

The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci

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A network of three classes of proteins consisting of bHLH and MYB transcription factors, and a WD40 repeat protein, TRANSPARENT TESTA GLABRA1 (TTG1), act in concert to activate trichome initiation and patterning. Using YFP-TTG1 translational fusions, we show that TTG1 is expressed ubiquitously in *Arabidopsis* leaves and is preferentially localized in the nuclei of trichomes at all developmental stages. Using a conditional transgenic allele, we demonstrate that TTG1 directly targets the same genes as the bHLH protein GLABRA3 (GL3). In vivo binding of the R2R3-MYB protein GLABRA1 (GL1) to the promoters of GLABRA2 (GL2), TRANSPARENT TESTA GLABRA2 (TTG2), CAPRICE (CPC) and ENHANCER OF TRIPTYCHON AND CAPRICE1 (ETC1) establishes that these genes are major transcriptional targets for the TTG1-bHLH-MYB regulatory complex. By co-precipitation, we confirm that TTG1 associates with GL3 and GL1 in vivo, forming a complex. The loss of TTG1 and GL1 through mutation, affects the subcellular distribution of GL3. Using particle bombardment, we show that TTG1, GL3, GL1 and the homeodomain protein GL2 do not move between adjacent epidermal cells, while the R3-MYB, CPC, does move to neighboring cells. These data support a model for the TTG1 complex directly regulating activators and repressors and the movement of repressors to affect trichome patterning on the *Arabidopsis* leaf.

KEY WORDS: Epidermis, Pattern formation, Trichome, Gene regulation, Cell differentiation, Leaf, *Arabidopsis thaliana*, Cell fate, Transcription, TTG1

INTRODUCTION

Studies on the regulation of cell fate and function on the plant epidermis continue to provide important insights into how plant cells are organized, how patterning develops, and how developmental and biochemical pathways interact. Trichome initiation in the model plant *Arabidopsis thaliana* has been an important model for understanding cell fate and patterning. Trichomes (leaf hairs) are large branched single cells that initiate and develop on young leaves in a regular spacing pattern (Larkin et al., 1997; Marks, 1997; Hulskamp and Schnittger, 1998; Hulskamp et al., 1999). Trichome patterning is not random or dependent on other cell types or position on the leaf, but is thought to be generated de novo by intercellular communication (Larkin et al., 1996; Schnittger et al., 1999). The model assumes that inhibitors, which are activated by self-enhanced activators, can move between cells to mediate competition between equivalent cells, resulting in the pattern formation (Larkin et al., 2003; Pesch and Hulskamp, 2004).

Years of genetic and molecular studies have enabled the identification of components of this trichome patterning machinery. Three classes of interacting regulators [including the R2R3-MYB transcription factor GLABRA1 (GL1) (Oppenheimer et al., 1991), the basic helix-loop-helix (bHLH) proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne et al., 2000; Zhang et al., 2003), and the WD40 repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Walker et al., 1999)] are postulated to form a combinatorial regulatory complex. Evidence comes from yeast two-hybrid studies showing that TTG1 and GL1 physically interact with

GL3/EGL3 but not with each other (Payne et al., 2000; Zhang et al., 2003). GLABRA2 (GL2) is a direct target of GL3 and EGL3 (Morohashi et al., 2007) and TRANSPARENT TESTA GLABRA2 (TTG2) is directly regulated by GL1 (Ishida et al., 2007). This activation is believed to be through the formation of TTG1-GL3-GL1 and TTG1-EGL3-GL1 (TTG1-bHLH-GL1) regulatory complexes (Szymanski et al., 1998), thereby regulating trichome cell fate. GL2, a homeodomain (HD-Zip), and TTG2, a WRKY transcription factor, are required for normal trichome development (Rerie et al., 1994; Johnson et al., 2002). Some levels of GL2 overexpression can result in trichome clusters, indicating that this HD-Zip may function in the regulation of trichome spacing (Ohashi et al., 2002).

To date, a group of at least four homologous single MYB proteins [TRIPTYCHON (TRY) (Schellmann et al., 2002), CAPRICE (CPC) (Wada et al., 1997), and ENHANCER OF TRY and CPC1 and 2 (ETC1 and 2) (Kirik et al., 2004a; Kirik et al., 2004b)] have been identified as negative regulators of trichome initiation and patterning. The *try cpc* double and the *try cpc etc1* triple mutants (Kirik et al., 2004a; Schellmann et al., 2002) display a greatly enhanced 'clustered-trichome' phenotype, indicating that lateral inhibition is disrupted. These inhibitory proteins contain no recognizable transcription activation domain. Therefore, they could work as negative transcriptional regulators. Protein interaction analysis in yeast has suggested that TRY or CPC would interrupt the functionality of the 'activating' TTG1-bHLH-GL1 complex by competitive interaction with the bHLH (Esch et al., 2003; Zhang et al., 2003). Additionally, the individual members of this inhibitory protein family may function differently. There is evidence that TRY might be more important in short-range inhibition, while CPC and particularly ETC1 may be important for long-range inhibition (Schellmann et al., 2002; Kirik et al., 2004a).

As described above, the identification of these positive and negative trichome regulators has laid an excellent foundation for understanding trichome patterning. However, a large amount of the

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data that elucidate the molecular mechanism of these regulators is either indirect or obtained from another similar pathway – root hair patterning. For example, evidence for the existence of the TTG1-bHLH-MYB complex is based entirely on protein interaction studies in yeast (Payne et al., 2000; Zhang et al., 2003; Zimmermann et al., 2004). Furthermore, the only evidence demonstrating the ability of a single MYB inhibitor to move between cells is that CPC-GFP fusion protein is detected both in the trichoblasts and in the atrichoblasts in roots when its transcript is found only in hairless cells (Wada et al., 2002). More importantly, the regulatory events triggered by the TTG1-bHLH-MYB active complex mostly remain unknown. The expression of *CPC* in the root epidermis is *GL3/ EGL3* dependent (Bernhardt et al., 2005) and directly regulated by the MYB WEREWOLF (WER) (Lee and Schiefelbein, 2002; Koshino-Kimura et al., 2005; Ryu et al., 2005), a GL1 equivalent protein in root hair patterning (Lee and Schiefelbein, 2001).

