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The Trihelix Transcription Factor GTL1 Regulates Ploidy-Dependent Cell Growth in the *Arabidopsis* Trichome [W] [OA]

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Leaf trichomes in *Arabidopsis thaliana* develop through several distinct cellular processes, such as patterning, differentiation, and growth. Although recent studies have identified several key transcription factors as regulating early patterning and differentiation steps, it is still largely unknown how these regulatory proteins mediate subsequent trichome development, which is accompanied by rapid cell growth and branching. Here, we report a novel trichome mutation in *Arabidopsis*, which in contrast with previously identified mutants, increases trichome cell size without altering its overall patterning or branching. We show that the corresponding gene encodes a GT-2-LIKE1 (GTL1) protein, a member of the trihelix transcription factor family. GTL1 is present within the nucleus during the postbranching stages of trichome development, and its loss of function leads to an increase in the nuclear DNA content only in trichomes that have completed branching. Our data further demonstrate that the *gtl1* mutation modifies the expression of several cell cycle genes and partially rescues the ploidy defects in the cyclin-dependent kinase inhibitor mutant *siamese*. Taken together, this study provides the genetic evidence for the requirement of transcriptional regulation in the repression of ploidy-dependent plant cell growth as well as for an involvement of GTL trihelix proteins in this regulation.

INTRODUCTION

Plant trichomes are highly specialized epidermal cells implicated in several important functions, including transpiration control, freezing tolerance, and protection against insects, diseases, and UV light (Johnson, 1975; Mauricio and Rausher, 1997). Trichomes are also of special commercial interest because they have a unique secondary metabolism, allowing the production and secretion of various useful natural compounds (Wang et al., 2008; Besser et al., 2009). Leaf trichomes in *Arabidopsis thaliana* develop through several distinct cellular processes, and over the last decade, they have become an ideal model system to study various developmental processes, such as cell patterning, differentiation, and morphogenesis, at the single cell level (Hulskamp et al., 1994).

Trichomes are the first epidermal cells that undergo cell specification in the *Arabidopsis* leaf primordia (Hulskamp et al., 1994; Larkin et al., 1996). The subsequent gradual development of leaf trichomes, ranging from their initiation at the leaf base to maturation at the leaf tip, can be easily followed on a single, young leaf (Hulskamp et al., 1994; Szymanski and Marks, 1998). From several mutagenesis screens in the past, a vast number of mutations affecting trichome development have been isolated (Hulskamp et al., 1994; Marks, 1997), and their molecular and genetic analyses have helped dissecting the pathways that underlie different phases of trichome development. Trichome patterning is governed by positive and negative regulators of trichome initiation. Mutations affecting the positive regulators *TRANSPARENT TESTA GLABRA1* (*TTG1*) or *GLABRA1* (*GL1*) result in impaired trichome initiation (Oppenheimer et al., 1991; Galway et al., 1994), and the corresponding *TTG1* and *GL1* loci encode WD40 protein and MYB-related transcription factors, respectively (Oppenheimer et al., 1991; Larkin et al., 1994; Walker et al., 1999). Strong *ttg1* and *gl1* mutants fail to develop large epidermal trichome precursor cells with polyploid nuclei, suggesting that endoreduplication cycles are a prerequisite for trichome initiation (Hulskamp et al., 1999). Further studies have shown that the basic helix-loop-helix transcription factor *GL3* and its close homolog *ENHANCER OF GLABRA3* (*EGL3*) are also positive regulators of trichome initiation. Single mutants for *GL3* and *EGL3* exhibit a small or no reduction in trichome number, whereas the *gl3 egl3* double mutants are completely trichomeless, demonstrating that *GL3* and *EGL3* function in a partially redundant manner (Payne et al., 2000; Zhang et al., 2003). *GL3*, together with *TTG1* and *GL1*, is known to activate the expression of downstream target genes, including an HD-Zip transcription factor *GL2* and a WRKY transcription factor *TTG2*, which also act as positive regulators of trichome initiation (Rerie et al., 1994; Johnson et al., 2002; Ishida et al., 2007; Morohashi et al., 2007; Zhao et al., 2008). By contrast, the *TRIPTYCHON* (*TRY*) gene encodes a single-repeat MYB protein that acts as a negative

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regulator of trichome initiation (Schellmann et al., 2002). In *try* mutants, trichome initiation in cells adjacent to trichomes is not repressed, leading to the formation of trichome clusters. Recent studies have shown that five other single-repeat MYB homologs, *CAPRICE* (*CPC*), *ENHANCER OF TRY AND CPC1* (*ETC1*), *ETC2*, *ETC3/CPC-LIKE-MYB3*, and *TRICHOMELESS1* (*TCL1*), also participate in trichome development, and all, except TCL1, which is required for the stem and pedicel trichome formation, act redundantly with TRY in leaf trichome initiation (Esch et al., 2004; Kirik et al., 2004a, 2004b; Schellmann et al., 2007; Wang et al., 2007; Tominaga et al., 2008; Wester et al., 2009).

After cell fate specification, trichome cells enter a highly complex differentiation program that proceeds through two major growth phases (Melaragno et al., 1993; Hulskamp et al., 1994; Schnittger et al., 1998). During the first phase, cells undergo several rounds of endocycles, leading to a final ploidy of 32C in most trichomes and substantial increase in cell size. The first growth phase is also defined by radial expansion and epidermal outgrowth, which leads to two successive branching events (Folkers et al., 1997). The branching process is then followed by the second growth phase, which is defined by rapid cell expansion mainly driven by vacuolization (Hulskamp et al., 1994; Szymanski et al., 1999). Previous genetic studies suggest that the typical three-dimensional branching pattern of trichomes is regulated by several intersecting genetic pathways that modify ploidy, cell size, and/or cytoskeletons (Hulskamp et al., 1994; Folkers et al., 1997; Perazza et al., 1999; Sugimoto-Shirasu et al., 2002; Ilgenfritz et al., 2003). In addition to the trichome patterning defects, *try* mutants exhibit over-branched, over-endoreduplicated trichomes with an overall increase in trichome cell size (Hulskamp et al., 1994; Schnittger et al., 1998). By contrast, *gl3* mutants display decreased ploidy, decreased trichome size and under-branching (Hulskamp et al., 1994), suggesting that TRY and GL3 also participate as negative and positive regulators, respectively, in the trichome development beyond the initial patterning and differentiation stage. Furthermore, several other mutations, such as *kaktus* (*kak*), *rastafari* (*rfi*), *polychome* (*pym*), and *spindly* (*spy*), that also cause alterations in trichome ploidy, size, and branching have been identified (Hulskamp et al., 1994; Perazza et al., 1999), highlighting the strong coupling between ploidy, cell size, and branching in trichome development.

The tight coupling between endocycling and trichome development is also supported by several lines of experimental evidence demonstrating that the up- or downregulation of key cell cycle genes directly modifies the trichome patterning and/or morphogenesis. For example, mutations in the *SIAMESE* (*SIM*) gene, encoding a plant-specific cyclin-dependent kinase (CDK) inhibitor, result in clustered multicellular trichomes with individual nuclei having reduced ploidy levels (Walker et al., 2000; Churchman et al., 2006). The SIM proteins function within the D-type cyclin (CYC)-CDKA;1 complex, which normally promotes the progression of mitotic cycles (Schnittger et al., 2002), and the interaction between SIM and the CYCD-CDKA complex appears to result in the repression of mitotic cycles, thus allowing an entry into the endocycle (Churchman et al., 2006). CDKA;1 is also known to form a regulatory complex with an A-type cyclin, CYCA2;3, in developing trichomes to repress the progression of endocycles (Imai et al., 2006). Correspondingly, mutations in CYCA2;3 cause an increase in ploidy and trichome over-branching (Imai et al., 2006). Another key regulator implicated in the regulation of endocycling is the CCS52/Fizzy-Related (FZR) family protein, which functions as an activator of anaphase-promoting complex (Cebolla et al., 1999; Lammens et al., 2008; Vanstraelen et al., 2009). A recent study demonstrates that at least one of the *Arabidopsis* homologs, CCS52A1/FZR2, is involved in the promotion of trichome endocycling (Larson-Rabin et al., 2009).

In this study, we identified a novel trichome mutant in *Arabidopsis*, which unlike all other previously described trichome mutants, only affects trichome size without altering their overall patterning or branching. We demonstrate that the corresponding gene encodes a plant-specific trihelix transcription factor, GT-2-LIKE1 (GTL1), and that it functions as a negative regulator of ploidy-dependent cell growth during postbranching phases of trichome development.

RESULTS

Cauliflower Mosaic Virus 35S Promoter–Driven Expression of Full-Length or Truncated GTL1 cDNA Leads to an Increase in Trichome Cell Size

In a screen of the *Arabidopsis* FOX (full-length cDNA over-expression) collection (Ichikawa et al., 2006) for gain-of-function mutants that display aberrant sizes of trichome cells, we identified a transgenic line, F26519, that shows substantial increase in trichome cell size (Figure 1A). The observed phenotype segregated dominantly within the original T2 population (43 seedlings with large trichomes and 14 seedlings with wild-type trichomes, $n = 57$, $\chi^2 = 0.003$, $P > 0.9$), and PCR genotyping using primers specific for FOX vectors revealed cosegregation of the genotype and the phenotype, suggesting that the observed phenotype is caused by the overexpression of F26519 cDNA in the FOX vector. Furthermore, the cauliflower mosaic virus 35S promoter (CaMV35S)–driven expression of F26519 cDNA in wild-type background recapitulated the large trichome phenotype among all selected transformants (Figure 1A, R26519), confirming the identity of the causal gene. Sequencing analysis revealed that F26519 cDNA corresponds to a truncated version of the *Arabidopsis GTL1* cDNA that lacks 750 bp in the central region and 4 bp in the C-terminal region. These deletions are likely to be an artifact caused during the construction of the full-length cDNA library (Seki et al., 2002), rather than the products derived from alternative splicing in vivo, since the borders of the deleted cDNA fragments do not match conventional eukaryotic splice sites. Consistently, our RT-PCR using RNA from 2-week-old wild-type seedlings amplified a 2010-bp cDNA that corresponds to the annotated full-length *GTL1*.

