Genetic evidence suggests that GIS functions downstream of TCL1 to regulate trichome formation in Arabidopsis

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Abstract

Background: Trichome formation in Arabidopsis is regulated by a MBW complex formed by MYB, bHLH and WD40 transcriptional factors, which can activate GLABRA2 (GL2) and the R3 MYB transcription factor genes. GL2 promotes trichome formation, whereas R3 MYBs are able to block the formation of the MBW complex. It has been reported that the C2H2 transcription factor GIS (GLABROUS INFLORESCENCE STEMS) functions upstream of the MBW activator complex to regulate trichome formation, and that the expression of TCL1 is not regulated by the MBW complex. However, gis and the R3 MYB gene mutant tcl1 (trichomeless 1) have opposite inflorescence trichome phenotypes, but their relationship in regulating trichome formation remained unknown.

Results: By generating and characterization of the gis tcl1 double mutant, we found that trichome formation in the gis tcl1 double and the tcl1 single mutants were largely indistinguishable, but the trichome formation in the 35S:TCL1/gis transgenic plant was similar to that in the gis mutant. By using quantitative RT-PCR analysis, we showed that expression level of GIS was increased in the triple mutant tcl1 try cpc, but the expression level of TCL1 was not affected in the gis mutant. On the other hand, trichome morphology in both gis tcl1 and 35S:TCL1/gis plants was similar to that in the gis mutant.

Conclusions: In summary, our results indicate that GIS may work downstream of TCL1 to regulate trichome formation, and GIS has a dominant role in controlling trichome morphology.

Keywords: GIS, TCL1, Trichome formation, Transcription factor, Arabidopsis

Background

Trichome formation in Arabidopsis has been shown to be a good model for the investigation of plant cell fate determination [16, 20, 21, 23, 34]. Accumulated evidence suggest that trichome formation in Arabidopsis is mainly regulated by several key transcription factors, including the WD40-repeat protein TTG1 (TRANSPARENT TESTA GLABRA1) [31], the R2R3 MYB transcription factor GL1 (GLABRA1) [14], the bHLH transcription factors GL3 (GLABRA3) and EGL3 (ENHANCER OF GLABRA3) [15, 42], the homeodomain protein GL2 (GLABRA2) [18], and several R3 MYB transcription factors [34, 35]. Genetic and molecular evidence indicates that TTG1, GL1, GL3 or EGL3 and GL2 positively regulate trichome formation [14, 15, 18, 31, 42], whereas the R3 MYB transcription factors including TRY (TRIPTYCHON), CPC (CAPRICE), TCL1 (TRICHOMELESS 1), TCL2, ETC1 (ENHANCER OF TRY AND CPC 1), ETC2 and ETC3 negatively regulate trichome formation [5, 10, 11, 19, 22, 28–30, 35, 36, 39].

It has been proposed that TTG1 and GL1 interacted with GL3 or EGL3 to form a MBW (MYB-bHLH-WD40) transcriptional activator complex, which is able to activate GL2, whereas GL2 is required for trichome formation [13, 16, 17, 21]. The same MBW activator complex can also activate the R3 MYB genes TRY, CPC, ETC1 and ETC3, but not TCL1, TCL2 and ETC2 [5, 34, 35]. R3 MYBs, in turn, can move from the trichome precursor cells to their surrounding cells, where they compete with GL1 for binding of GL3 or EGL3, resulting in the inhibition of the formation of MBW...
complex. As a result, GL2 cannot be activated, and trichome formation will be inhibited [4, 8, 9, 16, 19, 21, 34].

