TRICHOMELESS1 regulates trichome patterning by suppressing GLABRA1 in Arabidopsis

Shucai Wang1, Su-Hwan Kwak2, Qingning Zeng1, Brian E. Ellis3, Xiao-Ya Chen4, John Schiefelbein2,* and Jin-Gui Chen1.*

The patterning of epidermal cell types in Arabidopsis is a simple and useful model for studying the molecular basis of cell specification in plants. The distribution of different cell types in the Arabidopsis epidermis is regulated by a lateral inhibition mechanism that relies on interactions between transcription factors. However, it is unclear how temporal- or organ-specific differences in epidermal patterning are achieved. Here we identify TRICHOMELESS1 (TCL1) as a new and major single-repeat MYB-type transcription factor that negatively regulates trichome formation in the inflorescence epidermis. A dominant mutant with elevated expression of TCL1 has a glabrous (trichomeless) phenotype, whereas a loss-of-function mutation in TCL1 uniquely confers ectopic trichome formation on inflorescence stem and pedicels. Genetic analyses demonstrate that TCL1 and CAPRICE work synergistically to regulate trichome patterning on these organs. Interestingly, overexpression of TCL1 specifically suppresses the expression of GLABRA1 (GL1), a crucial component in the trichome initiation complex, whereas loss-of-function of TCL1 enhances GL1 expression. Chromatin immunoprecipitation results show that TCL1 can be recruited to the cis-acting regulatory elements of GL1. These results provide the first molecular and genetic evidence that an R3 MYB may negatively regulate trichome cell specification in a novel manner by directly suppressing the transcription of GL1.

KEY WORDS: Lateral inhibition, MYB, Pattern formation, Trichome, TRICHOMELESS1

INTRODUCTION

The patterning of epidermal cell types in Arabidopsis has become one of the best models for studying the molecular basis of cell specification in plants (Schiefelbein, 2003; Pesch and Hülskamp, 2004; Serna, 2005; Schellmann et al., 2007). Trichome patterning in Arabidopsis is controlled by several transcription factors. According to their effect on trichome initiation, these transcription factors can be divided into two groups: positive regulators and negative regulators. Positive regulators include the WD-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Galway et al., 1994; Walker et al., 1999), the R2R3 MYB-type transcription factor GLABRA1 (GL1) (Oppenheimer et al., 1991), the basic helix-loop-helix (bHLH) transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne et al., 2000; Zhang et al., 2003) and the homeodomain protein GLABRA2 (GL2) (Rerie et al., 1994; Masucci et al., 1996). The negative regulators include several small single-repeat R3 MYB transcription factors, such as TRIPTYCHON (TRY) (Schnittger et al., 1999; Schellmann et al., 2002), CAPRICE (CPC) (Wada et al., 1997; Wada et al., 2002) and ENHANCER OF TRY AND CPC 1 and 2 (ETC1 and ETC2) (Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b).

These positive and negative regulators work together to control trichome initiation and patterning in Arabidopsis. The R2R3 MYB-type transcription factor GL1, a bHLH transcription factor (GL3 or EGL3), and TTG1 form a complex to induce the expression of GL2, which in turn induces trichome formation in shoots (reviewed by Schiefelbein, 2003; Pesch and Hülskamp, 2004). The small MYB transcription factors TRY, CPC, ETC1 and ETC2, inhibit the trichome cell type in the shoot, presumably by competing with GL1 for binding GL3, thereby limiting the transcriptional activity of the trichome initiation and patterning activator complex (Hülskamp et al., 1994; Wada et al., 1997; Schellmann et al., 2002; Esch et al., 2003).

Root hair patterning is largely controlled by the same components, except that GL1 is replaced by another R2R3 MYB-type transcription factor, WEREWOLF (WER), to form a complex with TTG1 and GL3/EGL3 to induce GL2 expression (Bernhardt et al., 2003; Bernhardt et al., 2005; Lee and Schiefelbein, 1999). However, the positive regulators for trichome initiation function to inhibit root hair initiation, whereas negative regulators for trichome initiation function to promote root hair initiation (Schiefelbein, 2003; Pesch and Hülskamp, 2004).