In recent work, we have shown that *GL2*, *CPC* and *ETC1* are directly activated by GL3, and this targeting is GL1 dependent (Morohashi et al., 2007). The work presented here is aimed at further testing and refining details of the trichome development model under the control of the TTG1-bHLH-MYB complex. Here, we show that the trichome activators *GL2* and *TTG2*, and repressors *CPC* and *ETC1* are major transcriptional targets for the complex. In addition, we also demonstrate the existence of the TTG1-bHLH-MYB complex in plants and show that loss of TTG1 or GL1 disrupts the distribution of GL3. Furthermore, we demonstrate that the CPC protein moves in the leaf epidermis, whereas none of the activators tested move. These results support major aspects of the model and also add novel perspectives to the current model for trichome patterning.

MATERIALS AND METHODS

Plasmids

Plasmid descriptions are below. Details of plasmid constructions will be provided on request. Sequences of primers used can be provided on request.

pTTG1::YFP-TTG1 and *pTTG1::TTG1-cMYC* contain a *TTG1* genomic fragment, including 1 kb of 5' and 3' regulatory sequences, and either the *YFP*-coding region without a stop codon inserted in frame with the *TTG1* start codon or five copies of the *cMYC* epitope inserted in frame with *TTG1* with the stop codon removed.

pGL1::GL1-YFP-cMYC and *pGL1::GL1-YFP-6His* contain a *GL1* genomic fragment, including 1.45 kb of 5' and 1 kb of 3' regulatory sequences, and either a *YFP-cMYC* (*5XcMYC*) or *YFP-6His* fusion inserted in frame with *GL1* with the stop codon removed.

p35S::HA-GL3-6His contains the CaMV35S promoter driving the *GL3* genomic coding region with both the *HA* epitope in frame with the *GL3* start codon and the *6His* epitope in frame with *GL3* with the stop codon removed.

p35S::GL3-YFP, *p35S::GL1-YFP*, *p35S::GL2-YFP* and *p35S::YFP-CPC* contain the entire *GL3* or *GL1* genomic coding regions or the *GL2*-coding cDNA, with the stop codons removed, or the entire *CPC* cDNA cloned into appropriate CaMV35S-YFP fusion cassette vectors.

pEGL3::EGL3-YFP contains a 6 kb *EGL3* genomic fragment containing 3 kb upstream of the start with a deleted stop codon cloned in frame to *YFP*.

Plant materials and growth conditions

Ler/pGL3::GUS and *Ler/pEGL3::GUS* have been described previously (Zhang et al., 2003). *gl3 egl3/p35S::GL3-GR* has been described previously (Morohashi et al., 2007). *ttg1/p35S::TTG1-GR* seeds (Baudry et al., 2006) were generously provided by Dr Loïc Lepiniec. *gl3-2/pGL3::GL3-YFP* was previously described (Bernhardt et al., 2005). To generate *gll/pGL3::GL3-YFP* and *ttg1/pGL1::GL1-YFP-cMYC*, *gl3-2/pGL3::GL3-YFP* was crossed to *gll* and *gll/pGL1::GL1-YFP-cMYC* was crossed to *ttg1*. Plants expressing both *TTG1::TTG1-cMYC* and *GL1::GL1-YFP-6His* fusions were created by crossing *gll/pGL1::GL1-YFP-6His* to *ttg1/pTTG1::TTG1-cMYC*. F2 plants were confirmed by YFP fluorescence microscopy and

western blots probed with an anti-cMYC antibody. Lines expressing both *TTG1::TTG1-cMYC* and *35S::HA-GL3-6His* fusions were created by transforming *ttg1/pTTG1::TTG1-cMYC* plants with *p35S::HA-GL3-6His*. Transformants were identified by kanamycin and BASTA double resistance. All transgenic plants were created by floral dip transformation. Standard plant crosses were carried out with two homozygotes and the F1 were selfed to identify proper progeny. *Arabidopsis* plants were grown on soil at 21°C in continuous white light.

Gene expression analyses

Seedlings were grown on MS media containing 3% sucrose at 21°C in continuous white light. Four-day-old seedlings were treated with 20 µM dexamethasone (DEX) or mock-treated with 0.001% ethanol for 4 hours, washed with water and frozen in liquid nitrogen. Cycloheximide (CHX, 100 µM) treatment was used when appropriate. Total RNA was prepared according to Morohashi et al. (Morohashi et al., 2007). RNA (4 µg) was used in 20 µl reverse transcription reactions containing 250 nM actin and target gene-specific reverse primers. Parallel 25 µl PCR reactions were prepared using cDNA reactions as templates with half volume of 2× SuperPower Syber mixture (ABI) and run on a spectrofluorometric thermal cycler (ABI 7900HT). For each target, five PCR reactions containing 400 nM primers and 3 µl first strand target gene cDNA as template were performed alongside four actin control PCR reactions containing 200 nM actin primers and 1 µl first strand actin cDNA. The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System). Each experiment was performed twice for each target with consistent results. Results of representative experiments are presented.

Chromatin immunoprecipitation (ChIP) experiments

ChIP experiments were performed as described previously (Morohashi et al., 2007).

Microscopy

The histochemical analyses of *Promoter::GUS* reporter genes were performed with at least five seedlings for each strain essentially as described (Masucci et al., 1996).

Imaging of YFP fusions was performed on a Leica SP2 AOBs confocal laser scanning microscope with excitation (488 or 514 nm) and emissions (530–600 nm for YFP and 675–800 for chlorophyll). Collected images were processed for maximum intensity projection.

Microprojectile bombardment

Tungsten particles (1.5 mg) were coated with ~5 µg of each plasmid DNA as directed by the manufacturer's instructions (BioRad). Young leaves from *gl3 egl3* double mutant plants were excised, placed on MS plates and bombarded at 1100 psi with a flight distance of 15 cm using a Bio-Rad PDS-1000. Bombarded leaves were placed overnight under white light and imaged on the confocal microscope. At least three independent bombardment experiments were performed with each construct with multiple bombardment events in each experiment so that over 50 events were observed for each construct.

