A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*

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Summary

***GLABRA3* (*GL3*) encodes a bHLH protein that interacts with the WD repeat protein, TTG1. GL3 overexpression suppresses the trichome defect of the pleiotropic *ttg1* mutations. However, single *gl3* mutations only affect the trichome pathway with a modest trichome number reduction. A novel unlinked bHLH-encoding locus is described here, *ENHANCER OF GLABRA3* (*EGL3*). When mutated, *egl3* gives totally glabrous plants only in the *gl3* mutant background. The double bHLH mutant, *gl3 egl3*, has a pleiotropic phenotype like *ttg1* having defective anthocyanin production, seed coat mucilage production, and position-dependent root hair spacing. Furthermore, the triple bHLH mutant, *gl3 egl3 tt8*, phenocopies the *ttg1* mutation. Yeast two-hybrid and plant overexpression studies show that EGL3, like GL3, interacts with TTG1, the myb proteins GL1, PAP1 and 2, CPC and TRY, and it will form heterodimers with GL3. These results suggest a combinatorial model for TTG1-dependent pathway regulation by this trio of partially functionally redundant bHLH proteins.**

Key words: bHLH, TTG1, Glabra3, *Arabidopsis thaliana*

Introduction

The control of cell-fate determination has long been a central question in developmental biology. *Arabidopsis* trichome (hair) initiation and development has emerged as an important model system for cell-fate determination and developmental studies in plants. The trichome is a large, branched, single cell, easily visible with the naked eye that is expendable in the laboratory. Many mutants that affect form and spacing have been isolated. However, only two classical mutations exist in loci that are required for trichome initiation, i.e. positive regulators of the actual cell-fate decision event. These are a highly pleiotropic locus, *TRANSPARENT TESTA GLABRA1* (*TTG1*) (Koornneef, 1981) and a trichome specific locus, *GLABRA1* (*GL1*) (Koornneef et al., 1982), which encode for a WD repeat-containing protein (Walker et al., 1999) and a myb element (Oppenheimer et al., 1991), respectively.

Mutations in *TTG1* are suppressed by the expression of the maize bHLH transcription factor, R (Lloyd et al., 1992; Galway et al., 1994). We recently showed that the *GLABRA3* (*GL3*) locus encodes an R-like bHLH protein and that overexpressing the *GL3* genomic copy in the *ttg1* mutant weakly suppresses the trichome defect (Payne et al., 2000). We also showed that GL3 interacts with GL1 in plants and GL3 interacts with GL1 and TTG1 in yeast two-hybrid studies, but that TTG1 and GL1 do not interact in yeast. Thus, GL3 appears to supply an R-like activity in the trichome development pathway and is probably a physical link between the two cell-fate regulators, TTG1 and GL1. Paradoxically, *gl3* mutants are not glabrous. The most severe allele only produces a modest reduction in trichome initiation. Furthermore, *gl3* mutants do not appear to be defective in any of the other TTG1-dependent pathways. Recently the *TRANSPARENT TESTA8* (*TT8*) locus was identified as encoding a bHLH protein (Nesi et al., 2000). Thus part of the R-like bHLH activity that is required for seed coat pigmentation is supplied by TT8. *TT8* mutations confer a transparent testa because of phenylpropanoid pigment defects leading to the absence of condensed tannins in the seed coat. However, similar to the incomplete affects of *gl3* mutations on trichome initiation, *tt8* mutant plants produce substantial amounts of the phenylpropanoid pigment, anthocyanin. It appeared that either the R-like activities supplied by GL3 and TT8 enhanced, but were not absolutely required for the activation of trichome and anthocyanin production, or there were one or more partially functionally redundant loci responsible for the remainder of the bHLH protein requirement in these pathways. In addition, no bHLH locus had been identified in the position-dependent cell-fate pathway at work in root hair hairless cell file differentiation, or in the seed coat differentiation pathway that leads to mucilage production, both of which are also controlled by TTG1 (Galway et al., 1994; Koornneef, 1981).

In order to test the redundancy hypothesis, we performed a genetic enhancer screen in the *gl3-1* background. A novel unlinked locus was identified, that gave totally glabrous plants when combined with the *gl3-1* mutation. Because our previous work showed that overexpression of the bHLH locus, At1G63650, could suppress *ttg1* mutations (Payne et al., 2000), we focused on this locus as potentially redundant with *GL3*. We have identified this *ENHANCER OF GLABRA3* (*EGL3*) locus as the bHLH-encoding gene, At1G63650. *EGL3* is required along with *GL3* for trichome initiation. In addition, the double mutant was found to be *ttg1*-like, having altered root hair positioning, reduced mucilage production and reduced anthocyanin production. Furthermore, the triple bHLH mutant, *gl3-1 egl3-1 tt8-1*, is essentially phenotypically indistinguishable from the most severe *ttg1* mutations. These results explain why ectopic expression of the maize anthocyanin-specific bHLH regulator, R, suppresses all of the defects of the *ttg1* mutant and define roles for a set of three, partially functionally redundant, endogenous bHLH proteins in all of the TTG1-dependent pathways of *Arabidopsis*.

Materials and methods

Microscopy

Scanning electron microscopy was performed as previously described (Payne et al., 2000; Windsor et al., 2000).

Arabidopsis strains

All strains were in the Landsberg *erecta* (L*er*) ecotype unless noted otherwise. The *gl3-1*, *gl3-2* and *ttg1-1* strains have been described previously (Payne et al., 2000; Walker et al., 1999). The *gl3-1 egl3-1* and *gl3-1 egl3-2* strains were created by EMS mutagenesis of the *gl3-1* mutant background. 6,000 *gl3-1* seeds were treated with 0.3% EMS according to the method of Lightner and Caspar (Lightner and Caspar, 1998). The selfed progeny from groups of 1,000 mutagenized parents were pooled and 8,000 M_2 plants from each of the six pools were screened. In this group of 48,000 seedlings, we identified eleven enhancer mutants that appeared to have completely glabrous early leaves. Genetic complementation tests revealed that one enhancer was mutated in *GL2*, three were mutated in *TTG1*, and the other eight fell into two new complementation groups. Seven of the eight fell into a new complementation group identified as having lesions in the At1G63650 basic helix-loop-helix (bHLH). It is interesting to note that no mutations in *GL1* were isolated although we know that *gl3 gl1* double mutants are hairless and viable. The *gl3* enhancer complementation group with the single member has not been characterized.

The single *egl3-1* mutant was isolated by identifying wild-type-appearing F_2, from a *gl3-1 egl3-1* to wild-type cross that segregated three wild type to one completely glabrous in the F_3. These F_2 lines had to be homozygous for *egl3-1* and heterozygous for *gl3-1*. F_3 individuals were identified that segregated only wild-type-appearing progeny in the F_4 and PCR products were sequenced to verify the *egl3-1* homozygous genotype.

gl3-2 egl3-1 was identified by crossing an *egl3-1* homozygote to *gl3-2*, selfing the F_1 and identifying completely glabrous F_2 progeny. The genotype was verified by genetic noncomplementarity with *gl3-1 egl3-1*.

Genotypes that included *tt8* (Enkheim accession) were identified by crossing, selfing the F_1, and identifying F_2 with the appropriate trichome phenotype and a transparent testa. The *tt8 egl3* double mutant was verified by sequencing *egl3,* and the others by test crosses.

The GL1 (Larkin et al., 1994) and PAP1 (Borevitz et al., 2000) overexpression lines (both in Col0) were described previously.