GTL1 belongs to the *GT-2* gene family, a subfamily of the plant-specific trihelix transcription factor family (Smalle et al., 1998; Zhou, 1999). The *Arabidopsis* genome encodes 29 trihelix proteins, each of which contains at least one trihelix DNA binding domain, and depending on additional conserved motifs, they are classified into several subfamilies (Gao et al., 2009). In addition to *GTL1*, six other trihelix proteins, including previously described

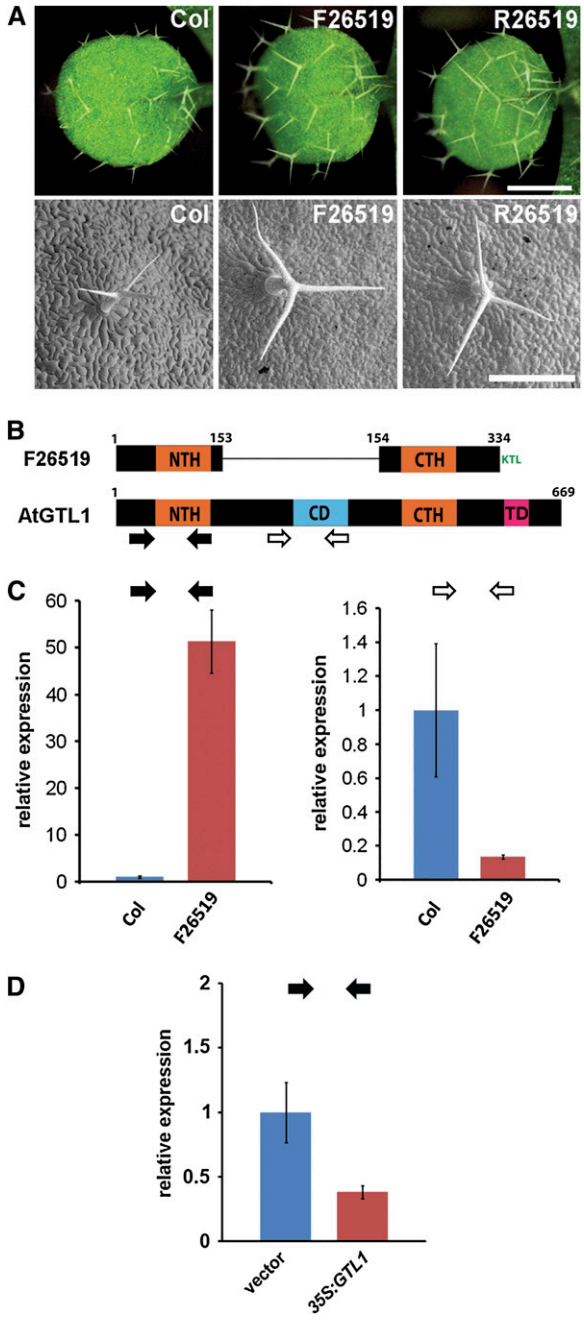


Figure 1. *CaMV35S* Promoter–Driven Expression of Full-Length or Truncated *GTL1* cDNA Increases Trichome Cell Size.

(A) Bright-field and scanning electron microscopy of the first true leaves of 10-d-old wild type (Columbia [Col]), FOX (F26519), and wild type retransformed with *GTL1*^{F26519} (R26519). Bars = 1 mm (top panels) and 400 μm (bottom panels).

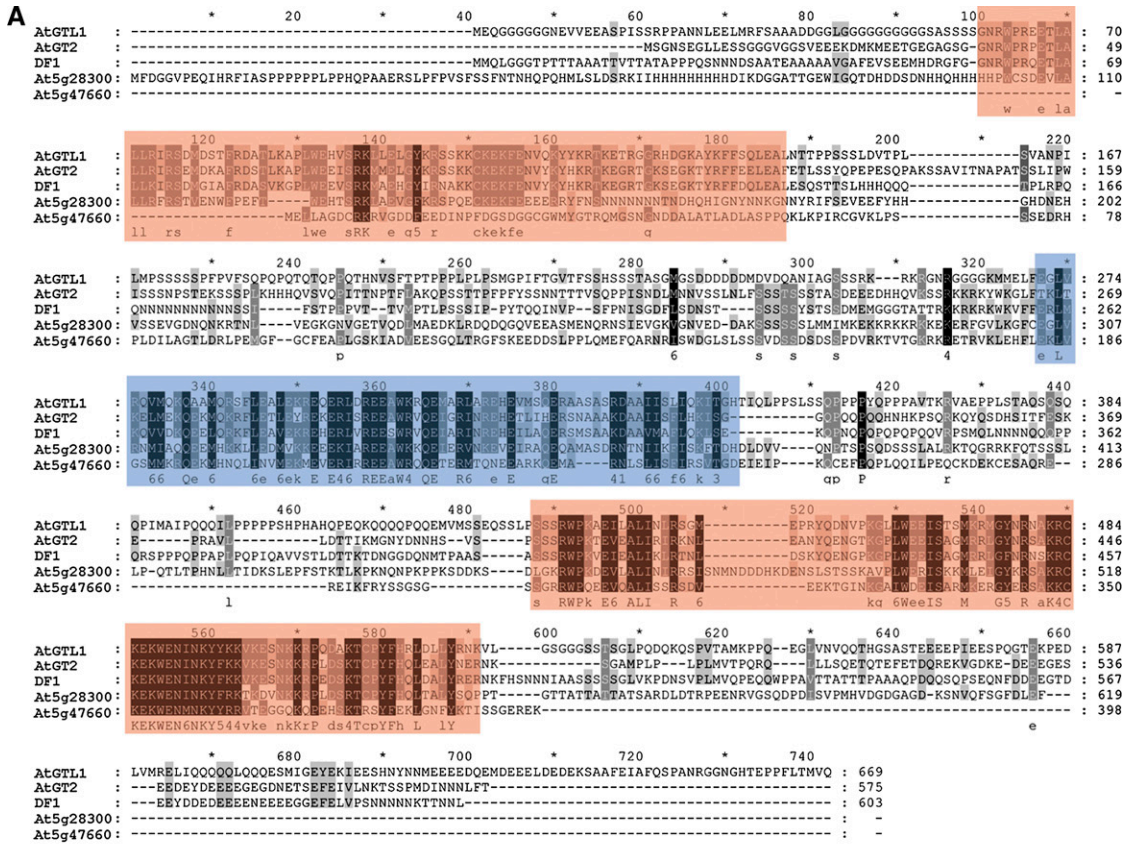
(B) Schematic representation of wild-type GTL1 and truncated GTL1^{F26519} proteins. NTH, N-terminal trihelix domain; CD, central domain; CTH, C-terminal trihelix domain; TD, putative transactivation domain; KTL, substituted amino acids caused by the 4-bp deletion in GTL1^{F26519}. Black and white arrows represent approximate positions of oligonucleotides used for quantitative PCR analysis.

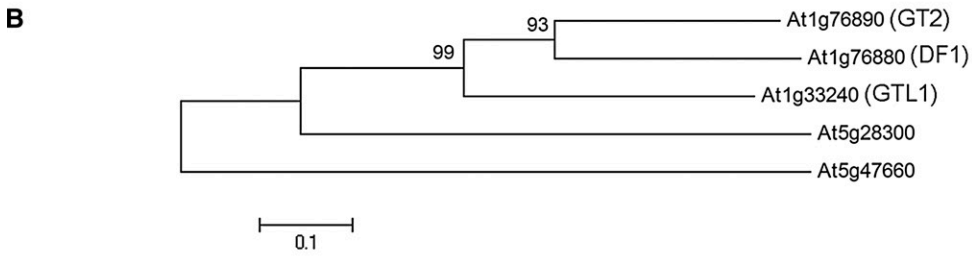
GT-2, *DF1*, and *PETAL LOSS* (*PTL*), are designated within the *GT-2* subfamily in *Arabidopsis*. Phylogenetic analysis of the five genes most closely related in the *GT-2* family demonstrates that *GT-2*, *GTL1*, and *DF1* form a small clade, while two other homologs encoded by At5g28300 and At5g47660 are more distantly related (Smalle et al., 1998; Nagano et al., 2001; Figure 2; see Supplemental Data Set 1 online). All five proteins, however, share common features with highly conserved N- and C-terminal trihelix DNA binding domains, which are unique to the *GT-2* family, and another well-conserved domain of unknown function in the central region (Figures 1B and 2A). Further sequence analysis using a bioinformatic tool (http://www.at.embnet.org/toolbox/9aatad/) also identified, in the C-terminal region of the GTL1 protein, a potential 9–amino acid transactivation domain (DLVMRELIQ, Figure 1B), which gives a 100% match to 9–amino acid transactivation domains previously identified in eukaryotic and viral transcription factors (Piskacek et al., 2007). Alignment of the deduced amino acid sequences of wild-type GTL1 and GTL1^{F26519} proteins revealed that the deletions in GTL1^{F26519} proteins cause a truncation of 250 amino acids within the central region and a frame shift in the C-terminal region, resulting in a triple amino acid substitution from PED to KTL and a premature translational stop codon that removes the C-terminal 82 amino acids (Figure 1B). These large truncations are expected to remove the entire central domain and the putative transactivation domain of GTL1 proteins, while leaving their two trihelix domains intact.

Intriguingly, our RT-PCR analysis using RNA isolated from trichome-enriched plant materials (Jakoby et al., 2008) revealed that while the CaMV35S promoter–driven expression of the *GTL1*^{F26519} cDNA causes ~50-fold overexpression of *GTL1*^{F26519}, it also leads to strong downregulation of endogenous *GTL1* expression with only ~10% of wild-type *GTL1* transcripts present in F26519 trichomes (Figure 1C). Furthermore, we found that the CaMV35S promoter–driven expression of full-length *GTL1* cDNA also results in the reduction of total *GTL1* levels in trichomes (Figure 1D) and increased trichome cell size (34 out

(C) Relative expression of endogenous *GTL1* and *GTL1*^{F26519} in wild-type (Col) and F26519 trichomes. The left panel shows the combined expression of endogenous *GTL1* and overexpressed *GTL1*^{F26519} amplified with oligonucleotides marked by black arrows in **(B)**, while the right panel shows expression of only endogenous *GTL1* amplified with oligonucleotides marked by white arrows. Quantitative PCR is repeated for three biological replicates, and the average expression levels are normalized against the expression in wild-type trichomes. Error bars represent ± SD of the means.

(D) Quantitative PCR analysis of *GTL1* transcripts in trichomes isolated from fourth to sixth true leaves of 3-week-old wild-type plants transformed with empty pBIG2113SF vectors (vector) or *pBIG2113SF:GTL1* vectors (*35S:GTL1*). The level of *GTL1* transcripts is reduced in trichomes of transgenic plants carrying *35S:GTL1* constructs. Total trichome RNA was pooled from ~80 young leaves of transgenic plants, and *GTL1* transcripts were amplified using oligonucleotides marked by black arrows in **(B)**. Shown values represent combined *GTL1* expression levels from the endogenous *GTL1* gene and *35S:GTL1* vectors. The error bars show ± SD of the mean from three biological replicates, and the average expression levels are normalized against the expression in trichomes of wild-type plants transformed with empty pBIG2113SF vector.