Co-precipitation experiments

Three-week-old *Arabidopsis* green tissue was ground into fine powder in liquid nitrogen. Protein extract was prepared by thorough mixing of 0.1 g powder with 1 ml ice-cold buffer A (50 mM Tris, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1 mM DTT, 1% Triton-X100, 1 mM PMSF, 1 µg/ml each of (Leupeptin, Antipain, Pepstatin A, Aprotinin), 5 mM imidazole, pH 7.3) in a 1.5 ml eppendorf tube. The mixture was centrifuged twice at 13,000 rpm for 10 minutes, and the supernatant was used as input extract. Input extract (0.9 ml) was applied to a pre-equilibrated His-select column (with buffer A), washed (with buffer A containing 45 mM imidazole) and eluted (with buffer A containing 300 mM imidazole) as directed by the manufacturer (Sigma). The elution was concentrated with Microcon Y-M30 filter (Millipore). Input extracts and concentrated eluates were mixed with loading buffer to final volume of 100 µl and boiled for 5 minutes prior to loading the SDS-PAGE gel (Bio-Rad). Input (2 µl) and 5 µl of elution loading samples were used for western blots, which were probed by anti-

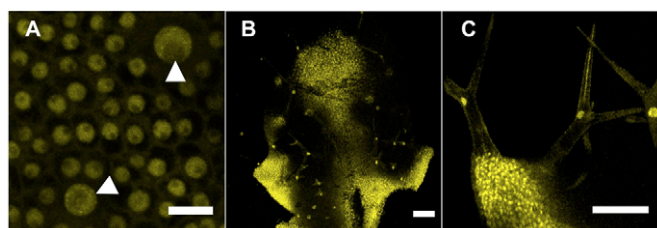


Fig. 1. Expression pattern of YFP-TTG1 fusion in the leaf epidermis. Maximum intensity projection images of confocal stacks of a TTG1::YFP-TTG1 construct in developing leaves of 20-day-old *ttg1* mutant seedlings. (A) Trichome initials (arrows) and surrounding cells. (B) Overview of a developing young leaf. This leaf is not flat so that in some areas the pavement cells are in focus and in other areas, focus is higher up on the trichomes. (C) Mature trichomes and surrounding cells. Scale bars: 10 μ m in A; 100 μ m in B; 50 μ m in C.

cMYC monoclonal antibody 9E10 (Santa Cruz Biotechnology) and visualized by Western Lightning Chemiluminescence Reagents (Amersham Biosciences).

RESULTS

TTG1 is expressed ubiquitously in *Arabidopsis* leaves

The transcription of TTG1 is detected in all major organs of *Arabidopsis* (Walker et al., 1999). To study the expression of the TTG1 protein during the process of trichome initiation and patterning, we examined YFP fluorescence of a YFP-TTG1 fusion protein under the control of the native *TTG1* promoter in the *ttg1* mutant background (*ttg1*/pTTG1::YFP-TTG1). The transgenic *ttg1*/pTTG1::YFP-TTG1 plants showed wild-type trichome formation (Fig. 1), as well as normal anthocyanin production, and seed coat pigment and differentiation (not shown), indicating that the translational YFP-TTG1 fusion was functional. At early stages, strong YFP signal is detected in the nuclei of trichome initials and of all pavement cells, with a much weaker YFP signal in the cytoplasm of these cells (Fig. 1A). Fig. 1B shows that the TTG1 protein is present in all epidermal cells and trichomes at all developmental stages. This ubiquitous and persistent expression pattern was further confirmed by a close-up view of the YFP expression in stage 5 trichomes (Szymanski et al., 1998) and their surrounding epidermal cells (Fig. 1C). TTG1 protein appears to be expressed at all stages of leaf and trichome development.

TTG1 regulates GL3 target genes

GL3 has been reported to target genes that regulate trichome development directly, both trichome activators and repressors (Morohashi et al., 2007). To better define the trichome genes regulated by the TTG1-bHLH-MYB regulatory complex, the expression changes of previously identified GL3 targets, including *GL3*, *GL2*, *ETC1* and *CPC* (Morohashi et al., 2007) were investigated by quantitative PCR (Q-PCR) in DEX-treated *ttg1*/p35S::TTG1-GR plants, in the presence or absence of CHX. The TTG1-GR fusion complements *ttg1* mutants only with the addition of DEX. Simultaneous treatment with DEX and CHX blocks de novo protein production and allows only the direct targets to be transcribed (Sablowski and Meyerowitz, 1998). This same TTG1-GR line has been used to show that the bHLH TT8 was directly activated by TTG1 in siliques (Baudry et al., 2006).

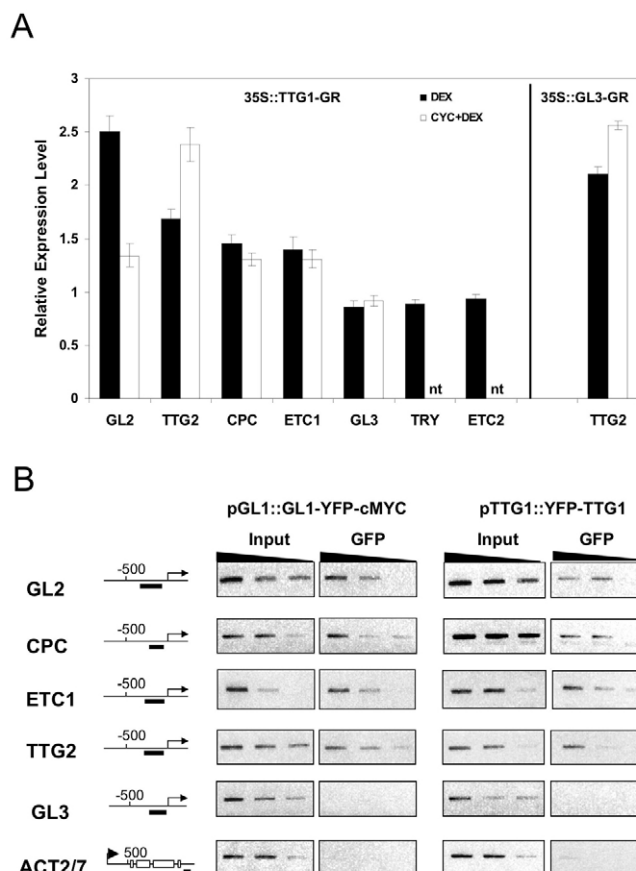


Fig. 2. Direct activation of GL3 target genes by 35S::TTG1-GR.

(A) Gene expression levels were measured by relative quantitative PCR. The results were calculated using the comparative Ct method (ABI bulletin) and presented as fold changes compared with the mock or CHX treatment, which were standardized to the level of actin expression. The induced expression levels of *GL2*, *TTG2*, *CPC* and *ETC1* were statistically significantly different from those of control treatments ($P < 0.05$); error bars indicate the ranges of expression change; nt, not tested. (B) Semi-quantitative PCR of ChIP experiments using *ttg1*/pTTG1::YFP-TTG1 (left) or *gl1*/pGL1::GL1-YFP-cMYC (right). PCRs were performed on three fourfold serial dilutions of the immunoprecipitated material, represented by the black slope.

In other work, we have shown that this fusion provides DEX-dependent activation of the late anthocyanin structural genes (Gonzalez et al., 2008).

