Control of Plant Trichome and Root-Hair Development by a Tomato (*Solanum lycopersicum*) R3 MYB Transcription Factor

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Abstract

In *Arabidopsis thaliana* the CPC-like MYB transcription factors (CAPRICE (CPC), TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC 1, 2, 3/CPC-LIKE MYB 3 (ETC1, ETC2, ETC3/CPL3), TRICHOMELESS 1, 2, 3/CPC-LIKE MYB 4 (TCL1, TCL2/CPL4)) and the bHLH transcription factors (GLABRA3 (GL3) and ENHANCER OF GLABRA 3 (EGL3)) are central regulators of trichome and root-hair development. We identified TRY and GL3 homologous genes from the tomato genome and named them *SlTRY* and *SlGL3*, respectively. Phylogenetic analyses revealed a close relationship between the tomato and Arabidopsis genes. Real-time reverse transcription PCR analyses showed that *SlTRY* and *SlGL3* were predominantly expressed in aerial parts of developing tomato. After transformation into Arabidopsis, *CPC::SlTRY* inhibited trichome formation and enhanced root-hair differentiation by strongly repressing GL2 expression. On the other hand, GL3::SlGL3 transformation did not show any obvious effect on trichome or non-hair cell differentiation. These results suggest that tomato and Arabidopsis partially use similar transcription factors for epidermal cell differentiation, and that a CPC-like R3 MYB may be a key common regulator of plant trichome and root-hair development.

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Introduction

Epidermal cell differentiation, including trichome and root-hair formation, in *Arabidopsis thaliana* is a popular model system for studying cell fate determination. Several regulatory factors are known to be involved in this event. The CAPRICE (CPC) gene encodes an R3 type MYB transcription factor that has been identified as a key regulator of root-hair differentiation [1]. Arabidopsis has several additional CPC-like MYB genes in its genome, including TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC 1 and 2 (ETC1 and ETC2), ENHANCER OF TRY AND CPC 3/CPC-LIKE MYB 3 (ETC3/CPL3), and TRICHOMELESS 1 and 2/CPC-LIKE MYB 4 (TCL1 and TCL2/CPL4) [2–10]. The TRY protein has a regulatory role mainly in trichome differentiation [2,11]. ETC1 and ETC2 enhance the functions of CPC and TRY [3–5]. TCL1 and TCL2/CPL4 negatively regulate trichome formation on the inflorescence stems and pedicels [8–10].

The GLABRA3 (GL3) gene encodes a bHLH transcription factor that is also involved in trichome and root-hair differentiation in Arabidopsis [12]. A GL3 homologous gene, ENHANCER OF GLABRA 3 (EGL3), functions in a redundant manner with GL3 in Arabidopsis [13]. The GLABRA2 (GL2) gene, which encodes a homeodomain leucine zipper protein, is thought to act farthest downstream in the epidermal cell fate regulatory pathway in Arabidopsis [1,14–17]. Transcription of GL2 is controlled by a protein complex that includes the WEREWOLF (WER), GL3/ EGL3 and TRANSPARENT TESTA GLABRA1 (TTG1) proteins [18]. WER encodes an R2R3 type MYB transcription factor and promotes the the differentiation of Arabidopsis root epidermal cells into non-hair cells [16]. The TTG1 gene, which encodes a WD-40 protein, is also required for the formation of non-hair cells [14]. Two bHLH proteins, GL3 and EGL3, interact with WER [13] and TTG1 [12,19,20]. The WER homologous gene GLABRA1 (GL1) is also thought to form a transcriptional complex with GL3/EGL3 and TTG1 to promote GL2 expression [12,20–22]. The CPC and CPC-like MYB proteins interact with GL3/EGL3 and may serve as epidermal cell fate determinants [7].

Although both tomato and Arabidopsis have trichomes, tomato trichomes are distinct from Arabidopsis trichomes. Arabidopsis has non-glandular three-branched unicellular trichomes that form on stem and leaf surfaces to an extent that depends on the ecotype [23,24]. On the other hand, tomato trichomes are highly diverse in morphology and chemistry [25–27]. Tomato trichomes are classified into types I–VII, with types I, IV, VI and VII being glandular, and types II, III and V being non-glandular [26,28]. Glandular trichomes contain various sticky or toxic chemicals that may resist herbivores [27], whereas non-glandular trichomes may function in defense by physically limiting herbivores [29].
Trichome morphology and root-hair patterning are different in tomato and Arabidopsis. Arabidopsis root-hair cells are located over two underlying cortical cells, whereas non-hair cells are positioned over a single cortical cell [14,30]. This position-dependent pattern results in rows of root-hair cells along the longitudinal root axis and has been found in Brassicaceae and other eudicot families [31–34]. This striped root-hair pattern (Type 3) is one of three types of root-hair cell distribution patterns [31–33,35–38]. Tomato belongs to the Type 1 root-hair pattern group, in which all the root epidermal cells have the potential to produce root-hairs. This type of pattern appears to be the most widespread in plants [39].