Liquid phase whole-mount RT-PCR in situ hybridization

This in situ protocol is a combination of the RT-PCR protocol of Koltai and Bird (Koltai and Bird, 2000) and the whole-mount protocol of Engler et al. (Engler et al., 1998).

Tissue fixation

Soil-grown seedlings were fixed in 1:1 heptane:fixation buffer (0.08 M EGTA, 5% formaldehyde and 10% DMSO) for 30 minutes, dehydrated twice for 5 minutes in absolute methanol and three times for 5 minutes in absolute ethanol. Samples were stored 1-3 days in ethanol at –20°C.

Tissue permeabilization and postfixation

Samples were rinsed once in absolute ethanol and incubated for 30 minutes in 1:1 absolute ethanol:xylene, washed twice for 5 minutes in absolute ethanol, twice for 5 minutes in absolute methanol, and once for 5 minutes in 1:1 methanol:PBT (phosphate buffered saline + 0.1% Tween 20). Samples were postfixed for 30 minutes in PBT containing 5% formaldehyde followed by one rinse with PBS and two rinses with double distilled water (ddH_2O).

Liquid phase RT-PCR on whole tissues

RNase inhibitor, M-MuLV RT, and either the *GL3* or *EGL3* gene-specific reverse primer were used to reverse transcribe the *GL3* or *EGL3* message. PCR reactions were performed with primers listed below with digoxigenin-labeled dUTP to yield a labeled PCR product of about 850 bp for *GL3* and 650 bp for *EGL3*.

GL3 primer sequence: forward 5′TGGTTGTGCAACGCT-CATACGGCG3′; reverse 5′TCCCAGTTTCATCTCTGGCTTCTG3′

EGL3 primer sequence: forward 5′AACGCTGAAACCGCC-GATAGC3′; reverse- 5′TCTCTCCCAATGTTTTCACA3′

Staining and detection

Immediately after PCR, samples were washed twice for 5 minutes in PBT and blocked for 30 minutes in PBT containing 3% BSA. Preabsorbed alkaline phosphatase conjugated anti-digoxigenin monoclonal antibody (Boehringer Mannheim/Hoffmann-La Roche) was diluted 1:1500 in blocking solution. Samples were incubated overnight at 4°C in 1 ml of diluted antibody. Antibody solution was replaced by fresh blocking solution and incubated for 10 minutes. Samples were washed five times in PBT for 15-30 minutes and placed in 35×10 mm Petri plates with 1 ml of washing buffer (10 mM Tris, 15 mM NaCl, pH 9.5) containing 150 μg/ml 4-nitro blue tetrazolium chloride and 370 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim/Hoffmann-La Roche). Color development was monitored by microscopy and stopped by rinsing with ddH_2O.

LUX RT-PCR

Total RNA was prepared from 100 mg aliquots of 5-day-old seedlings grown on germination medium (MS salts, Gamborg's B5 vitamins, 3% sucrose, 0.8% agar, pH 5.8) using a Qiagen RNeasy plant mini kit. 0.75 μg of total RNA was reversed transcribed in 20 μl reactions using a SuperScript II RT kit (Invitrogen).

Unlabeled and fluorophore-labeled primers were designed with the help of LUX web-based primer design software (www.invitrogen.com/lux). Primers amplifying target (*CHS* and *DFR*) and endogenous control (*APRT*) sequences were FAM- and JOE-labeled, respectively. The labeled T is in bold type.

CHS primers: forward, 5′CACCTGCCAGCGATCCTAGACC-AGG**T**G 3′; reverse, 5′ACGTGTCGCCCTCATCTTCT 3′

DFR primers: forward, 5′CTACATTTCTGCCGGAACCGTT-AATG**T**AG 3′; reverse, 5′CACGCTGCTTTCTCCGCTAA 3′

APRT primers: forward, 5′CAACGTGGCCCTCCTATTGCG**T**TG 3′; reverse, 5′CCGAAATAACCTTCCCAGGTAGC 3′

2 μl of cDNA template was amplified in 50 μl PCR reactions containing 100 nM target primers, 125 nM *APRT* primers, and 60 nM ROX reference dye using platinum Taq (Invitrogen) according to the manufacturer's instructions. Reactions were conducted and fluorescence was monitored in a spectrofluorometric thermal cycler (ABI PRISM 7700). The comparative cycle threshold (C_T) method was used to analyze the results of quantitative PCR (User Bulletin 2, ABI PRISM 7700 Sequence Detection System). Relative transcript levels of target genes are reported normalized to an endogenous

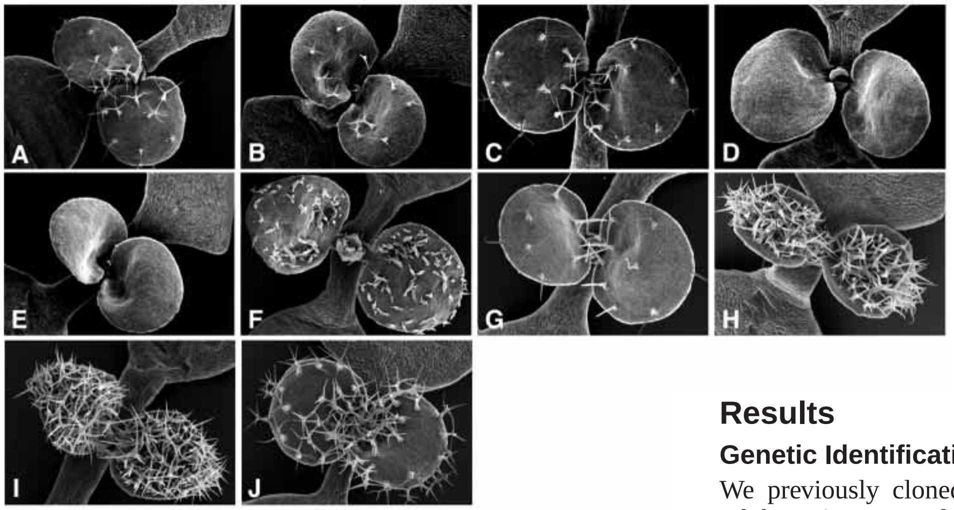

Fig. 1. SEM of seedlings at the expanded first and second leaf stage. (A) Wild type. (B) *gl3-1*. (C) *egl3-1*. (D) *gl3-1 egl3-1* double mutant. (E) *ttg1-1*. (F) *CaMV35S::GL3* cDNA in *ttg1-1*. (G) *CaMV35S::EGL3* genomic clone in *ttg1-1*. (H) *CaMV35S::GL3*/ *CaMV35S::EGL3* in *ttg1-1*. (I) *CaMV35S::GL3* cDNA in wild type. (J) *CaMV35S::EGL3* genomic clone in wild type. All plants are in the L*er* background.

reference, *APRT* (Moffatt et al., 1994; Cowling et al., 1998), and relative to a reference calibrator.

Constructs

Many of the *GL3*, *GL1* and *TTG1* constructs have been described previously (Payne et al., 2000). All others are briefly described here and cloning details are available upon request. All PCR amplification products used in construction were completely sequenced.

pGL3STR contains the *CaMV35S::GL3* cDNA from the start to stop codons in the plant overexpression vector, pLBJ21 (Payne et al., 2000). pEGL3E contains the *CaMV35S::EGL3* genomic fragment from the start to stop codons in the plant overexpression vector, pKYLX71 (Schardl et al., 1987). pGL3PGUS contains 2.5 kb of DNA upstream of the *GL3* coding region inserted in front of the *GUS* gene in pBI101.3 (Clontech). pEGLPGUS contains 3 kb of DNA upstream of the *EGL3* coding region inserted in front of the *GUS* gene in pBG1.1 (Gray-Mitsumune et al., 1999).

