the sequence alignment is the amino acid encoded for by the majority of the genes. These amino acids are boxed.

explained by the existence of a second R-homologous bHLH factor with a partially redundant and complementary function. Perhaps the truncated GL3 produced by *gl3-1* is interfering with the interactions required by a partially functionally redundant bHLH factor, specifically, those interactions with TTG1 and GL1. Further work will be directed toward testing this hypothesis.

As shown in the alignment of bHLH proteins (Figure 5), 146D23T7 and GL3 are highly homologous. A difference in length of 40 amino acids exists, but the additional residues in the GL3 protein are evenly distributed rather than present in a block that might figure as an additional domain or function acquired by GL3 relative to 146D23T7. If GL3 and 146D23T7 are functionally redundant, this distribution suggests a gradual drift in portions of the sequence not constrained by function after the seminal gene was duplicated. Overexpression of 146D23T7 will also suppress the *ttg1-1* mutation whereas overexpression of the more divergent bHLH protein ATMYC1 (Urao *et al.* 1996) does not (F. Zhang and A. M. Lloyd, unpublished results). Some *gl3-1* transgenics in which antisense-oriented 146D23T7 sequence has been overexpressed are virtually devoid of trichomes; preliminary data indicate that these also have root hair defects (F. Zhang and A. M. Lloyd, data not shown). These results are difficult to interpret, since the specificity and quantity of the antisense suppression are difficult to control but are consistent with the notion that the two loci are partially functionally redundant. The redundancy hypothesis would explain why *gl3* mutants devoid of trichomes have not been isolated.

Until the Arabidopsis genome is completely sequenced, we cannot know with certainty the extent of the R-homologous bHLH protein family represented by GL3 and 146D23T7. Separate bHLH factors may regulate the various TTG1-dependent pathways or have partially or wholly redundant functions. Analyses of expression patterns currently underway should enable the determination of whether and to what extent the transcription of GL3 and 146D23T7 overlap. It is also likely that the specificity of bHLH-controlled regulation will be determined in each pathway by the availability of a MYB-class partner. Two-hybrid analyses indicate the 146D23T7 is also capable of interacting with GL1 and TTG1 in yeast and that 146D23T7 and GL1 interact synergistically when both are overexpressed in Arabidopsis (F. Zhang and A. M. Lloyd, data not shown).

Near-neighbor inhibition: Analyses of wild-type trichome patterns reveal that trichomes are almost never produced by adjacent cells (Larkin *et al.* 1996). A near-neighbor inhibition pathway for trichome differentiation is often invoked to explain this pattern (see, for example, Larkin *et al.* 1996; Szymanski *et al.* 2000). In fact at least two loci have been identified as possible mediators of near-neighbor inhibition: *Tryptichon*, whose mutations produce clustered trichomes (Schnittger *et al.* 1999) and *Caprice*, whose overexpression leads to glabrous plants (Wada *et al.* 1997). Overexpression of the group of bHLH genes including *GL3*, *146D23T7*, and *R* leads to increased numbers of trichomes and substantial numbers of clustered trichomes. These genes are the only genes we know of that increase trichome number far beyond wild-type levels with solo overexpression. Because the number of trichomes produced appears to be a function of the concentration of the bHLH regulator, we feel that the endogenous Arabidopsis bHLH trichome regulators are likely targets for down-regulation by the near-neighbor inhibition pathway. This hypothesis is strengthened by the fact that Caprice is a MYB-type regulator without an acidic activation domain. Caprice (or Tryptichon) may bind to GL3, displacing GL1, and produce a nonproductive complex. There is precedence for this in the dominant inhibitor of anthocyanin biosynthesis, *C1-I*. This allele of *C1* is a MYB element that is missing the transcriptional activation domain similar to *CPC*. The protein has been proposed to act as an inhibitor by competitive binding to DNA sites and heterodimeric bHLH partners (Paz-Ares *et al.* 1990; Goff *et al.* 1992).

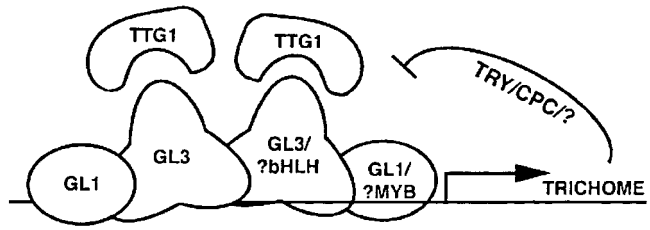

Figure 6.—Model for the regulation of the trichome and other TTG1-dependent pathways. Gl3 can form potential complexes with GL1 and TTG1. It is not known whether these GL1 and TTG1 can bind GL3 simultaneously. GL3 can homo- or heterodimerize with other bHLH regulators, which in turn could have their own interactions. As a consequence, there are many possible regulatory complexes. Inhibitors such as CPC or TRY could feed back on the pathway to inhibit near-neighbor cells from assuming the trichome cell fate.

