

Note

Analysis of *TTG1* and CPC-like MYB genes during *Arabidopsis* epidermal cell differentiation

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Abstract The development of *Arabidopsis thaliana* epidermal cells includes the differentiation of trichomes and root hairs. The *TRANSPARENT TESTA GLABRA 1* (*TTG1*) gene encodes a WD40 protein that induces trichome differentiation and reduces root hair formation in *Arabidopsis*. The *CAPRICE* (*CPC*) gene family includes *CPC*, *ENHANCER OF TRY AND CPC1* (*ETC1*), *ENHANCER OF TRY AND CPC2* (*ETC2*), and *CPC LIKE MYB3* (*CPL3*), which encode R3-type MYB transcription factors that inhibit trichome differentiation and promote root hair formation. *CPC* expression is positively regulated by a transcriptional complex that includes *TTG1*. To determine whether *ETC1*, *ETC2*, and *CPL3* are also regulated by the *TTG1* complex, we examined the functional relationship between *TTG1* and *CPC-like MYB* genes. Double mutant analysis showed that the *ttg1* mutant is epistatic to the *cpc*, *etc1*, *etc2*, and *cpl3* mutants in trichome cell fate determination but not in root hair development. In roots, the *cpc* mutant is epistatic to the *ttg1* mutant in root epidermal cell fate determination. Promoter-GUS analysis indicated that *TTG1* is necessary for the expression of *ETC1* and *CPL3*, but not for *ETC2* expression. These results indicate that *TTG1* had a stronger effect on trichome formation than *CPC-like MYBs*. By contrast, *CPC* had a stronger effect on root hair formation than *TTG1*. Our results suggest that *ETC1* and *CPL3* are also regulated by the *TTG1* complex as is the case for *CPC*; however, *ETC2* is not regulated by this complex. We concluded that *ETC2* does not have a role in trichome and root hair formation.

Key words: *Arabidopsis*, MYB, root hairs, trichomes, *TTG1*.

Trichomes and root hairs are single cell extensions that originate from leaf or root epidermal cells in *Arabidopsis thaliana*. Numerous regulatory factors involved in epidermal cell differentiation have been identified. The *TRANSPARENT TESTA GLABRA1* (*TTG1*) gene encodes a WD40-repeat protein that induces trichome differentiation and reduces root hair formation in *Arabidopsis* (Galway et al. 1994; Walker et al. 1999). The *CAPRICE* (*CPC*) gene encodes an R3-type myeloblastosis (MYB) transcription factor in *Arabidopsis* (Wada et al. 1997). Several other *CPC-like MYBs* are known: *ENHANCER OF TRY AND CPC1* (*ETC1*); *ENHANCER OF TRY AND CPC2* (*ETC2*); and *CPC LIKE MYB3/ENHANCER OF TRY AND CPC3* (*CPL3/ETC3*). Overexpression of these transcription factors inhibits trichome differentiation and promotes root hair formation in *Arabidopsis* (Esch et al. 2004; Kirik et al. 2004a, 2004b; Simon et al. 2007; Tominaga et al. 2008).

The R2R3-type MYB transcription factors, WEREWOLF (WER) and GLABRA 1 (GL1), are also involved in epidermal cell differentiation in *Arabidopsis* (Lee and Schiefelbein 1999; Oppenheimer et al. 1991). The *TTG1*, *CPC-like MYB*, WER, and GL1 proteins interact with the bHLH proteins, GLABRA 3 (GL3)

and ENHANCER OR GLABRA 3 (EGL3), and act as a transcription regulatory complex of MYB-bHLH-WD40 in *Arabidopsis* epidermal cells (Bernhardt et al. 2003; Esch et al. 2003; Payne et al. 2000; Tominaga et al. 2008; Zhang et al. 2003). The WER-GL3/EGL3-*TTG1* transcription complex activates the expression of *GLABRA 2* (*GL2*) and induces non-hair cell fate (Bernhardt et al. 2003; Hung et al. 1998; Lee and Schiefelbein 1999; Payne et al. 2000). Conversely, the *CPC-like MYB-GL3/EGL3-TTG1* transcriptional complex is proposed to inactivate expression of *GL2* (Schiefelbein and Lee 2006; Tominaga-Wada and Wada 2014). Further, the WER-GL3/EGL3-*TTG1* transcriptional complex positively regulates the expression of *CPC* and *ETC1* genes (Bernhardt et al. 2003; Lee and Schiefelbein 2002; Simon et al. 2007).