In addition to the key transcription factors mentioned above, transcription factors from other families have also been shown to involve in the regulation of trichome formation in Arabidopsis. These transcription factors including the C2H2 proteins GIS (GLABROUS INFLORESCENCE STEMS), GIS2 and GIS3 [6, 7, 24], the ZINC FINGER proteins ZFP5, ZFP6 and ZFP8 [1, 7, 43, 44], the squamosa promoter binding type protein SPL9 (SQUAMOSA PROMOTER BINDING PROTEIN LIKE 9) [40], and the membrane binding NAC protein NTL8 (NTM(NAC with transmembrane motif1)-like 8) [25]. However, the functions of these transcription factors in regulating trichome formation are achieved by regulating, directly or indirectly the expression of the key transcription factor genes. For instance, GIS, GIS3, ZFP5 and ZFP8 were found to be able to regulate some MBW genes [6, 7, 24, 43, 44]. On the other hand, SPL9 and NTL8 have been reported to be able to activate R3 MYB genes TRY and TCL1 [25, 40], although SPL9 and NTL8 may function in different pathway to regulate the expression of TRY and TCL1 [25].

Accumulated evidence in recent years indicates that trichome formation regulating transcription factor regulatory network is much more complicated than previously thought. For example, it had been reported that the expression of GLI was directly suppressed by the R3 MYB transcription factor TCL1 [36]. The conserved motif in the R3 domain of GLI that is required for its interaction with GL3, has recently been shown to involve in the binding of the GLI to its target genes [2]. It has also been reported that R3 MYB proteins may regulate trichome formation at the absence of GL2 [32].

Previous genetic evidence suggests that GIS may function upstream of the MBW complex in regulating trichome formation in Arabidopsis [6]. However, gis and tcl1 mutants showed a opposite inflorescence trichome phenotypes [6, 36], suggesting that GIS and TCL1 may function in the same pathway in regulating trichome formation. By using genetic and molecular techniques, we dissected the relationship between GIS and TCL1, we found that GIS may function downstream of TCL1 to regulate trichome formation in Arabidopsis.

**Results**

**Ectopic trichome formation in the gis tcl1 double mutant is similar to that in the tcl1 mutant**

Previously we showed that in the tcl1 mutant, ectopic trichomes were produced on the upper part of the inflorescence including stem internodes and pedicels, whereas the 35S:TCL1 transgenic plants showed a glabrous phenotype [36]. The gis single mutant, on the contrary, produced fewer trichomes on stems of the upper inflorescence, but the 35S:GIS transgenic plants have more stem trichomes [6]. The opposite phenotypes observed in the tcl1 and gis mutants indicate that GIS and TCL1 may have opposite functions in controlling trichome formation in Arabidopsis.

Previously researches showed that GIS was able to affect the expression of some MBW complex genes including the R2R3 MYB gene GLI, and the bHLH genes GL3 and EGL3 [6]. Considering that some R3 MYB transcription factor genes are regulated by the MBW complex, it is reasonable to assume that GIS may affect the expression of those R3 MYB genes. However, our previous results have shown that the expression TCL1 was not activated by the MBW complex [35], and TCL1 was able to repress the expression of GLI. Thus it will be of great interesting to examine whether GIS and TCL1 may coordinate to regulate trichome formation in Arabidopsis.

To do that, we generated gis tcl1 double mutant by crossing gis and tcl1 single mutants, and compared trichome phenotypes in the double and the single mutants by growing them side by side. We found that, stem trichome formation in the double mutant gis tcl1 was largely indistinguishable to that in the single mutant tcl1 (Fig. 1a). Quantitative analysis showed that numbers of trichomes on the second and third stem internode were greatly decreased in the single mutant gis (Fig. 1b), a result similar to reported previously [6], but increased significantly in the single mutant tcl1 (Fig. 1b). Although all the stem internodes in the double mutant gis tcl1 produced fewer trichomes when compared with that in the single mutant tcl1, the second and third stem internodes in the double mutant gis tcl1 produced much more trichomes when compared with that in the single mutant gis (Fig. 1b). In another word, similar to that in the Col-0 wild type plants, trichomes numbers along the stems decreased sharply in the single mutant gis, but slowly in the single mutant tcl1 and the double mutant gis tcl1 (Fig. 1). Ectopic trichomes were also found in the pedicels of the gis tcl1 double mutant (Fig. 2a), although with less numbers when compared with that in the single mutant tcl1 (Fig. 2b).