We report here the identification and functional analysis of a new negative regulator for trichome initiation and patterning, TRICHOMELESS1 (TCL1). TCL1 represents a previously unknown member of the single-repeat R3 MYB transcription factor family. We demonstrate that overexpression of TCL1 completely abolishes trichome formation on all organs examined, whereas a loss-of-function mutation in TCL1 confers unique, ectopic trichome formation along inflorescence stems and pedicels. These trichome phenotypes have not been reported in mutants of any other members of the single-repeat R3 MYB transcription factor family. Furthermore, we provide genetic evidence that TCL1 and CPC work synergistically to regulate trichome formation on inflorescence stems and pedicels. In an unexpected finding, we show that TCL1 is likely to act by negatively regulating GL1 expression. This provides new insight into the organ-specific control of epidermal patterning and suggests the existence of a novel regulatory loop in trichome patterning.

1Department of Botany, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. 2Department of Molecular, Cell, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA. 3Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. 4National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China.

*Authors for correspondence (e-mails: schiefel@umich.edu; jingui@interchange.ubc.ca)

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Isolation of the tcl1-1D mutant and molecular cloning of TCL1

A dominant mutant with glabrous leaves was isolated from an activation-tagged mutant population (~10,000 plants) produced in a gpa1-2 mutant background (Ullah et al., 2001), and designated as tcl1-1D. A plasmid rescue technique was used to clone the TCLI gene locus. Genomic DNA (20 µg) isolated from the tcl1-1D mutant was digested with PstI, which left the right-border of the T-DNA intact. The digestion products were purified, ligated, and transformed into Escherichia coli DH5α. The transformants were selected on LB plates containing 100 µg/ml ampicillin. Two independent colonies were selected and the plasmid DNA was sequenced using T-DNA left-border (5'-TTGACATGAGCACAATTGC-3') and right-border (5'-ATGTTGATATCTGATCCGAAAC-3') primers. The tcl1-1D phenotypes were subsequently confirmed by recification experiments.

Isolation of the loss-of-function allele tcl1-1

A T-DNA insertion mutant allele of TCLI, SALK_055460, was identified from the SALK T-DNA Express Database (http://signal.salk.edu/cgi-bin/tDNAexpress). In this allele, the T-DNA insertion site is in the second intron of the TCLI gene. The insertion was confirmed by PCR and sequencing using a TCLI-specific primer (5'-ATGGATAAACACAAACCGTC-3') and the T-DNA-specific primer JMLB1 (5'-GGCAAACTCA- GCTGTGTCGCCGTCCTACGTTG-3'), and the mutant allele was designated tcl1-1. Loss of detectable full-length TCLI transcript in the tcl1-1 mutant was verified by RT-PCR.

Plasmid construction

To generate the 35S::HA-TCLI construct, the full-length open reading frame (ORF) of the TCLI gene (GenBank accession number AT2g30432) was amplified by RT-PCR using primers designed to amplify from 10-day-old light-grown Arabidopsis seeds. The PCR fragment was then cloned in frame with an N-terminal HA tag into the pUC19 vector under the control of the double 35S enhancer promoter of CaMV. RT-PCR was used to confirm the 35S::HA-TCLI construct and to validate the TCLI promoter (a fragment that covers the region –1535 to +1 of the start codon of TCLI). The P35S::GUS construct was cloned by replacing the P35S promoter in P35S::GUS (Wang et al., 2007) with the TCLI promoter. Corresponding constructs in pUCh19 were digested with EcoRI, then subcloned into the binary vector pZP211 or pZP221 for plant transformation (Hajdukiewicz et al., 1994).

Phylogenetic analysis

The phylogenetic tree for TCLI, TRY, CPC, ETC1, ETC2 and At4g01060 (Fig. 2D) was generated using AlienBee - Multiple Alignment software, release 2.0 (http://www.genebee.msu.su/services/malign_reduced.html).

Plant transformation and selection of transgenic plants

Plants of about 5 weeks of age and with several mature flowers on the main inflorescence were transformed with various constructs via Agrobacterium tumefaciens GV3101 by the floral dip method (Clough and Bent, 1998).
RESULTS

TRICHOMELESS1 encodes a single-repeat R3 MYB transcription factor

A dominant mutant with glabrous leaves was identified in an activation-tagged mutagenized population of Arabidopsis. The null mutant of the heterotrimeric G-protein α subunit, gpa1-2 (Ullah et al., 2001), was used as the parental genotype. This glabrous mutant was named trichomeless I-1 Dominant (tcl1-1D) (Fig. 1). Compared with wild type and its parent, the tcl1-1D mutant does not have any trichomes on the first pair of rosette leaves (Fig. 1A, Table 1), nor on successive leaves, inflorescence stems, cauline leaves or floral organs (data not shown). However, all other aspects of plant growth and development studied were unaffected in the tcl1-1D mutant.