In the present study, we sought to elucidate the relationship for transcriptional regulation between *TTG1* and the *CPC-like MYB* genes *ETC1*, *ETC2*, and *CPL3* in *Arabidopsis*. Previously, *CPC* expression was reported to be enhanced in roots by a transcriptional complex that included *TTG1* (Bernhardt et al. 2003). However, the precise change in expression of *ETC1*, *ETC2*, and *CPL3* induced by the *TTG1* transcriptional complex has not

been determined. To clarify the epistatic interactions during epidermal cell differentiation, we analyzed homozygous double mutant combinations of *ttg1 etc1*, *ttg1 etc2*, and *ttg1 cpl3*. To elucidate the relationship between *TTG1* and *CPC-like MYB* genes more precisely, we introduced the *ETC1::GUS*, *ETC2::GUS*, or *CPL3::GUS* transcriptional reporters into the *ttg1* mutant lines.

The *Arabidopsis thaliana* ecotype Columbia (Col-0), Landsberg *erecta* (Ler), and Wassilewskija (WS) were used as the wild type for these experiments. The *ttg1-1* (Ler background) (Koornneef et al. 1982), *ttg1-10* (Col-0 background) (Larkin et al. 1994), *cpc-1* (WS background) (Wada et al. 1997), *etc1-1* (Col-0 background), *etc2-2* (Col-0 background) (Tominaga-Wada et al. 2013), *cpl3-1* (Col-0 background) mutants, and the *35S::CPL3* (Col-0 background) transgenic plants (Tominaga et al. 2008) were used in the present study. Double mutants of *ttg1* and *cpc-like myb* mutants were screened from F2 progeny using PCR to identify homozygous *ttg1-10 etc1-1*, *ttg1-10 etc2-2*, *ttg1-10 cpl3-1*, *ttg1-1 cpc-1*, and *ttg1-1 cpl3-1* double mutants. *35S::CPL3* was introduced into *ttg1-1* mutant by a traditional cross and F2 seedlings were analyzed by PCR to identify *ttg1-1 35S::CPL3* lines. The *ETC1::GUS*, *ETC2::GUS*, and *CPL3::GUS* constructs (Tominaga et al. 2008) were introduced into the *ttg1-1*, and *ttg1-10* mutants by conventional crossing and F2 seedlings were analyzed by PCR.

Promoter::GUS plants were immersed in a solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -glucuronide), 1 mM $K_3Fe(CN)_6$, 1.0 mM $K_4Fe(CN)_6$, 100 mM NaPi (pH 7.0), 100 mM EDTA, and 0.1% Triton X-100. Primary roots of five-day-old seedlings were incubated at 37°C overnight. Aerial parts of two-week-old seedlings were incubated at 37°C for 3 h.

For the observation of seedling phenotypes, seeds were surface sterilized and sown on 1.5% agar plates using a method described previously (Okada and Shimura 1990). Seeded plates were incubated at 4°C for two days and then transferred to 22°C under continuous white light (50–100 $\mu\text{mol m}^{-2}\text{s}^{-1}$). For each mutant transgenic line, at least five two-week-old third leaves were observed for trichome formation, and at least ten individual five-day-old seedlings were assayed for root hair formation. Mutant and transgenic plants were observed using the Leica MZ16FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were recorded using a high-sensitivity CCD color camera system (Keyence VB 7010, Osaka, Japan).