C

Amino acid sequence identity of the N- and C-terminal trihelix DNA-binding motifs between AtGTL1, AtGT2 and AtDF1

	AtGTL1 / AtGT2	AtGTL1 / AtDF1	AtGT2 / AtDF1
N-terminal	77%	71%	80%
C-terminal	73%	74%	84%

Figure 2. *GTL1* Belongs to the *Arabidopsis GT-2* Family.

(A) Protein alignment of GTL1 and its four closest homologs in *Arabidopsis*. Orange boxes highlight the N- and C-terminal trihelix DNA binding domains, and a blue box indicates the conserved central domain. Note that the sequence of the N-terminal trihelix DNA binding domain is highly conserved among GTL1, GT-2, and DF1 but to a lesser extent to the products of the loci At5g28300 and At5g47660.

(B) The phylogenetic tree, generated using MEGA version 4 (Tamura et al., 2007), shows that GTL1, GT-2, and DF1 form a subclade among the GT-2 family proteins. Bootstrap values (1000 trials) above 90% are shown on branches. Bar represents 0.1 substitutions per site.

(C) Sequence identity of the N- and C-terminal DNA binding motifs between GTL1, GT2, and DF1.

of 36 selected T1 transformants showed large trichome phenotypes). These results suggest that 35S-driven expression of full-length *GTL1* or *GTL1*^{F26519} may cause knockdown phenotypes with respect to trichome development.

GTL1 Represses Trichome Cell Growth

To further investigate the function of GTL1 in plant development, we identified one Wisconsin DsLox transposon insertion allele, *gtl1-1* (WiscDsLox413-416C9), and two SALK T-DNA insertion alleles, *gtl1-2* (SALK_005965) and *gtl1-3* (SALK_005966), for the *GTL1* locus (see Supplemental Figure 1A online). Both *gtl1-2* and *gtl1-3* mutants contain a T-DNA insertion in the first exon, disrupting the N-terminal trihelix domain, while the *gtl1-1* mutant contains a transposon insertion in the second exon, disrupting the conserved central domain. The progenies of heterozygous parental lines exhibited classic Mendelian segregations for the *gtl1* phenotypes (11:35 for *gtl1-1*, $\chi^2 = 0.014$, $P > 0.9$; 12:35 for *gtl1-2*, $\chi^2 = 0.004$, $P > 0.9$; 11:37 for *gtl1-3*, $\chi^2 = 0.055$, $P > 0.8$), indicating that all three alleles represent recessive loss-of-function mutations. RT-PCR analysis using primers spanning the *GTL1* coding sequence further demonstrates that the full-length *GTL1* transcripts are not detectable in all three homozygous mutants (see Supplemental Figure 1B online).

Our phenotypic observation reveals that homozygous mutations for all three alleles lead to an increase in trichome size indistinguishable from original F26519 plants (Figures 1A and 3A). Further quantitative analysis using trichomes isolated from the fourth and fifth leaves of 24-d-old plants indeed demonstrates that the trichome branch length is increased to very similar degrees in *gtl1-1*, *gtl1-2*, and F26519 (Figure 3B). Although all previously described large trichome mutants, including *try*, *kak*, *rfi*, and *pym*, also have over-branching phenotypes, *gtl1* trichomes do not show significant alterations in their branching patterns (Figure 3A), and, as in the wild type, the majority of trichomes possess three branches (Figure 3C). Quantification of epidermal cell numbers and trichome numbers in individual leaves also demonstrates that the *gtl1* mutations do not affect their relative frequencies (Table 1). These results suggest that GTL1 is involved in the regulation of trichome cell growth through pathways uncoupled from those mediating trichome initiation or branching. The third and fourth leaves of 21-d-old *gtl1* plants, which are still expanding, appear to be slightly smaller than those of the wild type (Table 1), but after their full development, we do not find significant differences in their sizes (see Supplemental Figure 2A online).

An Increase in Trichome Cell Size Is Accompanied by an Increase in Nuclear DNA Content in *gtl1* Mutants

Many recent studies have shown that trichome cell size is often positively linked with their ploidy level (Hulskamp et al., 1994; Perazza et al., 1999; Sugimoto-Shirasu et al., 2005; Breuer et al., 2007). To test whether an increase in trichome cell size in *gtl1* is accompanied by an increase in trichome ploidy, we visualized the nuclear DNA of fully mature trichomes isolated from the fourth and fifth leaves of 24-d-old plants using 4′,6-diamidino-2-phenylindole (DAPI) staining. As shown in Figure 3D, our fluorescence microscopy shows that the DAPI-stained nuclear size in these trichomes is significantly increased in *gtl1-1*, *gtl1-2*, and F26519 trichome cells compared with wild-type trichomes at the equivalent developmental stage. Quantitative analysis of the nuclear DNA content indeed demonstrates that endoreduplication proceeds beyond wild-type levels in *gtl1-1*, *gtl1-2*, and F26519 trichome cells (Figure 3E). Our flow cytometry analysis using the whole third and fourth leaves of 26-d-old wild-type, *gtl1-1*, and *gtl1-2* plants did not reveal major differences in the overall ploidy distributions (see Supplemental Figure 2B online), further supporting that GTL1 mainly functions in trichome development in leaves.

GTL1 Is Required for the Repression of Endocycles after Trichomes Complete Branching

Our data show a clear uncoupling of cell growth and endocycling from branching in *gtl1*, and one possible explanation for this phenotype is that GTL1 is required for the repression of cell growth and/or endocycling only after trichomes complete branching. To test this hypothesis, we measured the DNA content of DAPI-stained nuclei in young trichomes that have completed branching but not their subsequent cell growth (Figure 4A). If GTL1 functions only after the completion of trichome branching, *gtl1* trichomes should not undergo an additional round of endocycling before they form three branches. Consistent with this idea, the *gtl1* trichomes have comparable amounts of nuclear DNA relative to wild-type trichomes at the equivalent developmental stage (Figure 4B). These observations suggest that GTL1 is required for endocycle repression after trichomes complete their branching.

GTL1 Is a Nuclear Protein Expressed during the Postbranching Stages of Trichome Development

To investigate whether the expression pattern of the *GTL1* gene supports its predicted function in postbranching stages of trichome development, we first generated transgenic lines carrying *GTL1pro:β-glucuronidase* (*GUS*) reporter constructs. All examined transgenic lines, derived from at least 10 independent T1 transformants, show strong GUS expression in developing trichomes (Figure 5A). Interestingly, we do not detect any GUS signal in small trichomes that have just initiated or in those that are still undergoing the branching process at the base of developing young leaves. The GUS signal becomes visible, however, in large trichomes with three branches and appears to peak as trichomes reach their final size. To test whether GTL1 proteins also show developmental-specific expression patterns, we expressed GTL1:green fluorescent protein (GFP) or GFP:GTL1 fusion proteins under the same *GTL1* promoter. We recovered multiple independent transgenic lines that show full complementation of the *gtl1* mutant phenotypes by expression of either GTL1:GFP or GFP:GTL1 proteins, indicating that both N and C terminus fusion proteins are functional in planta. Our fluorescence microscopy further demonstrates that, as with its promoter activity, GFP:GTL1 proteins are expressed only after trichomes complete their second branching event (Figure 5B), supporting our hypothesis that GTL1 acts in postbranching

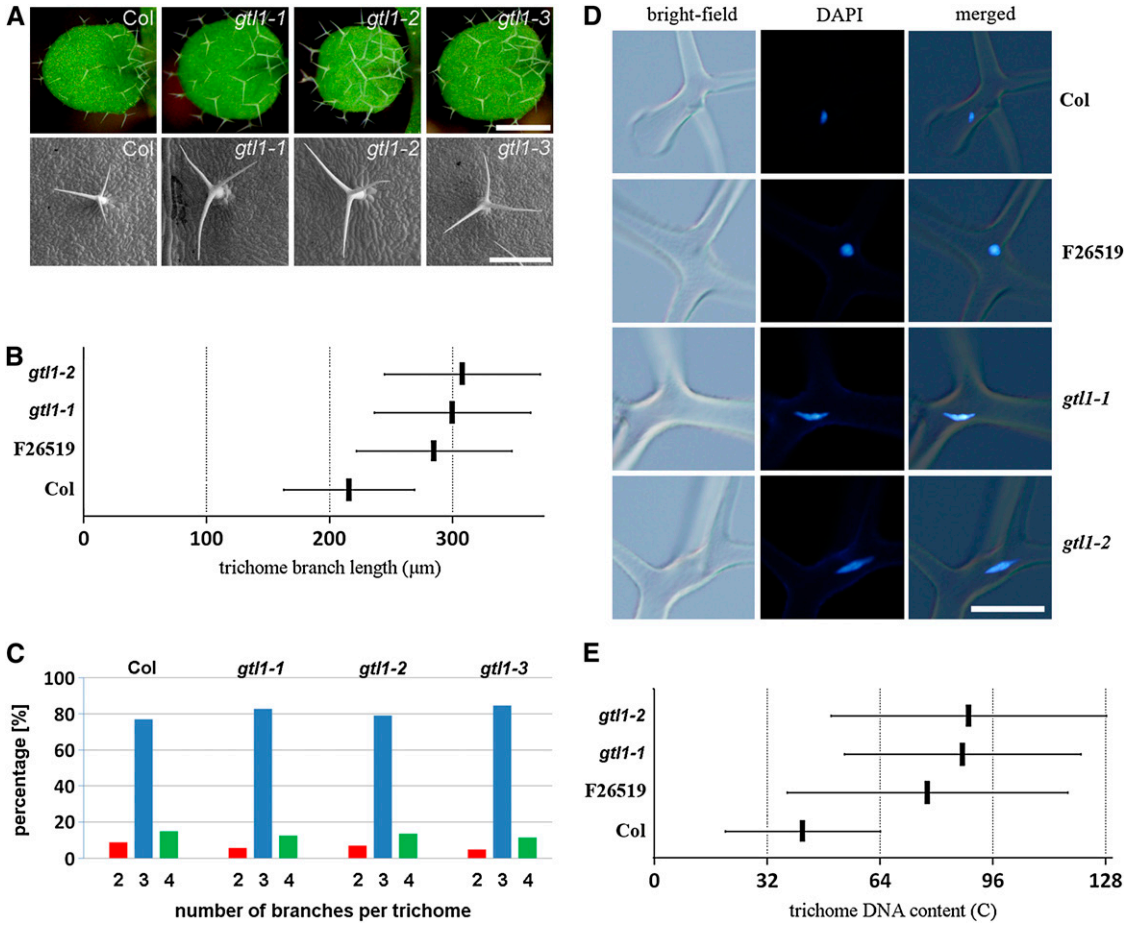


Figure 3. Homozygous Mutations in *gtl1-1*, *gtl1-2*, and *gtl1-3* Lead to Increased Trichome Cell Size and Nuclear DNA Content without Affecting the Number of Trichome Branches.