A plasmid rescue procedure (Weigel et al., 2000) was used to identify the T-DNA insertion site in the tcl1-1D mutant. As shown in Fig. 2A, in the tcl1-1D mutants the T-DNA was inserted in chromosome 2 at a position that is 2115 bp upstream of the start codon of the gene locus At2g30432, and 1339 bp downstream of the stop codon of the gene locus At2g30432, with the four outward-facing 35S enhancers oriented toward the gene locus At2g30432. RT-PCR analysis revealed that the transcript level of gene locus At2g30432 was elevated (Fig. 2B), indicating that the TCL1 gene locus is most likely to be at At2g30432.

BLAST search analysis indicated that TCL1 encodes a protein that is closely related to proteins encoded by the previously characterized genes TRY, CPC, ETC1, ETC2, and by an uncharacterized gene locus At4g01060 (Fig. 2C) (Kirik et al., 2004b; Serna and Martin, 2006). All these proteins have a conserved MYB region that is most closely related to the R3 MYB domains of the R2R3 MYB gene family members in plants (Wada et al., 1997; Schellmann et al., 2002; Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b). Therefore, TCL1 represents a previously unknown member of the single-repeat R3 MYB transcription factor family. Results from phylogenetic analysis suggested that TCL1 is more closely related to CPC than to TRY (Fig. 2D).

To confirm that the phenotypes observed in the tcl1-1D mutant were caused by elevated expression of TCL1, we transformed wild-type Columbia (Col) plants with a binary vector containing the N-terminal HA-tagged full-length ORF of TCL1 expressed from the strong 35S promoter of the cauliflower mosaic virus (35S:HA-TCL1). As expected, overexpression of TCL1 recapitulated the tcl1-1D phenotypes (Fig. 1B). The transcript level of TCL1 in the transgenic lines was confirmed by RT-PCR using HA-specific and TCL1-specific primers (Fig. 1B).

TCL1 does not appear to affect root hair formation and patterning. The number and pattern of root hair cells in the tcl1-1D mutant or in plants overexpressing TCL1 were indistinguishable from those of wild-type plants (Fig. 1C, and see Table S1 in the supplementary material). Because overexpression of the other single-repeat R3 MYBs (including CPC, TRY, ETC1 and ETC2) using the 35S promoter induces ectopic root hair cells (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004a; Kirik et al., 2004b), this result indicates that the TCL1 protein may differ functionally from other R3 MYBs.

Loss-of-function mutation in TCL1 promotes trichome formation on inflorescence stems and pedicels

To further analyze the function of TCL1 in trichome formation, we took a reverse genetic approach to seek loss-of-function alleles of TCL1. By searching the SALK T-DNA Express Database (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al., 2003), we found one mutant allele, SALK_055460, in which TCL1 is interrupted by a T-DNA insertion within its second intron (Fig. 3A). The presence of the T-DNA at the expected location was further verified by sequencing, and plants homozygous for the T-DNA insertion at this locus were isolated by PCR-based screening (data not shown). This mutant allele was named tcl1-1. The expression of TCL1 was undetectable in tcl1-1 mutants by RT-PCR (Fig. 3B), indicating that tcl1-1 is likely to be a loss-of-function mutant allele of TCL1.