Semi-quantitative RT-PCR analysis was performed. Total RNA was prepared from roots, shoots, stems, siliques, inflorescences, and rosette leaves using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). On-column DNase I digestion was performed during RNA purification following the protocol described in

the RNeasy Mini Kit handbook. First-strand cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mixture using the Prime Script RT Reagent Kit (Takara). Semi-quantitative RT-PCR reaction was conducted as described by Kurata et al. (2003). The *CPC* and *ETC2* fragments were amplified with RT128/RT129 and RT124/RT125 primer pairs, respectively (Tominaga-Wada and Nukumizu 2012). The *CPL3* and *EF* fragments were amplified with RT73/RT92 and EF1a-F/EF1a-R primer pairs, respectively (Tominaga et al. 2008). The *ETC1* fragment was amplified with the RT122 GCG ATC GTA AAT CTT TGT GTA CTA AG/RT123 CTC AGG AAC AAA ACT GCA GAA TTA C primer pair.

To investigate the interaction of the *TTG1* gene and the *CPC-like MYB* genes in epidermal cell development, plants carrying the *ttg1-10* and *ttg1-1* mutant alleles were crossed with *cpc-1*, *etc1-1*, *etc2-2*, or *cpl3-1* mutants, or the *35S::CPL3* transgenic line (Table 1). As previously reported, the *ttg1-10* single mutant has a dramatically reduced number of trichomes and a slightly increased number of root hairs, and the *ttg1-1* single mutant has no trichomes and a greatly increased number of root hairs compared to the wild type (Figure 1, Table 1). These observations were consistent with previous descriptions of the *ttg1* mutant alleles and suggest that between the two alleles, the *ttg1-1* allele presents a more severe phenotype than the *ttg1-10* allele (Larkin et al. 1994). The *ttg1-10 etc1-1*, *ttg1-10 etc2-2*, and *ttg1-10 cpl3-1* double mutants showed a dramatically reduced number of trichomes compared to wild type (Figure 1, Table 1). Based on these double mutant leaf phenotypes, *ttg1-10* is epistatic to *etc1-1*, *etc2-2*, and *cpl3-1* in trichome formation. However, compared to the *ttg1-10* single mutant, the *ttg1-10 etc1-1* and *ttg1-10 cpl3-1* double

Table 1. Number of leaf trichomes and root hairs on wild type and mutant seedlings.

Genotype	Trichome number/leaf	Root hair number/mm
Col-0	48.0 \pm 3.8	44.7 \pm 2.2
Ler	33.2 \pm 4.3	36.1 \pm 1.6
WS	51.6 \pm 6.1	37.0 \pm 1.2
<i>ttg1-10</i> (Col-0)	2.0 \pm 1.4	47.4 \pm 2.4
<i>ttg1-1</i> (Ler)	0	61.1 \pm 3.7
<i>cpc-1</i> (WS)	81.0 \pm 4.0	5.7 \pm 1.1
<i>etc1-1</i> (Col-0)	48.4 \pm 8.1	44.5 \pm 2.0
<i>etc2-2</i> (Col-0)	49.0 \pm 5.0	47.0 \pm 1.6
<i>cpl3-1</i> (Col-0)	59.6 \pm 2.3	31.3 \pm 2.0
<i>35S::CPL3</i> (Col-0)	0 \pm 0	59.6 \pm 1.9
<i>ttg1-10 etc1-1</i>	7.3 \pm 1.6*	39.4 \pm 1.6**
<i>ttg1-10 etc2-2</i>	0.2 \pm 0.2	46.7 \pm 2.3
<i>ttg1-10 cpl3-1</i>	9.2 \pm 1.2**	29.6 \pm 2.7**
<i>ttg1-1 cpc-1</i>	0.2 \pm 0.2	0.5 \pm 0.4
<i>ttg1-1 cpl3-1</i>	0 \pm 0	46.5 \pm 3.2
<i>ttg1-1 35S::CPL3</i>	0 \pm 0	99.7 \pm 4.4

Data represent the mean \pm S.D. of at least 5 leaves or 10 roots per experiment. Student's *t*-test, * p <0.05, ** p <0.02 vs. *ttg1-10*.

