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Research

Distinct relationships between GLABRA2 and single-repeat R3 MYB transcription factors in the regulation of trichome and root hair patterning in Arabidopsis

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Summary

- The patterning of epidermal cell types in Arabidopsis is an excellent model for studying the molecular basis of cell specification. Trichome and root hair formation is controlled by a transcriptional activator complex that induces the homeobox gene *GLABRA2 (GL2)* and some single-repeat R3 MYB genes (single *MYB*). However, it remains unclear how the actions of GL2 and single MYBs are coordinated to regulate epidermal patterning.
- GL2 is thought to act downstream of single MYBs to regulate trichome and root hair development. In order to test this hypothesis genetically, double and higher order mutants between *gl2* and single *myb* were generated.
- In these mutants, the glabrous phenotypes observed in the *gl2* single mutants were partially recovered, suggesting that single MYBs may not act solely through GL2 to regulate trichome development. On the other hand, double and higher order mutants between *gl2* and single *myb* phenocopied the root hair phenotype of *gl2* single mutants, suggesting that GL2 and single MYBs act in a common pathway to regulate root hair patterning.
- These findings reveal distinct relationships between GL2 and single MYBs in the regulation of trichome vs root hair development, and provide new insights into the molecular mechanism of epidermal patterning.

Introduction

Trichome and root hair cell patterning in Arabidopsis is controlled by several different classes of transcription factor (Hülskamp *et al.*, 1994). A WD-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Galway *et al.*, 1994; Walker *et al.*, 1999), an R2R3 MYB-type transcription factor GLABRA1 (GL1) or WEREWOLF (WER) (Oppenheimer *et al.*, 1991; Lee & Schiefelbein, 1999) and bHLH transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne *et al.*, 2000; Zhang *et al.*, 2003) have been proposed to form a transcriptional activator complex to control the expression of *GLABRA2* (*GL2*), which encodes a homeodomain protein (Rerie *et al.*, 1994; Masucci *et al.*, 1996). GL2, in turn, promotes trichome formation in shoots and inhibits root hair formation in roots (reviewed by Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008). The same TTG1–GL3/EGL3–GL1/WER complex can also activate the expression of some single-repeat R3 MYB genes (single MYB) (reviewed by Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008). So far, a total of six single MYBs has been identified in Arabidopsis, including TRIPTYCHON (TRY) (Schnittger *et al.*, 1999; Schellmann *et al.*, 2002), CAPRICE (CPC) (Wada *et al.*, 1997, 2002), TRICHOMELESS1 (TCL1) (Wang *et al.*, 2007) and ENHANCER of TRY and CPC 1, 2 and 3 (ETC1, ETC2 and ETC3/CPL3) (Esch *et al.*, 2004; Kirik *et al.*, 2004a,b; Simon *et al.*, 2007; Tominaga *et al.*, 2008). Accumulating evidence supports the notion that single MYBs act largely in a redundant manner to negatively regulate trichome formation in shoots, but positively regulate root hair formation in roots, although functional diversity of single MYBs also exists (Simon *et al.*, 2007; Wester *et al.*, 2009). Therefore, GL2 and single MYB transcription factors have opposing roles in trichome and root hair development.

Currently, the opposing role of GL2 and single MYBs is believed to be a result of the inhibitory effect of single MYBs on the activity of the TTG1–GL3/EGL3–GL1/WER transcriptional activator complex (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). All single MYBs discovered so far, including TRY, CPC, TCL1, ETC1, ETC2 and ETC3, interact with GL3 (Esch *et al.*, 2003; Zhang *et al.*, 2003; Kirik *et al.*, 2004b; Zimmermann *et al.*, 2004; Tominaga *et al.*, 2008; Wang *et al.*, 2008). Furthermore, in yeast three-hybrid assays, it has been shown that single MYBs, such as TRY and ETC1, can compete with GL1 in binding to GL3 (Esch *et al.*, 2003, 2004). This property has been proposed to enable single MYBs, which move from a trichome precursor cell to its neighboring cell in shoots, or move from a hairless cell (N cell) to a hair cell (H cell) in roots, to compete with GL1 (in shoots) or WER (in roots) for the binding of GL3 (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). This competitive binding reduces the abundance of the TTG1–GL3/EGL3–GL1/WER transcriptional activator complex, which decreases the expression of *GL2* and leads to the inhibition of trichome formation and the promotion of root hair formation (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). In a previous study, we have confirmed that each of these six single MYBs can indeed interact with GL3 in plant cells, and that an activator complex between GL1/WER and GL3/EGL3 is required and sufficient to activate the expression of *GL2* and a subset of single MYB genes (Wang & Chen, 2008; Wang *et al.*, 2008).