As shown in Fig. 2A, *GL2*, *CPC* and *ETC1* were upregulated in response to the 4-hour induction by DEX, while the expression of *GL3*, *TRY* and *ETC2* did not change. This experiment was repeated with a DEX plus CHX treatment. *GL2*, *CPC* and *ETC1* again were upregulated, but to a lower level (Fig. 2A). A two-sided *t*-test indicates that these induction levels are significantly greater than uninduced levels ($P < 0.05$). To confirm that these expression results are due to direct activation, chromatin immunoprecipitation (ChIP) experiments were performed with *ttg1*/pTTG1::YFP-TTG1 plants, using antibodies against GFP which cross-react with YFP. Similar to what was previously described for *GL3* (Morohashi et al., 2007), YFP-TTG1 was recruited to the promoters of *GL2*, *CPC* and *ETC1* in vivo (Fig. 2B). These results show that *GL2*, *CPC* and *ETC1* are immediate direct targets of TTG1, indicating that TTG1 and GL3 share many of the same targets.

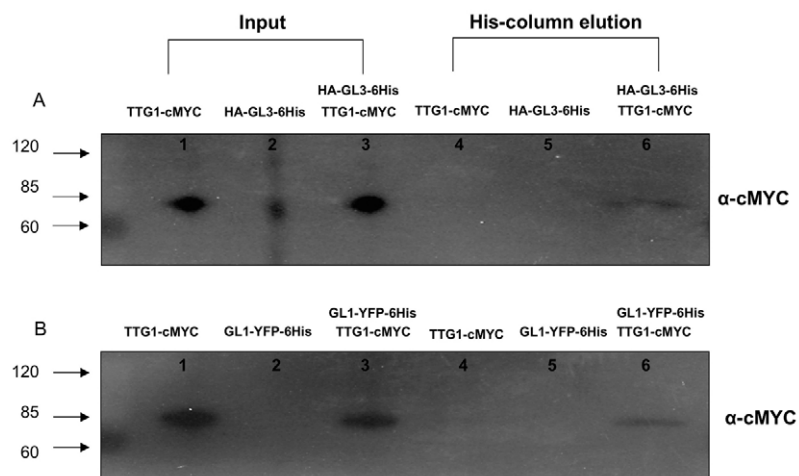


Fig. 3. Co-precipitation of TTG1-cMYC with HA-GL3-6His and GL1-YFP-6His from seedling extracts. His-Select Ni columns were used to pull down 6His-tagged fusion proteins. Input and eluted proteins were separated by SDS-PAGE gels in the order labeled. Membranes were probed with anti-cMYC mAb. In vivo interaction between TTG1 and GL3 is indicated in **A** and between TTG1 and GL1 in **B**.

***TTG2* is an immediate direct target of TTG1 and GL3**

Genetic data show that the expression of *TTG2* requires *TTG1* (Johnson et al., 2002), suggesting that TTG1 and GL3 directly control *TTG2* expression in vivo. We analyzed *ttg1/p35S::TTG1-GR* and *gl3 egl3/p35S::GL3-GR* transgenic seedlings for expression changes in *TTG2* after DEX induction. Four-hour DEX induction of TTG1-GR and GL3-GR resulted in the upregulation of *TTG2* (Fig. 2A). Inclusion of DEX and CHX also resulted in the significant induction ($P < 0.05$, two-sided *t*-test) of *TTG2*, and identified it as a direct target of both TTG1 and GL3. This finding is confirmed by ChIP results, unequivocally demonstrating that TTG1 binds to the promoter of *TTG2* in vivo (Fig. 2B).

GL1 participates in the regulation of *GL2*, *TTG2*, *CPC* and *ETC1*

It has previously been shown that WER binds the *CPC* promoter in vitro (Koshino-Kimura et al., 2005; Ryu et al., 2005) and it has been recently reported that GL1 directly regulates *TTG2* (Ishida et al., 2007). We have shown that GL3 regulates and binds the promoters of *GL2*, *CPC*, *ETC1* and *GL3* in vivo. The binding of GL3 to the *CPC* and *GL2* promoters is dependent on the presence of GL1, while binding to its own promoter is not (Morohashi et al., 2007). We used *gll/pGL1::GL1-YFP-cMYC* plants to perform ChIP experiments to investigate the in vivo binding of GL1 to the promoters of these known GL3 targets. GL1 was found to bind to the promoters of *CPC*, *ETC1* and *GL2*, as well as that of *TTG2*, but not of *GL3* (Fig. 2B). These results suggest that GL1 participates with GL3 in the regulation of *GL2*, *CPC*, *ETC1* and *TTG2*, but not in the auto-regulation of *GL3*. Taken together with the finding that *GL2*, *TTG2*, *CPC* and *ETC1* are direct transcriptional targets of both GL3 and TTG1, while *GL3* is regulated only by GL3, it is most likely that *GL2*, *TTG2*, *CPC* and *ETC1* are activated by a complex containing TTG1, GL3 and GL1.

It is interesting that the QRT-PCR analyses with cycloheximide seem to reveal additional, non-TTG1-dependent regulatory effects with GL2 being activated and *TTG2* being repressed by other factors. In these experiments, we only conclude that a gene is a direct target if the RT-PCR and the ChIP experiments are in agreement.

TTG1 associates with GL3 and GL1 in vivo

The gene expression studies presented above support the hypothesis that TTG1 participates in a TTG1-bHLH-MYB activation complex but do not directly demonstrate that TTG1 and

GL1 co-exist in a complex. To detect this complex in vivo, we performed co-precipitation assays to test whether TTG1 associates with GL3. The *TTG1::TTG1-cMYC* and *35S::HA-GL3-6His* fusions are functional in promoting trichome differentiation in *ttg1* and *gl3 egl3* mutants respectively. As shown in Fig. 3A, the TTG1-cMYC fusion was detected in the input protein extractions of plants containing this construct (lanes 1 and 3) using an anti-cMYC monoclonal antibody. However, when His-select Ni columns were used to affinity purify the HA-GL3-6His fusion protein from these extracts, TTG1-cMYC was detected only in the line containing both fusion proteins (Fig. 3A, lane 6), demonstrating that TTG1 associates with GL3 in vivo.

Using the same approach, we also tested for an association between TTG1 and GL1 in vivo. Strikingly, TTG1-cMYC was also purified by the His-select Ni columns only when it was co-expressed with GL1-YFP-6His (Fig. 3B, lane 6), while TTG1-cMYC was not detected in the samples processed from *ttg1/pTTG1::TTG1-cMYC* or *gll/pGL1::GL1-YFP-6His* (Fig. 3B, lanes 4, 5), demonstrating that TTG1 associates with GL1 in vivo.