How does TTG1 regulate GL3? The data presented here support a combinatorial model for the regulation of epidermal pathways dependent on TTG1. In this model the WD repeat protein TTG1 interacts with an R homolog; the latter also interacts with a MYB-class transcription factor specific to the pathway regulated (Figure 6). For example, GL3 might interact with GL1 in the case of trichome development and with PAP1 in the case of anthocyanin synthesis. Whether these interactions are simultaneous or sequential remains to be determined, but the assumption is made that GL1 and GL3 (and other MYB:MYC-like transcription factor pairs) bind to promoters of downstream genes. The functional significance of the TTG1:GL3 interaction has not been established.

The most striking characteristic of the TTG1 protein is that a considerable portion is composed of WD repeats. Proteins containing four or more adjacent WD

repeats are thought to assume a β-propeller fold in which each blade corresponds to parts of two WD repeats (Smith *et al.* 1999). The number of WD repeats present in the TTG1 sequence would appear to be five using the criteria of Neer *et al.* (1994), which is the number determined for the highly homologous AN11 protein, an anthocyanin regulator isolated from *P. hybrida* (de Vetten *et al.* 1997). We have found that AN11 expression efficiently complements the *ttg1-1* mutation (C. T. Payne and A. M. Lloyd, data not shown). Walker *et al.* (1999) chose to model a TTG1 structure based on the heterotrimeric G-protein β-subunit, which possesses a sevenfold symmetry (Sondek *et al.* 1996), and infer that like the G-protein, TTG1 may be a signal transduction component. But, as those authors note, WD repeat-containing proteins have been implicated in a variety of eukaryotic cellular processes other than direct involvement in signal transduction, including RNA processing, gene regulation, vesicular trafficking, and cell cycle control. Common to all is the function of the repeats themselves, which appears to be the assumption of a structure facilitating protein-protein interactions.

Our two-hybrid data indicate that TTG1 interacts physically with the GL3 protein in yeast, and taken together with genetic data this implies that TTG1 activates, modifies, stabilizes, or in some other fashion has a positive effect on the capacity of the GL3 protein to activate trichome structural gene transcription. One attractive hypothesis discussed by Walker *et al.* (1999) is that TTG1 regulates by controlling nuclear localization of relevant transcription factors. Precedents for this exist, for instance, the Arabidopsis WD repeat protein CONSTITUTIVE PHOTOMORPHOGENESIS1/FUSCA1 (COP1/FUS1) differentially localizes in response to light and has been shown to interact with the bZIP transcription factor HY5 (Ang *et al.* 1998). However, the COP1/FUS1 protein contains readily identifiable functional domains in addition to the WD repeats (Deng *et al.* 1992), and the latter have been found to serve as autonomous repressor domains not related to the protein's localization (Torii *et al.* 1998). Interestingly, the findings of Miséra *et al.* (1994) indicate that TTG1 is regulated by COP1/FUS1. PRL1, another Arabidopsis WD repeat protein and a repressor of glucose responsive genes, localizes to the nucleus (Németh *et al.* 1998) and in a two-hybrid screen conducted by those authors interacted with a novel α-importin, ATHKAP2, which did not bind to conventional plant NLSs. We tested the ATHKAP2 construct against TTG1 in the two-hybrid assay but detected no interaction (not shown), suggesting that there is no generalizable relationship between WD repeat-containing regulatory factors and this novel class of α-importins.

Database searches using the 341-amino-acid TTG1 sequence as a query have not detected functionally defined motifs other than the WD repeats themselves. In the absence of catalytic domains it may be hypothesized that TTG1's function relative to GL3 is merely binding. The consequence of binding might be modification of GL3 by another protein bound to TTG1. A two-hybrid screen conducted in our lab has identified several proteins that potentially interact with TTG1 *in planta*. Indeed, it would be remarkable if a WD repeat protein with multiple binding surfaces like TTG1 participated in only one physical interaction, especially when precedents for multiple interaction partners for this class of proteins are to be found in the literature (Smith *et al.* 1999). A directly correlated relationship between GL3 concentration and trichome number is indicated by overexpression experiments performed in a wild-type genetic background. TTG1 overexpression in a wild-type background does not result in the development of supernumerary trichomes (C. T. Payne and A. M. Lloyd, data not shown). Taken together, these data suggest that bHLH protein concentration is limiting for the trichome developmental pathway. TTG1 binding (and theoretical modification by other bound proteins) might be expected to stabilize the GL3 protein in protodermal cells destined to become trichomes.

Overexpression experiments have frequently been used to address gene function in plants (for example, Tamagnone *et al.* 1998). The results of such experiments are necessarily preliminary, because ectopic expression may place the protein being studied outside of its normal context and in quantities that potentially swamp post-transcriptional or translational regulatory mechanisms. Heterologous overexpression experiments must be interpreted with greater caution, since their utility rests on the assumption that function is conserved between species (Payne *et al.* 1999). The findings described in this article generally validate overexpression studies for preliminary functional analysis of gene products. Furthermore, we confirmed our earlier overexpression analysis that suggested that TTG1 activates an R homologue (Lloyd *et al.* 1992).

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