One of the central remaining questions is how single MYBs and GL2 are coordinated to regulate trichome and root hair formation, given that the expression of both *GL2* and some single MYB genes is activated by the same TTG1–GL3/EGL3–GL1/WER complex. For example, do single MYBs simply act by regulating the expression of *GL2* through limitation of the transcriptional activity of the TTG1–GL3/EGL3–GL1/WER complex, or do they influence epidermal cell differentiation via additional mechanisms? In the present study, we generated and analyzed double and higher order mutants between *gl2* and single *myb* mutants. Our genetic analysis reveals a previously unrecognized relationship between GL2 and single MYBs in trichome development, and provides new insights into the molecular mechanism of transcription factors in regulating epidermal patterning in Arabidopsis.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis thaliana* L. Heynh single mutants *try_29760* (Esch *et al.*, 2003), *tcl1-1* (Wang *et al.*, 2007) and *etc1-1* (Kirik *et al.*, 2004a) are in the Columbia (Col) ecotypic background. A *gl2* allele in the Col ecotypic background was used in our study. This allele is a T-DNA insertional mutant, *gl2-3* (SALK_039825), which harbors a T-DNA in the second intron of the *GL2* gene, at a very similar site as that in *gl2-2* (SALK_130213) (Guan *et al.*, 2008). The *gl2-3* mutant displays an identical, glabrous phenotype to *gl2-2*, forms ectopic root hair and does not produce seed coat mucilage, suggesting that it is probably a null allele. The *cpc-1* mutant is in the Wassilewskija (WS) ecotypic background (Wada *et al.*, 1997). The single *myb* double and higher order mutants have been described previously (Wang *et al.*, 2008). The *gl2-3 cpc-1* double mutant was generated by crossing single mutants *gl2-3* and *cpc-1*, examining the F_2 progeny for the putative mutant phenotype and confirming their double mutant status by genotyping in F_2 and subsequent generations. The *gl2-3 cpc-1 tcl1-1* and *gl2-3 try-29760 cpc-1* triple mutants were generated by crossing *cpc-1 tcl1-1* or *try-29760 cpc-1* with *gl2-3 cpc-1* double mutants. The *gl2-3 try-29760 cpc-1 tcl1-1* quadruple mutant was generated by crossing *gl2-3 try-29760 cpc-1* and *try-29760 cpc-1 tcl1-1*. The *gl2-3 try-29760 cpc-1 etc1-1* quadruple mutant was generated by crossing *gl2-3 try-29760 cpc-1* and *try-29760 cpc-1 etc1-1*. For simplicity, the *gl2 cpc*, *gl2 try cpc*, *gl2 try cpc etc1*, *gl2 cpc tcl1*, *gl2 try cpc tcl1* nomenclatures in this report refer specifically to the *gl2-3 cpc-1*, *gl2-3 try-29760 cpc-1*, *gl2-3 try-29760 cpc-1 etc-1*, *gl2-3 cpc-1 tcl1-1*, *gl2-3 try-29760 cpc-1 tcl1-1* mutants, respectively.

Seedlings used for phenotypic analyses were obtained either by growing surface-sterilized seeds on 0.6% (w/v) phytoagar (Plantmedia, Dublin, OH, USA)-solidified ½ Murashige & Skoog (MS) basal medium with vitamins (Plantmedia) and 1% (w/v) sucrose, or by sowing seeds directly into soil. Plants were grown at 23°C with a 14 h : 10 h photoperiod at *c.* 120 μmol m^{-2} s^{-1}.

Trichome and root hair analyses

Trichome analysis was performed using both 10-d-old seedlings and soil-grown plants. In 10-d-old seedlings, the number of trichomes was counted from the first two leaves. In soil-grown plants, the rosette leaves in bolting plants and the inflorescence stems, pedicels and floral organs in flowering plants were used for trichome analysis. Ten-day-old seedlings grown vertically on Petri dishes were used for root hair analysis. The pattern of root epidermal cell types was determined as described previously (Lee & Schiefelbein, 2002; Kirik *et al.*, 2004a,b).

Mucilage staining

Seeds were stained by shaking in 0.01% (w/v) Ruthenium Red (Sigma-Aldrich, St. Louis, MO, USA) for 2 h and then

mounted in water. Mucilage was viewed and photographed using a dissecting microscope.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the leaf mesophyll protoplasts of wild-type and transgenic plants overexpressing the GL1–GL3 fusion protein (Wang & Chen, 2008), the various tissues and organs of Col wild-type plants and the 10-d-old seedlings of single *myb* mutants using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada). cDNA was synthesized using 1 μg of total RNA by Oligo(dT)-primed reverse transcription using an OMNISCRIPT RT Kit (Qiagen). RT-PCR was used to examine the expression of *GL2* and single *MYB* genes. The primers and procedure used for examining the expression of six single *MYB* genes and *GL2* have been described previously (Wang & Chen, 2008; Wang *et al.*, 2008).