These results do not indicate that TTG1 directly touches GL1 and when combined with yeast 2-hybrid analysis (Payne et al., 2000; Zhang et al., 2003), these results indicate that TTG1 and GL1 associate in a complex by both binding to GL3 or EGL3 as intermediates.

Loss of TTG1 and GL1 disrupts the nuclear distribution of GL3

Experiments were performed to test whether TTG1 and GL1 affect the GL3 protein distribution pattern in the leaf epidermis. A functional *GL3::GL3-YFP* fusion (Bernhardt et al., 2005) was examined in the *gl3-1* mutant plant. We detected GL3-YFP signal restricted to the nuclei of trichome cells with an evenly distributed fluorescence pattern (Fig. 4A). When *GL3::GL3-YFP* was introduced into the *ttg1* mutant background, no obvious changes in the partitioning of GL3 to the nucleus was observed. However, the GL3-YFP protein was unevenly distributed into speckles in the nuclei of young epidermal cells and this increases with age (Fig. 4B). GL3::YFP eventually fades away as these cells mature. By contrast, young epidermal cells of the *ttg1* mutant showed evenly distributed GL1-YFP with only a couple of speckles in the nucleus (Fig. 4C). These results suggest that TTG1 is required for the proper subnuclear distribution of GL3. Although it is difficult to quantitatively compare these images, it does not appear that loss of TTG1 affects the stability of the GL3-YFP fusion.

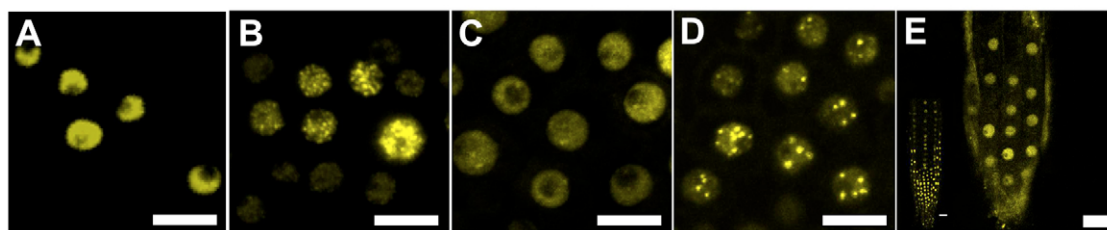


Fig. 4. Speckled nuclear distribution of GL3 in the leaf epidermis of *ttg1* and *gl1* mutants. Confocal images of (A) *gl3*/pGL3::GL3-YFP, (B) *ttg1*/pGL3::GL3-YFP, (C) *ttg1*/pGL1::GL1-YFP-cM, (D) *gl1*/pGL3::GL3-YFP and (E) *gl1*/pGL3::GL3-YFP. GL3-YFP is unevenly distributed and forms nuclear speckles in leaf epidermal cells of *ttg1* and *gl1* mutants (B,D). GL1-YFP formed only a couple of speckles in nuclei in occasional leaf epidermal cells (C). Uniform GL3-YFP distribution in *gl3* leaf epidermal cells (A), and *gl1* root epidermal cells (inset picture shows GL3::GL3-YFP accumulation in hairless cell files forming wild type-looking stripes) (D). Scale bars: 10 μ m.

To test whether mutations in GL1 might affect the distribution of GL3, we examined the subcellular localization of GL3-YFP in the *gl1* mutant. When *GL3::GL3-YFP* was expressed in the *gl1* mutant, GL3 still partitioned to the nucleus. However, just like the *ttg1* mutant, GL3 formed speckles in the nuclei of leaf epidermal cells (Fig. 4D). In the roots of the same transgenic plant, where GL1 function is replaced by WER, GL3-YFP showed wild-type patterning with no speckles (Fig. 4E). These results suggest that GL1 is specifically required for the normal distribution of GL3 within the nuclei of *Arabidopsis* leaf cells.

Taken together, our studies on in vivo protein associations and the subcellular localization of fluorescent fusion proteins show that TTG1, GL3 and GL1 form a nuclear complex in vivo. Moreover, the loss of TTG1 or GL1 leads to an abnormal speckled distribution of GL3, a key complex member.

CPC moves in leaf epidermal cells

It has been shown that GL3 and CPC traffic from cell to cell in the *Arabidopsis* root epidermis to specify near neighbor cell fate (Wada et al., 2002; Bernhardt et al., 2005). We hypothesized that similar

movements might be required during trichome patterning events. YFP fusions to TTG1, GL3, GL1, CPC and GL2 were used to examine whether any of these proteins could move from cell to cell in the leaf epidermis. The fusion genes were introduced into developing leaf tissue by microprojectile bombardment and were scored after overnight expression. We also bombarded a *35S::GUS* reporter and we did not detect any area with clusters of transformed GUS-expressing cells, indicating that the probability of bombarding adjacent cells is very low (data not shown).

We repetitively observed extensive trafficking of the YFP-CPC fusion into adjacent cells, as evidenced by cytoplasmic and nuclear YFP signal (CPC moved in 32 of 76 bombardment events), generating clusters of up to 15 fluorescent cells in the *Arabidopsis* leaf epidermis (Fig. 5D; see Fig. S1 in the supplementary material). By contrast, we did not observe the same fluorescent pattern with any of the other fusion proteins, which were expressed in isolated single cells (Fig. 5); at least 50 bombardment events were observed with each gene. These results show that CPC can move in the leaf epidermis, but that GL3 does not. Our results, showing that CPC but