Two-hybrid constructs

The original *EGL3* EST, 146d23T7, encodes a spurious stop codon at predicted codon 248 (GenBank Accession Number, AF027732). A new *EGL3* cDNA was prepared from WS wild-type inflorescence by RT-PCR. The product encodes a 596 amino acid peptide.

pEGL3NA encodes for the 367 amino acid EGL3 amino end in activation domain vector, pGAD424 (Clontech).

pEGL3CTA encodes for the 229 amino acid EGL3 carboxy end from residue 368 through 596 in pGAD424.

pCPCDB encodes for the full length CPC protein in DNA binding domain vector, pGBT9 (Clontech).

pTRYDB encodes for the full- length TRY protein in pGBT9.

pP1MDB encodes for the PAP1 myb domains, amino acids 1-113, in DNA binding domain vector, pAS2-1 (Clontech).

pP2MDB encodes for the PAP2 myb domains, amino acids 1-113, in pAS2-1.

The *GL3*, *GL1* and *TTG1* two-hybrid constructs used here have been fully described (Payne et al., 2000) and are briefly described here.

pGL3A encodes for the full-length GL3 protein in pGAD424.

pGL3NTA encodes for the 400 amino acid GL3 amino end in pGAD424.

pGL396A encodes for amino acids 97-635 of GL3 in pGAD424.

pGL3211A encodes for amino acids 212-635 of GL3 in pGAD424.

pGL3CTA encodes for amino acids 401-635 of GL3 in pGAD424.

pGL1NTA encodes for amino acids 1-121 of GL1 in pGAD424.

pTTG1B encodes for the full-length TTG1 protein in pAS2-1.

Results

Genetic Identification of enhancers of *gl3-1*

We previously cloned the *Arabidopsis* R-like bHLH locus, *Glabra3* (Payne et al., 2000). Ectopic overexpression of GL3 in wild-type plants gives phenotypes similar to ectopic R expression and GL3 overexpression partially suppresses the *ttg1* mutation. *gl3* mutants do not have a truly glabrous phenotype and we speculated that there was another, partially functionally redundant bHLH protein involved in trichome initiation. We screened for mutations that result in completely hairless plants in the *gl3-1* mutant background. Screening of M_2 seedlings from EMS-treated *gl3-1 Arabidopsis* seeds resulted in the isolation of 20 independent glabrous lines. Complementation testing revealed two new complementation groups. Of these two new groups, one was hit only once and one was hit seven times.

The large new complementation group was designated Enhancer of Glabra3 (*EGL3*, Fig. 1 compare A, B, and D). When the *gl3-1 egl3-1* double mutant was crossed to the *gl3-1* progenitor, the F_1 looked like the *gl3* parent. When crossed to wild type, the F_1 looked wild type, indicating that the *egl3* mutation is qualitatively recessive.

EGL3 encodes for a bHLH protein similar to GL3

In order to test whether the *GL3*-like locus, At1G63650, was mutated in the new *gl3-1* enhancer complementation groups, we sequenced PCR generated fragments. Lesions at this locus that result in premature stop codons were identified in both sequenced alleles of the large complementation group. The stop in *egl3-1* is codon 26, TGG to TGA. The stop in *egl3-2* is codon 266, CAA to TAA.

EGL3 encodes a putative protein of 596 amino acids, 39 amino acids shorter than GL3. The length difference is distributed throughout the coding region. Like *GL3*, *EGL3* appears to have six introns and the two proteins are approximately 75% similar at the amino acid level. An alignment of GL3 and EGL3 has been presented elsewhere (Payne et al., 2000).

gl3 egl3 double mutant is pleiotropic

Mutations in *GL3* have a moderate effect on trichome initiation and a strong effect on reducing trichome branching, endoreduplication and cell size (Hulskamp et al., 1994; Payne et al., 2000) but no apparent effect on non-trichome pathways. However, ectopic expression of GL3, EGL3 or R will suppress most or all of the defects caused by mutations in *TTG1* (Lloyd