On the other hand, trichome formation on rosette leaves in the single mutants gis and tcl1, and the double mutant gis tcl1 was largely similar to that in the Col-0 wild type (Fig. 3a). However, more trichomes were observed on the cauline leaves of the single mutant tcl1 and double mutant gis tcl1 when compared with that in the Col-0 wild type and the single mutant gis (Fig. 3b, c). Quantitative analysis showed that numbers of trichomes on cauline leaves reduced gradually along the stems in all the plants examined. However, the numbers of trichomes in the single mutant gis and Col-0 wild type was similar, whereas that in the double mutant gis tcl1 and the single mutant tcl1 was similar (Fig. 3c).

GIS may function downstream of TCL1 to regulate trichome formation

The above results indicate that GIS and TCL1 may coordinate to regulate trichome formation in Arabidopsis. However,
Fig. 1 Trichome formation on stems of Col-0 wild type and the gis, tcl1 and gis tcl1 mutant plants. (a) Trichomes on the main stem internodes of the Col-0 wild type and the gis, tcl1 and gis tcl1 mutants. Photographs were taken from the first three internodes on the main inflorescence stems of 5-week-old soil-grown plants. Note that the trichome patterning on the main inflorescence stem of the gis tcl1 mutant was similar to that of the tcl1 mutant, but the morphology of the trichomes on the main inflorescence stem of the gis tcl1 mutant was similar to that of the gis mutant. (b) Trichome density on the first three internodes on the main inflorescence stems of the Col-0 wild type and the gis, tcl1 and gis tcl1 mutants. Number of trichomes on the each internode was count, the length of the internodes was measured, and the trichome density was calculated. Data represent the mean ± standard deviation (SD) of 10 plants. *Significantly different from that in the gis mutant plants (P < 0.0001).

Fig. 2 Trichome formation on inflorescences of the Col-0 wild type and the gis, tcl1 and gis tcl1 mutant plants. (a) Trichomes on the main inflorescences of the Col-0 wild type and the gis, tcl1 and gis tcl1 mutants. Photographs were taken from the main inflorescences of 5-week-old soil-grown plants. (b) Trichome numbers on the main inflorescence pedicel of the Col-0 wild type and the gis, tcl1 and gis tcl1 mutants. Trichomes on the first seven pedicels of the main inflorescence were count. Data represent the mean ± SD of 10 plants. Significantly different from that in the tcl1 mutant plants (*P < 0.0001, #P < 0.05).
since GIS positively, whereas TCL1 negatively regulate trichome formation [6, 36], it may seems difficult to judge from the above results the functional sequence of GIS and TCL1 in regulating trichome formation. We thus generated 35S:TCL1 transgenic plant in the gis mutant background (35S:TCL1/gis) by crossing the 35S:TCL1 transgenic with the gis single mutant plants, and compared trichome formation in the transgenic plant with the 35S:TCL1 transgenic plant and the gis single mutant by growing them side by side. As shown in Fig. 4a, the 35S:TCL1 transgenic seedlings showed a glabrous phenotype, a result similar to reported previously [36]. However, trichome production was resumed in the 35S:TCL1/gis transgenic seedlings, to a level similar to that in the Col-0 wild type or the single mutant gis (Fig. 4a).

Observation of mature plants showed that cauline leaves of the 35S:TCL1 transgenic plant failed to produce any trichomes, however, that of the 35S:TCL1/gis transgenic plant were able to do so, similar to that of the Col-0 wild type or the single mutant gis (Fig. 4b). Quantitative analysis showed that trichome numbers on cauline leaves of the 35S:TCL1/gis transgenic plants and the Col-0 wild type or the single mutant gis were largely similar (Fig. 4c).

Because trichome formation on rosette and cauline leaves in the single mutant gis was largely indistinguishable to that in the Col-0 wild type, whereas the gis single mutant produced fewer trichomes on the stem internodes of the upper part inflorescence [6], we further examined trichome formation in the inflorescences of the 35S:TCL1/gis transgenic plants. We found that trichome formation in the inflorescences of the 35S:TCL1/gis transgenic plants was also largely indistinguishable from that in the single mutant gis (Fig. 5). These results indicate that GIS may function downstream of TCL1 to regulate Arabidopsis trichome formation.