Results

Confirmation of the activation of *GL2* and single *MYB* genes by GL1 and GL3

Previously, we used an Arabidopsis protoplast transient expression system to examine the activation of transcription of single *MYB* genes, and found that co-transfection of GL1/WER and GL3/EGL3 was sufficient to activate the transcription of a subset of single *MYB* genes (Wang *et al.*, 2008). In a subsequent study, we used an Arabidopsis protoplast transfection system with an integrated reporter gene (P_{GL2}*::GUS*) to examine the activation of *GL2*, and found that co-transfection of GL1 and GL3 could activate the expression of the integrated P_{GL2}*::GUS* reporter gene (Wang & Chen, 2008). Ectopic expression of the P_{GL2}*::GUS* reporter gene was also observed in stable transgenic lines overexpressing the GL1–GL3 fusion protein (*35S::GL1–GL3*) (Wang & Chen, 2008). In the present study, we wanted to examine further whether the transcription of endogenous *GL2* and single *MYB* genes was activated in *35S::GL1–GL3* stable transgenic lines. We found that the transcripts of *GL2* and single *MYB* genes were undetectable in mesophyll protoplasts of wild-type plants (Fig. 1). In *35S::GL1–GL3* transgenic plants, however, an RT-PCR-derived product was obtained for *GL2* and a subset of single *MYB* genes, including *TRY*, *CPC*, *ETC1* and *ETC3*, but not *TCL1* and *ETC2* (Fig. 1). These results are in agreement with those of Arabidopsis protoplast transient expression assays (Wang & Chen, 2008; Wang *et al.*, 2008), and are also consistent with the recent finding that *GL2* and *TRY*, *CPC*, *ETC1* and *ETC3* are direct targets of GL3/GL1 (Morohashi & Grotewold, 2009). These results confirm that *GL2* and a subset of single *MYB* genes can indeed be activated by the same transcriptional activator complex.

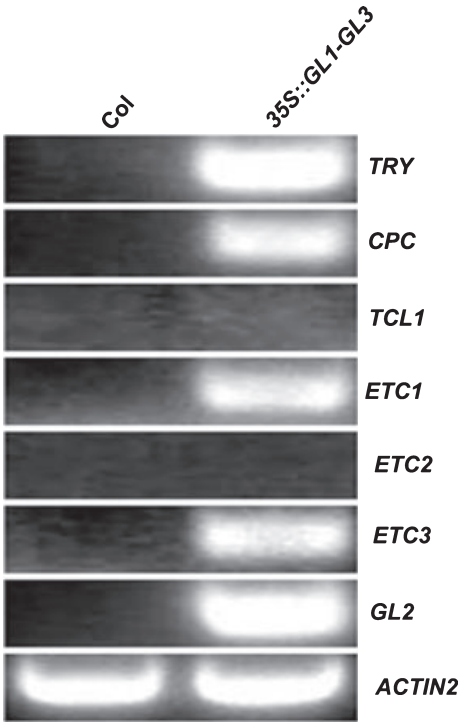

Fig. 1 Overexpression of GL1–GL3 fusion protein activates the transcription of *GL2* and a subset of single *MYB* genes. Total RNA was extracted from the mesophyll protoplasts of *Arabidopsis thaliana* wild-type (Col) or stable transgenic plants overexpressing the GL1–GL3 fusion protein (*35S::GL1–GL3*). RT-PCR analyses of the transcript of *GL2* and single *MYB* genes are shown. The expression of *ACTIN2* provided a control.

Loss-of-function mutation in a single *MYB* gene, *CPC*, partially restores trichome formation in the *gl2* mutant

As described in the Introduction, it has been proposed that single MYBs compete with GL1 or WER for the binding of GL3, thus limiting the activity of the TTG1–GL3/EGL3–GL1/WER transcriptional activator complex and reducing the expression of *GL2* (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). In this context, *GL2* is thought to act downstream of single *MYB* genes to regulate trichome and root hair formation. To test this hypothesis genetically, we generated double mutants between *gl2* and single *myb* mutants. In particular, we generated the *gl2 cpc* double mutant. As reported previously (Rerie *et al.*, 1994; Masucci *et al.*, 1996; Wada *et al.*, 1997; Schellmann *et al.*, 2002), the *cpc* single mutant displays increased trichome formation on leaves and reduced root hair formation in roots, whereas the *gl2* single mutant displays reduced trichome formation on leaves and increased root hair formation in roots (Tables 1 and 2).

We predicted that the *gl2 cpc* double mutant would phenocopy the *gl2* single mutant because, according to current models (reviewed by Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008), *GL2* acts downstream of *CPC* in epidermal patterning. First, we focused on the examination of trichome formation in leaves. We found that, in young seedlings, the *gl2 cpc* double mutants do not appear to differ from the *gl2* single mutants (Table 1). However, in bolting plants, the *gl2 cpc* double mutants produce more trichomes on rosette leaves than do the *gl2* single mutants (Fig. 2). A more dramatic difference between the *gl2 cpc* double mutant and the *gl2* single mutant in trichome formation was observed in the inflorescence stems (Fig. 3). Although the *gl2* single mutant had glabrous stems, trichome formation was restored in the inflorescence stems of the *gl2 cpc* double mutant (Fig. 3). Similarly, more trichomes were also observed in the sepals of *gl2 cpc* double mutants, compared with the *gl2* single mutant (Fig. 4). Taken together, these results suggest that the loss of function of *CPC* can partially restore trichome formation in the *gl2* mutant.