Because trichome initiation in the tcl1-1D mutant was suppressed, we expected to see an increase in trichome initiation or altered trichome patterning in the tcl1-1 mutant. We first checked trichome production on rosette leaves. However, both trichome initiation and patterning in the tcl1-1 mutant were indistinguishable from those in wild-type plants (Table 1). Interestingly, we observed a dramatic increase in trichome...
formation on the inflorescence stems in the tcl1-1 mutant (Fig. 3C, Fig. 4). In wild-type plants, in addition to a decline in adaxial trichome production on successive cauline leaves (Telfer et al., 1997), a decline of trichome production on successive inflorescence stem internodes was also observed (Gan et al., 2006). We found that no matter how many internodes (usually 3–4) Col wild-type plants produced on the main inflorescence stem, trichome production was restricted to the region below the first flower on the main inflorescence stem. Only very few, or no trichomes were formed on the internode just adjacent to the first flower (Fig. 3C, Fig. 4). However, in the tcl1-1 mutant, the number of trichomes on the main inflorescence stem internodes was dramatically increased (Fig. 3C, Fig. 4). Moreover, trichomes also formed beyond the site of the first flower branch (Fig. 3C, Fig. 4). Trichome distribution on the internodes of lateral branches was similar to that of the main inflorescence stem (data not shown). Such ectopic trichome formations on the inflorescence stems have not been reported for mutants of any other members of the single-repeat R3 MYB transcription factor family.

In addition to the ectopic formation of trichomes on inflorescence stems, tcl1-1 mutants also produced trichomes on pedicels (Fig. 3C, Fig. 4). No trichomes were found on the pedicels of wild-type plants (Fig. 3C). Similar to the pattern on inflorescence internodes, trichome production declined on successive pedicels in the tcl1-1 plants (Fig. 4). Again, ectopic trichome formation on pedicels has not been reported in mutants of any other members of the single-repeat R3 MYB transcription factor family. Mutations in ETC2, one member of this gene family, confer ectopic formation of trichomes on the leaf petioles but not on the inflorescence stems or pedicels (Kirik et al., 2004b). Based on these results, we conclude that TCL1 has a unique role in regulating epidermal cell patterning by controlling trichome cell specification on the inflorescence stems and pedicels.

To confirm that the phenotype we observed in tcl1-1 mutants was due to the loss-of-function of TCL1, we transformed tcl1-1 mutants with a binary vector containing the full-length ORF of TCL1 fused in frame with GFP, driven by TCL1’s own promoter (P_{TCL1}:TCL1-GFP). We used a genomic DNA fragment that covers the region −1535 to +1 of the start codon of TCL1 to provide putative regulatory sequences for TCL1. When expressed from this putative regulatory region, the TCL1 ORF was able to complement the tcl1 mutant (Fig. 3C), indicating that this putative regulatory sequence is sufficient for normal TCL1 expression, and that the TCL1-GFP fusion protein is most likely functional. The complete rescue of the tcl1-1 phenotype by P_{TCL1}:TCL1-GFP shows that the trichome phenotype in the tcl1-1 mutant is indeed due to the loss-of-function of TCL1.

Microscopic examination of the P_{TCL1}:TCL1-GFP transgenic plants revealed that TCL1 is localized in the nucleus of epidermal cells, but TCL1-GFP fluorescence could also be detected in regions near to, or at, the plasma membrane (Fig. 3D).

### Synergistic effect between TCL1 and CPC on trichome formation

Four single-repeat R3 MYB proteins, CPC, TRY, ETC1 and ETC2, have been shown to repress trichome initiation in a redundant manner (Schellmann et al., 2002; Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b). Overexpression of TCL1 also repressed trichome initiation (Fig. 1A), implying that TCL1 might function redundantly with other single-repeat MYB proteins in regulating trichome formation. Because TRY and CPC are the best characterized of the known single-repeat R3 MYB transcription factors, we focused on testing functional redundancy between TCL1 and TRY or CPC.

We generated double mutants between tcl1-1 and try or cpc. As shown in Table 1, tcl1-1 try double mutants have no significant difference in the number of trichomes or trichome clusters on their

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**Table 1. Leaf trichome production in wild-type, mutant and transgenic lines**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of trichomes per leaf</th>
<th>Frequency of trichome clusters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Col)</td>
<td>50.7±10.2</td>
<td>0</td>
</tr>
<tr>
<td>WT (WS)</td>
<td>39.1±5.4</td>
<td>0</td>
</tr>
<tr>
<td>tcl1-1D</td>
<td>0±0*</td>
<td>0</td>
</tr>
<tr>
<td>35S:HA-TCL1</td>
<td>0.1±0.1*</td>
<td>0</td>
</tr>
<tr>
<td>P_{TCL1}:TCL1-GFP/tcl1-1</td>
<td>49.7±11.7</td>
<td>0</td>
</tr>
<tr>
<td>tcl1-1</td>
<td>55.8±9.2</td>
<td>0</td>
</tr>
<tr>
<td>cpc</td>
<td>83.7±16.6*</td>
<td>0.1</td>
</tr>
<tr>
<td>try</td>
<td>44.1±8.2*</td>
<td>7.1*</td>
</tr>
<tr>
<td>tcl1-1 cpc</td>
<td>86.9±12.5*</td>
<td>0.2</td>
</tr>
<tr>
<td>tcl1-1 try</td>
<td>45.8±8.5</td>
<td>8.3*</td>
</tr>
<tr>
<td>cpc try</td>
<td>181±24*</td>
<td>85*</td>
</tr>
<tr>
<td>tcl1-1 cpc try</td>
<td>164±28*</td>
<td>81*</td>
</tr>
</tbody>
</table>