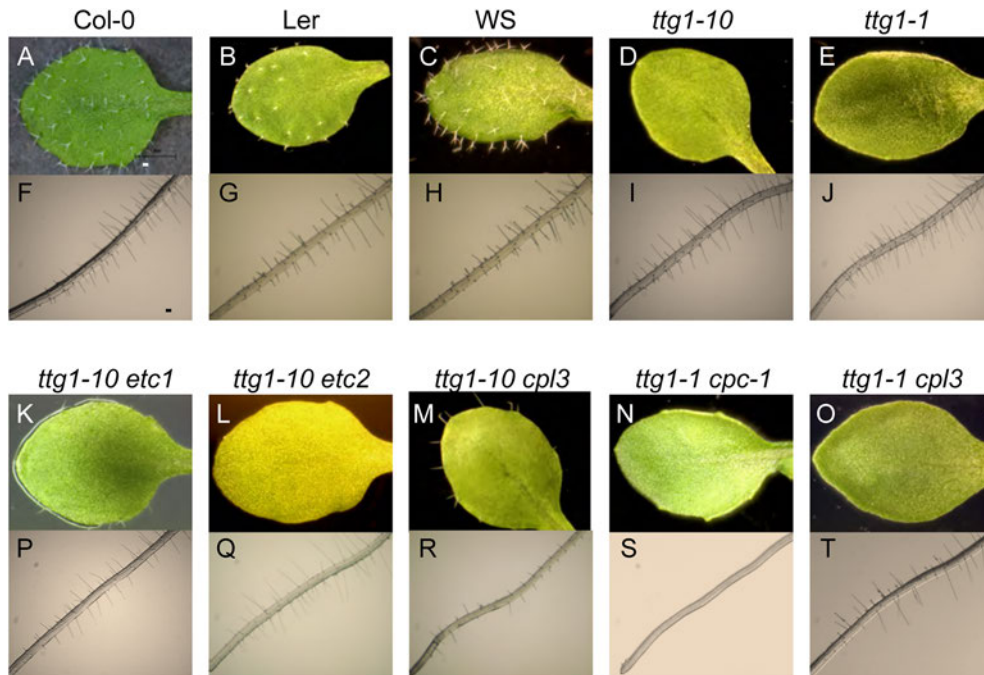


Figure 1. Leaf and root phenotypes of *Arabidopsis* mutants. Trichome formation on the third leaves of two-week-old *Arabidopsis* seedlings of Col-0 (A), Ler (B), WS (C), *ttg1-10* (D), *ttg1-1* (E), *ttg1-10 etc1-1* (K), *ttg1-10 etc2-2* (L), *ttg1-10 cpl3-1* (M), *ttg1-1 cpc-1* (N), and *ttg1-1 cpl3-1* (O). Root hair formation in five-day-old *Arabidopsis* seedlings of Col-0 (F), Ler (G), WS (H), *ttg1-10* (I), *ttg1-1* (J), *ttg1-10 etc1-1* (P), *ttg1-10 etc2-2* (Q), *ttg1-10 cpl3-1* (R), *ttg1-1 cpc-1* (S), and *ttg1-1 cpl3-1* (T). Scale bars: 1 mm in (A)–(E) and in (K)–(O); 200 μ m in (F)–(J) and in (P)–(T).

mutants produced significantly more trichomes (Figure 1, Table 1). Although most parts of the leaves of double mutants were glabrous, *ttg1-10 etc1-1* and the *ttg1-10 cpl3-1* double mutants produced several trichomes, especially at the leaf margin (Figure 1K, M). In addition, the *ttg1-10 etc1-1* and *ttg1-10 cpl3-1* double mutants had significantly reduced numbers of root hairs compared to that of the *ttg1-10* single mutant (Figure 1, Table 1). These results suggest that the mutation in *ETC1* and *CPL3* have opposing effects to *TTG1* on *Arabidopsis* root and leaf epidermal cell development. On the other hand, the *ttg1-10 etc2-2* double mutant showed similar trichome and root hair phenotypes to those of the *ttg1-10* single mutant (Figure 1, Table 1). These observations indicate that the *etc2* mutation did not have a noticeable effect on the trichome and root hair development in a *ttg1-10* mutant background. As shown in Figure 2J, *ETC2* was not expressed in roots. This result strongly indicates that *ETC2* is not involved in root hair formation. Although the *ETC1*, *ETC2*, and *CPL3* genes encode functionally equivalent R3 MYB proteins (Kirik et al. 2004a, 2004b; Tominaga et al. 2008), mutations in these genes showed different effects on the *ttg1-10* mutant, suggesting their functions may not be redundant.