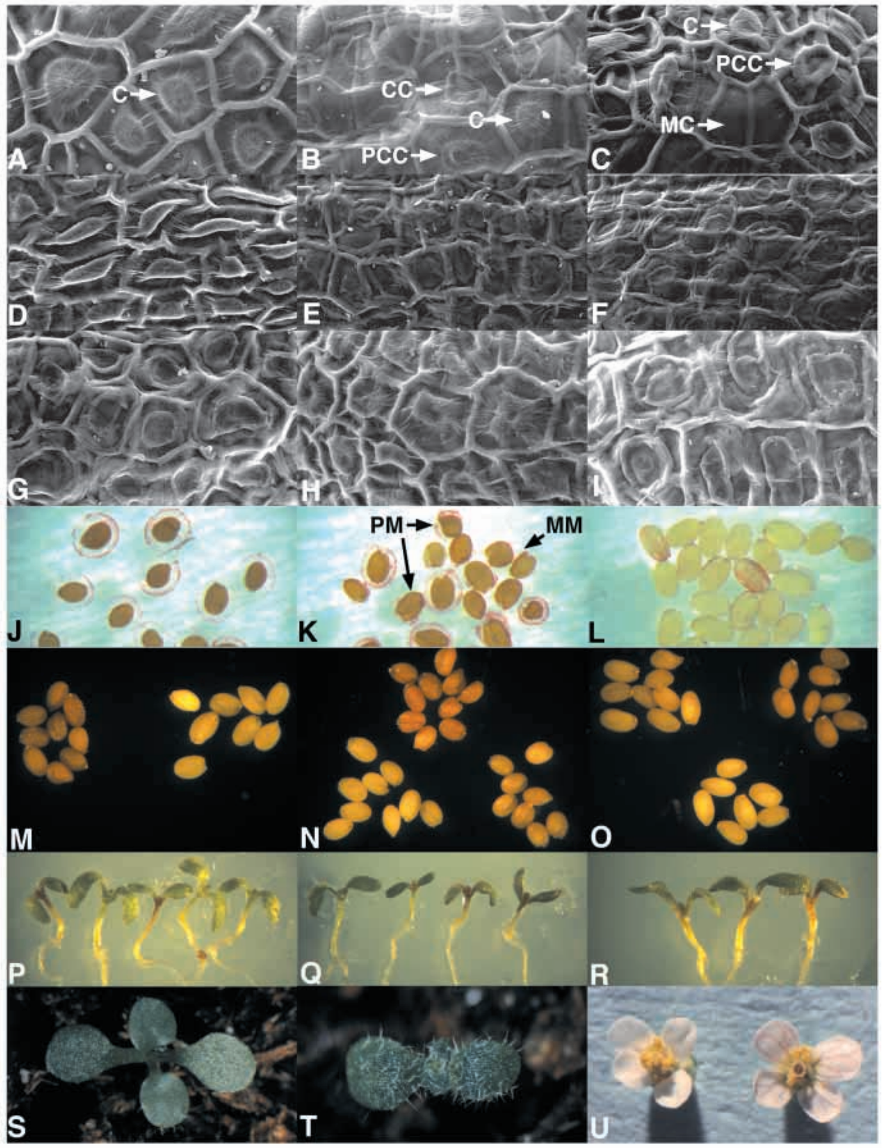

Fig. 2. Seed coat, seedling and flower phenotypes of mutants and transformants. (A-I) Scanning electron micrographs of seed coats illustrating the columella phenotypes. (J-L) Ruthenium red-stained seed coat mucilage phenotypes. (M-O) Seed coat pigment phenotypes. (P-R) 5-day-old seedlings. (S,T) First and second leaf stage seedlings. (U) Single flowers. (A) *gl3-1* single mutant (looks like wild type). (B) *gl3-1 egl3-1* double mutant. (C) *egl3-1* single mutant. (D) *tt8* single mutant (looks like wild type). (E) *egl3-1 tt8* double mutant. (F) *egl3-1 gl3-1 tt8* triple mutant. (G) *ttg1-1*. (H) *CaMV35S::EGL3* in *ttg1-1*. (I) *CaMV35S::GL3* in *ttg1-1*. (J) *gl3-1* single mutant (looks like wild type); (K) *gl3-1 egl3-1* double mutant (looks like *egl3-1* single mutant); (L) *egl3-1 tt8* double mutant. (M) Left: wild type; right: *ttg1-1* mutant. (N) Lower left: *CaMV35S::GL3* in *ttg1-1*; lower right: *CaMV35S::EGL3* in *ttg1-1*; upper center: hybrid expressing *CaMV35S::GL3* and *CaMV35S::EGL3* in *ttg1-1*. (O) Lower center: *tt8*; upper left: *CaMV35S::GL3* in *tt8*; upper right: *CaMV35S::EGL3* in *tt8*. (P-R) Seedlings in order left to right: (P) wild type, *ttg1-1*, *gl3-1*, *egl3-1*, *gl3-1 egl3-1* double mutant; (Q) *ttg1-1*, *CaMV35S::GL3* in *ttg1-1*, *CaMV35S::EGL3* in *ttg1-1*, hybrid expressing *CaMV35S::GL3* and *CaMV35S::EGL3* in *ttg1-1*; (R) *tt8*, *CaMV35S::GL3* in *tt8*, *CaMV35S::EGL3* in *tt8*. (S) *CaMV35S::GL1* in wild type. (T) Hybrid expressing *CaMV35S::GL1* and *CaMV35S::EGL3* in wild type. (U) Left: *PAP1-D* flower (wild type expressing *CaMV35S::PAP1*, activation tagged); right: flower of hybrid coexpressing *CaMV35S::PAP1* and *CaMV35S::EGL3*. Arrows in the first three panels point to examples of columellae types: C, normal round-topped columella; CC, collapsed columella; PCC, partially collapsed columella; MC, missing columella; PM, patchy mucilage; MM, missing mucilage.

et al., 1992; Galway et al., 1994; Payne et al., 2000) (this work). One possible explanation is that there are multiple TTG1-dependent bHLH proteins responsible for the different pathways and that ectopic expression of any one of them will bypass the need for TTG1 in many of the pathways. We characterized the three TTG1-dependent non-trichome pathways in the *gl3 egl3* double mutant to determine whether the absence of both endogenous bHLH proteins conferred pleiotropic defects.

Pigment analysis

The seeds coats, or testa, of *gl3 egl3* are brown, not transparent like the many *transparent testa* mutants including *ttg1* (Fig. 2M). However, a clear-cut qualitative anthocyanin deficit is seen in the hypocotyls and cotyledons of 5-day-old *gl3 egl3* seedlings (Fig. 2P). Anthocyanins are commonly highly expressed in the hypocotyls (Kubasek et al., 1992) and Fig. 2P compares the wild-type strain, *ttg1-1*, *gl3-1*, *egl3-1* single and the *gl3 egl3* double mutants. It is clearly evident that the double mutant and *ttg1-1* have no observable purple anthocyanin compared to the parental line. The *gl3* mutant looks more or less wild type and the *egl3* mutant has reduced anthocyanin content.

Seed coat morphology differentiation

Wild-type seeds produce copious mucilage during seed coat differentiation and this mucilage is released from dry seeds imbibed in water (Windsor et al., 2000; Western et al., 2000). The *ttg1* and *glabra2* (*gl2*) mutants (Koornneef, 1981; Koornneef et al., 1982) do not produce releasable mucilage. The *gl3* mutants produce normal amounts of mucilage as seen by Ruthenium red staining (Sterling, 1970) of seeds imbibed in water (Fig. 2J). However, both the *egl3* single (not shown) and the *gl3 egl3* double (Fig. 2K) mutants produce less mucilage. This phenotype displays incomplete penetrance however, with some seeds producing almost normal amounts of mucilage while most produce none or patches of mucilage. The *ttg1*, *gl2*, *ap2* (Jofuku et al., 1994) and *myb61* (Penfield et al., 2001) mutants fail to produce columellae in the their mature outer seed coat cells and this failure is directly correlated with the absence of releasable mucilage. Fig. 2 compares the seed

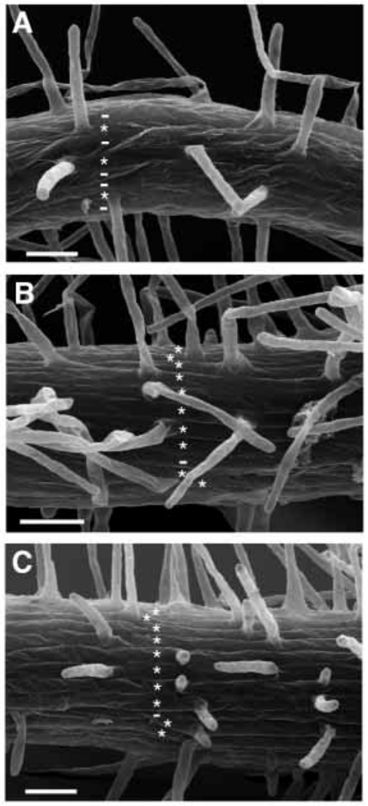

Fig. 3. SEM of root hair phenotypes. (A) Wild type. (B) *gl3-1 egl3-1* double mutant. (C) *ttg1-1* mutant. All are in the L*er* background. Hair files are marked with *, non-hair files are marked with –. Scale bar: 50 μm.

coat morphology of *gl3-1* (Fig. 2A, indistinguishable from wild type), *gl3 egl3* (Fig. 2B), *egl3* (Fig. 2C) and *ttg1* (Fig. 2I). Both the *egl3* single and the *gl3 egl3* double mutants produce many seeds with a testa that exhibits a mosaic of cells with and without columellae and collapsed columellae. Examples of these are illustrated in Fig. 2B,C. The influence of *TT8* on mucilage cell differentiation is discussed below.

Position dependent root hair differentiation

Wild-type *Arabidopsis* produce root hairs (trichoblasts) in files of epidermal cells that lie over the radial wall between two cortical cells. There are normally eight cortical cells with eight separating radial walls and therefore eight files of trichoblast cells separated by a variable number of non-hair cell files (Dolan et al., 1994; Galway et al., 1994). Mutations in *TTG1*, *GLABRA2* and *WEREWOLF* (Lee and Schiefelbein, 1999) cause essentially all root epidermal cells to assume a hair cell fate, ablating position-dependent differentiation. The *gl3 egl3* double mutant exhibits this same *ttg1*-like phenotype. We have not done extensive quantification of root hair production, but it is clear that the *gl3 egl3* double mutant (Fig. 3B) differentiates root hairs in all cell files, as does *ttg1* (Fig. 3C), showing that the double mutant has lost the ability to limit root hair differentiation to specific cell files (Fig. 3A). Qualitative observations of *gl3* and *egl3* single mutants show at most, a mild loss of root hair position dependency (not shown).