Values indicate means±s.d. of at least ten rosette leaves for each line. * P<0.05, relative to the corresponding wild-type line.
rosette leaves, as compared with the *try* single mutant. Furthermore, the *tcl1-1 try* double mutant had no significant change in root epidermal cell pattern (see Table S1 in the supplementary material). Similarly, *tcl1 cpc* double mutants were indistinguishable from the *cpc* single mutant in leaf trichome formation or in H- and N-root epidermal cell pattern (see Table S1 in the supplementary material).

We next examined trichome formation on the inflorescence stems and pedicels in adult plants of *tcl1-1 try* and *tcl1-1 cpc* double mutants to determine whether TRY and CPC have overlapping function with TCL in trichome cell specification on these organs. As shown in Fig. 4, the number of trichomes on the inflorescence stem and pedicels in *tcl1-1 try* double mutants was statistically equivalent to that in the *tcl1-1* single mutant. However, the number of trichomes on the inflorescence stem was increased 2- to 5-fold in *tcl1-1 cpc* double mutants, as compared with the *tcl1-1* single mutant. The number of trichomes on the pedicels in *tcl1-1 cpc* double mutants increased even more dramatically. For example, a >10-fold increase in trichome number was observed in the sixth flower/silique pedicel on the main inflorescence stem of *tcl1-1 cpc* double mutants, as compared with *tcl1-1* single mutants (Fig. 4C). Furthermore, a significant number of trichomes were also found in the seventh pedicel along the main inflorescence stem of *tcl1-1 cpc* double mutants, whereas no trichomes were found at the same site in the *tcl1-1* single mutant. These results indicate that TCL1 and CPC can work synergistically to regulate trichome formation on the inflorescence stem and pedicels. Because the number of trichomes on the inflorescence stems and pedicels in *try* or *cpc* single mutants do not differ from those in wild-type, these results confirmed that TCL1 is the major player of the single-repeat R3 MYB transcription factor family in regulating trichome cell specification on inflorescence stem and pedicels.

We generated *tcl1-1 cpc try* triple mutants and found that they did not differ significantly from the *cpc try* double mutants in terms of the number of trichomes and trichome clusters on their rosette leaves (Table 1), or in root epidermal cell pattern (see Table S1 in the supplementary material). As expected, *tcl1-1 cpc try* triple mutants formed trichome clusters on the inflorescence stems beyond the site of the first flower branch, and on pedicels (Fig. 5). These results support a predominant role for TCL1, compared to that of other members of the single-repeat R3 MYB transcription factor family, in regulating trichome cell specification on inflorescence stem and pedicels.
TCL1 suppresses the expression of GL1

To gain insight into the mechanism by which TCL1 affects trichome initiation and patterning, we examined the expression of the known trichome initiation and patterning positive regulators GL1 and GL3. Consistent with this, we found that the expression of GL1::GUS reporter was dramatically reduced in plants overexpressing TCL1 (Fig. 6B). These results raise the possibility that TCL1 affects trichome formation by directly suppressing GL1 expression. In this scenario, the reduced expression of GL2 in plants overexpressing TCL1 might be a consequence of reduced expression of GL1, because a reduced availability of GL1 would decrease the transcriptional activity of the overall activator complex. To investigate this further, we examined the expression levels of GL1 and GL2 in the developing inflorescence of tcl1-1 mutants by RT-PCR, because TCL1 was expressed at a relatively higher level in inflorescence than in other tissues/organs (Fig. 6C). GL1 and GL2 transcripts were found to be increased in the tcl1-1 mutant, as compared with the wild type (Fig. 6D), supporting the possibility that TCL1 negatively regulates GL1 in the developing inflorescence epidermis.