Table 1 Leaf trichome production in wild-type and mutant *Arabidopsis thaliana*

Genotype	No. trichomes per leaf	Frequency of trichome clusters (%)
WT (Col)	25.3 ± 3.9	0
WT (WS)	27.2 ± 4.7	0
gl2-3	2.7 ± 1.5*	0
cpc-1	39.7 ± 9.5*	0
gl2-3 cpc-1	2.5 ± 1.1*	0
cpc-1 tcl1-1	41.5 ± 7.5*	1.1
gl2-3 cpc-1 tcl1-1	4.5 ± 2.5*	0
try-29760 cpc-1	78 ± 21*	82*
gl2-3 try-29760 cpc-1	7.2 ± 2.8*	51*
try-29760 cpc-1 tcl1-1	77 ± 24*	91*
gl2-3 try-29760 cpc-1 tcl1-1	8.1 ± 2.2*	41*
try-29760 cpc-1 etc1-1	89 ± 32*	96*
gl2-3 try-29760 cpc-1 etc1-1	6.5 ± 3.7*	45*

Values indicate mean ± SD of at least 10 leaves for each line.
*$P < 0.05$, relative to the corresponding wild-type line.

Single *MYB* genes act redundantly to negatively regulate trichome formation even in the absence of a functional *GL2*

Previous studies have shown that single *MYB* genes largely function redundantly to regulate trichome formation (Schellmann *et al.*, 2002; Esch *et al.*, 2004; Kirik *et al.*, 2004a,b; Wang *et al.*, 2007, 2008; Tominaga *et al.*, 2008; Wester *et al.*, 2009). We wanted to examine further whether a combination of loss of function in several single *MYB* genes could have more profound effects on the restoration of trichome formation in the *gl2* mutant background. Therefore, we generated a triple mutant between *gl2* and *try cpc*, and a quadruple mutant between *gl2* and *try cpc etc1*. As reported previously (Schellmann *et al.*, 2002; Kirik *et al.*, 2004a; Wang *et al.*, 2008), the *try cpc* double mutant and the *try cpc etc1* triple mutant produced more trichomes, which were mostly present as clusters, compared with the *cpc* single mutant (Fig. 2, Table 1). In the *gl2 try cpc* triple mutant and the *gl2 try cpc etc1* quadruple mutant, more trichomes appeared to be formed on leaves when compared with those in the *gl2 cpc* double mutant (Fig. 2). Moreover, trichome cluster formation was also observed (Fig. 2).

Table 2 Root hair and non-hair cell specification in the root epidermis of wild-type and mutant *Arabidopsis thaliana*

		H cell position		N cell position	
Genotype	Hair cells in epidermis (%)	Hair cells (%)	Non-hair cells (%)	Hair cells (%)	Non-hair cells (%)
WT (Col)	40.5 ± 1.9	97.5 ± 1.9	2.5 ± 1.9	0.4 ± 1.0	99.6 ± 1.0
WT (WS)	41.4 ± 3.2	96.6 ± 2.1	3.4 ± 2.1	0.5 ± 0.8	99.5 ± 0.8
gl2-3	92.2 ± 5.4*	99.3 ± 1.8	0.7 ± 1.8	86.2 ± 5.1	13.8 ± 5.1
cpc-1	15.1 ± 2.8*	27.7 ± 3.9	72.3 ± 3.9	0.9 ± 1.5	99.1 ± 1.5
gl2-3 cpc-1	90.1 ± 6.8*	100 ± 0	0 ± 0	79.8 ± 6.5	20.2 ± 6.5
cpc-1 tcl1-1	17.2 ± 3.3*	29.3 ± 4.6	70.7 ± 4.6	0.5 ± 1.1	99.5 ± 1.1
gl2-3 cpc-1 tcl1-1	93.2 ± 2.7*	98.5 ± 3.1	1.5 ± 3.1	89.2 ± 4.5	10.8 ± 4.5
try-29760 cpc-1	0 ± 0*	0 ± 0	100 ± 0	0 ± 0	100 ± 0
gl2-3 try-29760 cpc-1	92.0 ± 3.1*	99.3 ± 1.0	0.7 ± 1.0	85.1 ± 5.0	14.9 ± 5.0
try-29760 cpc-1 tcl1-1	0 ± 0*	0 ± 0	100 ± 0	0 ± 0	100 ± 0
gl2-3 try-29760 cpc-1 tcl1-1	90.8 ± 4.6*	97.6 ± 2.1	2.4 ± 2.1	86.8 ± 5.2	13.2 ± 5.2
try-29760 cpc-1 etc1-1	0 ± 0*	0 ± 0	100 ± 0	0 ± 0	100 ± 0
gl2-3 try-29760 cpc-1 etc1-1	88.7 ± 5.1*	99.5 ± 1.7	0.5 ± 1.7	77.9 ± 6.8	22.1 ± 6.8

Values indicate mean ± SD of at least 10 roots for each line. In all strains, *c.* 40% of epidermal cells are in the H position.
*$P < 0.05$, relative to the corresponding wild-type line.