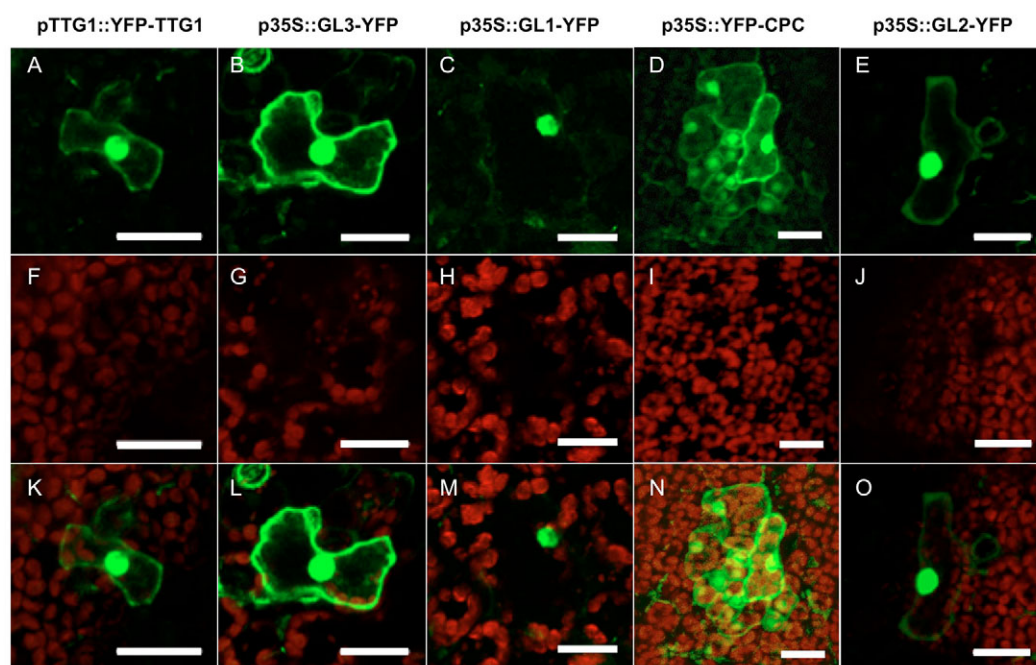


Fig. 5. Intercellular trafficking of YFP-CPC in the leaf epidermis. Confocal images of bombarded YFP fusion proteins. (A-E) YFP fluorescence (green). (F-J) Chlorophyll fluorescence (red). (K-O) Merged images. Only YFP-CPC shows cell-to-cell movement forming a cluster of fluorescent cells (D,N). TTG1, GL3, GL1 and GL2 fusions are cell autonomous, as fluorescence is restricted to single cells. Guard cells, as in the upper left of B, are often autofluorescent under these conditions. Scale bars: 10 μ m.

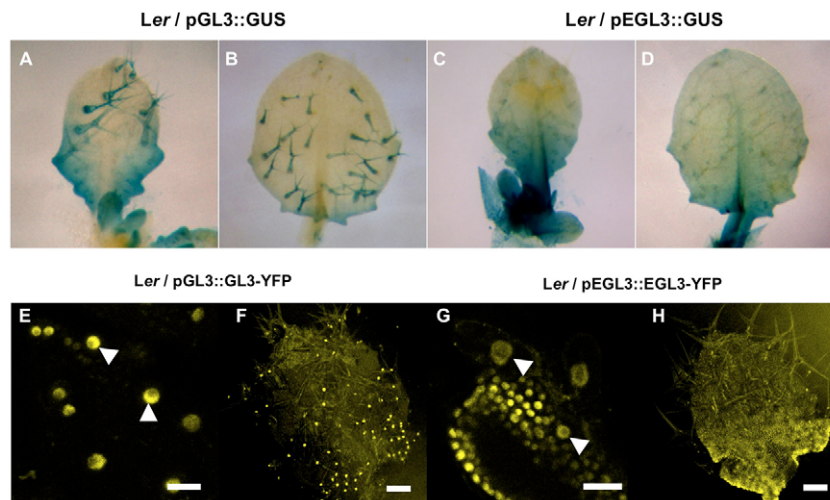


Fig. 6. GL3 and EGL3 have overlapping but distinct expression patterns. (A-D) Transcription patterns of *GL3* and *EGL3* promoter::GUS in wild-type leaves. (A,C) At a very young stage, both *GL3* and *EGL3* are strongly transcribed in undifferentiated leaf primordia; in later differentiating leaves, high GL3 is observed in undifferentiated cells close to the basal edge of the leaf, while EGL3 is more widespread and not restricted to the edge; trichome initials show higher levels of GL3 and EGL3 than surrounding epidermal cells. (B,D) At a more mature stage, strong GL3 expression becomes restricted to trichomes, while EGL3 expression persists in pavement cells as well as in trichomes. (E-H) Protein accumulation patterns of GL3 and EGL3 in wild-type leaves. (E,F) GL3-YFP highly accumulates in trichome initials (arrowheads) and young trichomes. (G,H) At the same stages, strong EGL3-YFP was detected in the trichomes (arrowheads) and non-trichome cells. Scale bars: 20 μ m in E,G; 50 μ m in F,H.

not GL3 moves in the leaf epidermis, contrast with previous findings that they both move in roots (Bernhardt et al., 2005; Wada et al., 2002). This probably reflects that fact that trichome patterning and root hair patterning are not regulated by the same mechanisms, although they largely share the same hierarchy of regulatory genes. In roots, GL3 must move from the hair cell files, where it is transcribed, to the hairless files where it functions. In leaves, it is transcribed in the trichome initials, where it functions, and so is not required to move. It is possible that there is some developmental control of intercellular movement; however, we observed CPC movement no matter where on the leaf we bombarded (see Fig. S1 in the supplementary material) while GL3 never moved. We note that we were not able to successfully bombard the very youngest and smallest cells on the leaf epidermis.

GL3 and EGL3 have overlapping but distinct expression patterns

Our previous studies showed that *GL3* and *EGL3* are partially redundant in regulating trichome initiation. The *gl3 eg13* double mutant is completely glabrous (Zhang et al., 2003). However, single *gl3* mutants show a much more severe reduction in trichome initiation and branching than *egl3* (Zhang et al., 2003), and we have shown that *GL3* but not *EGL3* participates in an auto-regulatory loop (Morohashi et al., 2007). In order to begin to characterize the functional differences between GL3 and EGL3, we carefully examined the *Promoter::GUS* expression and protein accumulation patterns of *GL3* and *EGL3* during trichome development in wild-type plants.

Maximum GUS activity was observed in young leaf primordia for both *GL3* and *EGL3* (Fig. 6A,C). In young developing leaves, GL3::GUS activity is observed especially in the region close to the basal edge of the leaf (Fig. 6A). In the same age leaves, high EGL3::GUS activity is observed in the basal one-third of the leaf and is not restricted to the edge (Fig. 6C). In both lines, maturing and mature trichomes show significantly higher levels of GUS

activity than surrounding epidermal cells (Fig. 6A,C). In more mature leaves, strong GL3::GUS activity becomes restricted to trichomes (Fig. 6B), while EGL3::GUS persists at low levels in pavement cells as well as in trichomes (Fig. 6D). Compared with *GL3*, *EGL3* exhibits a more widely distributed transcription pattern with higher GUS activity in the epidermal pavement cells than GL3 and lower GUS activity in trichomes than GL3. High EGL3::GUS activity is also observed in the petioles of leaves, while GL3::GUS is not. Taken together, GL3 and EGL3 show overlapping, yet distinct, transcription patterns during trichome development.

The *GL3::GL3-YFP* and *EGL3::EGL3-YFP* fusions were constructed and shown to be fully functional by rescuing *gl3 eg13* mutants (not shown). The analysis of the YFP fluorescence profiles of representative wild-type *GL3::GL3-YFP* and *EGL3::EGL3-YFP* containing transgenic plants shows that the protein expression profiles of GL3 and EGL3 generally match well with their transcription patterns, respectively, with some notable differences.