(A) Bright-field and scanning electron microscopy of the first true leaves from 10-d-old wild-type (Col), *gtl1-1*, *gtl1-2*, and *gtl1-3* seedlings. Bars = 1 mm (top panels) and 400 μm (bottom panels).

(B) Quantitative analysis of trichome branch length in wild-type, F26519, *gtl1-1*, and *gtl1-2* plants. Trichomes were removed from fourth and fifth leaves of 24-d-old seedlings following the procedure described previously (Zhang and Oppenheimer, 2004). The isolated trichomes were digitally recorded by light microscopy, and the length of individual branches was measured using ImageJ. Vertical and horizontal lines represent means and SD (n = 200 from at least 10 plants for each genotype), respectively.

(C) Quantitative analysis of trichome branch number in wild-type, *gtl1-1*, *gtl1-2*, and *gtl1-3* plants. Branch numbers were counted for all trichomes in the first pair of leaves from 24-d-old plants. A total of 450 trichomes from at least 10 different plants were used for each genotype, and their relative frequencies are shown as a percentage.

(D) Bright-field and fluorescence microscopy of isolated trichomes and their DAPI-stained nuclei from fourth and fifth leaves of 24-d-old seedlings. Bar = 40 μm.

(E) Quantitative analysis of nuclear DNA content in wild-type, F26519, *gtl1-1*, and *gtl1-2* trichomes. DAPI-stained nuclei in isolated trichomes were recorded at fixed optical settings by fluorescence microscopy and their nuclear DNA content, defined as total pixel intensities of DAPI signals per individual nucleus, was quantified using ImageJ. DNA content values are normalized relative to the 2C DNA content of stomatal guard cell nuclei. Vertical and horizontal lines in represent means and SD (n = 120 from at least 10 plants per genotype), respectively.

processes of trichome development. Once trichomes complete their full growth, GTL1 expression declines and eventually ceases since we do not detect any GFP signals in older trichomes (Figure 5C). As expected for a putative transcription factor, we found that GFP:GTL1 proteins are localized exclusively within the nucleus (Figure 5D).

We also explored whether GFP:GTL1 proteins are expressed in any other cell types in leaves, but we did not detect any GFP signals in cells other than trichomes under our standard growth conditions. By contrast, we did detect some weak nuclear accumulation of GFP:GTL1 in developing roots and petals (see Supplemental Figure 3 online). Interestingly, GFP:GTL1 proteins do not accumulate in proliferating cells at the root meristem, and its expression is induced as cells start expanding (see Supplemental Figures 3A and 3B online). We found that GFP:GTL1 proteins are also expressed in growing root hairs (see

Table 1. Comparison of Adaxial Epidermal Cell Number and Trichome Number in the Wild Type (Col), *gtl1-1*, and *gtl1-2*

	Col	*gtl1-1*	*gtl1-2*
Average size of third and fourth true leaves (mm^2)[a]	28.97 ± 2.73	24.28 ± 2.43	23.77 ± 3.10
Size of adaxial epidermal cells (μm^2)[b]	3869 ± 1294	3295 ± 1378	3188 ± 1618
Estimated total number of adaxial cells[c]	7487	7369	7456
Number of trichomes/leaf	34.75 ± 5.00	33.13 ± 3.19	34.05 ± 4.30
Ratio adaxial cells/trichome	215.5	222.4	219.0

All measurements were carried out on the third and fourth leaves of 3-week-old seedlings. Indicated values are mean ± SD

[a] $n = 12$ for the wild-type, *gtl1-1*, and *gtl1-2*.

[b] Cell areas of 200 epidermal cells, excluding stomatal guard cells, from at least eight leaves were measured for each genotype.

[c] To estimate the total number of adaxial cells, the average size of the third and fourth true leaves was divided by the average size of adaxial epidermal cells.

Supplemental Figures 3C and 3D online). Within young petals, GFP:GTL1 proteins are present in a gradient fashion, with its highest expression level being detected at the tip of petals (see Supplemental Figures 3E and 3F online). In fully expanded petals, GFP:GTL1 proteins can be found in all epidermal cells (see Supplemental Figures 3E and 3F online).

The Expression and Function of GTL1 Require the Progression of Trichome Differentiation, Which Is Largely Dependent on TTG1 and GL2

Trichome cell fate is specified at an early stage by genetic pathways that involve several transcription factors, such as GL1-3, TTG1, and TRY (Oppenheimer et al., 1991; Galway et al., 1994; Larkin et al., 1994; Rerie et al., 1994; Walker et al., 1999). The expression of GTL1 during much later stages of trichome development suggests that GTL1 may act downstream of these early regulatory pathways. To test this hypothesis, we first generated double mutants between *gtl1-1* and *ttg1-10* or *gl2-130213*. Mutations in the *TTG1* gene result in a dramatic decrease in the number of trichome initiation sites, and only a few trichomes develop at the leaf margins (Larkin et al., 1994). Phenotypic analysis of *gtl1-1 ttg1-10* double mutants demonstrates that the *ttg1-10* mutation is epistatic to the *gtl1-1* mutation, with their double mutants showing *ttg1-10*–like patterning defects (Figure 6A). Mutations in *gl2* do not affect specification of trichome initiation sites but severely impair perpendicular epidermal outgrowth and endoreduplication (Rerie et al., 1994). The *gl2-130213* mutants, like *ttg1-10*, are epistatic to the *gtl1-1* mutation since neither cell size nor ploidy of *gtl1-1 gl2-130213* trichomes are increased compared with those of *gl2-130213* trichomes (Figures 6A to 6D).

To further explore the functional relationship between GTL1 and TTG1 or GL2, we introduced the *GTL1pro:GUS* reporter construct into the *ttg1-10* or *gl2-130213* mutant backgrounds and asked whether these mutations modify the promoter activity of the *GTL1* gene. As shown in Figure 6E, *GTL1* promoter activity is completely abolished by the *ttg1-10* or *gl2-130213* mutations in almost all leaf epidermal cells of 16-d-old plants, although some GUS signals can be detected in *ttg1-10* trichomes that occasionally form at the leaf margins. The leaves of 24-d-old *gl2-130213* plants sometimes develop one or two unbranched trichomes, and we detected GUS signals in a fraction of these unbranched trichomes (Figure 6F).

While our data are largely consistent with our hypothesis that GTL1 acts downstream of TTG1 and GL2, the GUS activity detected in the *ttg1-10* and *gl2-130213* trichomes suggests that the expression of GTL1 is not absolutely dependent on TTG1 or GL2 function. An alternative scenario that can explain these observations is that GTL1 expression requires a certain stage of trichome development, which usually depends on TTG1 or GL2. As the *ttg1-10* and *gl2-130213* mutant phenotypes show (Figure 6A), both of these mutations are leaky, and trichomes that manage to initiate in the absence of TTG1 or GL2 appear to proceed to the developmental stage where GTL1 expression is induced. We found that the trichomes in *gtl1-1 ttg1-10* double mutants are slightly larger and have higher ploidy than those in *ttg1-10* (Figure 6A; see Supplemental Figure 4 online), indicating that the GTL1 proteins expressed in *ttg1-10* trichomes do function to repress cell growth and endocycles. These results support the idea that the progression of trichome development to a certain stage is the prerequisite for the expression and function of GTL1.

The *gtl1 gl3* and *gtl1 try* Mutants Display Additive Trichome Cell Size and Ploidy Phenotypes

The two patterning transcription factors, GL3 and TRY, also act as positive and negative regulators of ploidy-dependent trichome cell growth and branching, respectively. (Hulskamp et al., 1994; Folkers et al., 1997; Schnittger et al., 1998; Perazza et al., 1999). Accordingly, leaf trichomes in *gl3* mutants are reduced in ploidy, are smaller in size, and are under-branched, while *try* mutations cause larger, over-branched trichomes with increased ploidy. To test the genetic relationship between GTL1, GL3, and TRY, we generated the corresponding double mutant combinations. As shown in Figure 7, mature trichomes of *gtl1-1 gl3-7454* double mutants display intermediate phenotypes between their parental lines with respect to trichome cell size and ploidy (Figures 7A to 7C) but *gl3*-like phenotypes with respect to trichome branching (Figures 7A and 7D), further supporting an involvement of GTL1 in the regulation of cell growth and ploidy. Similarly, the *gtl1-1 try-29760* double mutants show additive phenotypes for trichome cell size and ploidy (Figures 7A to 7C) while their over-branching phenotype resembles that of single *try-29760* mutants (Figures 7A and 7D). The expression of TRY is invoked from a very early stage of trichome development (Schellmann et al., 2002). Consistent with this, young

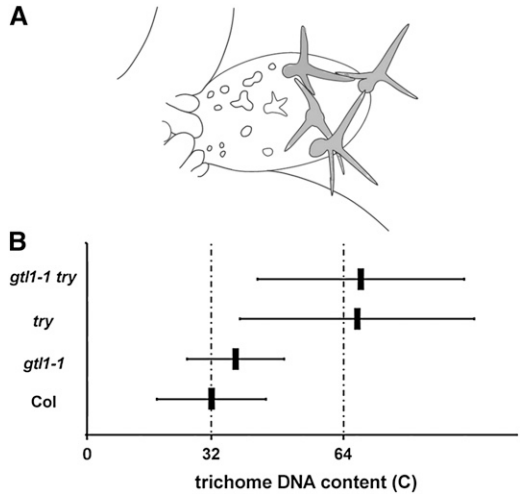


Figure 4. The Nuclear DNA Content of Young Three-Branched Trichomes Is Comparable between Wild-Type and *gtl1-1* Trichomes.

(A) A schematic representation of a young leaf showing the gradual progression of early trichome development from the base to the tip of the leaf. Trichomes equivalent to those marked in gray were used for the measurement of nuclear DNA content.