The *ttg1-1 cpc-1* double mutant had a dramatically reduced number of trichomes and root hairs compared to that measured in wild type plants (Figure 1, Table 1). The *ttg1-1 cpc-1* double mutant leaves were nearly lacking all trichomes and resembled that of the *ttg1-1*

single mutant leaves, suggesting epistasis of *ttg1-1* to *cpc-1* in leaf trichome formation. The non-hair phenotype of the *ttg1-1 cpc-1* double mutant roots resembled that of the *cpc-1* single mutant roots, suggesting epistasis of *cpc-1* to *ttg1-1* on root hair formation. The *ttg1-1 cpl3-1* double mutant had a glabrous leaf phenotype resembling that of the *ttg1-1* single mutant, and possessed an intermediate number of root hairs (46.5 ± 3.2) compared to *ttg1-1* (61.1 ± 3.7) and *cpl3-1* (31.3 ± 2.0) single mutants (Figure 1, Table 1). These results suggest that the *ttg1-1* mutant is epistatic to the *cpc-1* and *cpl3-1* mutants regarding to trichome formation, whereas, the *cpc-1* mutant is epistatic to the *ttg1-1* mutant during root hair differentiation. However, because of genetic background differences, it is difficult to simply compare *ttg1-1 cpl3-1* and *ttg1-10 cpl3-1*. Consistent with the *ttg1* single mutant phenotypes, a relatively stronger effect of *ttg1-1* than *ttg1-10* was found even in double mutants with *cpl3-1* (Table 1). Although *ttg1-1 cpl3-1* had a no-trichome *ttg1-1*-like phenotype, *ttg1-10 cpl3-1* had a slightly increased number of trichomes compared with *ttg1-10*. The double mutant *ttg1-1 cpl3-1* possessed an intermediate number of root hairs compared to *ttg1-1* and *cpl3-1*; however, *ttg1-10 cpl3-1* produced a comparable number of root hairs to *cpl3-1*. Therefore, the *ttg1* mutation is epistatic to the *cpl3-1* mutation for trichome formation, whereas the *cpl3* mutation may be epistatic to the *ttg1* mutation for root hair formation in both *ttg1-1 cpl3-1* and *ttg1-10 cpl3-1* mutants.

When expressed in *ttg1-1* transgenic plants, the *35S::CPL3* construct produced an increased number of root hairs compared to untransformed parental lines, *35S::CPL3* or *ttg1-1* mutant. These results suggest a

synergistic effect between *CPL3* overexpression and the *ttg1-1* mutation on root hair formation (Table 1).

To explore the relationship between *TTG1* and CPC-like MYBs further, we introduced *ETC1::GUS*,