In the basal region of the developing leaf, where trichomes continue to initiate, strong GL3-YFP signal was detected in the nuclei of unbranched trichome initials, while only a very weak GL3-YFP signal was occasionally detected in the neighboring non-trichome cells (Fig. 6E arrows, F). As a trichome matures, the level of GL3-YFP intensity keeps decreasing until it completely disappears (not shown). Like GL3-YFP, EGL3-YFP was also found to increase in the nuclei of trichome initials in the leaf basal region but not as high as GL3. However, EGL3-YFP was also detected in the nontrichome cells throughout the epidermal layer of a developing leaf (Fig. 6G,H).

A comparison of patterns of *GL3::GUS* and *GL3::GL3-YFP* reveals a difference between the transcription pattern and the protein expression pattern of GL3. Significant GL3::GUS activity was observed in the epidermal cells that neighbor young trichomes where GL3 protein is absent (compare Fig. 6A with 6E). Taken together with the finding that EGL3 gene is expressed and the EGL3

protein accumulates in both trichome and non-trichome cells, these data imply that EGL3 functions within the non-trichome cell in the maintenance of the non-trichome cell fate, while GL3 does not.

DISCUSSION

The *Arabidopsis* *TTG1* locus encodes a WD40 protein containing four WD40 repeat motifs but no recognizable nuclear localization signal, DNA-binding motif or transcriptional activation domain (Walker et al., 1999). A common function of WD40 repeat motifs is to facilitate protein-protein interactions. A preponderance of indirect evidence indicated that TTG1 interacts with bHLH proteins (GL3, EGL3 and TT8) in regulating all TTG1-dependent development pathways. The evidence includes: (1) *gl3 egl3 tt8* triple mutant phenocopies *ttg1* mutant; and (2) TTG1 physically interacts with GL3, EGL3 and TT8 in the yeast-two hybrid system (Payne et al., 2000; Zhang et al., 2003; Baudry et al., 2004). TTG1 may form a complex with bHLH proteins for nuclear import or retention and/or act as a transcriptional co-regulator. Prior to the present study, it was also possible that TTG1 was located only in the cytoplasm, possibly as a signal transduction component to regulate bHLH proteins. A cytoplasmic location would be in agreement with the reported location of AN11 (de Vetten et al., 1997), a petunia WD40 protein that is highly similar to TTG1 and complements the *ttg1* mutation (not shown). In this paper, we report that TTG1 is preferentially localized in the nucleus in the *Arabidopsis* leaf epidermis (Fig. 1), with apparently a lower, yet significant, amount of TTG1 in the cytoplasm. This result indicates that TTG1 could function both as a transcriptional co-regulator in the nucleus and as a protein-interacting factor in the cytoplasm.

The TTG1-bHLH-MYB regulatory complex

Although we have demonstrated that TTG1 associates with GL3 in vivo (Fig. 3), the biological significance of the TTG1-bHLH interaction still remains to be elucidated. Our previous genetic data (Zhang et al., 2003), together with the results discussed in this paper, favors the possibility that TTG1 functions as a transcription co-regulator. TTG1 may modify, stabilize or in some other fashion positively affect GL3/EGL3 in their capacity to activate the transcription of downstream target genes. Our work on the regulation of the anthocyanin pathway shows that GL3 and TTG1 regulate the same set of anthocyanin biosynthetic target genes (Gonzalez et al., 2008). It would not be surprising that TTG1 and GL3 regulate the same target genes in the trichome development pathway. Our results using a TTG1-GR inducible system show that *GL2*, *CPC* and *ETC1* are also direct targets of TTG1, because the transcription of these genes increased significantly in response to TTG1-GR induction even in the absence of de novo protein synthesis. We have also identified *TTG2* as an immediate direct target of both TTG1 and GL3 (Fig. 2), which is consistent with the finding that *TTG2* is directly regulated by GL1 (Ishida et al., 2007). These data show that TTG1 largely regulates the transcription of the same regulatory loci as GL3 during trichome cell fate specification. It also supports the notion that TTG1 regulates the trichome pathway through affecting the activation capacity of bHLH proteins.

Interestingly, we failed to detect any changes in *GL3* expression after TTG1-GR induction, as opposed to the finding that *GL3* is repressed by GL3-GR (Morohashi et al., 2007). It has been reported that GL3 binds to and activates *GL2*, *CPC* and *ETC1* in a GL1-dependent manner, but the GL3 self-repression is GL1-independent (Morohashi et al., 2007). In our ChIP experiments with *gll/pGL1::GL1-YFP-cMYC*, we detected the in vivo recruitment of GL1 to the *GL2*, *TTG2*, *CPC* and *ETC1* promoters but not to the

promoter of *GL3* (Fig. 2B). These data suggest that the GL1 DNA-binding activity is required for the TTG1-bHLH complex to select target genes and that GL3 self-repression may be both GL1 and TTG1 independent. Additionally, the in vivo association of TTG1 and GL1 (Fig. 3B) fits perfectly with the model that TTG1, bHLH and R2R3-MYB proteins form a TTG1-bHLH-MYB regulatory complex in vivo. The TTG1-bHLH-MYB complex seems to only activate the transcription of downstream targets but not the transcription of bHLH or R2R3-MYB proteins in the trichome pathway.

We could not detect changes in the expression of *TRY* or *ETC2* by the induction of *gll3 egl3/p35S::GL3-GR* (Morohashi et al., 2007) or *ttg1/p35S::TTG1GR* (Fig. 2A). These results demonstrate that although *TRY* and *ETC2* are largely redundant with *CPC* and *ETC1*, they are regulated differently, perhaps by GL2 for example, which is consistent with their different levels of expression in different tissues (Kirik et al., 2004b).

How does TTG1 function?

GL3 transcripts can be easily detected in the *ttg1* and *gll* mutants (Payne et al., 2000), indicating that they are not required for the transcription of *GL3*. We wanted to determine whether TTG1 might regulate the subcellular localization of GL3. In the *ttg1* mutant, we found that the GL3-YFP protein was still located entirely in the nucleus. Surprisingly, however, the loss of TTG1 caused GL3 to be abnormally distributed within the nucleus of leaf epidermal cells. GL3 protein forms unevenly distributed ‘speckles’ (Fig. 4B). By contrast, the nuclear distribution pattern of GL1-YFP-cMYC in *ttg1* is very similar to the wild-type pattern – a more or less even nuclear distribution. One or two GL1 speckles were found in a single nucleus (Fig. 4C). These results suggest that functional TTG1 protein is required for the appropriate bHLH distribution in the nucleus but is largely not necessary for GL1 distribution.