In this study, we have identified Arabidopsis TRY and GL3 homologous genes from tomato. Transformants expressing the tomato TRY homologous gene (STRI) in Arabidopsis had no trichomes and a greater number of root-hairs, a phenotype similar to that seen in over-expressors of CPC-like MYB genes. On the other hand, transformants expressing the tomato GL3 homologous gene (SIL3) in Arabidopsis had no obvious GL3-like effects on trichome and non-hair cell differentiation. We concluded that tomato and Arabidopsis use similar transcription factors for trichome and non-hair cell differentiation and that the STRI-like R3 MYB may be a key common regulator of plant trichome and root-hair development.

Materials and Methods

Plant Materials and Growth Conditions

Tomato, Solanum lycopersicum L. cv. Micro-Tom, was used. Seeds were surface-sterilized with 10% commercial bleach including detergent (Kitchen Haiter, Kao, Tokyo, Japan), for 20 min and then rinsed with sterilized water three times for 5 min each and sown on 1.5% agar plates containing 0.5xMS medium [40]. Seeded plates were kept at 4°C for 2 d and then incubated at 25°C under constant white light (50–100 μmol m−2 s−1) for 7 days to produce seedlings for DNA and RNA extraction. Some 7-day-old third leaves were assayed for trichome number.

Arabidopsis thaliana ecotype Columbia (Col-0) and, cognate GL3-7454 mutant plants were used. Seeds were surface-sterilized, sown on 1.5% agar plates containing 0.5xMS medium [40]. Seeded plates were kept at 4°C for 2 d and then incubated at 22°C under constant white light (50–100 μmol m−2 s−1). For each transgenic line, at least ten individual 5-day-old seedlings were assayed for over-expressors of GL3 homologous genes from tomato. Transformants expressing the GL3-7454 homologous genes from tomato.

Construct Gene Constructs

All primer sequences used in this paper are listed in Table 1.

Table 1. Primer sequences used in this study.

| Primer Name | Sequence (5’ to 3’) |
|-------------|---------------------|
| RTSTIRY-F   | 5’-CGATGGTGGACGACCTAAGAAGA-3’ |
| RTSTIRY-R   | 5’-TGTCGAACACCGCCTATTCACT-3’ |
| RTSGL3-1-F  | 5’-AAATTACCTCCTCTTGGGAATG-3’ |
| RTSGL3-1-R  | 5’-AAAGAATCTTGCTTTTTTTTTCATAC-3’ |
| LeActin-F    | 5’-CTTGCGTATTTAAGGCTTGGTAT-3’ |
| LeActin-R    | 5’-CGATTTAAATACGACCAAGCAAT-3’ |
| GL2-F        | 5’-ATCGCTCAACACCGCATGAC-3’ |
| GL2-R        | 5’-CAGCGCCCTTCTGCTGCTCA-3’ |
| GFP-F        | 5’-CAGTCCGCGCTGAGCAAAGAC-3’ |
| GFP-R        | 5’-CCCTGTCACCTGATCTGGTA-3’ |
| Act2-F       | 5’-CAGTTACGTGGTTGCTCATC-3’ |
| Act2-R       | 5’-CCTGAGCTCCTTCTATC-3’ |
| STIRY-F01    | 5’-TGAAGGAGAATCTGCTATGCA-3’ |
| STIRY-R01    | 5’-TGTTTGTTGTTGTTTGTTTAC-3’ |
| STIRY-F02    | 5’-GATTAGTGGTGATCGAAGACCA-3’ |
| STIRY-R02    | 5’-CGGGCGGCTGAGTGAATGACATGT-3’ |
| STIRY-F03    | 5’-TCTACACCACAGCTCCATTCCTGAC-3’ |
| STIRY-R03    | 5’-ACGAATGTGCTGGTCCGGTCCATTAC-3’ |
| STIRY-F01    | 5’-CGGGGACACTCCAGCTAACAC-3’ |
| STIRY-R01    | 5’-CCTATGACCTTGTTTCTTCTC-3’ |
| STIRY-F02    | 5’-TGAAGAACAATCTGGCTGTTGCA-3’ |
| STIRY-R02    | 5’-TGTTTGTTGTTGTTTGTTTATC-3’ |
| STIRY-F03    | 5’-ATGAAATCTCCTCTATTCCATTTCATAC-3’ |
| STIRY-R03    | 5’-CGATGACCTGGTCCGGTCCATTAC-3’ |
| STIRY-F02    | 5’-GATTAGTGGTGATCGAAGACCA-3’ |
| STIRY-R02    | 5’-CGGGCGGCTGAGTGAATGACATGT-3’ |
| STIRY-F03    | 5’-CAGTTACGTGGTTGCTCATC-3’ |
| STIRY-R03    | 5’-ACGAATGTGCTGGTCCGGTCCATTAC-3’ |
| STIRY-F01    | 5’-CGGGGACACTCCAGCTAACAC-3’ |
| STIRY-R01    | 5’-CCTATGACCTTGTTTCTTCTC-3’ |
| STIRY-F02    | 5’-TGAAGAACAATCTGGCTGTTGCA-3’ |
| STIRY-R02    | 5’-TGTTTGTTGTTGTTTGTTTATC-3’ |
| STIRY-F03    | 5’-ATGAAATCTCCTCTATTCCATTTCATAC-3’ |
| STIRY-R03    | 5’-CGATGACCTGGTCCGGTCCATTAC-3’ |
| STIRY-F02    | 5’-GATTAGTGGTGATCGAAGACCA-3’ |
| STIRY-R02    | 5’-CGGGCGGCTGAGTGAATGACATGT-3’ |
| STIRY-F03    | 5’-CAGTTACGTGGTTGCTCATC-3’ |
| STIRY-R03    | 5’-ACGAATGTGCTGGTCCGGTCCATTAC-3’ |