Trichome phenotype of *egl3* single mutant

Observations of the F_2 progeny from the *gl3 egl3* × wild type outcross implied that *egl3* had no trichome phenotype by itself, i.e. *gl3* is epistatic to *egl3*. However, it is possible that *egl3* single mutants exhibited a subtle phenotype not easily observed with qualitative observation.

Table 1. Leaf trichome phenotypes for wild-type and egl3-1 mutant *Arabidopsis*

Genotype	Leaf number	Branching phenotype 1	2	3	4	Average trichome number
L*er* wild type	1,2	0	0.2±0.4	9.6±1.3	4.4±2.0	14.2±1.5
	3	0	0.4±0.5	19.4±4.8	15.4±1.1	35.2±5.3
	4	0	0.6±0.9	33.2±5.3	20.4±5.5	54.2±1.8
	Total no.	0	7	359	223	589
	% of total	0	1.2	60.1	37.9	100
L*er egl3-1*	1,2	0	0.8±0.8	10±1.8	1.1±1.6	11.9±1.7
	3	0	0.6±0.9	24.2±8.3	6.6±4.8	31.4±4.0
	4	0	1.4±1.3	29.2±2.0	8.4±1.5	39±2.3
	Total no.	0	18	367	86	471
	% of total	0	3.8	77.9	18.3	100

The trichomes on five plants were counted and branch number (1, spike; 2, 2 branches, etc.) was scored for each genotype. Total number is the total number of trichomes counted for a category. Leaves one and two are indistinguishable so the data for these were pooled. Data are average±s.d.

Single *egl3-1* mutant lines (Fig. 1C) were grown beside wild-type seedlings (Fig. 1A) and scored for trichome number and branching in leaves numbered on to four. Table 1 shows that the *egl3-1* line exhibits a significant reduction in trichome number (approximately a 20% drop) and a shift to fewer branches. The proportion of total four-branched trichomes dropped from 38% to 18% while three-branched trichomes increased from 60% to 78%.

One of these lines was crossed to *gl3-2* and the F_1 were selfed. Approximately one sixteenth of the F_2 were completely hairless. This corroborates the finding that mutations in two bHLH loci, *GL3* and *EGL3* are required to produce a truly glabrous trichome phenotype and that this interaction is not specifically dependent on the *gl3-1* allele.

Ectopic expression of EGL3 or GL3 suppresses *ttg1*

Prior to identifying enhancers of *gl3*, we overexpressed a PCR-generated genomic copy (including introns) of the At1G63650 locus in *ttg1-1* and wild type under the control of the CaMV 35S promoter. Overexpression of the At1G63650 /*EGL3* genomic clone initiated trichome differentiation in the *ttg1* background (Fig. 1E,G) and increased trichome initiation in wild type (Fig. 1J). However, like the *GL3* genomic clone (Payne et al., 2000), the overexpressed *EGL3* genomic clone was a weak suppressor of the *ttg1* trichome defect. *EGL3* also suppressed the anthocyanin defect of the *ttg1* mutation (Fig. 2Q), as did *GL3* (Fig. 2Q). *EGL3* genomic clone overexpression also suppressed the mucilage defect of the *ttg1* mutation, while neither *GL3* cDNA nor genomic clone was able to. This can be seen by comparing the collapsed columellae of *ttg1* that are not rescued by *GL3* overexpression but are by *EGL3* (Fig. 2G,H,I).

Similar to the suppression of the mucilage defect, overexpressed *EGL3* was able to suppress the transparent testa defect of *ttg1* while *GL3* was not (Fig. 2N). In addition, the *ttg1* lines overexpressing *GL3* cDNA consistently had a different trichome phenotype (Fig. 1F) than those overexpressing either the *EGL3* genomic (Fig. 1G) or the *GL3* genomic fragment. The only difference between the *GL3* cDNA and genomic DNA fragments is the inclusion of the introns. The *EGL3* genomic overexpression trichome phenotype looked very much like the overexpressed *GL3* genomic clone phenotype. These had fewer and less branched trichomes than wild type but the trichomes did not appear distorted, while the *GL3* cDNA produced many short fat distorted trichomes. Overexpressed *GL3* cDNA in wild type (Fig. 1I) was also a much stronger trichome initiator than the genomic clone. Root hairs have not been characterized in these overexpression lines.

Coectopically expressed *GL3* and *EGL3* interact synergistically in *ttg1*

The *EGL3* and *GL3* overexpression lines were crossed to create a *ttg1-1* mutant background overexpressing both genes. Together the two genes are extremely strong suppressors of the *ttg1* trichome defect (Fig. 1H), causing the plants to produce far more trichomes than wild type. This is similar to *ttg1* plants overexpressing *R*. Many of these trichomes are highly branched, like the *R*-induced trichomes. In addition, trichomes produced by coectopic expression are not distorted.

We also looked at seed coat pigment phenotypes when *GL3* or *EGL3* were overexpressed in *ttg1*. *EGL3* was able to restore some seed coat pigment while *GL3* was not (Fig. 2N). The *GL3/EGL3* co-overexpressing lines produced as much or more seed coat pigment as wild type (Fig. 2N, upper) indicating that GL3 can participate in seed coat pigment regulation.

Suppression of *gl3 egl3* and *tt8* by ectopic *GL3* or *EGL3* expression

As further demonstration that these bHLH proteins have overlapping regulatory capabilities, we overexpressed *GL3* and *EGL3* in the *gl3 egl3* double mutant and in *tt8*. Each construct was able to suppress the double mutant (not shown). Ectopic expression of the *EGL3* genomic clone strongly suppressed, while the *GL3* cDNA or genomic clone weakly suppressed the *tt8* seed coat pigment defect (Fig. 2O) similar to the differential affect seen in *ttg1*. We also found that either gene was able to increase the visible pigment content of the *tt8* mutant hypocotyls (Fig. 2R) but not by much.

The *gl3 egl3 tt8* triple mutant phenocopies *ttg1*

The *tt8* mutant has a transparent testa, like *ttg1*. However, unlike *ttg1* mutants, it produces substantial anthocyanins in the plant body. Mutants blocked early in the anthocyanin pathway lack anthocyanins in the plant as well as having a transparent testa. These include the regulatory mutant, *ttg1* and mutations in structural genes such as chalcone synthase (*tt4*) (Koornneef, 1990; Feinbaum and Ausubel, 1988) and dihydroflavanol reductase (*tt3*) (Koornneef, 1990; Shirley et al., 1992). Anthocyanins in the *tt8* plant body and seed coat in *gl3 egl3* indicates that there is flux through this pathway in both genotypes.

We produced *gl3-1 egl3-1 tt8-1* triple mutants and observed them for anthocyanin production by looking at young hypocotyls, a stage when anthocyanin production is relatively high. While the double mutant produced some visible pigment, no anthocyanin production was observed in any developmental stage of the triple mutant indicating that these three bHLH loci are partially redundant in regulating the anthocyanin pathway. Analysis of the regulation of *TT4* and *TT3* is presented below.

Columellae development and mucilage production were observed in the triple mutants. Recall that *egl3* single and *gl3 egl3* double mutants are partially defective in columellae development and mucilage production, but *tt8* does not appear to have any defect in this pathway (Fig. 2D) (Nesi et al., 2000). Fig. 2E, F and L shows that both the *gl3 egl3 tt8* triple and the *egl3 tt8* double mutants produce collapsed columellae with no releasable mucilage indicating these bHLH loci are partially redundant in mucilage pathway regulation. Our evidence indicates that *GL3* normally plays no role in mucilage production.