GL1 is a target gene of TCL1

The hypothesis that TCL1 might directly target GL1 was tested further. Because TCL1 is a single-repeat R3 transcription factor that does not contain an apparent transcription-activation domain, we first tested whether TCL1 itself could alter reporter gene expression in a protoplast transient-expression system (Tiwari et al., 2003; Schellmann et al., 2005). As expected, the TCL1 protein alone could not activate or repress reporter gene expression when it was recruited to the promoter region (Gal4) of the GUS reporter gene (Fig. 7A,B). However, when TCL1 was fused with a heterologous activator domain, VP16, the TCL1-VP16 fusion protein was able to function efficiently as an activator (Fig. 7A,B). We therefore generated transgenic plants overexpressing this TCL1-VP16 fusion protein.

We reasoned that if GL1 is the target for TCL1, one would expect that the expression of GL1 would be elevated in 35S:TCL1-VP16 plants owing to the direct binding of TCL1 to the cis-acting regulatory elements of GL1 and the concurrent activation of GL1 transcription by VP16. In addition, we would predict that the 35S:TCL1-VP16 plants should phenocopy plants overexpressing GL1. Indeed, multiple transgenic lines of 35S:TCL1-VP16 plants displayed dramatically reduced number of trichomes on their leaves and inflorescence stems, some of which had glabrous stems (Fig. 7C), thus phenocopying plants overexpressing GL1 (Larkin et al.,...
Consistent with this, we detected elevated levels of GL1 transcript (in contrast to largely unchanged levels of TTG1 and GL3 transcripts) in these transgenic lines (Fig. 7D). Taken together, these results support a working model in which TCL1 controls trichome patterning by suppressing the expression of GL1 (Fig. 8).

To test this directly, we used a chromatin immunoprecipitation (ChIP) assay to determine the association of TCL1 protein with the cis-acting regulatory sequence of GL1. 35S:HA-TCL1 plants and anti-HA antibodies were used. We detected a specific PCR product of the expected size amplified using primers specific to the first intron or the 3′-UTR region of GL1 were used in PCR reactions. ACTIN2 provided a control.

DISCUSSION

We report the identification and functional analysis of a previously unknown member of the single-repeat R3 MYB transcription factor family, TCL1. Several lines of evidence support the hypothesis that TCL1, like TRY, CPC, ETC1 and ETC2 (Hülskamp et al., 1994; Wada et al., 1997; Schellmann et al., 2002; Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b), acts as a negative regulator for trichome initiation. First, the dominant mutant tcll-1D isolated from an activation-tagged population displayed a glabrous phenotype, owing to TCL1 overexpression (Fig. 1, Table 1). Second, ectopic overexpression of TCL1 under the control of the CaMV 35S promoter in wild-type plants resulted in the loss of trichomes (Fig. 1, Table 1). Finally, a loss-of-function mutation in TCLI in Arabidopsis, GL1 encodes an R2R3 MYB-type transcription factor. GL3 encodes a bHLH-related transcription factor. TTG1 encodes a WD40 domain-containing protein. GL2 encodes a homeobox transcription factor. TCLI encodes a single-repeat R3 MYB transcription factor. Other known single-repeat MYB transcription factors, including TRY, CPC, ETC1 and ETC2, are not shown in the model. TTG1, GL3 and GL1 form an activator complex, whereas TCLI, TRY, CPC, ETC1 and ETC2 act as repressors that were proposed to bind GL3, thus limiting the interaction between GL1 and GL3, an interaction that is required to form the TTG1-GL3-GL1 activator complex that regulates the transcription of GL2. As shown in this study, TCLI can directly suppress the expression of GL1, thereby limiting the transcriptional activity of the TTG1-GL3-GL1 activator complex. It is unclear whether the TTG1-GL3-GL1 activator complex can promote the transcription of TCLI. Arrows indicate positive regulation and the blunt-ended line indicates negative regulation.