The pSMT3::CPC::SlTRY construct was sequenced using the STIRY-P2, -P3, -P4, -F02, -F03, -VP1 and -VP2 primers.

CPC::SlTRY Construct

A 1.0-kb PCR-amplified linear CPC promoter sequence (primers STIRY-F01/R01) from the Arabidopsis genome, a 0.8-kb PCR-amplified linear STIRY tomato genomic fragment (primers STIRY-F02/R02) and a 1.8-kb PCR-amplified 2xGFP fragment [41] (primers STIRY-F03/R03) using PrimeSTAR HS DNA Polymerase and TaKaRa LA Taq (Takara, Tokyo, Japan) were ligated into the XhoI and KpnI sites of pBHA212K binary vector [44] using an In-Fusion HD Cloning Kit (Takara, Tokyo, Japan) to create CPC::SlTRY. PCR-generated constructs were completely sequenced following isolation of the clones to check for amplification-induced errors. The plasmid of CPC::STIRY was sequenced using the STIRY-P2, -P3, -P4, -F02, -F03, -VP1 and -VP2 primers.

GL3::SlGL3 Construct

A 1.3-kb PCR-amplified linear GL3 promoter sequence (primers SlGL3-F01/R01) from the Arabidopsis genome, a 4.3-kb PCR-amplified linear SlGL3 tomato genomic fragment (primers SlGL3-F02/R02) and a 1.0-kb PCR-amplified GFP fragment [41]
segregation ratios for kanamycin resistance. and selected at least ten T2 and five T3 lines on the basis of their resistance. We isolated at least twenty T1 lines for each construct. Homozygous transgenic lines were selected based on kanamycin resistance. PCR-generated constructs were completely sequenced following isolation of the clones to check for amplification-induced errors. The plasmid of CPC::SlGL3 was sequenced using the SlGL3-P1, -P4, -P5, -F1, -F2, -F3, -F03, -R1, -R2, -R3, -VP1 and -VP2 primers.

Transgenic Plants
Genetic constructs were introduced into Agrobacterium tumefaciens C58C1. Arabidopsis plants (wild-type Col-0, cpc-2, and gl3-7454) were transformed by the floral dipping method [45] and screened on 0.8% agar plates containing diluted (50% v/v) Murashige and Skoog medium and 50 mg/L (for Col-0, and gl3-7454) background) kanamycin sulfate. Homozygous transgenic lines were selected based on kanamycin resistance. We isolated at least twenty T1 lines for each construct and selected at least ten T2 and five T3 lines on the basis of their segregation ratios for kanamycin resistance.

Real-time Reverse Transcription PCR Analysis
Total RNA from tomato or Arabidopsis tissues was extracted with MagDEA RNA 100 (GC) (PSS, Chiba, Japan) using a Magtrac System protocol. Plant tissue (100 mg) was homogenized with MagDEA RNA 100 (GC) (PSS, Chiba, Japan) using MagDEA RNA 100 (GC) (PSS, Chiba, Japan) using a TissueLyser II (Qiagen, Valencia, CA, USA) with 100 μl of RLT buffer (Qiagen, Valencia, CA, USA). Sample supernatants were applied to the instrument, and RNA was eluted with 50 μl of sterile distilled water.

First-strand cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mixture using the Prime Script RT Master Mix (Perfect Real Time) (Takara, Tokyo, Japan). Real-time PCR was performed using a Chromo4 Real-Time IQ5 PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Premix Ex Taq II (Takara, Tokyo, Japan). PCR amplification employed a 30 s denaturing step at 95°C, followed by 5 s at 95°C and 30 s at 60°C with 40 cycles for STIR1, SIGL3, LeActin, GL2, GFP and ACT2. Real-time PCR was used to analyze the mRNA expression level of each transcript encoding STIR1 and SIGL3 in tomato, and GL2 and GFP in Arabidopsis transformants. The relative expression of each transcript was calculated by the ΔΔCT method [46]. The expression levels of STIR1 and SIGL3 were estimated after being normalized to the endogenous control gene LeActin (TC116322). The expression levels of GL2 and GFP were estimated after being normalized to the endogenous control gene ACT2 (AB026654). The primers were: RTSTIR1-F and RTSTIR1-R for STIR1; RTSlGL3-F and RTSlGL3-R for SIGL3; LeActin-F and LeActin-R for LeActin [47]; GL2-F and GL2-R for GL2 [48]; GFP-F and GFP-R for GFP; and ACT2-F and ACT2-R for ACT2 [49].