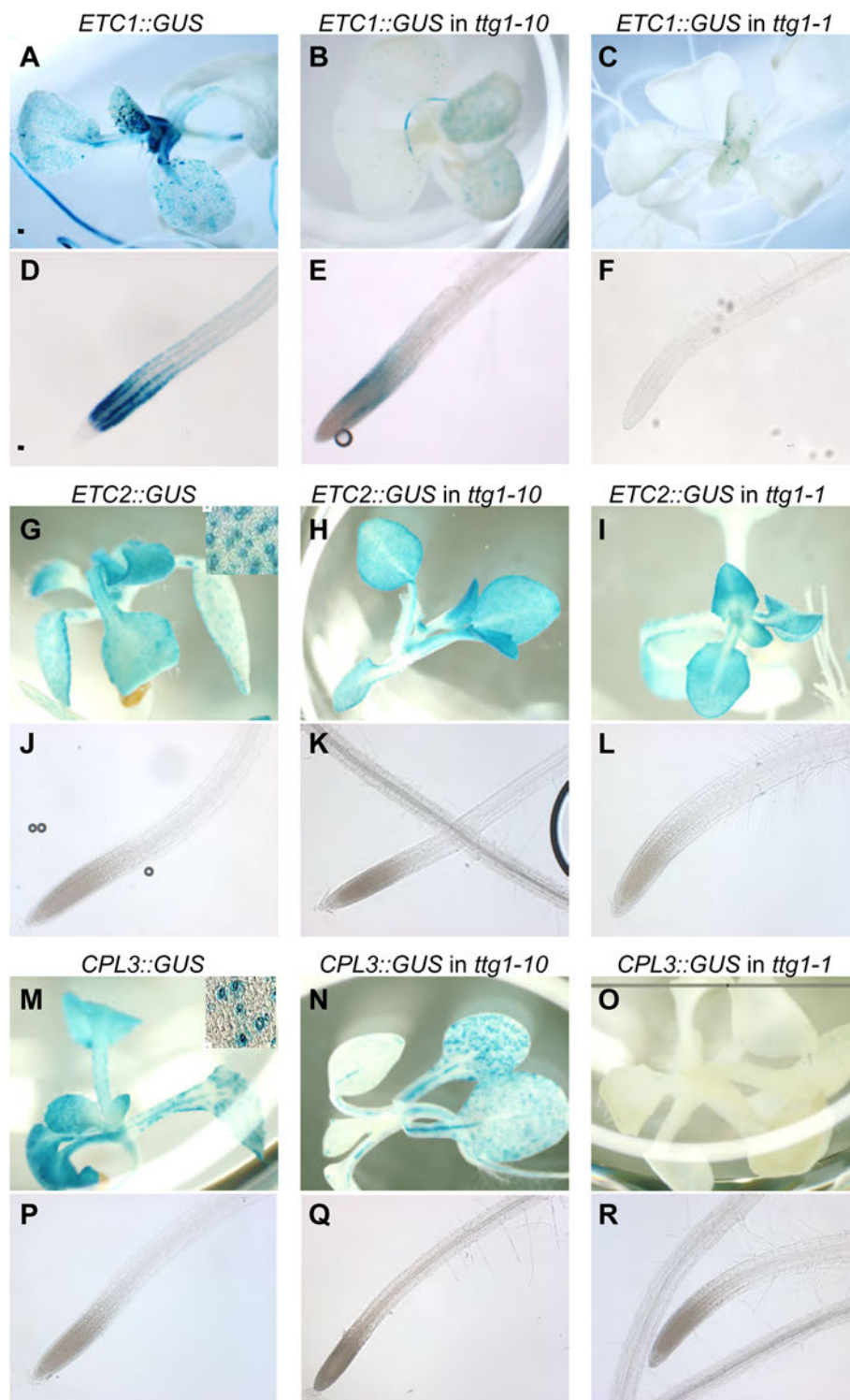


Figure 2. Histochemical staining of GUS activity in transgenic plants. Expression of the *ETC1::GUS* (A–F), *ETC2::GUS* (G–L), and *CPL3::GUS* (M–R) reporters in two-week-old leaves or in roots of five-day-old wild type Col-0 (A, D, G, J, M, and P), *ttg1-10* (B, E, H, K, N, and Q), and *ttg1-1* (C, F, I, L, O, and R) seedlings. Whole plants were stained with X-Gluc. Inset in G and M show magnified leaf epidermal cells ($\times 10$). Scale bars: 1 mm in (A, B, C, G, H, I, M, N, and O); $200\ \mu\text{m}$ in (D, E, F, J, K, L, P, Q, and R).