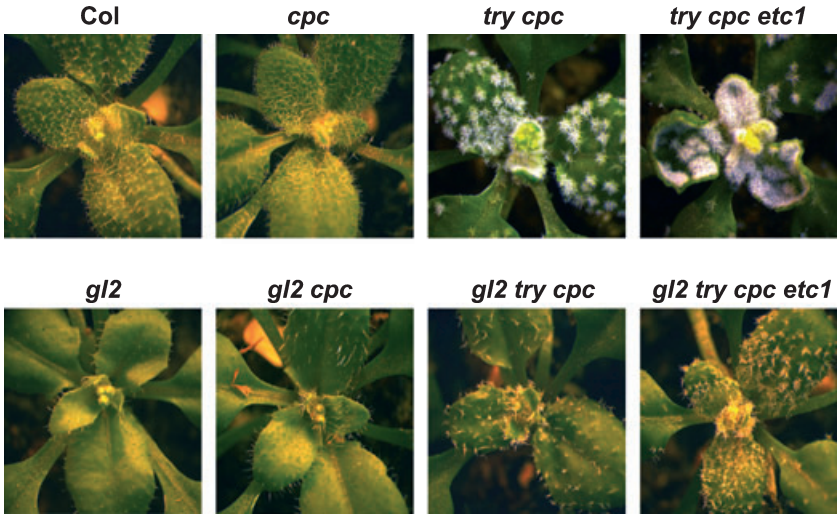

Fig. 2 Loss-of-function mutations in the single *MYB* genes partially restore trichome formation on rosette leaves in the *gl2* mutant background. Photographs were taken from 3-wk-old, soil-grown *Arabidopsis thaliana* plants.

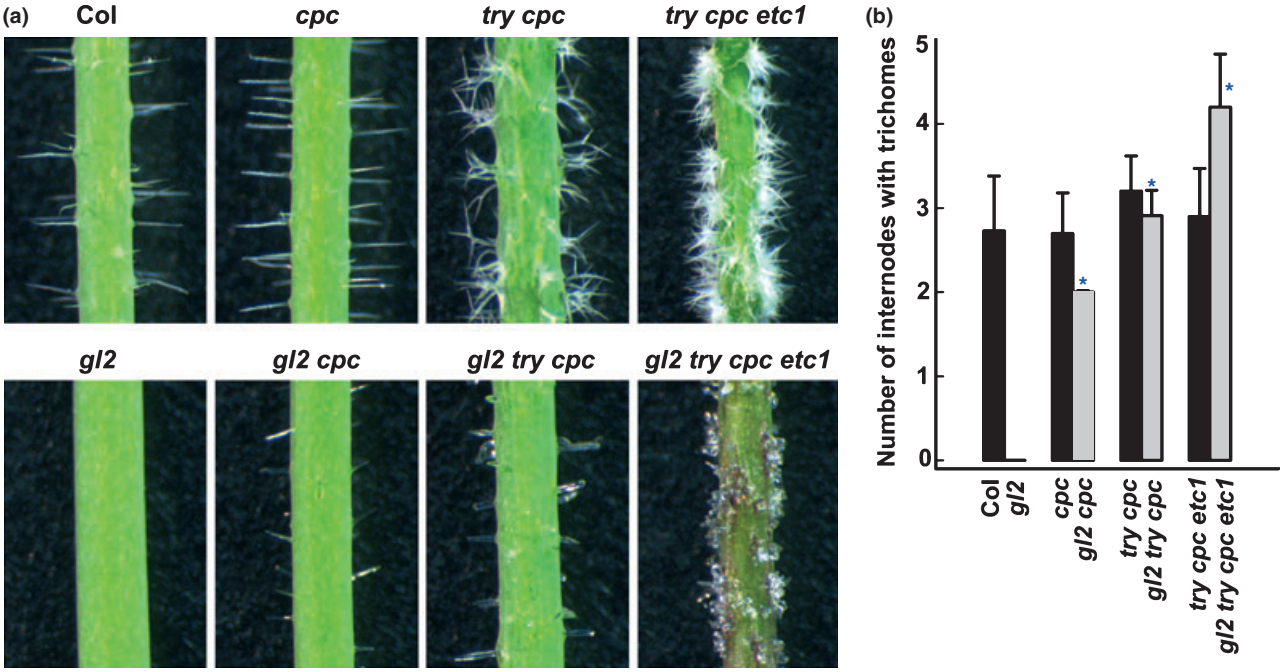

Fig. 3 Loss-of-function mutations in the single *MYB* genes partially restore trichome formation on the inflorescence stems of the *gl2* mutant. (a) Stem trichomes. Photographs were taken from 4-wk-old, soil-grown *Arabidopsis thaliana* plants. Note that there is no trichome formed on the inflorescence stems of the *gl2* single mutant. (b) Number of internodes on the main inflorescence stems with trichome formation. The means ± SD of at least 10 plants are shown for each genotype; $*P < 0.05$, significant difference from *gl2* single mutant.