EGL3 interacts with GL1 and PAP1 in plants

GL3 and R interact with GL1 when co-overexpressed in *Arabidopsis* to produce more trichomes (Larkin et al., 1994; Payne et al., 2000) and R and C1 interact to produce more anthocyanin pigment (Lloyd et al., 1992). Here we tested whether co-ectopic expression of EGL3 with either of two myb elements, GL1 and PAP1, would give synergistic phenotypes thus indicating interaction. GL1 overexpression alone suppresses trichome initiation on true leaves (Fig. 2S) (Oppenheimer et al., 1991). EGL3/GL1 co-overexpression produces supernumerary trichomes on the hypocotyls and cotyledons and excessive trichomes on the true leaves (Fig. 2T) indicating a synergistic interaction similar to the GL3/GL1 and R/GL1 interactions. EGL3 and PAP1 co-overexpression resulted in more anthocyanin production than in either parent alone indicating a synergistic interaction similar to the R/C1 interaction. This is most easily seen in the anthocyanins present in the normally white petals. PAP1 overexpression alone causes increased anthocyanin production, however, the petals are still essentially white (Fig. 2U, left) (Borevitz et al., 2000). When PAP1 and EGL3 were co-overexpressed, the petals were pink with dark pink veins (Fig. 2U, right). GL3/PAP1 co-overexpression gave the same result (not shown).

Pigment gene expression analysis

For molecular verification that members of this set of bHLH genes regulate anthocyanin production, expression of the anthocyanin biosynthetic genes, *chalcone synthase* (*CHS*) and *dihydroflavonol reductase* (*DFR*) was analyzed in various genetic backgrounds. Quantitative real time RT-PCR was performed on L*er* wild type, *ttg1*, *egl3 gl3* double mutant, and *egl3 gl3 tt8* triple mutant. The comparative C_T method was used to analyze the data. In Fig. 4F, the expression levels of *CHS* and *DFR* in *ttg1* were set to equal 1 and all other levels are relative to that. Kubasek et al. (Kubasek et al., 1992) showed that *TTG1* does not regulate transcription of *CHS*, the first step in the anthocyanin branch of the phenylpropanoid pathway, but it does regulate *DFR*, a later step. Our results agree with that finding. We also found that the bHLH regulators studied here play no appreciable role in regulating *CHS*. However, like *TTG1*, the bHLH regulators positively regulate *DFR* transcription. *DFR* expression in wild type is more than 900-fold greater than in *ttg1*, while *DFR* expression in *gl3 egl3* is 100-fold more (9-fold down from wild type) and *gl3 egl3 tt8* is 15-fold more than in *ttg1* (63-fold down from wild type).

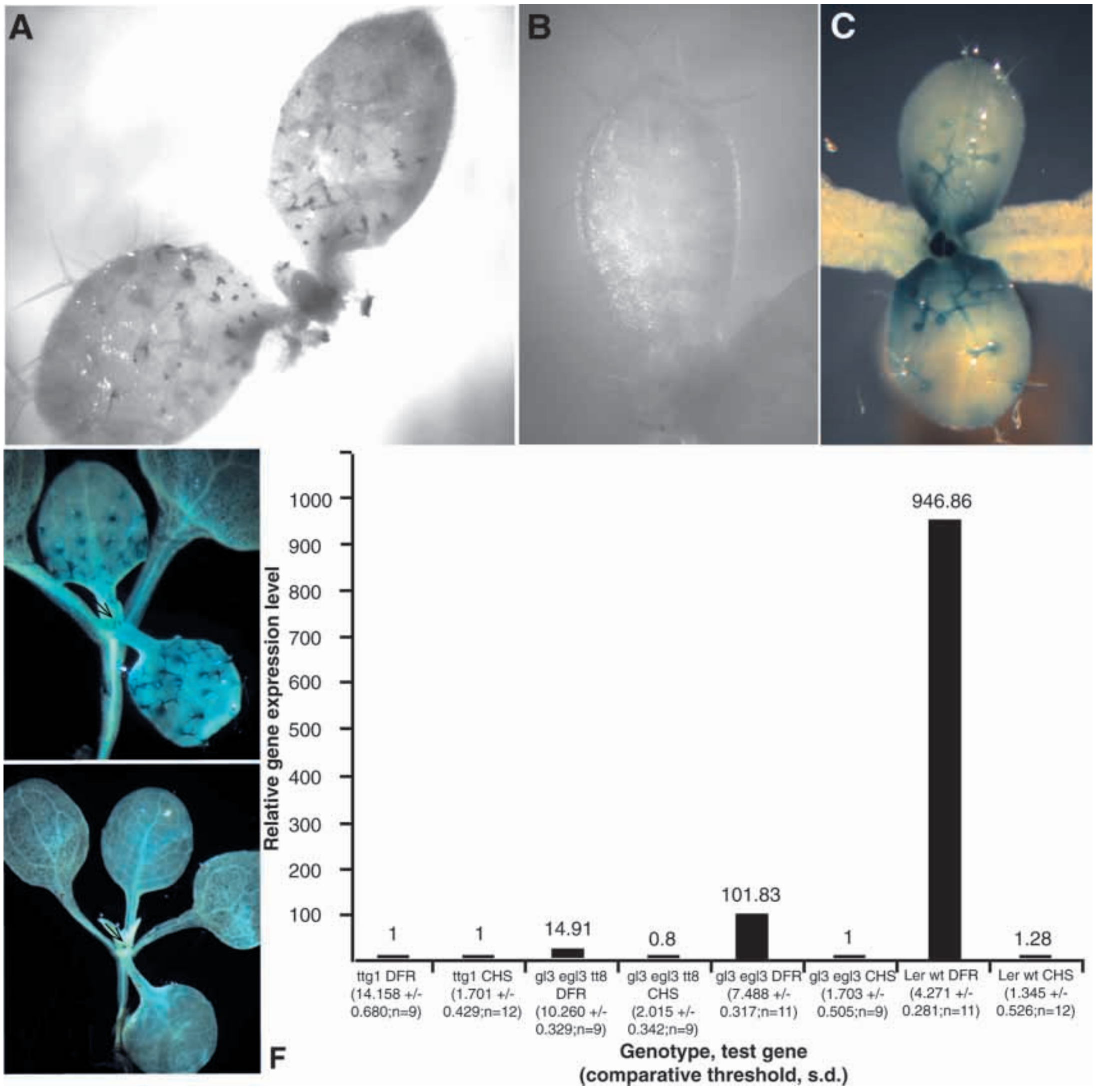

Fig. 4. Gene expression studies. (A-E) In situ RT-PCR of (A) *EGL3* expression, (B) control minus primers, (C) *GL3::GUS* expression pattern, (D) *GL2::GUS* expression in wild type and (E) *GL2::GUS* expression in *egl3 gl3* mutant. (F) LUX RT-PCR analysis of *CHS* and *DFR*. Gene expression levels relative to *ttg1* are given above the bars. C_T values and s.d. are given below the genotype and test gene labels.

Table 2. Positive and negative interaction in yeast two-hybrid analysis

	GL1NTB	P1MDB	P2MDB	CPCDB	TRYDB	GL3CTB	EGL3CTB	TTG1B
GL3A	+	+	+	+	+	+	+	+
GL3NTA	+	+	+	+	+	–	nt	+
GL396A	–	–	–	–	–	+	nt	+
GL3211A	–	–	–	–	–	+	nt	+
GL3CTA	–	–	–	–	–	+	+	–
EGL3NA	+	+	+	+	+	–	nt	+
EGL3CTA	–	–	–	–	–	+	+	–

Vectors and fragments are described in the Materials and methods. nt, not tested.