(B) Quantitative analysis of the nuclear DNA content in young three-branched trichomes from the sixth and seventh leaves of 24-d-old wild-type (Col), *gtl1-1*, *try-29760*, and *gtl1-1 try-29760* seedlings. Note that DNA content values in wild-type trichomes are artificially set to 32C since we cannot accurately measure DNA content of stomatal guard cell nuclei in the young leaves due to the high fluorescence levels in the background. These values should thus be used only for the context of this experiment. Vertical and horizontal lines represent means and SD (n = 150 from at least 10 different plants per genotype), respectively.

three-branched trichomes in *try-29760* possess significantly larger nuclei than those in wild-type or *gtl1-1* plants (Figure 4B), indicating that *try-29760* trichomes already undergo one additional round of endocycling before they complete branching. The average nuclear DNA content of *gtl1-1 try-29760* trichomes at these early developmental stages resembles that of *try-29760* (Figure 4B), further supporting the requirement of GTL1 in later stages of trichome development.

The *gl3* and *try* mutations also disrupt trichome initiation and/or patterning, leading to alterations in trichome number and/or spacing (Schnittger et al., 1999; Payne et al., 2000). The *gtl1-1* mutation does not appear to enhance or rescue either of these phenotypes in *gl3-7454* or *try-29760* plants (Figure 7E), further demonstrating that GTL1 does not play profound roles in trichome initiation or patterning.

The *gtl1* Mutation Modifies the Expression of Cell Cycle Genes and Partially Rescues the Ploidy Defects in *sim1* Mutants

Modified activities of core cell cycle regulators often have strong downstream consequences on trichome development (Ishida et al., 2008). It is thus plausible that the primary function of GTL1 is to modify the expression of cell cycle genes, preventing the progression of endocycles and trichome cell growth beyond wild-type levels. To explore this possibility, we first tested whether the phenotypes observed in *gtl1* trichomes associate with the misexpression of genes involved in cell cycle regulation. As shown in Figure 8, our quantitative RT-PCR analysis using RNA isolated from young trichomes of 28-d-old plants reveals that the expression of several cell cycle genes, many of which are implicated in trichome development, is modified moderately but significantly in *gtl1-1* trichomes. The strongest upregulation, of up to fourfold, is found for the expression of *CCS52A2/FZR1*, a positive regulator of endocycle progression in plants (Lammens et al., 2008; Vanstraelen et al., 2009), while the expression of its close homolog, *CCS52A1/FZR2*, which promotes endocycles in trichomes (Larson-Rabin et al., 2009), is only mildly increased (Figure 8). A plant-specific small CDK inhibitor protein, SIM, is required for the repression of endocycles in trichomes, and its

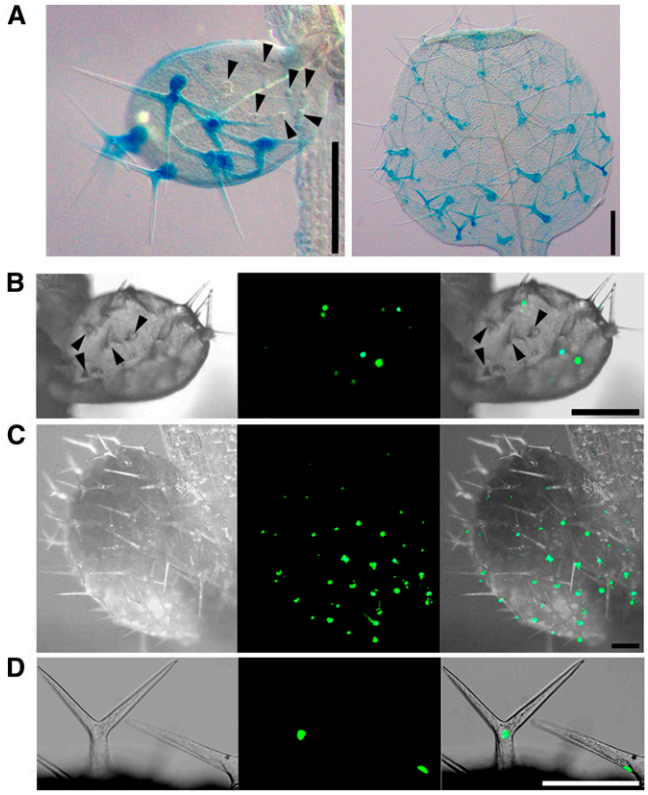


Figure 5. GTL1 Is Expressed during the Late Stages of Trichome Development and Is Targeted to the Nucleus.

(A) Expression patterns of *GTL1pro:GUS* in emerging young leaves (left panel) and slightly older expanding leaves (right panel) from 2-week-old, light-grown *Arabidopsis* seedlings.

(B) and **(C)** Expression patterns of *GTL1pro:GFP:GTL1* in emerging young leaves **(B)** and older expanding leaves **(C)** from 2-week-old, light-grown *Arabidopsis* seedlings.

(D) Nuclear localization of GFP:GTL1 proteins in developing leaf trichomes.

Black arrowheads in **(A)** and **(B)** mark young trichomes at the leaf base that have just initiated or those that have not completed branching. Bars = 200 μm.

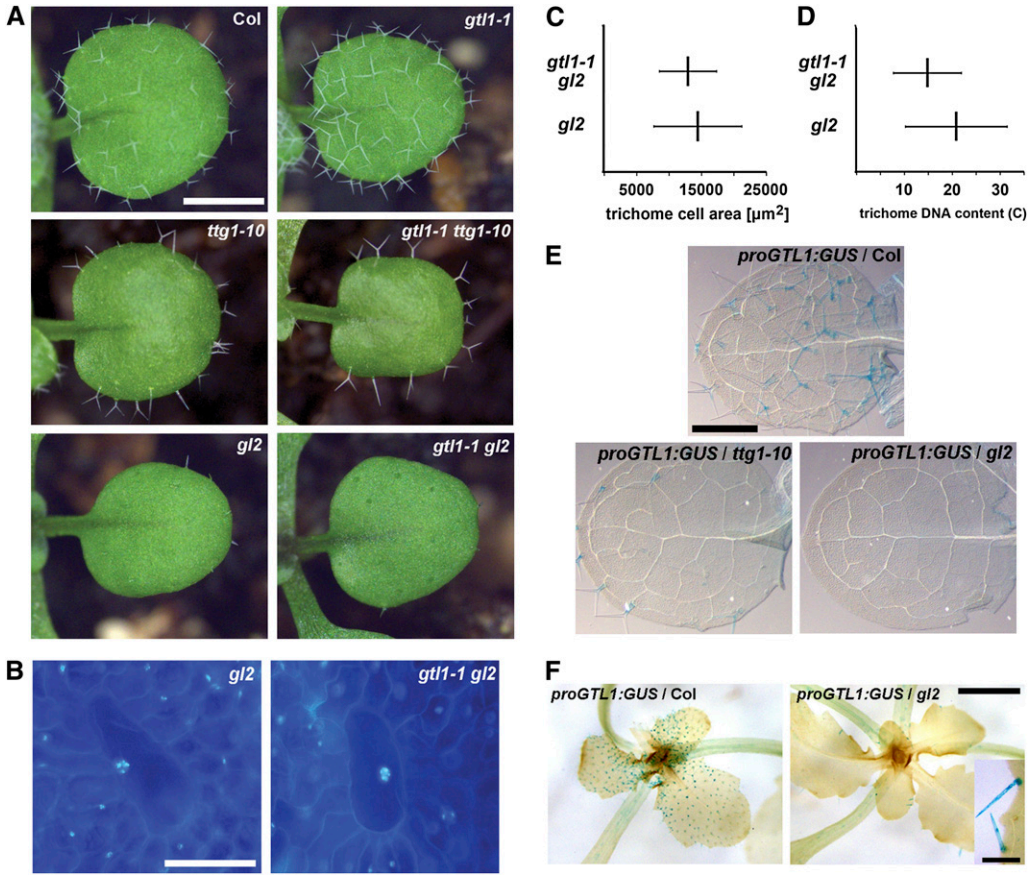


Figure 6. The *ttg1-10* and *gl2-130213* Mutants Are Epistatic to the *gtl1-1* Mutants.

(A) Bright-field microscopy of the first true leaf of 12-d-old wild-type (Col), *ttg1-10* (Col), *gl2-130213* (Col), *gtl1-1*, *gtl1-1 ttg1-10*, and *gtl1-1 gl2-130213* mutants.

(B) Fluorescence microscopy of DAPI-stained nuclei in the very small, unbranched trichome cells that occasionally form in *gl2-130213* and *gtl1-1 gl2-130213* mutants. Shown images represent nuclei typical for each genotype from the first true leaves of 12-d-old plants.

(C) Trichome cell area in the fourth and fifth leaves of 24-d-old *gl2-130213* and *gtl1-1 gl2-130213* seedlings. Error bars represent ± SD (*n* = 63 from at least five different plants per genotype).

(D) Quantitative analysis of nuclear DNA content in *gl2-130213* and *gtl1-1 gl2-130213* trichomes at the developmental stages equivalent to those in **(C)**. Error bars represent ± SD (*n* = 95 from at least five different plants per genotype).