ETC2::GUS, and *CPL3::GUS* constructs into the two *ttg1* mutant alleles, *ttg1-10* and *ttg1-1*. Consistent with previous studies, *ETC1::GUS* was expressed primarily in trichomes and non-hair cell files in wild type *Arabidopsis* roots (Figure 2A, D); and *ETC2::GUS* and *CPL3::GUS* were expressed in wild type *Arabidopsis* young leaves, especially in guard cells (Figure 2G, M, insets) (Tominaga et al. 2008). *ETC1::GUS* expression was repressed in the *ttg1-10* mutant leaves and roots comparing with that of wild type (Figure 2B, E). *ETC1::GUS* expression was also strongly repressed in *ttg1-1* trichomes and abolished in *ttg1-1* roots (Figure 2C, F). However, the *ETC2::GUS* expression level did not change in the *ttg1-10* and *ttg1-1* mutant background when compared to its expression in wild type plants (Figure 2H, I). *ETC2::GUS* was expressed in young leaves of *ttg1-10* and *ttg1-1* with approximately the same intensity as that measured in wild type leaves (Figure 2H, I). *ETC2::GUS* expression was not observed in wild type, *ttg1-10*, and *ttg1-1* roots (Figure 2J–L). Similar to *ETC1::GUS* expression, the *CPL3::GUS* signal was strongly reduced in *ttg1-10* mutant leaves compared to that in wild type leaves (Figure 2N). Moreover, the *CPL3::GUS* expression was not detected in the *ttg1-1* mutant leaves (Figure 2O). *CPL3::GUS* was not expressed in root epidermal cells of wild type, *ttg1-*

10, and *ttg1-1* (Figure 2P–R). These results suggest that the *TTG1* gene is necessary for the native expression of the *ETC1* and *CPL3* genes in *Arabidopsis*. In contrast, the expression level of *ETC2* was not affected by the loss of the *TTG1* gene.

It is remarkable that the *cpl3-1* single mutant and the *ttg1-10 cpl3-1* double mutant exhibited a root hair phenotype, even though *CPL3::GUS* expression was not detected in the root (Figure 2P). To clarify this apparent contradiction, we performed a semi-quantitative PCR analysis to examine expression of selected genes. As expected, strong expression of *CPC* and *ETC1* in roots and rosette leaves was found using 25 cycles of amplification (Figure 3). *CPC* was also strongly expressed in siliques (Figure 3). However, even after 35 cycles of amplification, distinct *ETC2* expression could be detected only in rosette leaves (Figure 3). Relatively strong *CPL3* expression was detected in rosette leaves (Figure 3). After 35 cycles of amplification, a low level of *CPL3* expression was detected in roots (Figure 3). *CPC3* expression was detected in all tissues examined in this experiment after

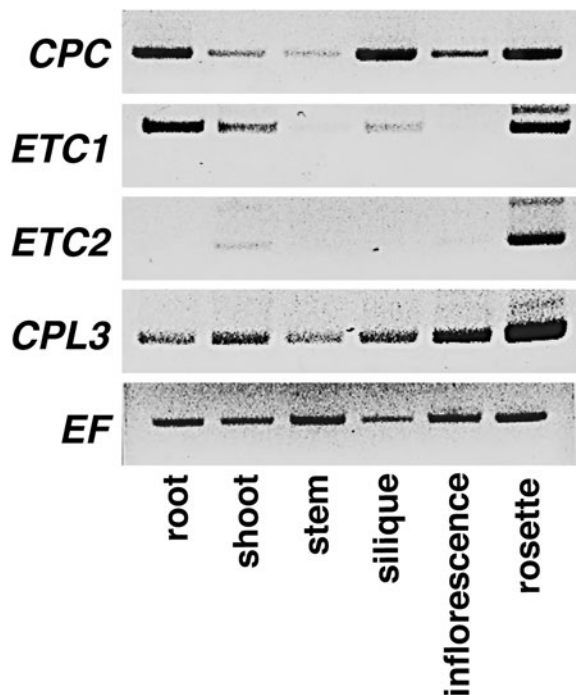


Figure 3. Semi-quantitative RT-PCR analysis of the expression of *CPC* family genes. Expression of *CPC*, *ETC1*, *ETC2*, and *CPL3* in different organs of *Arabidopsis*. Wild-type Col-0 was grown for 5 days. Total RNA was prepared from the root, shoot, stem, silique, inflorescence, and rosette leaf and subjected to semi-quantitative RT-PCR. Twenty-five amplification cycles were used for *CPC*, *ETC1*, and *EF*. Thirty-five amplification cycles were used for *ETC2* and *CPL3*. The expression of *EF* was used as a control.