EGL3 interacts with itself, GL3, TTG1 and myb elements in 2-hybrid analysis

We previously found that GL3 had three distinct protein-protein interaction domains (Payne et al., 2000). (1) The first 100 amino acids were required for interactions with myb proteins GL1, PAP1 and PAP2, CPC and TRY (the latter four presented here; Table 2), (2) approximately amino acids 200-400 mediated interaction with TTG1, and (3) a carboxy end fragment including the bHLH domain was able to interact with itself (homodimerize). We performed a similar but less extensive analysis of EGL3 two-hybrid interactions using two *EGL3* cDNA fragments. The amino fragment contains the first

367 amino acids and excludes the bHLH domain. The carboxy fragment is 229 amino acids long and contains the entire bHLH region. Two-hybrid studies (Table 2) showed that the EGL3 amino fragment interacted with full-length TTG1 and the myb domains of GL1, PAP1 and PAP2, CPC and TRY. The EGL3 carboxy fragment interacted with itself and the full-length and bHLH end of GL3. All EGL3 interactions observed were consistent with a model where EGL3 and GL3 are able to form essentially the same protein-protein interactions.

GL3 and *EGL3* expression pattern in developing leaves

We performed whole-mount in situ RT-PCR to observe the gene expression patterns of *EGL3* and *GL3* in seedlings with developing leaves and trichomes (*EGL3* shown in Fig. 4A). Both genes were expressed in young leaves prior to trichome initiation. They became more highly expressed in initiating and young trichome cells. Expression dropped in the pavement cells between trichomes, in the region of early trichome development, but remained relatively strong in the base of the leaf where active trichome initiation and development was occurring. The expression became very faint or nondetectable in mature trichomes. Both genes had this same pattern but interestingly, *EGL3* was consistently expressed at higher levels. Promoter-GUS fusion experiments showed the same overall expression pattern as well as the higher *EGL3* expression level (*GL3GUS* shown in Fig. 4C). These expression patterns are similar to those reported for *GLABRA1* (Larkin et al., 1993).

GLABRA2 expression in wt and *gl3 egl3* leaves

GL2 expression was observed by using a *GL2* promoter-*GUS* transgenic line that has been extensively used (Masucci et al., 1996; Szymanski et al., 1998). The *GL2GUS* fusion was isolated in the double mutant by crossing and isolating completely glabrous, kanamycin resistant F_2 plants. Fig. 4D shows the typical *GL2* expression pattern in wild-type plants. *GL2* is expressed in young and developing trichomes but is not strongly expressed in young leaves as opposed to *EGL3* and *GL3*. In the *gl3 egl3* mutant, *GL2* expression is not detected in the leaves (Fig. 4E). It is interesting that we see relatively strong *GL2* expression in the stipules of wild-type plants, which remains strong in the mutant (arrows in Fig. 4D,E). *GL1* is reported to be expressed in stipules (Oppenheimer et al., 1991; Larkin et al., 1993) but neither *GL3* or *EGL3* expression is obvious in stipules.

Discussion

EGL3 defines a role for bHLH proteins in all TTG1-dependent pathways

A preponderance of circumstantial evidence indicated that there should be bHLH-level control of all the TTG1-dependent processes in *Arabidopsis*. This evidence has been outlined above and includes: (1) the fact that ectopic expression of some bHLH proteins can suppress all or subsets of the *ttg1*-defective phenotypes and, (2) the fact that myb genes regulating these processes have been identified in *Arabidopsis* and that a postulated bHLH partner for most of them has not been identified. However, until now, genetics had revealed only two such bHLH genes, *GL3* and *TT8*, and these genes had only partial control over only a subset of the TTG1-dependent pathways.

We hypothesized that *GL3* was redundant with the At1G63650 locus in the trichome pathway at least. So an enhancer screen in the *gl3-1* mutant background was performed, looking for mutations in new loci that result in totally bald plants. Our strategy was to sequence the At1G63650 locus in any new complementation group that required the *gl3* mutation to show the hairless phenotype, i.e. mutated loci that were hypostatic to *gl3* mutations. A new complementation group was identified with lesions shown to be stop codons in exons of the At1G63650 (*EGL3*) locus.

Isolation of mutations in the *EGL3* locus allowed us to characterize the central role for bHLH proteins in all TTG1-dependent processes and show that this central role has been masked by partial functional redundancy, an increasingly common theme in *Arabidopsis*.

Although we initially only screened for a trichome defect, the *gl3 egl3* double mutant was noted to have defects in the other developmental pathways regulated by TTG1. These include anthocyanin production and the related seed coat tannin production, position-dependent root hair spacing, and seed coat mucilage production. It was found that the new double mutant was partially defective in anthocyanin production, defective in root hair spacing, partially defective in seed coat mucilage production, but apparently normal for the production of the tannin seed coat pigment.

The transparent testa or *tt* series of phenylpropanoid mutants are missing seed coat tannin. *TT8* was cloned and shown to encode a bHLH protein responsible for the seed coat tannin but no other phenotypes were reported (Nesi et al., 2000). We combined the *tt8* mutation with *gl3*, *egl3* and the *gl3 egl3* mutations and found that *tt8* and *egl3* are partially redundant for the seed coat mucilage production. *egl3-1* single mutant has a mucilage phenotype similar to the *gl3-1 egl3-1* double mutant and when *tt8* is combined with *egl3-1* as either double or triple mutant, the seed coats are devoid of releasable mucilage and the columellae are collapsed. The *tt8*, *gl3* single and *tt8 gl3* double mutants all produce apparently normal amounts of mucilage and have pronounced columellae, indicating that *GL3* plays little or no role in this process. As expected, all mutant combinations containing the *tt8* mutation have a transparent testa.

The *tt8* single mutant produces significant amounts of visible anthocyanin pigment in the seedling. However, the *egl3 gl3* and *egl3 gl3 tt8* mutant combinations do not. Molecular data indicate that including the *tt8* mutation drives down *DFR* expression more than 6-fold from the double to the triple mutant. This indicates that *tt8* probably plays some role in activating anthocyanin pathway genes in the plant, but this experiment is complicated by the fact that the *tt8-1* mutation used here is in a different ecotype and other genetic factors may be at work. Shirley et al. (Shirley et al., 1995) also found that the *tt8-1* mutant had downregulated *DFR* but not *CHS* in seedlings, but with the same ecotype caveat as the present work. Also consistent with our work, they showed that *DFR* but not *CHS* was even further downregulated in the *ttg1* mutant, but that *DFR* expression was still detectable.

bHLH proteins and genetic interactions

We previously reported interactions between GL3 and other proteins in the yeast two-hybrid system. These include interactions with GL1, TTG1 and self interactions. These interactions occurred through separate domains included in roughly the first 100 amino acids, amino acids 200-400 and the carboxy end including the bHLH region, respectively. A two-hybrid analysis with EGL3 demonstrates the same GL1 and TTG1 interactions as GL3, and that GL3 and EGL3 can form heterodimers, and that the EGL3 carboxy end forms homodimers. The myb-protein anthocyanin regulators PAP1 and PAP2 were both found to interact with EGL3 and GL3 through the same GL1 interacting protein fragments. Myb proteins have been identified that regulate all of the TTG1-dependent pathways and we predict that each of these will interact with one or more of the three bHLH proteins that are the subject of this paper. The mybs not tested here include the root hair position regulator WEREWOLF (Lee and Schiefelbein, 1999), the seed coat pigment regulator TT2 (Nesi et al., 2001), and the seed coat mucilage regulator MYB61 (Penfield et al., 2001). GL1, PAP1/2, WER, TT2 and MYB61 are so-called R2R3 mybs, containing two myb repeats and an acidic transcriptional activation domain. We have further shown that both GL3 and EGL3 interact with the single myb repeat repressors, TRY and CPC, and that all of these myb interactions occur through the same amino domains of GL3 and EGL3.