GIS plays a dominate role in controlling trichome morphology

In addition to regulate trichome formation, GIS also regulates trichome morphology [6]. The single mutant gis produced smaller but more branched stem trichomes (Fig. 1a). In contrast, the stem trichomes in the single mutant tcl1 were morphological similar to that in the Col-0 wild type. Similar to that in the single mutant tcl1, the double mutant gis tcl1 produced more stem trichomes on the internodes of the upper part inflorescence. However, the morphology of the stem trichomes in the double mutant gis tcl1 was still similar to that in the single mutant gis (Fig. 1a).
formation in the 35S:TCL1 transgenic plants (Fig. 5a), the stem trichomes of the 35S:TCL1/gis transgenic plants were morphological similar to that in the gis single mutant (Fig. 5a). These observation suggests that GIS plays a dominate role in controlling trichome morphology in Arabidopsis.

Expression of GIS is affected by R3 MYB transcription factors

Having shown that GIS may function downstream of TCL1 to regulate Arabidopsis trichome formation, we wanted to examine whether TCL1 may affect the expression of GIS. Total RNA isolated from seedlings of the 35S:TCL1 transgenic plant and the single mutant tcl1 was used to check the transcript level of GIS by quantitative RT-PCR. As shown in Fig. 6a, the expression level of GIS in both the 35S:TCL1 transgenic and the single mutant tcl1 seedlings was similar to that in the Col-0 wild type seedlings. It has been reported that R3 MYBs function redundantly to regulate Arabidopsis trichome formation [35], we thus further checked whether the expression of GIS in the seedlings of the double mutant tcl1 try and the triple mutant tcl1 try cpc may be affected.

We found that although no changes in the expression level of GIS was observed in the double mutant tcl1 try, an ~2 folds increase was observed in the triple mutant tcl1 try cpc (Fig. 6a), indicating that R3 MYBs may have redundant function in regulating the expression of GIS.

Considering that GIS functions down stream of TCL1 to regulate trichome formation, and the expression level of GIS was increased in the tcl1 try cpc triple mutant, we wanted to examine whether TCL1 may directly regulate the expression of GIS. To do that, we decided to use protoplast transient transfection assays. Previously we have successfully used these assays combined with RT-PCR or qRT-PCR analysis to examine the activation of endogenous genes by several different transcription factors [33, 35, 37], and we have shown that TCL1 functioned as a transcription activator when fused with VP16 (TCL1-VP), a strong transcription activator [36]. We thus transfected plasmid DNA of the TCL1-VP construct

**Fig. 4** Trichome formation on leaves of Col-0 wild type, the gis mutant and the 35S:TCL1 and 35S:TCL1/gis transgenic plants. (a) Trichomes on the first two rosette leaves of the Col-0 wild type, the gis mutant, and the 35S:TCL1 and 35S:TCL1/gis transgenic plants. Photographs were taken from the first two rosette leaves of 10-day-old soil-grown plants. Graph at the bottom showing the quantification of the trichomes on the first two rosette leaves. Data represent the mean ± SD of 10 plants. *significantly different form that in the 35S:TCL1 transgenic plants (P < 0.0001). (b) Trichomes on the first cauline leaves of the Col-0 wild type, the gis mutant and the 35S:TCL1 and 35S:TCL1/gis transgenic plants. Photographs were taken from the first cauline leaves of 5-week-old soil-grown plants. (c) Trichome numbers on the cauline leaves of the Col-0 wild type, the gis mutant and the 35S:TCL1 and 35S:TCL1/gis transgenic plants. Number of trichomes on the first three cauline leaves was count. Data represent the mean ± SD of 10 plants. Significantly different form that in the 35S:TCL1 transgenic plants (*P < 0.0001, #P < 0.0005)
into Arabidopsis protoplasts and examined the expression of GIS by RT-PCR. As shown in Fig. 6b, transfection of TCL1-VP only slightly increased the expression of GIS. As a control, transfection of GL1GL3-VP greatly activated the expression of GL2, a result has been reported previously [33].