TCL1 is a major negative regulator for trichome patterning on the inflorescence stems and pedicels

In wild-type plants, trichome initiation decreases on successive internodes with only very few or no trichomes on the internode subtending the site of the first flower on the main inflorescence stem (Gan et al., 2006), and no trichomes were found on the main inflorescence stem above the site of the first flower (Figs 3, 4). In the tcll-1 mutant, the number and patterning of trichomes on leaves were indistinguishable from those of wild type (Table 1). However, the number of trichomes was significantly increased in the internodes of tcll-1 mutants, and trichomes were also formed...
beyond the site of the first flower (Figs 3, 4). Loss-of-function of TCL1 also results in trichome formation on pedicels, which normally do not bear any trichomes (Figs 3, 4, 5). Because these trichome phenotypes on inflorescence stems and pedicels have not been reported in mutants of other members of the single-repeat R3 MYB transcription factor family, our data support the notion that TCL1 is the predominant member of this family for the regulation of trichome formation on these organs.

Although TCL1 functions as a trichome initiation repressor, as do TRY, CPC, ETC1 and ETC2 (Hulsinkamp et al., 1994; Wada et al., 1997; Schellmann et al., 2002; Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b), our results suggest that TCL1 might have a unique biochemical role in regulating epidermal cell specification because TCL1 does not appear to affect root hair formation and patterning. The position-dependent specification of root epidermal cells is not altered in either the gain- or loss-of-function mutants of TCL1 (Fig. 1, and see Table S1 in the supplementary material), whereas overexpression of the other single-repeat R3 MYBs tested, including CPC, TRY, ETC1 and ETC2, induces ectopic root hair cells (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004a; Kirik et al., 2004b). These results imply significant differences in the biochemical properties of these single-repeat R3 MYBs. Our results from genetic analysis of double and triple mutants among tcl1-1, cpc and try, suggested that TCL1 and CPC, but not TRY, have a synergistic effect on trichome formation on inflorescence epidermis (Figs 4, 5). These findings are consistent with the promoter-swap assays. We found that the expression of TCL1 under the control of the CPC promoter in a cpc mutant background (P<sub>cpc</sub>:HA-TCL1/cpc) could partially rescue the root hair phenotype of the cpc mutant while simultaneously repressing trichome initiation on leaves (see Fig. S1 in the supplementary material). On the other hand, trichome clusters, a phenotypic trait of the try mutant (Esch et al., 2003), were still present on the leaves of plants expressing TCL1 under the control of the TRY promoter in a try mutant background (P<sub>try</sub>:HA-TCL1/trv), although their frequency was significantly reduced (see Fig. S1 in the supplementary material). These results suggest that the TCL1 protein is not fully interchangeable with TRY in controlling trichome patterning on rosette leaves, and that TCL1 is functionally similar, but not identical, to CPC in the developmental context of regulation of epidermal cell specification. These results support the notion that the functional specificity of TCL1 largely derives from the protein coding sequence, rather than from its promoter activity.

The phenotype of ectopic trichome formations on the inflorescence stems observed in tcl1-1 mutants has also been reported for plants overexpressing GIS (Gan et al., 2006). GIS encodes a transcription factor of the C2H2 family, and acts upstream of the trichome initiation complex (Gan et al., 2006). Recently, it has been found that GIS and two other C2H2 transcription factors, ZFP8 and GIS2, play partially redundant and essential roles in inflorescence trichome initiation and are regulated by the plant hormones, gibberellins and cytokinins (Gan et al., 2007). It is unclear whether TCL1 can work together with GIS to regulate trichome formation on inflorescence epidermis.

Although TCL1 is a major negative regulator for trichome patterning on the inflorescence stems and pedicels, it is possible that TCL1 might also have a role in leaf epidermal development. For example, TCL1 transcript can be detected in the leaf (Fig. 6C), and overexpression of TCL1 represses trichome formation (Fig. 1). A higher-order combination of mutations in single-repeat R3 MYBs might help clarify the exact role of TCL1 in leaf trichome formation.