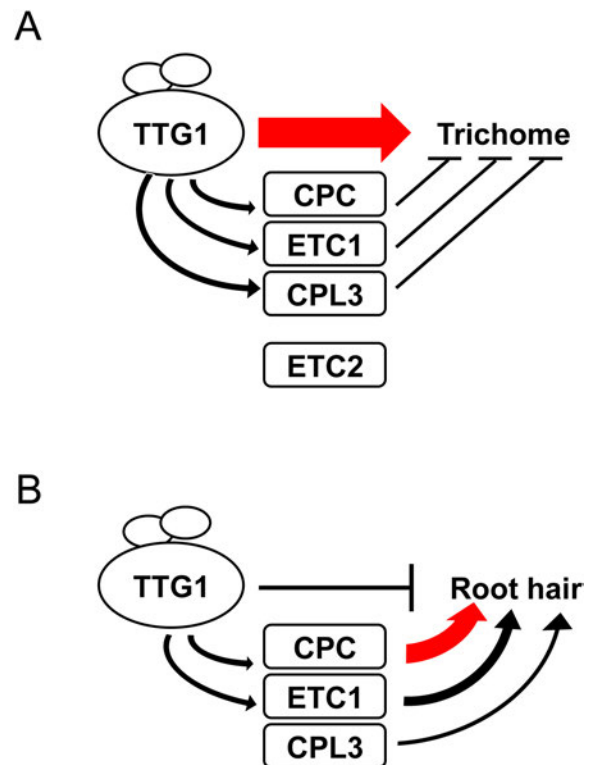


Figure 4. Model of root epidermal cell specification showing the regulation and proposed role of *TTG1*, *CPC*, *ETC1*, *ETC2*, and *CPL3*. (A) Trichome formation is strongly induced by a transcriptional complex that includes *TTG1*. This *TTG1* complex induces *CPC*, *ETC1*, and *CPL3* expression in leaves. *CPC*, *ETC1*, and *CPL3* participate in inhibition of trichome formation. (B) The *TTG1* complex inhibits root hair formation, but *CPC* counteracts its effect and strongly promotes root hair formation. *TTG1* complex-induced *CPC* and *ETC1* expression in roots. *ETC1* and *CPL3* also enhance root hair formation. Arrows indicate positive effects. Red arrows indicate strongly positive effects. Blunt lines indicate negative effects.

35 cycles of amplification. These results suggest that a low level of expression of *CPL3*, which was not detected by the *CPL3::GUS* analysis, might occur in roots and contribute to root-hair formation and therefore explain the root hair phenotype of *cpl3-1* and *ttg1-10 cpl3-1* mutants.

In this study, we showed that *TTG1* is necessary for the expression of *ETC1* and *CPL3*, but does not for *ETC2* expression in *Arabidopsis* leaf and root epidermal cells (Figure 4A). Mutations in the *TTG1* gene are epistatic to that of the *CPC-like MYB* genes during trichome cell differentiation; however, the *cpc* mutation has a stronger effect on root epidermal cell differentiation than do *ttg1* mutant alleles (Figure 4B). The *ttg1-1 cpc-1* double mutant showed a totally glabrous phenotype both in leaves and roots. We concluded that *ETC1* and *CPL3* were regulated by a transcriptional complex, including *TTG1*, as in the case for *CPC* (Figure 4). By contrast, *ETC2* was not regulated by this *TTG1* complex and did not contribute to trichome or root hair formation (Figure 4). Our findings also indicate that subtle *CPL3* expression in roots may contribute to root hair formation against the effect of *TTG1*.

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