We indirectly tested for some of these interactions in plants by looking for hypermorphic phenotypic synergism between co-overexpressed bHLH and myb regulators. We found that we could detect interactions between EGL3 or GL3 and GL1 and PAP1 and between EGL3 and GL3. When co-overexpressed, each of these combinations gave phenotypes that were far more severe than can be explained by additive regulation leading us to conclude the regulators are interacting, probably at the protein-protein level.

Extensive work has been done to show required interactions between the myb proteins C1 or Pl and the bHLH containing proteins, R or B, to activate the anthocyanin pathway in maize and to show that these proteins interact in yeast two-hybrid assays (Goff et al., 1992). The general protein-protein interactions presented here are consistent with this earlier work. However, in maize and *Antirrhinum* (Goodrich et al., 1992), the bHLH anthocyanin regulators are not reported to be involved in the regulation of any other pathways. In *Petunia*, the myb/bHLH protein interactions also hold (Quattrocchio et al., 1998), however, as in *Arabidopsis*, at least one bHLH protein appears to regulate more than just one pathway. AN1 regulates anthocyanin production, vacuole pH and seed coat cell shape (Spelt et al., 2002).

Differential function for GL3, EGL3, and TT8

Our mutant analysis indicates that the three bHLH proteins have overlapping but different functions in *Arabidopsis*. TT8 alone regulates seed coat pigment, probably participates in regulating anthocyanin biosynthesis in the plant, and shares seed coat mucilage regulation with EGL3. We have not uncovered any evidence that TT8 functions in the trichome or root hair pathways. EGL3 functions with GL3 in the root hair pathway and trichome pathway but has a much smaller effect on trichome development than GL3 as a single mutant. GL3 does not appear to affect seed coat mucilage at all. GL3 and EGL3 together regulate anthocyanin accumulation in the hypocotyl with EGL3 apparently having a larger role.

The *GL3* cDNA gives very different trichome phenotypes when overexpressed in the *ttg1* mutant than either the *GL3* or *EGL3* genomic fragments. We have not yet tested the *EGL3* cDNA and it may behave like the *GL3* cDNA. It is also interesting that overexpressed *EGL3* genomic fragment is a much better suppressor of seed coat pigment defects of the *tt8* and *ttg1* mutations and the mucilage defect of the *ttg1* mutation than either *GL3* genomic or cDNA fragments. It may be that GL3 does not normally function in the seed coat and that it is unable to productively interact with the seed coat mybs that regulate pigment and mucilage production.

Model for epidermal cell-fate and differentiation

The data presented in this and other papers indicate that the TTG1 protein directly interacts with a set of three bHLH proteins and these bHLH proteins directly interact with a larger set of myb elements (Fig. 5). The genetic evidence is that the pleiotropic spectrum narrows as one moves down this regulatory hierarchy. Mutations in *TTG1* affect the maximum number of pathways while mutations in the bHLH proteins affect overlapping subsets of the pathways that TTG1 regulates. Apparently none of the bHLH proteins affect all of the pathways and none are specific to one. As far as we can tell, none of the three regulate pathways that are not regulated by TTG1.

Much of the specificity for pathways and tissues seems to lie with the myb proteins. For example, *gl1* and *wer* mutations only affect trichome and root hairs respectively, although they can substitute for each other when cis-regulatory regions are swapped (Lee and Schiefelbein, 2001). The PAP mybs cause increased anthocyanin production when overexpressed but they do not affect trichome initiation or root hair production (mucilage has not been observed). *TT2* and *Myb61* myb

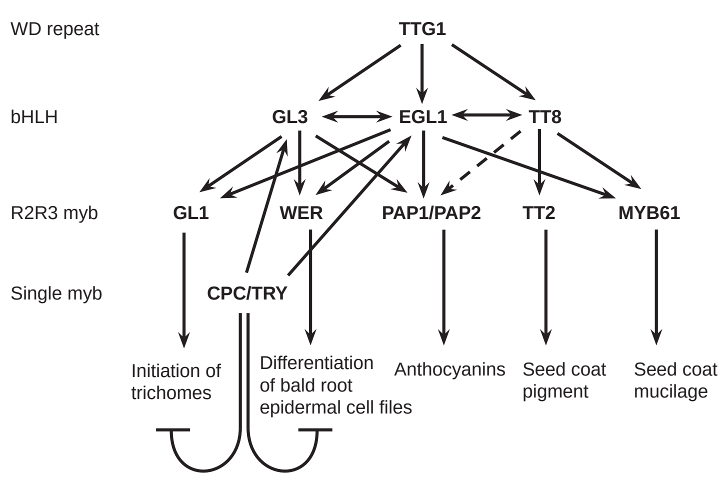

Fig. 5. Regulatory network model. This reticulated model shows all of the known bHLH and myb transcriptional regulators that function the TTG1-dependent pathways. The model illustrates the potential (broken lines) and demonstrated (unbroken lines) interactions among the proteins and genes, the narrowing of the specificity of the regulatory function as one goes from WD to bHLH to myb, and the redundancies demonstrated at the bHLH and single myb levels.

mutations also appear to be specific for seed coat pigment and mucilage production respectively. The exceptions to this specificity rule are the single myb repeat proteins Tryptichon and Caprice. These partially redundant proteins repress near neighbor root and shoot epidermal cells from assuming the same differentiation states. Double *cpc try* mutants exhibit both trichome and root hair defects that are not seen in the single mutants (Schellman et al., 2002). The myb activators appear to affect one specific pathway regulated by TTG1, and like the bHLH proteins, none of the mybs affect pathways not regulated by TTG1.

A possible mechanism by which single MYB repeat proteins cause inhibition of a particular pathway is by competition with R2R3 MYBs for binding to bHLHs. In a cell destined to be a trichome or a root non-hair cell, an activator complex consisting of TTG1s-bHLHs-MYBs activates transcription of genes required for cell fate differentiation and possibly the repressor genes. The repressors then move to neighboring cells where they bind to bHLHs and form a non-activating or repressive complex consisting of TTG1s-bHLHs-single MYB repressor. Evidence for this model comes from the fact that the repressors of cell fate are transcribed in cells that have adopted the trichome or non-root hair cell fate (Schellmann et al., 2002), and in the case of root cells, CPC repressor protein accumulates in root hair (repressed) cells (Wada et al., 2002). Evidence for bHLH proteins as the binding targets of single MYB repressors is suggested by yeast-two-hybrid results demonstrating that MYBs can interact with bHLHs but not with TTG1 or each other.

The discovery of EGL3 and additional functions for GL3 and TT8 completes the search for the missing bHLH proteins required in the regulation of all TTG1-dependent pathways. These proteins have been hypothesized to exist (Lloyd et al., 1992; Lee and Schiefelbein, 1999; Payne et al., 2000; Schellmann et al., 2002) but have been largely disguised by functional redundancy within the genome. This analysis raises many new questions for this reticulated regulatory hierarchy. Identification of the bHLH components of TTG1-dependent regulation will allow the study of how these key developmental complexes function in the plant.

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