We also examined whether the expression of TCL1 may be affected by GIS, by examining the transcript level of TCL1 in the single mutant gis. We found that the transcript level of TCL1 in the single mutant gis remained largely unchanged when compared that in the Col-0 wild type (Fig. 6c).

Discussion

The seven Arabidopsis R3 MYBs showed highly similarity at amino acid level [34], they all have [D/E]Lx2[R/K]x3Lx6Lx3R, the amino acid signature that is required for protein-protein interaction of MYB with bHLH proteins [41], and WxM, a motif has been shown to be required for cell-to-cell movement of the R3 MYB protein CPC [12]. Consistent with these features, all the seven R3 MYB transcription factors were able to interact with GL3/EGL3 in plant cells [5, 35], over-expression any of the R3 MYB genes resulted in glabrous phenotypes in Arabidopsis [5, 19, 22, 29, 30, 36], and characterization of the double, triple and higher order mutants of the R3 MYB genes indicated that R3 MYBs functioned in a highly redundant manner in regulating trichome formation in Arabidopsis ([3–5, 10, 11]; Tominage et al. 2008; [35, 36, 39]).

Among the seven R3 MYBs, TCL1 is unique in several different aspects. First, in addition to interact with GL3/ EGL3, TCL1 has been shown to be able to directly suppress the R2R3 MYB gene GL1 [36]. Second, unlike TRY, CPC, ETC1 and ETC3, the expression of TCL1 was not regulated by the MBW activator complex [5]. Third, TCL1 has been shown to be a direct target gene of SPL9 and NTL8 [25, 40]. We provide evidence in this study that GIS function downstream of TCL1 in regulating Arabidopsis trichome formation.

According to previously publications, the gis and tcl1 mutants showed opposite phenotypes in stem trichome formation [6, 36], indicating that GIS and TCL1 may function in a same pathway in regulating trichome formation. Therefore a genetic approach was used to dissect the relationship between GIS and TCL1. We found that trichome
patterning in the double mutant gis tcl1 was largely indistinguishable to that in the single mutant tcl1 (Fig. 1, Fig. 2, Fig. 3). Judged only from this observation, it may seems that TCL1 acts downstream of GIS in regulating trichome formation. However, because GIS positively regulates trichome formation. Consistent with this, the expression of GIS was increased in the triple mutant tcl1 try cpc, whereas the expression of TCL1 in the single mutant gis was largely unaffected (Fig. 6).

On the other hand, both the gis tcl1 double mutant and the 35S:TCL1/gis transgenic plant produced small, more branched trichomes, a phenotype similar to that observed in the gis mutant (Fig. 1, Fig. 4), suggesting that GIS functions dominantly in controlling trichome morphology.

It should be noted that although the expression of GIS was increased in the tcl1 try cpc mutant, its expression level in the tcl1 mutant and the 35S:TCL1 transgenic plants remained largely unchanged (Fig. 6). Together with the observation that tranfection of TCL1-VP into Arabidopsis only slightly increased the expression level of GIS, our results indicate that it is unlikely that TCL1 can directly regulate the expression of GIS.

It has been shown that the expression of TCL1 is directly regulated by SPL9 and NTL8 [25, 40], whereas TCL1 is able to regulate GL1 expression directly [36]. Even though our results could not support a role of TCL1 in regulating GIS expression, the observation that GIS function downstream of TCL1 added another regulation loop into the transcription factor regulating networks that control trichome formation in Arabidopsis.

Conclusions
GIS acts downstream of TCL1, and possible other R3 MYB proteins to regulate trichome formation, and GIS play a dominant role in regulating trichome morphology.