In *gll* mutants, we also detected a speckled GL3-YFP distribution specifically in the leaf epidermis (Fig. 4D,E). However, GL3 forms fewer but more clearly isolated nuclear speckles in *gll* than in *ttg1* (compare Fig. 4B and 4D). We previously showed that in a *gll* mutant, GL3 is no longer recruited to the promoter of its major trichome targets, *GL2* and *CPC* (Morohashi et al., 2007). Taken together, we conclude that GL1 is responsible for GL3 or the TTG1-bHLH complex tethering to the promoters of specific downstream targets, and TTG1 may function as a ‘helper’ for the bHLH::GL1 interaction. Loss of proper DNA and/or protein interactions leads to aberrant bHLH distribution.

Besides participating in the TTG1-bHLH-MYB regulatory complex, TTG1 may regulate trichome genes through other mechanisms. It was recently shown that TTG1 physically interacts with GEM, a protein that modulates cell division and represses the expression of *GL2* and *CPC* in *Arabidopsis* roots. Overexpression of *GEM* caused increased root hair and decreased leaf trichome densities (Caro et al., 2007). Overexpressed GEM is shown to bind to the promoters of *GL2* and *CPC*, and is associated with the acquisition and/or maintenance of histone H3K9me2 (typical of silent heterochromatic regions) at these two genes. These data imply that the interaction between TTG1 and GEM could prevent GEM from joining a complex that represses the expression of *GL2* and *CPC* or other trichome genes.

Trichome patterning

In theoretical models (Meinhardt, 1994; Meinhardt and Gierer, 2000), it is proposed that de novo patterning often requires the local self-enhancement of activators in combination with lateral inhibition by inhibitors. Based on this theory, a common model is

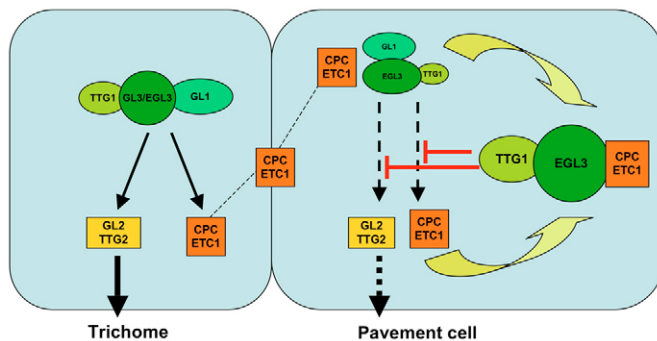


Fig. 7. Model for *Arabidopsis* trichome/non-trichome cell fate specification. Regulators of trichome fate are green shades, activators (GL2/TTG2) are yellow and inhibitors (CPC/ETC1) are orange. Black arrows indicate transcriptional activation. In trichome cells, the inhibitors are directly activated by the activating complex and move (broken lines) into neighboring cells, where they and endogenous inhibitors block the activity of the activating complex thereby decreasing the expression of GL2/TTG2 to below a required initiating threshold level (broken arrows). Thus, the trichome cell fate is not triggered.

proposed for the *Arabidopsis* trichome and root hair patterning, in which single MYB repressors (CPC and TRY) are thought to be able to move (faster than activators if activators can also move) into neighboring cells (Pesch and Hulskamp, 2004). In support of such a model is the fact that although CPC-GFP proteins are expressed in non-root hair cell files, the CPC-GFP protein is also detected in the neighboring root hair files (Wada et al., 2002). In this paper, our microprojectile bombardment experiment with 35S::YFP-CPC directly demonstrates the ability of CPC to move in the leaf epidermis for the first time (Fig. 5), strongly supporting the current trichome patterning model from this perspective. YFP-CPC protein was detected in clusters of epidermal cells, generally one cell but up to two cells away from the bombardment center, suggesting CPC could move from one cell to another (Fig. 5D). As we discussed, long-range repressors, CPC and ETC1 (Kirik et al., 2004a), are directly activated by the TTG1-bHLH-MYB complex, while the short-range repressor TRY is not. This may indicate that the accumulation of the active TTG1-bHLH-MYB complex in the trichome initials triggers primarily long-range inhibition but not short-range inhibition.

In addition, we also tested the movement potential of TTG1, GL1 and GL2, and we find that these proteins do not move in the leaf epidermis under the same condition where CPC moves. Another issue deserving special attention is that GL3 did not move from cell to cell in the leaf epidermis, in contrast to our earlier finding that GL3 moves between root cell files (Bernhardt et al., 2005). By examining the protein accumulation and *Promoter::GUS* expression patterns, we find that GL3 is transcribed and the protein accumulates in the trichome initials and GL3 is transcribed in the surrounding epidermal cells but the GL3 protein is not detectable there (Fig. 6). The apparent lack of GL3 movement in the leaf, coupled with the transcription and protein pattern may indicate that the absence of GL3 protein in epidermal cells is not caused by GL3 trafficking into developing trichomes, but rather by some form of post-transcriptional or translational regulation.

The current model of trichome patterning is largely based on genetic analysis and on molecular data obtained from the root hair system. The data presented in this paper demonstrate that a similar

molecular mechanism by a TTG1-bHLH-MYB regulatory complex directly activating downstream targets is responsible for trichome patterning. Based on this mechanism, we have refined the model for trichome patterning. As shown in Fig. 7, a functional activating complex TTG1-GL3/EGL3-GL1 activates trichome activators (GL2 and TTG2) and single MYB repressors (CPC and ETC1) in the cell chosen to be a trichome. CPC and ETC1 then move into the neighboring cells where they, together with locally expressed repressors, compete with GL1 for binding to EGL3, forming an inactivating complex, TTG1-EGL3-CPC/ETC1. This inactivating complex disrupts the function of the activating complex. The decreased concentration of the TTG1-EGL3-GL1 complex in these surrounding epidermal cells is not enough to activate GL2 and TTG2 beyond a required initiating threshold level, and the trichome cell fate is not triggered.

Our results also show differences between the trichome and root hair pathways at the molecular level: GL3 is preferentially transcribed in the cells where it functions during trichome development, while GL3 is transcribed in root hair cell files, and accumulates and functions in non-root hair cell files during root hair patterning. This raises many new questions for this regulatory network. Identification of the molecular components that mediate the differentiation of bHLH expression patterns in different tissues will allow the study of how these key developmental complexes are regulated in the plant.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/11/1991/DC1>

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