Light Microscopy
To observe trichomes, images were recorded with a VC4500 3D digital fine microscope (Ommron, Kyoto, Japan) or a digital microscope (VH-8000; Keyence, Osaka, Japan). At least five 2-week-old third true leaves were analyzed for trichome number for each transgenic line. Root phenotypes were observed using an Olympus Previs AX70 microscope and an Olympus SZH binocular microscope. For each transgenic line, at least ten individual 5-day-old seedlings were analyzed for root-hair number.

Results
Identification of the STIR1 and SIGL3 Genes
To find transcription factors regulating trichome and root-hair differentiation of tomato epidermis, we searched a tomato genome database (http://solgenomics.net/). We identified tomato homologs of the Arabidopsis CPC and GL3 genes and named them STIR1 (Soly01g095640.1) and SIGL3 (Soly01g081140.2). Members of the CPC family encode R3 type MYB transcription factor proteins in Arabidopsis [1–10]. The STIR1 encoded protein is more closely related to TRY than CPC (Figure 1A). Alignment of the amino acid sequences showed that the full length STIR1 protein shares 53% amino acid identity with TRY, and 50% with CPC. The R3 MYB motif of STIR1 shares 73% amino acid identity with that of TRY, and 71% with that of CPC.

To provide a framework for examining R3 MYB transcription factor evolution, we estimated the phylogeny of CPC-like R3 MYB transcription factor proteins from Arabidopsis (CPC, TRY, ETC1, ETC2, ETC3/CPL3, TCL1 and TCL2/CPL4), rice (Oryza sativa) (Os1g43180 and Os1g43230) and tomato (Solanum lycopersicum) based on their deduced amino acid sequences (Figure 1B). STIR1 was more closely related to TRY than CPC, which belongs to a cluster that includes TCL1, TCL2/CPL4, ETC2, and TRY (Figure 1B). ETC1, ETC3/CPL3 and CPC belong to another cluster branching from the TRY subgroup. Consistent with previous reports, phylogenetic analyses using the entire amino acid sequence showed that the CPC-like MYB family can be divided into two groups: TRY, ETC2, TCL1 and TCL2/CPL4 in one group and CPC, ETC1 and ETC3 in the other [6–9]. As previously described, the two rice orthologs, O01g43180 and O01g43230, form a distinct clade from the Arabidopsis CPC-like R3 MYB family (Figure 1B) [7].

The SIGL3 encoded protein is closely related to the Arabidopsis bHLH transcription factor proteins encoded by GL3 and EGL3 (Figure 2A). Alignment of the amino acid sequences showed that the full length SIGL3 protein shares 45% amino acid identity with GL3, and 46% with EGL3. The bHLH motif of SIGL3 shares 46% amino acid identity with that of GL3, and 50% with that of EGL3. Based on the amino acid sequences of the bHLH regions, the Arabidopsis bHLH transcription factors are classified into 12 groups (I-XII) [50]. Group III contains 6 subgroups (IIIa-f), and GL3 and EGL3 belong to the IIIe subgroup [50].

To characterize SIGL3, we evaluated the phylogeny of bHLH transcription factor proteins (Figure 2B). Clustering in a phylogenetic tree constructed from subgroups IIIa (AtbHLH003, AtbHLH013, AtbHLH014 and AtbHLH017), IIIe (AtbHLH004, AtbHLH005, AtbHLH006 and AtbHLH028), IIIF (GL3, EGL3, TT8 and AtMYC1) and IVa (AtbHLH018, AtbHLH020 and AtbHLH025) was similar to the clustering in previously reported phylogenetic trees [50–52] (Figure 2B). SIGL3 belongs to the IIIe subgroup and is more closely related to GL3 and EGL3 than AtMYC1 and TT8 (Figure 2B).

Expression Patterns of the STIR1 and SIGL3 Genes in Tomato
Expression of STIR1 and SIGL3 was examined in tomato tissues using real-time reverse transcription PCR. STIR1 was strongly expressed in stem and cotyledons of 7-day-old seedlings (Figure 3A). The relative expression level of STIR1 in cotyledon tissues was approximately 5 times greater than that in seedling root tissues (Figure 3A). The strongest expression of SIGL3 was observed in 5-week-old plant leaves (Figure 3B). The relative expression level of SIGL3 in true leaf tissues was approximately 5 times greater than that in seedling root tissues (Figure 3A).
Both SlTRY and SlGL3 were more strongly expressed in aerial tissues (including stem, cotyledon, leaf, bud and flower) than in roots. These results suggest that both SlTRY and SlGL3 act in both shoot and root tissues and might have relatively strong functions in the aerial parts of plants.