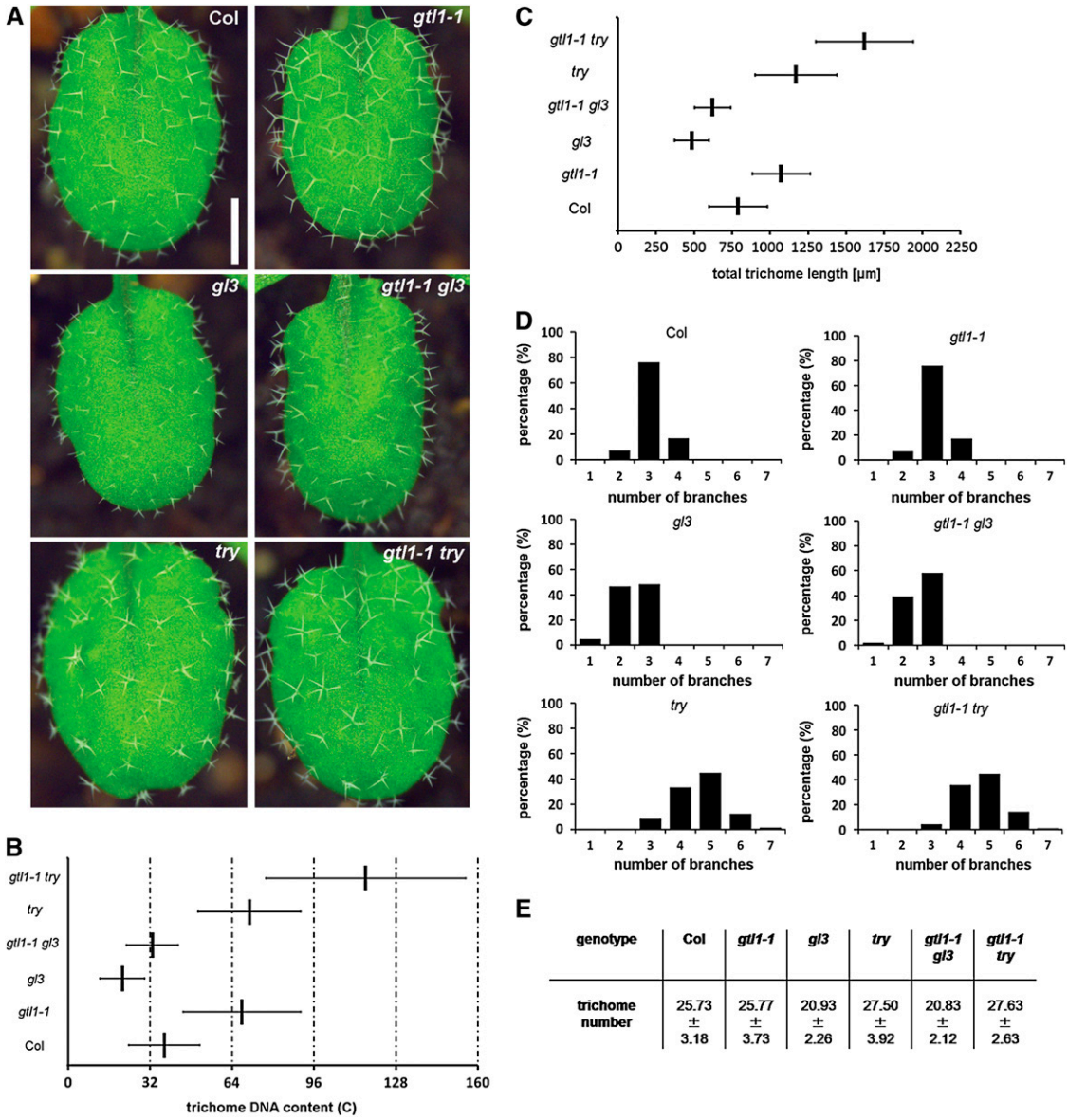
(E) Expression of the *GTL1pro:GUS* reporter in the third true leaf of wild-type (Col), *ttg1-10*, and *gl2-130213* plants.

(F) Expression of the *GTL1pro:GUS* reporter in young rosette leaves of 24-d-old wild-type (Col) and *gl2-130213* plants. A magnified view of the *GTL1pro:GUS* reporter expression in unbranched trichomes in *gl2-130213* leaves is shown as an inset.

Bars = 2 mm in **(A)**, 100 μm in **(B)**, 500 μm in **(E)**, and 2 mm and 100 μm for the large image and inset in **(F)**, respectively.

loss-of-function mutation leads to the development of multicellular trichomes with reduced ploidy (Walker et al., 2000; Churchman et al., 2006). The level of SIM transcripts is not significantly changed, while the expression of its interacting proteins, CDKA;1 and CYCD4;1, is reduced by 25 to 35% compared with the wild type (Figure 8). Downregulation of *CYCA2;3* is previously shown to cause extended endocycling (Imai et al., 2006), and, consistent with this, the *CYCA2;3* transcript level is reduced by 25% in *gtl1-1* trichomes (Figure 8). Cell cycle proteins required for the licensing of DNA replication, including CDT1a and CDC6a, are expressed in developing trichomes, and overexpression of these proteins promotes an additional round of endocycling in trichomes (Castellano et al., 2001). We found that the transcript levels of CDT1a, its close homolog CDT1b, and CDC6a are moderately increased in *gtl1-1* (Figure 8).

To further investigate the possibility that GTL1 functions in endocycle control, we introduced the *gtl1-1* mutation into the *sim-1* mutant background and tested whether the function of GTL1 interferes with that of SIM. Although many trichomes in *sim-1* are multicellular (Walker et al., 2000), their overall size is comparable with that of wild-type trichomes (Figure 9A). By contrast, the majority of trichomes in the *gtl1-1 sim-1* double mutants appear to be slightly larger than those in *sim-1* (Figure 9A), although we cannot accurately measure the trichome size of *gtl1-1 sim-1* plants due to the irregular branching patterns of trichome in these plants (Churchman et al., 2006). As previously



genotype	Col	gtl1-1	gl3	try	gtl1-1 gl3	gtl1-1 try
trichome number	25.73 ± 3.18	25.77 ± 3.73	20.93 ± 2.26	27.50 ± 3.92	20.83 ± 2.12	27.63 ± 2.63

Figure 7. The *gtl1-1 gl3-7454* and *gtl1-1 try-29760* Mutants Display Additive Trichome Cell Size and Ploidy Phenotypes.

(A) Bright-field microscopy of the third true leaves of 15-d-old wild-type (Col), *gl3-7454* (Col), *try-29760* (Col), *gtl1-1*, *gtl1-1 gl3-7454*, and *gtl1-1 try-29760* mutants. Bar = 2 mm.

(B) Quantitative analysis of the nuclear DNA content in fully mature wild-type, *gl3-7454*, *try-29760*, *gtl1-1*, *gtl1-1 gl3-7454*, and *gtl1-1 try-29760* trichomes isolated from fourth and fifth leaves of 24-d-old plants. Vertical and horizontal bars represent mean values and SD (n = 120 from at least 10 different plants), respectively. Nuclear DNA content is normalized relative to DNA content in guard cell nuclei.

(C) Total branch length of wild-type (Col), *gl3-7454* (Col), *try-29760* (Col), *gtl1-1*, *gtl1-1 gl3-7454*, and *gtl1-1 try-29760* trichomes. The length of individual trichome branches was measured using trichomes isolated from fourth and fifth leaves of 24-d-old plants, and their sum was calculated for each trichome (n = 60 trichomes from at least five different plants per genotype).

(D) Distribution of trichome branch numbers in wild-type (Col), *gl3-7454* (Col), *try-29760* (Col), *gtl1-1*, *gtl1-1 gl3-7454*, and *gtl1-1 try-29760* leaves. Branch numbers were counted for all trichomes on the first pair of leaves from 24-d-old plants (n = 450 trichomes from at least 15 different plants per genotype).

(E) Average number of trichomes on the third and fourth leaves of 24-d-old seedlings (n = 30 from at least 15 different plants per genotype).

reported, the DNA content of individual nuclei in *sim-1* trichomes is strongly reduced compared with that in wild-type trichomes (Walker et al., 2000; Figure 9B). We found that the average nuclear DNA content in *gtl1-1 sim-1* trichomes is significantly greater than in wild-type trichomes (Figure 9B), demonstrating that the *gtl1-1* mutation can partially bypass the endocycle defects caused by the *sim-1* mutation. These results strongly support our hypothesis that GTL1 participates in the regulation of successive endocyling in trichome development.

DISCUSSION

GTL1 Is a Repressor of Cell Growth and Endocycling in Trichome Development

We have demonstrated that a novel *Arabidopsis* trihelix protein, GTL1, participates in the repression of ploidy-dependent cellular growth in trichome development. Recent molecular and genetic studies have uncovered the transcriptional networks involved in trichome patterning and differentiation, but the mechanism underlying subsequent growth and development is still poorly understood. Two trichome patterning transcription factors, *TRY* and *GL3*, are also implicated in the control of cell growth and branching (Hulskamp et al., 1994; Folkers et al., 1997; Schnittger et al., 1998; Perazza et al., 1999), indicating that some of the early acting transcriptional regulators also participate in trichome development beyond the differentiation stage. Our data show that the expression of *GTL1* largely depends on the early patterning/differentiation genes, *TTG1* and *GL2*, strongly suggesting that GTL1 acts as part of the regulatory mechanisms linking

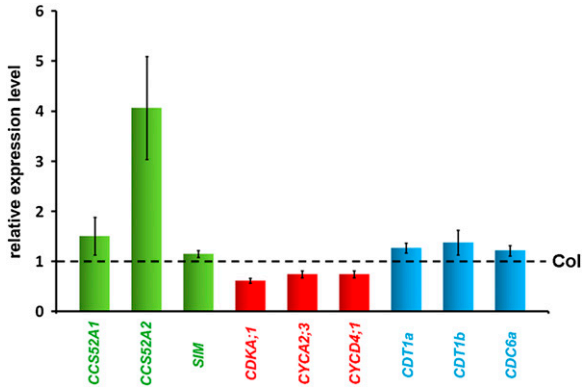


Figure 8. The Expression of Cell Cycle Genes Is Modified in *gtl1-1* Trichome Cells.

The quantitative PCR analysis of *CCS52A1/FZR2*, *CCS52A2/FZR1*, *SIM*, *CDKA;1, CYCA2-3*, *CDT1a*, *CDT1b*, and *CDC6a* expression in wild-type and *gtl1-1* trichomes. The *CCS52A1/FZR2*, *CCS52A2/FZR1*, and *SIM* genes, labeled in green, and *CDKA;1*, *CYCD4-1*, and *CYCA2-3*, labeled in red, are implicated in the promotion and repression, respectively, of endocycles in plants. Those labeled in blue (*CDT1a*, *CDT1b*, and *CDC6a*) participate in the licensing of DNA replication. RNA was isolated from trichomes of young leaves of 28-d-old seedlings. Mean expression levels of four technical repeats in *gtl1-1*, relative to those of the wild type (Col), are shown with SD.

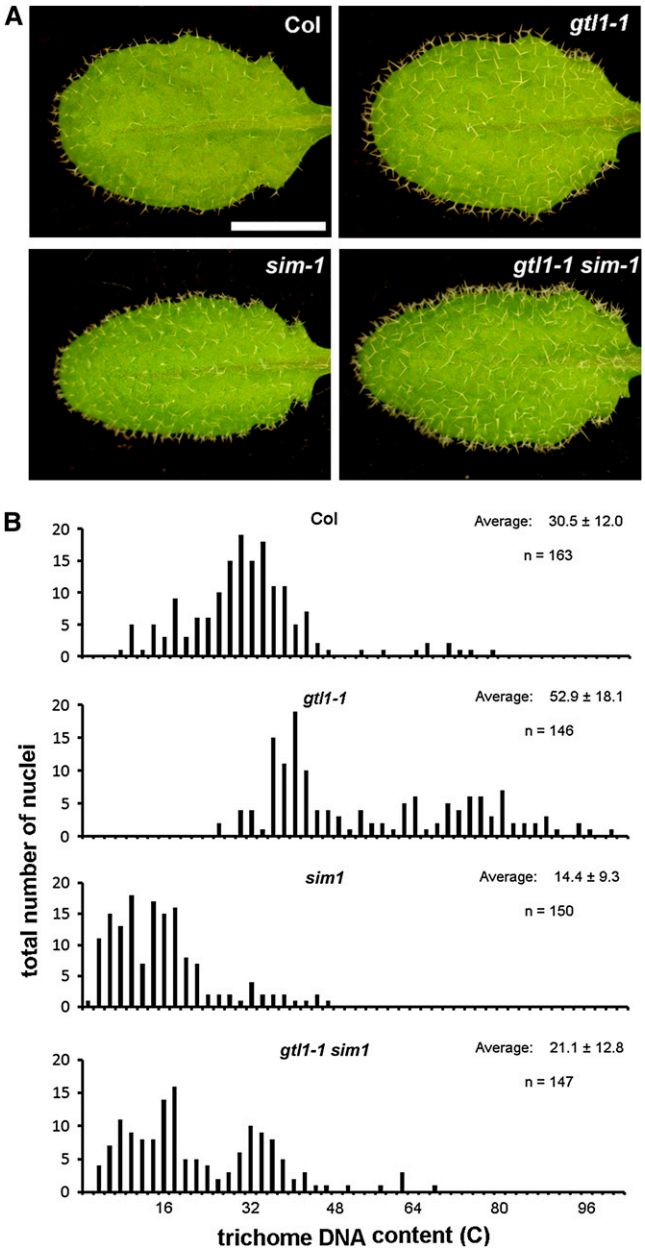


Figure 9. The *gtl1* Mutation Partially Rescues the Ploidy Defects in *sim-1* Trichomes.