Similarly, more trichomes were formed in the inflorescence stems of the *gl2 try cpc* triple mutant and the *gl2 try cpc etc1* quadruple mutant than in the *gl2 cpc* double mutant (Fig. 3). Typically, on the main inflorescence stems of wild-type plants, trichomes were formed in the first three internodes (Fig. 3b). Although the inflorescence stems of the *gl2* single mutant do not bear any trichomes, trichome formation was restored in the first two internodes (lower inflorescence stems) of the *gl2 cpc* double mutant (Fig. 3b). In the *gl2 try cpc* triple mutant and the *gl2 try cpc etc1* quadruple mutant, trichomes were also formed in the third and fourth internodes (Fig. 3b). The morphology of trichomes was also altered (discussed further below). These results suggest that single *MYB* genes can still act redundantly to negatively regulate trichome formation in the absence of a functional *GL2*.

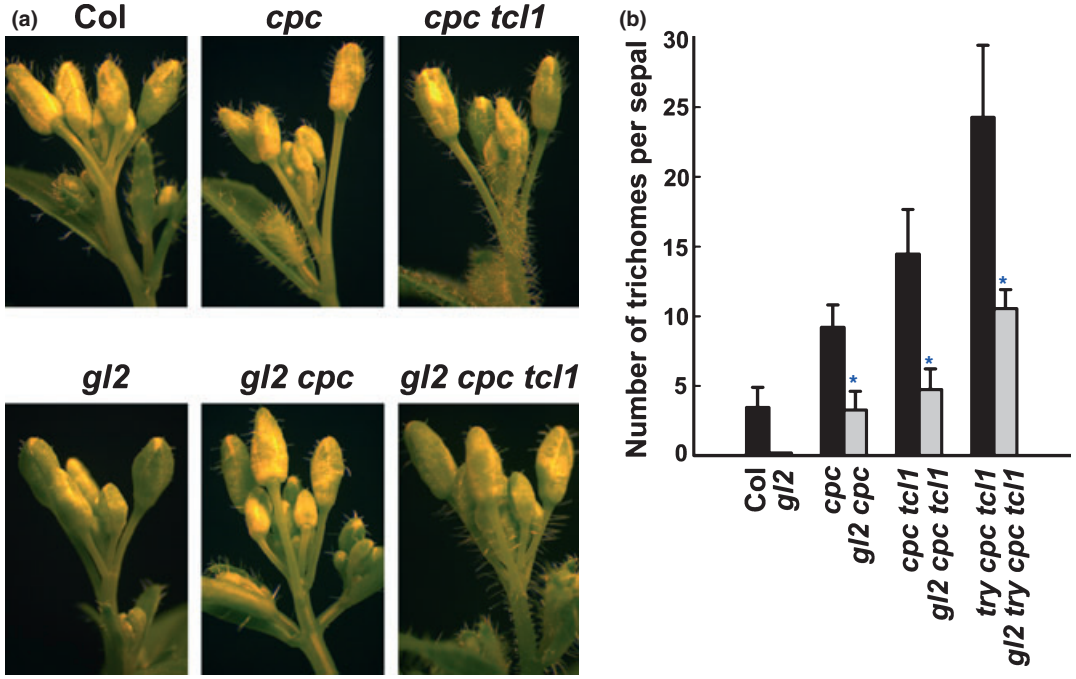

Fig. 4 Loss-of-function mutations in the single *MYB* genes partially restore trichome formation on the inflorescences of the *gl2* mutant. (a) Trichomes on floral organs. Photographs were taken from 4-wk-old, soil-grown *Arabidopsis thaliana* plants. (b) Number of trichomes on sepals. Trichomes were counted from the sepals of the first flower of each plant. The means ± SD of at least 10 plants are shown for each genotype; $*P < 0.05$, significant difference from the *gl2* single mutant.