**Figure 1. Amino acid sequence and phylogenetic tree of CPC-like R3 MYB proteins.** (A) Sequence alignment of SlTRY (Solyc01g095640.1.1), TRY (AC007288) and CPC (FJ268773). Shaded letters indicate identical residues. R3 MYB domains are indicated by a line above the sequences. (B) Phylogenetic tree based on deduced amino acid sequences of CPC-like R3 MYB proteins [SlTRY, TRY, CPC, ETC1 (NM100020), ETC2 (FJ972652), ETC3/CPL3 (AB264292), TCL1 (FJ972675), TCL2/CPL4 (FJ972681)], Os1g43180 and Os1g43230 were aligned with a multiple alignment program (Genetyx ver. 16.0.2 software, Genetyx, Tokyo, Japan), and a dendrogram was created using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Branch length indicates relative evolutionary distances. Numbers above branches are genetic distances based on 10,000 bootstrap replicates. Distances are shown as the p-distance.

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**SlTRY Gene Functions in Trichome and Root-hair Development in Arabidopsis**

To see if SlTRY is functionally similar to the CPC family of MYB transcription factors, we introduced SlTRY into Arabidopsis wild-type Col-0 plants under the control of the CPC promoter (CPC::SlTRY). The CPC-like MYB genes are thought to function redundantly in trichome and root-hair formation. For example, 35S::CPC, 35S::ETC1, and 35S::ETC3 transgenic plants are all trichome-deficient and have a greater number of root-hairs.
Figure 2. Amino acid sequence and phylogenic tree of bHLH proteins. (A) Sequence alignment of SIGL3 (Solyc08g081140.2.1), GL3 (AF246291) and EGL3 (NM20235). Shaded letters indicate identical residues. bHLH regions are indicated as line above the sequences. (B) Phylogenic
tree based on deduced amino acid sequences of bHLH proteins (SIGL3, GL3, EGL3, TT8 (AJ277509), AtMYC1 (AF251697), AtbHLH003 (AF251688), AtbHLH004 (AF251689), AtbHLH005 (AF251690), AtbHLH006 (X95548), AtbHLH013 (AY120752), AtbHLH014 (AJ619812), AtbHLH017 (AY094399), AtbHLH018 (AF488562), AtbHLH025 (AF488567) and AtbHLH028 (AF252636) aligned with a multiple alignment program (Genetyx ver. 16.0.2 software, Genetyx, Tokyo, Japan). The dendrogram was created using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Branch length indicates relative evolutionary distances. Numbers above branches are genetic distances based on 10,000 bootstrap replicates. Distances are shown as the p-distance. Subdivision groups of Arabidopsis bHLH proteins (Group IIId, Ille, IIIf and IVa) are shown to the right of the gene names.

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Figure 3. Tomato SITRY and SIGL3 gene expression. (A) Real-time reverse transcription PCR analysis of SITRY gene expression in tomato organs. (B) Real-time reverse transcription PCR analysis of SIGL3 gene expression in tomato organs. Total RNA was isolated from the indicated tissues from 7-day-old seedlings and 5-week-old plants. Expression levels of SITRY and SIGL3 in each organ relative to those in the seedling root were shown. The experiments were repeated three times. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the seedling root and the other organs by Student’s t-test (P<0.050).

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[1,3,4,7]. Consistent with these previous observations, all homozygous CPC::SlTRY transgenic lines (#1–#6) have the no-trichome phenotype, although wild-type Col-0 produces approximately 30 trichomes on the adaxial surface of the third true leaf (Figure 4A, E, G). To see a STRY function more clearly, we introduced CPC::STRY into the qpc-2 mutant. As previously reported, the qpc-2 mutant has a greater number of trichomes than wild-type [7] (Figure 4B, F). All homozygous CPC::STRY in qpc-2 transgenic lines (#1–#6) show the no-trichome phenotype (Figure 4A, G) as observed in CPC::STRY in a wild-type background (Figure 4A). These results indicate that the tomato STRY protein has a function similar to the Arabidopsis CPC-like MYB proteins in regulating trichome development.

On the other hand, all homozygous CPC::STRY transgenic lines (#1–#6) produced greater numbers of root-hairs compared with wild-type Col-0, which produces approximately 40 root-hairs per mm (Figure 4C, I, K). This result is similar to the root-hair numbers of CPC-like MYB over-expressors [1,3,4,7]. Homozygous CPC::SlTRY in qpc-2 transgenic lines (#1–#6) also showed a greater number of root-hairs compared with wild-type and qpc-2, a mutant that produces a decreased number of root-hairs. These results are similar to the previously reported results using CPC::CPC in qpc-2 or GL2::CPC in qpc-1 transgenic plants [53,54] (Figure 4D, J, L). These results indicate that the tomato protein STRY has a function similar to Arabidopsis CPC-like MYB proteins in regulating root-hair development. Together, our results show that STRY functions similar to CPC-like MYBs both in trichome and root-hair formation in Arabidopsis.