(A) Bright-field microscopy of the sixth true leaves of 28-d-old wild-type (Col), *gtl1-1*, *sim-1* (Col), and *gtl1-1 sim-1* mutants. Bar = 2 mm.

(B) Quantitative analysis of the nuclear DNA content in wild-type, *gtl1-1*, *sim-1*, and *gtl1-1 sim-1* trichomes isolated from the fourth and fifth leaves of 28-d-old plants. The values on the *y* axis represent total number of nuclei counted for each genotype. Nuclear DNA content is normalized relative to that in guard cell nuclei.

early trichome differentiation to final growth and development. Given that we occasionally detect some *GTL1* expression in *ttg1* or *gl2* trichomes, *TTG1* or *GL2* itself is not likely to be the absolute requirement for *GTL1* expression. Our data, instead, suggest it is the progression of trichome development to a certain stage that induces the expression of *GTL1*. We do not yet know what specifies the developmental stage to allow *GTL1* expression, but at least our data suggest that the level of trichome branching is not the prerequisite for this, since unbranched or under-branched trichomes can express *GTL1* in *ttg1* or *gl2.*

A key difference between GTL1 and previously identified trichome growth regulators, such as KAK, RFI, PYM, and SPY, is that GTL1 represses trichome cell growth without altering its branching pattern. The tight coupling between cell size and branching in many trichome mutants previously led to the idea that the pathways regulating these two cellular features are closely linked. Our new data, however, clearly demonstrate that at least part of these regulatory pathways can be uncoupled. One possible mechanism that can uncouple trichome cell growth from branching is that trichome cells continue to have growth potential when they reach their normal maximum size while their branching potential ceases much earlier during their development (Figure 10). Unlike TRY and GL3, which are both expressed throughout trichome development, GTL1 is expressed only after trichome cells complete branching, allowing its specific repression of trichome cell growth at the late stage of trichome development. In agreement with this idea, young three-branched trichomes in *gtl1* have not undergone additional rounds of endocycling as opposed to those in *try*, which already possess significantly greater nuclear DNA content than the wild type. Thus, TRY and GTL1 appear to exert their function at different stages of trichome development, with TRY acting early, while cells are still undergoing branching and GTL1 only after cells complete branching. It is worth noting that 35S promoter–driven overexpression of GL1 is previously reported to increase trichome ploidy levels without modifying its branching (Larkin et al., 1994; Schnittger et al., 1998). However, this is only in the context of overexpression studies, and in combination with other mutations, GL1 overexpression does lead to altered trichome branching. We therefore believe that the mechanism underlying these events is probably distinct from that of GTL1.

How *35S:GTL1* or *35S:GTL1*^{F26519} expression leads to the trichome phenotype resembling the loss-of-function mutants is not clear, but our data show that 35S-driven expression of either *GTL1* or *GTL1*^{F26519} leads to the downregulation of full-length *GTL1* expression (Figure 1). It is plausible that the ectopic expression of a growth repressor in cell types essential for the plant survival (e.g., meristem cells) leads to lethality, preventing the recovery of transgenic plants with high *GTL1* expression. It is intriguing that *35S:GTL1*^{F26519} expression, by contrast, allows ~50-fold accumulation of *GTL1*^{F26519} cDNA in F26519 trichomes. Overexpression of these heavily truncated variants

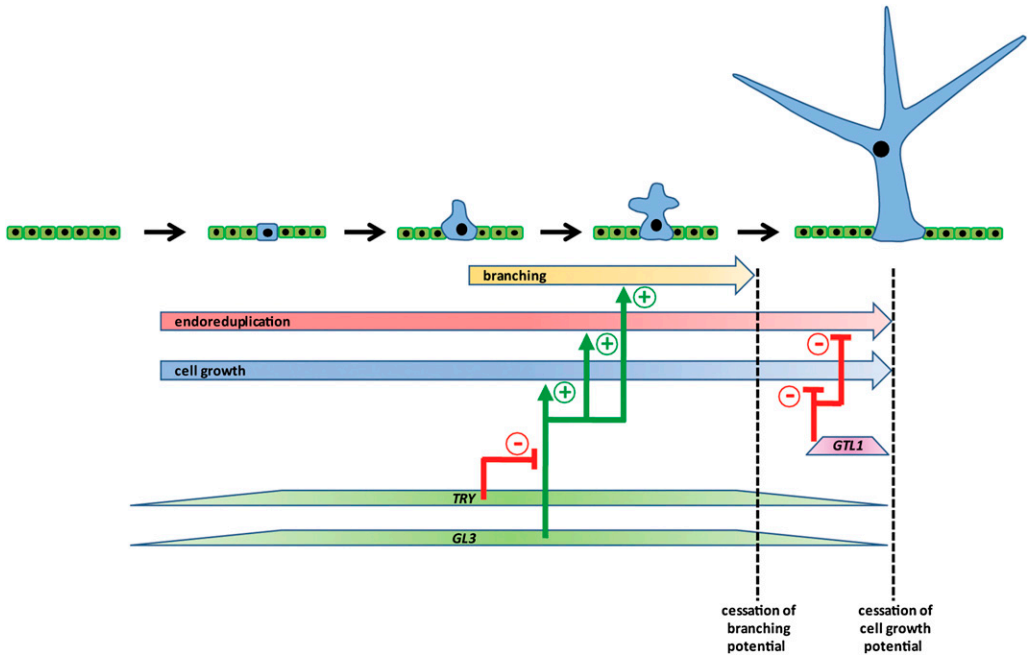


Figure 10. A Schematic Model Illustrating How GTL1 May Function in Trichome Development.

Two trichome patterning genes, *GL3* and *TRY*, expressed throughout trichome development, function as a positive and negative regulator, respectively, of endoreduplication, cell growth, and branching. How they participate in these postinitiation processes is not well defined, but previous genetic studies suggest that TRY acts as a negative regulator of GL3 function in the regulation of cell growth. By contrast, *GTL1*, expressed only after trichomes complete branching, specifically represses endoreduplication and cell growth toward the end of trichome development. The uncoupling of cell growth and branching in *gtl1* implies that the growth potential of trichome cells is maintained until much later stages than their branching potential, allowing the specific repression of cell growth at the time of GTL1 expression.

may cause dominant-negative or potentially gain-of-function phenotypes, and these possibilities should be investigated in future studies.

Transcriptional Regulation of Growth Repression and Its Link to Endocycling

The loss of GTL1 function permits trichome cell growth beyond wild-type levels, and this phenotype can be caused by either faster rates of cell growth and/or extended periods of cell growth compared with the wild type. Although technical limitations do not allow clear judgments of these two possibilities, the temporally restricted GTL1 expression after the completion of trichome branching and the *gtl1* growth phenotypes observed at the corresponding developmental stage support the latter. These data suggest that normal trichome development in *Arabidopsis* requires previously uncharacterized regulatory steps to actively terminate their cell growth. We further show that the enhanced cell growth in *gtl1* is coupled with additional cycles of endoreduplication and that the expression of several cell cycle genes implicated in endocycle control is modified in *gtl1*. We cannot yet distinguish whether these altered patterns of gene expression are the cause or downstream consequence of the enhanced endocycles, but these observations raise the possibility that GTL1 participates in the regulation of cell cycle gene expression to terminate endocycles. An involvement of GTL1 in cell cycle regulation is further substantiated by our observation that the *gtl1* mutation can partially rescue the ploidy defects caused by the *sim1* mutation. Our data of *gtl1 sim1* double mutants, as well as of *gtl1 ttg1* or *gtl1 gl3* double mutants, show that GTL1 can repress early cycles (i.e., 4C-to-32C) of endoreduplication, suggesting that GTL1 can act as a general repressor of endocycles upon its expression. Identification of direct targets of GTL1 and their functional analyses should help us to gain further insights into how trichome cell growth is regulated and how endoreduplication contributes to this process.

An important next question is whether other plant cell types also require similar transcriptional regulation to repress their growth. Because many plant cell types have unique, and often very constant, final cell sizes, the extent of their growth and/or the timing of growth cessation are likely to be tightly controlled. Although we know very little about these controls, one recent study reports that a basic helix-loop-helix transcription factor, *BIGPETALp*, is required to limit petal cell growth in *Arabidopsis* (Szecsi et al., 2006), implying a more ubiquitous involvement of transcriptional regulators in the repression of plant cell growth. Our data show that GTL1 proteins are also expressed in growing petals and roots. Under our standard growth conditions, we do not see any obvious developmental defects in these organs, but, as discussed below, this could be due to the potential functional redundancies among *GT-2* family genes.