Methods
Plant materials used in this study and growth conditions
The wild type Arabidopsis ecotype Col-0 was used as a control in phenotypic assays and for protoplast isolation. The single mutants gis and tcl1, double mutant tcl1 try, triple mutant tcl1 try cpc and the 35S:TCL1 transgenic plants in the Col-0 background have been reported previously [6, 35, 36]. The double mutant gis tcl1 was obtained by crossing the single mutants gis with tcl1, examining the putative mutant phenotypes in the F2 progeny, and confirming their double mutant status by genotyping. The 35S:TCL1/gis transgenic plant was obtained by crossing the 35S:TCL1 transgenic plant with the gis mutant, examining the putative mutant and transgenic plant phenotypes in the F2 progeny, and confirming the gis mutant and 35S:TCL1 overexpression status by genotyping.

For RNA isolation, sterilized seeds were grown on 1/2 MS (Murashige & Skoog) plates with vitamins (Plantmedia) and 1% (w/v) sucrose, and solidified with 0.6% phytoagar
(Plantmedia). To obtain plants for phenotypic analysis and protoplast isolation, Arabidopsis seeds were sown directly into soil filled pots. All the Arabidopsis plants were grown in a growth room with a temperature at 22 °C and a 16/8 h photoperiod with light density at about 120 μmol m⁻² s⁻¹.

**RNA isolation, PCR and quantitative RT-PCR (qRT-PCR)**

Total RNA from 10-day-old Arabidopsis seedlings was isolated by using EasyPure Plant RNA Kit and following the instruction provided by the manufacturer (TransGen Biotech). Two μg RNA was subjected to complementary DNA (cDNA) synthesis via reverse transcription primed by Oligo(dT). The cDNA was synthesized by using DNA Synthesis Super Mix provided by TransGene Biotech in a reaction volume of 20 μl. For qRT-PCR, the synthesized cDNA was diluted 10 times with TE buffer (pH = 8.0), and 1 μl of the dilute solution was then used for each PCR reaction. The qRT-PCR was carried out on an Applied Biosystems StepOnePlus™ Real-Time PCR system by using the TransStart™ Top Green qPCR SuperMix reagent (TransGen Biotech). Total RNA from transfected protoplasts was isolated, and cDNA was synthesized by following the procedures described previously [37], with exceptions that an EasyPure Plant RNA Kit was used for RNA isolation, and EasyScript First-Strand DNA Synthesis Super Mix was used for cDNA synthesis. The primers used to examine the expression of GIS, TCL1, GL2 and ACT2 have been reported previously [6, 25, 45].

**Constructs**

The reporter construct Gal4-GLUS and the effect constructs GD (Gal4 DNA binding domain), GD-VP, GD-SPL9, TCL1-VP, GL1GL3-VP, and CAT used for protoplasts transfection have been reported previously [25–27, 33, 36].

**Plasmid DNA preparation, Arabidopsis protoplast isolation, protoplast transfection**

Plasmid DNA of the effector was isolated using the GoldHi EndoFree Plasmid Maxi Kit by following the procedures provided by the manufacturer (Kangwei).

Protoplasts were isolated and transfected by following the procedures described previously [37, 38]. Briefly, rosette leaves were collected from 3- to 4-week-old Col-0 wild type plants grown in soil pots and used for protoplast isolation. Isolated protoplasts were transfected with plasmid DNA isolated. The protoplasts were then incubated in darkness for 20–22 h at room temperature, and then subjected to RNA isolation, or GUS activity assays on a BioTEK Synergy™ HT microplate reader.

**Microscopy**

Trichome formation and trichome morphology in Arabidopsis plants was analyzed under a microscope (Motic K) and photographed with a digital camera (EOS 1100D) attached to the microscope. Trichome formation on rosette leaves was observed using the first two true leaves of the wild type and mutant seedlings grown on soil pots. Trichome formation on inflorescences and cauline leaves was observed using adult soil-grown plants.

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**Availability of data and materials**

Not Applicable.

**Authors’ contributions**

SW conceived the study and designed the experiments. NZ, LY, SL, XW, WW, YC, HT, KZ, and LC performed the experiments, NZ, LY and SW analyzed the data, SW drafted the manuscript, and all the authors participated in the revision of the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not Applicable.

**Consent for publication**

Not Applicable.

**Competing interests**

The authors declare no competing interests.

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