**SGL3 does not Function in Trichome and Root-hair Development in Arabidopsis**

To see if SGL3 is functionally similar to Arabidopsis GL3 or EGL3, we introduced SGL3 into Arabidopsis wild-type Col-0 under the control of the GL3 promoter (GL3::SGL3). The GL3 and EGL3 genes are thought to be involved in trichome and root-hair formation. GL3 or EGL3 over-expressors produce greater numbers of trichomes and reduced numbers of root-hairs [12,13]. Most of homozygous GL3::SGL3 transgenic lines produced the similar number of trichomes as that of wild-type (Figure 5A, F). Unlike the previous observation from complementation analysis of the gl3-1 mutant by GL3::GL3 [12], homozygous GL3::SGL3 in gl3-7454 transgenic plants did not have an increased number of trichomes compared with the gl3-7454 mutant (Figure 5B, E, G). On the contrary, one of the GL3::SGL3 in gl3-7454 transgenic lines (#5) had significantly fewer trichomes in comparison with the gl3-7454 mutant (Figure 5B). These results suggest that tomato SGL3 may not have a function same to Arabidopsis GL3.

In addition to the effects on trichome number, GL3 is known to affect the trichome branching phenotype [12]. As observed in the gl3-1 mutant (Ler background) [12], trichomes of the gl3-7454 mutant (Col-0 background) had fewer branches than wild-type Col-0 (Table 2). Introduction of the GL3::SGL3 gene did not rescue the decreased branch number phenotype of the gl3-7454 mutant trichomes (Table 2). This result suggests that the SGL3 gene does not have a function similar to GL3 in the induction of trichome branching.

Three of five homozygous GL3::SGL3 transgenic lines (#3–#5) produced significantly fewer root-hairs compared with wild-type (Figure 5C, I). This result is similar to the tendency for fewer root-hairs in the GL3 or EGL3 over-expressors [13], but the effect of SGL3 was weaker than that of GL3 and EGL3. In contrast, three of five homozygous GL3::SGL3 plants in gl3-7454 transgenic lines (#3–#5) produced significantly higher numbers of root-hairs compared with wild-type Col-0 and gl3-7454 (Figure 5D, H, J).

**Expression of the GL2 Gene in STRY expressing Plants**

To determine whether CPC::STRY functions (Figure 4) were due to epistatic effects of STRY on GL2 activity, we carried out real-time reverse transcription PCR analyses using GL2 primers (Figure 6). The GL2 gene is thought to act downstream of the MYB-bHLH transcriptional complex to promote trichome formation and inhibit root-hair formation [1,14–17]. Consistent with the CPC::STRY transgene phenotype (Figure 4A), GL2 expression was strongly repressed in all CPC::STRY transgenic lines (Figure 6A). In the CPC::STRY in qpc-2 transgenic lines, GL2 expression was also strongly repressed compared with wild-type and qpc-2 as was the case in the wild-type background (Figure 6B). To compare gene expression levels of the introduced gene among transgenic lines, we checked GFP expression since GFP was fused to the C-terminal region of STRY (Figure S1A). Although the transgene expression levels varied depending on the lines (Figure S1A), expression in all lines was strong enough to repress GL2 expression.

**Expression of the GL2 Gene in SGL3 expressing Plants**

To determine whether CPC::SGL3 functions (Figure 5) were due to epistatic effects of SGL3 on GL2 activity, we also carried out real-time reverse transcription PCR analyses using GL2 primers (Figure 7). Inconsistent with the GL3::SGL3 transgene phenotypes (Figure 5A), significant GL2 expression changes were observed only in GL3::SGL3 line #1 compared with wild-type Col-0 (Figure 7A). Apparently, SGL3 does not have a remarkable effect on GL2 expression. In GL3::SGL3 in gl3-7454 transgenic plants, a significant increase in GL2 expression was observed in lines #2 and #3 compared with that in the gl3-7454 mutant; however, these GL2 expression levels did not reach similar expression levels of GL2 in wild-type Col-0 (Figure 7B). A significant decrease in GL2 expression was observed in line #5 compared with that in the gl3-7454 mutant (Figure 7B). Thus, we checked GFP expression that should reflect the introduced SGL3 expression levels since the SGL3 construct was fused to GFP (Figure S1B). The GFP expressions varied greatly among GL3::SGL3 in gl3-7454 transgenic lines (Figure S1B). In addition, the relative expression levels of GFP in GL2:SGL3 in gl3-7454 lines were lower than that in the GL3::SGL3 lines (Figure S1B). These results suggest that SGL3 expression was unstable in the gl3-7454 mutant background.

**Discussion**

In this study, we identified tomato STRY and SGL3 genes that were orthologous to the Arabidopsis TRY and GL3 genes, respectively. Recently, a high-quality genome sequence of tomato was released by the Tomato Genome Consortium [55]. Since tomatoes are very distantly related to Arabidopsis evolutionarily, these sequence data may offer important information about plant evolution in the future. The functional analyses of the STRY and SGL3 genes in this study provide insights into tomato trichome and root-hair evolution. Branching of the STRY and CPC clusters from a common trunk in the phylogenetic tree suggests that the evolution of tomato and Arabidopsis CPC-like R3 MYB genes began with duplication of a single common ancestor after divergence from rice (Figure 1B). Based on the functions of known members of the Arabidopsis bHLH transcription factor family, it was hypothesized that different members participate in distinct developmental processes [50]. Among the genes, members of the III subgroup, including AtMYC1, TT8, GL3 and EGL3 function in trichome and root-hair development, flavonoid/anthocyanin metabolism, and/or mucilage biosynthesis.
Phylogenetic analyses predicted that tomato SlGL3 evolved from a common ancestor to Arabidopsis GL3 and EGL3 after divergence of the III subgroup from other bHLH subgroups (Figure 2B). Thus, we expected that SlTRY and SlGL3