Previously, we have identified TCL1 as a major single MYB transcription factor that negatively regulates trichome formation in the inflorescence epidermis (Wang *et al.*, 2007). Loss-of-function mutations in *TCL1* confer ectopic trichome formation on inflorescence stems and pedicels. These phenotypes have not been observed in the loss-of-function mutants of any other single *MYB* genes, although a synergistic effect between *TCL1* and *CPC* on trichome formation in these organs has been observed (Wang *et al.*, 2007). Because TCL1 appears to have diverged from the other single MYBs at the protein level (Wang *et al.*, 2007; Wester *et al.*, 2009), we wanted to examine whether TCL1 behaves similarly to other single MYBs on trichome formation in the absence of a functional *GL2*. Therefore, we generated a triple mutant between *gl2* and *cpc tcl1*, and a quadruple mutant between *gl2* and *try cpc tcl1*. As described previously, stem trichome formation was restricted to the region below the first flower on the main inflorescence stem and no trichomes were formed on the pedicels (Gan *et al.*, 2006; Wang *et al.*, 2007). Interestingly, ectopic trichome formation was found in the *gl2 cpc tcl1* triple mutant and the *gl2 try cpc tcl1* quadruple mutant, beyond the point at which the first flower on the main inflorescence stem appeared (Fig. 5). Similarly, ectopic trichome formation was also observed in the pedicels and sepals of *gl2 cpc tcl1* triple mutants (Figs 4 and 5). Because the *gl2* single mutant does not produce any trichomes on the upper part of inflorescence stems and pedicels, these results suggest that *TCL1*, like any other single *MYB* gene examined, can still execute its inhibitory effect on trichome formation in the absence of a functional *GL2*. Taken together, we conclude that single *MYB* genes may not act solely through *GL2* to execute their function, arguing for additional mechanisms of single MYBs and GL2 in the regulation of trichome formation in the shoot epidermis.

Expression of *GL2* in various tissues and organs

Our genetic studies have indicated that significant trichome formation can occur in the absence of a functional GL2 (in the single *myb* mutant backgrounds), which raises the possibility that trichome formation is not solely controlled by GL2. To examine this possibility further, we analyzed the expression of *GL2* in different tissues and organs of Arabidopsis wild-type plants. We reasoned that, if the expression of *GL2* is an indicator of trichome formation, we would expect to find a correlation between the location of *GL2* expression and the tissues and organs that normally produce trichomes. We found that the transcript of *GL2* could indeed be detected in the tissues and organs that normally produce trichomes, such as rosette leaves, cauline leaves, lower inflorescence stems (the region below the site of first flower branch) and floral organs (Fig. 6a). Further, the transcript of *GL2* was present at a very low or undetectable level in cotyledons and petioles (Fig. 6a), organs that normally do not bear any trichomes. However, the *GL2* transcript was also detected in hypocotyls, upper inflorescence stems (the region beyond the site of first flower branch), pedicels

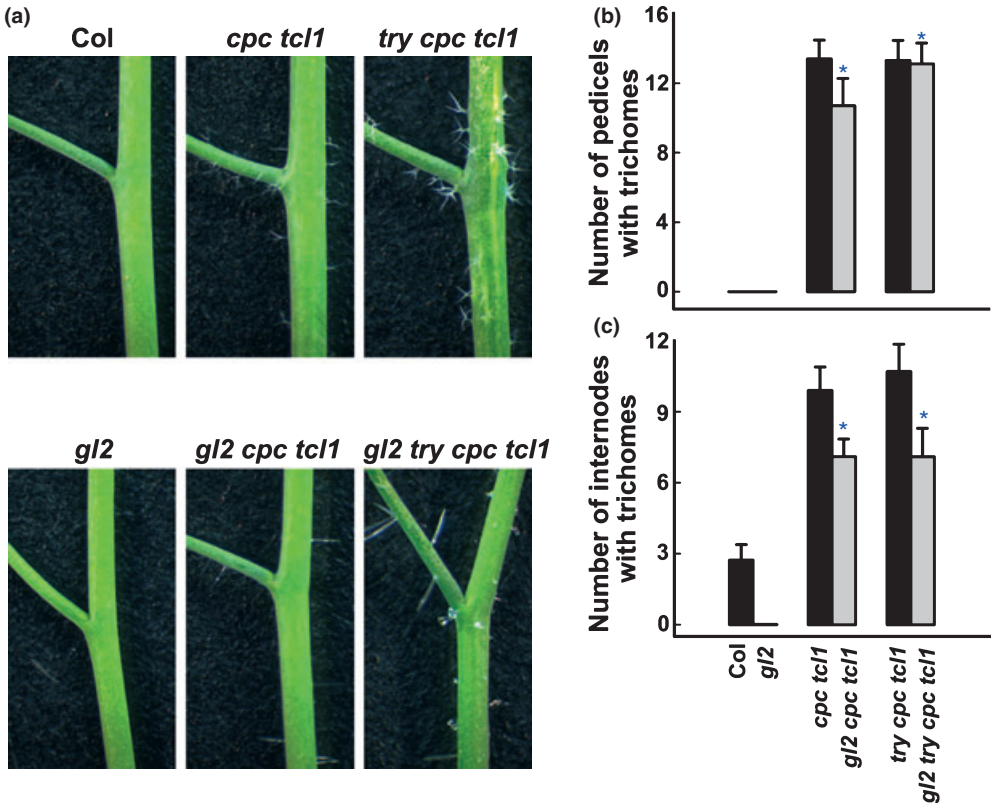

Fig. 5 Loss-of-function mutations in the single *MYB* genes induce ectopic trichome formation in the upper inflorescence stems of the *gl2* mutant. (a) Trichomes on the upper inflorescence. Photographs were taken from 5-wk-old, soil-grown *Arabidopsis thaliana* plants. Note that there is no trichome formed in the upper inflorescence stems or pedicels of the wild-type (Col) and *gl2* single mutant. (b) Number of pedicels with trichomes. (c) Number of internodes on the main inflorescence stems with trichomes. The means ± SD of at least 10 plants for each genotype are shown in (b) and (c); $*P < 0.05$, significant difference from the *gl2* single mutant.