A possible molecular mechanism for the action of TCL1 in the regulation of trichome patterning

Previous analyses in yeast two-hybrid assays have demonstrated that TRY, CPC, ETC1 and ETC2, can interact with GL3 to limit the interaction between GL1 and GL3 (Payne et al., 2000; Esch et al., 2003; Esch et al., 2004; Kirik et al., 2004b; Zimmermann et al., 2004), thus inhibiting the formation of an activator complex between TTG1, GL1 and GL3/EGL3, that is required for the activation of GL2 transcription (Schiefelbein, 2003). Our results showed that GL2 expression was reduced in plants overexpression TCL1 (Fig. 6), which could be explained if TCL1 operates by the same mechanism. However, overexpression of TCL1 also suppressed the expression of GL1 (Fig. 6). The suppression of GL1 by TCL1 appears to be specific to this member of the activator complex, because the expression levels of the other two components in the activator complex, TTG1 and GL3, were largely unaffected by overexpression of TCL1 (Fig. 6). One possible scenario is that TCL1 directly suppresses the expression of GL1 and, thereby, indirectly causes a reduction in GL2 expression (Fig. 8). This possibility is supported by the elevated levels of GL1 and GL2 transcripts in the developing inflorescence of tcl1-1 mutants (Fig. 6). Furthermore, the notion that GL1 might be a potential target gene for TCL1 was supported by the observation that plants overexpressing a TCL1-VP16 fusion protein, shown to be a transactivator in the protoplast transfection assay, have elevated expression levels of GL1 (but largely unchanged levels of TTG1 and GL3) and display phenotypes similar to plants overexpressing GL1 (Fig. 7). An increase in GL1 level in response to TCL1-VP16 overexpression presumably alters the titration balance among components of the GL1/GL3-1TTG1 activator complex, thereby disrupting efficient formation of the activator complex and, subsequently, its transcriptional activity. Finally, our ChIP assay demonstrates that TCL1 can be recruited to the cis-acting regulatory elements of GL1, suggesting that GL1 is indeed a target gene of TCL1. A study on GL1 promoter activity and GL1 protein localization at a subcellular level in a tcl1 mutant background might strengthen the proposed model, and is worth further investigation.

Little is known about the regulation of GL1 expression. Here we provide evidence that TCL1 might act to directly control GL1 expression. It is possible that, in addition, TCL1 competes with GL1 for binding to GL3, so as to inhibit the formation of the activator complex, as suggested for the other single-repeat R3 MYB transcription factors. It is worth noting that TCL1 has the amino acid signature [D/E]Lx2[R/K]x3Lx6Lx3R (Zimmermann et al., 2004) that is required for interacting with R/B-like bHLH transcription factors (Fig. 2C). TCL1 also has the conserved amino acids in the MYB domain (Kurata et al., 2005) that are crucial for cell-to-cell movement (Fig. 2C). Using transgenic plants expressing a TCL1-GFP fusion protein, we found that the TCL1-GFP can be detected in epidermal cells (Fig. 3D). Using transgenic plants expressing a TCL1 promoter-GUS fusion construct (P<sub>TCL1</sub>-GUS), we found that P<sub>TCL1</sub>-GUS was widely expressed (see Fig. S2 in the supplementary material). A detailed study on TCL1 mRNA expression pattern and TCL1 protein subcellular localization might help clarify the cell-to-cell movement ability of TCL1.

TCL1 is unique among the single-repeat R3 MYB proteins in that overexpression of TCL1 does not affect root epidermis cell fate, which does not depend on GL1 function. However, we found that overexpression of CPC could also dramatically suppress the expression of GL1 (see Fig. S3 in the supplementary material). Therefore, it is possible that other single-repeat R3 MYBs, such as CPC, can also function in a similar manner to TCL1 to directly suppress the transcription of GL1 and regulate trichome patterning.
It is worth noting that a similar mechanism has been proposed for root hair patterning in which CPC (single-repeat MYB) negatively regulates WER (R2R3 MYB) expression in hair-forming cells (Lee and Schiefelbein, 2002). WER has been proposed to be functionally equivalent to GL1 (Lee and Schiefelbein, 2001).

In summary, we identified TCI1 as a previously unknown member of the single-repeat R3 MYB transcription factor family. We provide evidence that TCI1 is a negative regulator of trichome initiation and patterning, and that TCI1 has a specific role in regulating epidermal cell specification on inflorescence stems and pedicels. This provides new insight into the way that organ-specific regulation of epidermal patterning might be achieved using a common mechanism and related transcription factor molecules.

Furthermore, we show that TCI1 is likely to negatively regulate trichome formation in a novel manner, by directly suppressing the expression of GL1 (Fig. 8). This suggests the existence of a novel regulatory loop in trichome patterning, and offers a fine-tuning mechanism for the interaction between the negative regulators and the activator complex.

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Supplementary material

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References