Figure 4. Trichome and root hair phenotypes of CPC::SlTRY transgenic plants. (A) Trichome formation on 2-week-old Arabidopsis third leaves of wild-type Col-0 and CPC::SlTRY (#1, #2, #3, #4 and #5). (B) Trichome formation on 2-week-old Arabidopsis third leaves of wild-type Col-0, cpc-2 mutant and CPC::SlTRY in cpc-2 (#1, #2, #3, #4 and #5). Number of trichomes per leaf was determined by counting a minimum of five 2-week-old third leaves from each line. (C) Root hair formation in 5-day-old Arabidopsis seedlings of wild-type Col-0 and CPC::SlTRY (#1, #2, #3, #4 and #5). (D) Root hair formation in 5-day-old Arabidopsis seedlings of wild-type Col-0, cpc-2 mutant and CPC::SlTRY in cpc-2 (#1, #2, #3, #4 and #5). The number of root hairs per mm was determined by counting a minimum of ten 5-day-old seedlings from each line. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the wild-type Col-0 and the transgenic lines (C), or the CPC-2 mutant and the transgenic lines (D) by Student’s t-test (P<0.050). Trichome phenotypes of wild-type Col-0 (E), cpc-2 (F), CPC::SlTRY (G) and CPC::SlTRY in cpc-2 (H). Root hair phenotypes of wild-type Col-0 (I), cpc-2 (J), CPC::SlTRY (K) and CPC::SlTRY in cpc-2 (L). Scale bars: 1 mm.

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Figure 5. Trichome and root hair phenotypes of GL3::SlGL3 transgenic plants. (A) Trichome formation on 2-week-old Arabidopsis third leaves of wild-type Col-0 and GL3::SlGL3 (#1, #2, #3, #4 and #5). (B) Trichome formation on 2-week-old Arabidopsis third leaves of wild-type Col-0, gl3-7454 mutant and CPC::SlTRY in gl3-7454 (#1, #2, #3, #4 and #5). Number of trichomes per leaf was determined by counting a minimum of five 2-week-old third leaves from each line. (C) Root hair formation in 5-day-old Arabidopsis seedlings of wild-type Col-0 and GL3::SlGL3 (#1, #2, #3, #4 and #5). (D) Root hair formation in 5-day-old Arabidopsis seedlings of wild-type Col-0, gl3-7454 mutant and CPC::SlTRY in gl3-7454 (#1, #2, #3, #4 and #5). The number of root hairs per mm was determined by counting a minimum of ten 5-day-old seedlings from each line. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the wild-type Col-0 and the transgenic lines [(A), (C)], or the gl3-7454 mutant and the transgenic lines [(B), (D)] by Student’s t-test (P<0.050). Trichome phenotypes of gl3-7454 (E), GL3::SlGL3 (F), and GL3::SlGL3 in gl3-7454 (G). Root hair phenotypes of gl3-7454 (H), GL3::SlGL3 (I), and GL3::SlGL3 in gl3-7454 (J). Scale bars: 1 mm.

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levels were normalized to repeated three times. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the wild-type Col-0 and the transgenic lines (A), or the GL1/WER-GL3/EGL3, leading to repression of the SlTRY protein may also disrupt the MYB-bHLH complex of CPC::SlTRY also repressed trichome and root-hair formation (Figure 4). Both similarly to the CPC-like MYB transcription factors in Arabidopsis GL2 expression (Figure 6). These results suggest that the SlTRY protein may also disrupt the MYB-bHLH complex of GL1/WER-GL3/EGL3, leading to repression of GL2 expression. In contrast to STRY, SIGL3 did not show clear GL3/EGL3-like functions for trichome and root-hair differentiation in Arabidopsis (Figure 5). Overexpression of GL3 and/or EGL3 induced a notable increase in trichome number and a decrease in root-hair number in Arabidopsis [12,13]. However, in our experiment, only two of five and three of five GL3::SIGL3 transgenic lines showed a significant increase in trichome number and a significant decrease in root-hair number compared with wild-type, respectively (Figure 5A and B). It is thus possible that tomato SIGL3 has an evolutionarily conserved, highly homologous amino acid sequence and only a partially similar function to Arabidopsis GL3/EGL3. Since trichome and root-hair structure differs between Arabidopsis and tomato, the SIGL3 gene may have acquired another function from GL3/EGL3 during evolution. The functional difference between SIGL3 and GL3/EGL3 may be derived from the relatively low amino acid homology region in the bHLH motifs (Figure 2A). Only one of five GL3::SIGL3 transgenic lines showed a significant increase in the GL2 expression level compared with wild-type Col-0 (Figure 7A). Thus, two possibilities exist. First, a low affinity of SIGL3 protein to WER/GL1 proteins may result in the formation of an incomplete MYB-bHLH protein complex that cannot activate GL2 expression. Recently, Zhao et al. reported that a single amino acid substitution in another GL3 homologous gene AmMYC1 leads to trichome and root-hair patterning defects by abolishing its interaction with partner proteins in Arabidopsis [61]. Arginine (R173) in the AmMYC1 protein is an essential amino acid residue for interaction with MYB proteins for proper functions [61]. We confirmed that there is a conserved Arg in the SIGL3 protein as in the GL3, EGL3 and AMYC1 proteins (Figure 2A). Thus, some amino acid substitution other than Arg may contribute to the functional difference between SIGL3 and GL3/EGL3. Second, SIGL3 might have lost the DNA binding ability to the GL2 promoter region or the ability to activate the GL2 promoter. For example, we previously reported that WER loses its DNA binding ability by at least two amino acid substitutions [53]. Tomato SIGL3 may have lost its DNA binding ability to the GL2 promoter region during evolution.