Role of GT-2 Family Trihelix Proteins in Plant Development

Because trihelix transcription factors, also referred to as GT proteins because of their binding to GT motifs, are found only in plants, they are thought to have functional roles in plant-specific processes. The *Arabidopsis* genome contains at least seven GT-2 family proteins, which are distinct from other GT proteins because of their duplicated trihelix domains. Earlier studies have demonstrated that GT-2 proteins from rice (*Oryza sativa*) and its closely related homolog, DF1, from pea (*Pisum sativum*) bind to the promoter region of light-regulated genes, implying their involvement in the light response (Dehesh et al., 1990; Nagano et al., 2001). GTL1 is closely related to both GT-2 and DF1, sharing 70 to ~80% identity in amino acid sequence in both N- and C-terminal trihelix domains (Figure 2C), suggesting that they may have some overlapping functions. The loss-of-function mutants of GT-2 and DF1 do not display obvious morphological defects under our standard growth conditions (our unpublished data), and it will be of interest in future studies to explore the genetic and functional relationships between GT-2 and DF1. Interestingly, it has been previously shown that another GT-2 homolog in *Arabidopsis*, PTL, which has less homology to GT-2 compared with DF1 or GTL1, sharing 40 to ~60% identity in amino acid sequence in the trihelix domains, is involved in the repression of petal growth in developing flowers (Brewer et al., 2004). Furthermore, 35S promoter–driven expression of *PTL* causes strong inhibition of seedling growth, indicating that PTL can act as a general growth repressor. How PTL represses growth at the cellular level, for instance whether it retards cell proliferation or cell growth, is not currently known, but these observations raise an interesting possibility that one of the primary functions of GT-2 family trihelix proteins might be to repress plant growth in a tissue- or cell-specific manner.

METHODS

Plant Material and Growth Conditions

The original FOX mutant collection, and the *gl3-7454* and *try-29760* mutants (Col background), were described previously (Ichikawa et al., 2006; Ishida et al., 2007; Tominaga et al., 2008). The three *gtl1* mutant alleles and *gl2-130213* (SALK_130213) were obtained from the ABRC Stock Center (Ohio State University), and *ttg1-10* (Col background; Larkin et al., 1994) and *sim-1* (Col background; Walker et al., 2000) were provided by John Larkin (Louisiana State University). Plants were either germinated and grown on plates containing Murashige and Skoog salts, pH 5.7, 1% sucrose (w/v), and 0.5% phytogel (w/v) or on a 1:1 mixture of soil Supermix A (SAKATA) and vermiculite (VS Kako) in continuous light at 22°C.

Amino Acid Sequence Alignment and Phylogenetic Analysis

Amino acid sequences of the full-length proteins were aligned using the online tool MULTALIN with default settings of the following parameters: gap penalty value, −1; gap length value, −1; extremity gap penalty, no penalty; scoring method, absolute (Corpet, 1988). The phylogenetic tree was constructed using the neighbor-joining algorithm MEGA version 4 (Tamura et al., 2007) with 1000 bootstrap trials performed.

Genotype Determination of Mutants

The original *Arabidopsis thaliana* FOX line (F26519) was genotyped with a pair of oligonucleotides, *GS17* 5′-GTACGTATTTTTACAACAATTACCAACAAC-3′ and *GS18* 5′-GGATTCAATCTTAAGAAACTTTATTGCCAA-3′, designed for the FOX vector. The T-DNA/transposon mutant lines for *GTL1* were genotyped for the relevant insertions using the following oligonucleotide pairs: *WF627-F1* 5′-TTCTCGTCTCATAGCTCATCG-3′

and *WF627-R1* 5′-TGGCCATCTTGATGATGATGG-3′ for WiscDsLox413-416C9; *WF965-F1* 5′-TGATGGAGCAAGGAGGAGG-3′ and *WF965-R1* 5′-GGTGAAGATGCTTTGATCTCG-3′ for SALK_005965; and *WF966-F1* 5′-CCTTTCTCCTTCAGACCCCTCC-3′ and *WF966-R1* 5′-TGGATCTC-TATCAAGAAACACC-3′ for SALK_005966. The T-DNA/transposon insertions were identified with the insertion-specific oligonucleotides *LBcb1* 5′-ACCACCATCAAACAGGATTTTCGCCTGCTG-3′ for the SALK lines and *Wis1* 5′-AACGTCCGCAATGTGTTATTAAGTTGTC-3′ for the transposon line.

Vector Constructions and *Arabidopsis* Transformation

To recapitulate the FOX phenotype, the FOX construct was PCR amplified using the oligonucleotides *GS17* and *GS18*, digested with *Sfi*I, recloned into the binary vector pBIG2113SF (Ichikawa et al., 2006), and retransformed into wild-type (Col background) plants. To generate the *CaMV35S-GTL1* constructs, the full-length *GTL1* coding sequence was cloned into pBIG2113SF using oligonucleotides *GTL1-SfiF* 5′-ACAACTACATCTAGAGGCCAAATCGGCCATGGAGCAAGGAGGAG-GTGGTGG-3′ and *GTL1-SfiR* 5′-CGGGGATCCTCTAGAGGCCCTTA-TGGCCTTACTGAACCATTGTCAAGAAAGG-3′. For construction of the *GTL1pro:GUS* reporter, a 1.8-kb fragment of the 5′-untranslated region was cloned using the pENTR/D-TOPO cloning kit (Invitrogen) using oligonucleotides *PROF* 5′-CACCCACCTTCTTCTTCTTCACCTTC-3′ and *PROR* 5′-CATCAATCTTGATGATGTTTCAATC-3′ and subsequently cloned into the binary destination vector pGWB3 (Nakagawa et al., 2007). To express translational GTL1:GFP or GFP:GTL1 fusion proteins, a genomic 5.8-kb fragment was cloned into pENTR/D-TOPO using oligonucleotides *GTLGENF* 5′-CACCCACCTTCTTCTTCTTCACCTTC-3′ and *GTLGENR* 5′-CACCATTCATGACCTTAGTCTCTTC-3′. Additional *Sma*I restriction sites were inserted either downstream (adjacent to the translational start) or upstream (adjacent to the translational stop) of the coding sequence by site-directed mutagenesis using the following oligonucleotides: *GFPATGF* 5′-TTGATGCCCGGGGAGCAAGGAGG-AGGTGGT-3′ and *GFPATGR* 5′-CTCCCCGGGCATCAATCTTGATGAT-GTTTC-3′ (for N-terminal fusion), and *GFPTAAF* 5′-CAGCCCGGGTA-AAATCAGAATCATTGTTTC-3′ and *GFPTAAR* 5′-ATTTTACCCGGGCT-GAACCATTGTCAAGAA-3′ (for C-terminal fusion). An *Sma*I-digested GFP fragment was inserted either as an N- or C-terminal translational fusion, and the resulting fusion constructs were cloned into the binary destination vector pGWB1 (Nakagawa et al., 2007). All stable plant transformations were performed via floral dip (Clough and Bent, 1998).

RNA Extraction and Gene Expression Analyses

Total RNA was extracted from aerial parts of young seedlings or leaf trichomes using the Qiagen RNeasy plant mini kit. For extraction of total trichome RNA, trichomes of the fifth to seventh true leaf were collected from 3-week-old plants as previously described (Jakoby et al., 2008). Extracted RNA was treated with DNaseI (Takara Bio) following the manufacturer's instructions, and subsequent reverse transcription was performed using the Invitrogen Superscript III kit. Transcript levels were determined by quantitative real-time PCR using the SYBR Green Supermix kit (Toyobo). The following oligonucleotides were used for RT-PCR analysis of the full-length *GTL1* and the reference gene *APT1* (adenine-phosphoribosyl-transferase 1) expression in wild-type and *gtl1* mutants: *GTL1F* 5′-ATGAGCAAGGAGGAGGTGG-3′ and *GTL1R* 5′-TTACTGAAC-CATTGTCAAGAAAGG-3′, and *APT1F* 5′-GTGAAATGGCGACTGAA-GATGTGC-3′ and *APT1R* 5′-GCGACGTCTCTCCTAGTTTCTCCTT-3′. For quantitative PCR analysis of full-length *GTL1* expression in transgenic plants carrying *35S:GTL1*^{F26519} or *35S:GTL1* constructs, oligonucleotides *ENDOF* 5′-ACCTCCTTCTTTGTCATCTCAACC-3′ and *ENDOR* 5′-CTTG-AGGACGTTTCTTGTTGC-3′ (indicated by black arrows in Figure 1B) were used. For quantitative PCR analysis of total *GTL1* expression that is derived from both full-length *GTL1* and truncated *GTL1*^{F26519} genes in F26519 plants, oligonucleotides *ALLF* 5′-GAAAGAGAGAGTGTGTGTG-TAG-3′ and *ALLR* 5′-CCTGGAAACATGTTCCCAAAGAGG-3′ (indicated by white arrows in Figure 1B) were used. To normalize total RNA levels, Actin 2 (*ACT2*) mRNA concentrations were determined for each RNA sample using oligonucleotides *ACT2F* 5′-CTGGATCGGTGGTTCCA-TTC-3′ and *ACT2R* 5′- CCTGGACCTGCCTCATCATAC-3′. Quantitative PCR analysis of the cell cycle genes was performed using primer sets listed in Supplemental Table 1 online.

GUS Staining and Microscopy

To investigate the expression of *GTL1pro:GUS* reporter constructs, whole seedlings were fixed in acetone for 30 min at −30°C and then incubated in GUS staining solution for 5 to 12 h at 37°C as previously described (Malamy and Benfey, 1997). Stained plant tissue was washed in 70% ethanol for 4 h and cleared in chloral hydrate:H_2O:glycerol (8:2:1) overnight at 4°C. GUS signal was visualized using a Leica MZ 16 FA microscope equipped with a digital Leica DFC 500 camera.

Fluorescence Microscopy and Ploidy Measurements

The in vivo expression pattern and subcellular localization of the GTL1pro:GFP:GTL1 proteins were documented using an Olympus BX51 fluorescence microscope equipped with a digital Olympus DP70 camera and Olympus DP Manager software (version 1.2.1.107). The same equipment was used to record DAPI-stained nuclei in leaf trichomes (Zhang and Oppenheimer, 2004). Fluorescent signals were measured with ImageJ (version 1.37; NIH) as previously described (Schnittger et al., 1998; Sugimoto-Shirasu et al., 2005). Flow cytometry analysis was performed using the ploidy analyzer PA-I (Partec) as described previously (Sugimoto-Shirasu et al., 2002). At least 10,000 nuclei isolated from third and fourth leaves of 24-d-old seedlings were used for each ploidy measurement.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *GTL1*, At1g33240; *GT2*, At1g76890; *DF1*, At1g76880; At5g28300; and At5g47660.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Structure of the *GTL1* Gene Indicating the Position of T-DNA/Transposon Insertions.

Supplemental Figure 2. The *gtl1-1* Leaves Do Not Display Major Alterations in the Ploidy Distribution.

Supplemental Figure 3. GTL1 Is Expressed in Expanding Roots and Petals.

Supplemental Figure 4. Trichomes That Develop in *gtl1-1 ttg1-10* Leaves Are Slightly Larger and Possess Higher Ploidy Than Those in *ttg1-10* Leaves.

Supplemental Data Set 1. Protein Sequences Used to Generate the Phylogeny in Figure 2B.

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