Table 2. Trichome branch numbers.

| Genotype          | branches (br)/trichome (%) | 1 br | 2 br | 3 br | 4 br |
|-------------------|-----------------------------|------|------|------|------|
| Col-0             |                             | 0    | 12±2 | 86±4 | 2±1  |
| gl3               |                             | 26±9 | 71±10| 3±2  | 0    |
| GL3::SlGL3        |                             | 1±1  | 40±14| 57±13| 2±1  |
| GL3::SlGL3 in gl3 |                             | 30±13| 64±12| 6±4  | 0    |

Data, including s.d., were obtained from at least 10 two-week-old third leaves from each line.

Figure 6. GL2 expression in the CPC::STRY transgenic plants. Real-time reverse transcription PCR analyses of the GL2 gene in wild-type Col-0 and CPC::STRY (1#1, 2#2, 3#3, 4#4 and 5#5) (A), and wild-type Col-0, cpc-2 mutant and CPC::STRY in cpc-2 (1#1, 2#2, 3#3, 4#4 and 5#5) (B). Expression levels were normalized to Act2 expression. An expression level of GL2 in each line relative to that in wild-type was indicated. The experiments were repeated three times. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the wild-type Col-0 and the transgenic lines (A), or the cpc-2 mutant and the transgenic lines (B) by Student’s t-test (P<0.050).

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In order to compare our results in the same Col-0 background, we used the gl3-7454 mutant for the complementary experiment. The gl3-7454 mutant shows only a mild phenotype compared with the gl3-1 mutant [Ler: Landsberg erecta background] [12]. The gl3-7454 mutant shows no significant difference in trichome number or in root-hair number compared with wild-type Col-0 (Figure 5B and D). Unexpectedly, one of five GL3::SlGL3 in gl3-7454 transgenic lines showed significant decreases in trichome number compared with gl3-7454 (Figure 5B and D). Consistent with these unexpected phenotypes of GL3::SlGL3 in gl3-7454 transgenic plants, the relative expression levels of GL2 varied (Figure 7B). Two of five GL3::SlGL3 in gl3-7454 transgenic lines showed significantly higher GL2 expression levels compared with that in gl3-7454, but the level did not reach that in the wild-type Col-0 (Figure 7B). One of five GL3::SlGL3 in gl3-7454 transgenic lines showed significantly lower GL2 expression levels compared with that in the gl3-7454 mutant (Figure 7B). As checked by fusion of SlGL3 to GFP, expression of introduced SlGL3 was unstable and fluctuated depending on the lines (Figure S1). In addition, SlGL3 did not rescue the reduced number of trichome branches phenotype of gl3-7454 (Table 2). These data strongly suggest the functional divergence between tomato SlGL3 and Arabidopsis GL3/EG3L3. There are 158 bHLH genes in Arabidopsis [52, 62]. Tomato should have the similar or more number of the bHLH genes when the full annotations of tomato genes are determined. We concluded that there is other GL3 ortholog(s) in the unannotated tomato genomes or tomato uses other pathways to regulate the epidermal cell differentiation. Additional investigations to further determine the functions of R3-MYB and bHLH in trichome and root-hair differentiation in tomato are necessary.

Supporting Information

Figure S1  GFP expression in the transgenic plants. Real-time reverse transcription PCR analyses of the GFP gene in CPC::SlTRY (#1, #2, #3, #4 and #5) (A), CPC::SlGL3 in gl3-7454 (#1, #2, #3, #4 and #5) (B), CPC::SlGL3 (#1, #2, #3, #4 and #5) (C), and CPC::SlGL3 in gl3-7454 (#1, #2, #3, #4 and #5) (D). Expression levels were normalized to Act2 expression. Relative expression levels: expression levels of GFP in each line relative to that in wild-type were indicated. The experiments were repeated three times. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between the wild-type Col-0 and the transgenic lines (A), or the gl3-7454 mutant and the transgenic lines (B) by Student’s t-test (P<0.050).

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Author Contributions

Conceived and designed the experiments: RT TW SS. Performed the experiments: RT TW YN. Analyzed the data: RT YN. Wrote the paper: RT TW.

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