and siliques (Fig. 6a). These organs do not normally produce any trichomes. Although *GL2* expression in these organs may imply that *GL2* has other roles (e.g. different from trichome formation), it is known that these organs are capable of producing trichomes under certain specific conditions. For example, the loss-of-function mutations in *TCL1* conferred ectopic trichome formation on upper inflorescence stems and pedicels (Wang *et al.*, 2007). One possible explanation for the poor correlation between *GL2* expression and trichome development is that these organs (e.g. upper inflorescence stems) may require a higher threshold of *GL2* expression to produce trichomes. However, the fact that single *myb* mutants containing *tcl1* can still produce trichomes in these organs, even in the absence of *GL2* (e.g. in the *gl2 cpc tcl1* mutant), implies that *GL2* may not be the sole master gene required for trichome formation.

GL2 and single MYB transcription factors regulate trichome morphology

As mentioned above, during the process of analysis of trichome formation in double and higher order mutants between *gl2* and single *myb*, we observed that, in addition to the partial recovery of trichome formation, trichome morphology was altered in the inflorescence stems of the *gl2 try cpc* triple mutant and *gl2 try cpc etc1* quadruple mutant. As shown in Fig. 3, stem trichomes of wild-type plants usually are single branched, stem trichomes of the *cpc* mutant are indistinguishable from those of the wild-type, and stem trichomes of the *try cpc* double mutant and *try cpc etc1* triple mutant mostly have two to three branches, suggesting that single MYBs can also act redundantly to regulate trichome branching. Although no stem trichome was formed in the *gl2* single mutant, interestingly, significant differences were observed in the trichome morphology between the *try cpc* double mutant and *gl2 try cpc* triple mutant (Figs 3 and 7). Compared with the stem trichomes in the *try cpc* double mutant, trichomes in the *gl2 try cpc* triple mutant appeared to be shorter and were blunt, transparent and often single branched (Figs 3 and 7). More dramatic differences were observed between the *try cpc etc* triple mutant and the *gl2 try cpc etc1* quadruple mutant (Figs 3 and 7). These results suggest that single *MYB* genes function redundantly to regulate trichome branching and that *GL2* regulates both the outgrowth of the trichome and its branches. These results are consistent with the previously identified role of *GL2* in the

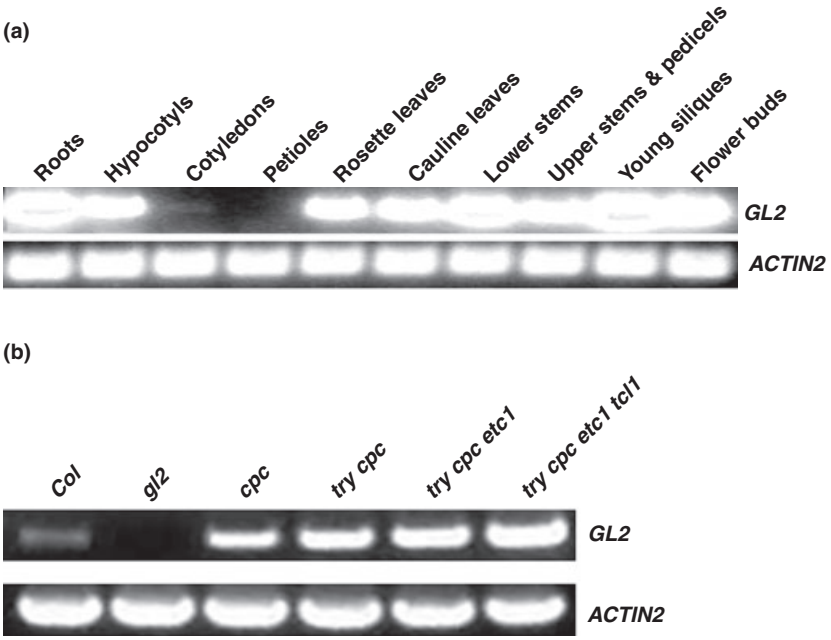

Fig. 6 RT-PCR analysis of *GL2* expression. (a) The transcript of *GL2* in various tissues and organs of wild-type *Arabidopsis thaliana* plants. (b) The transcript of *GL2* in single *myb* mutants. The expression of *ACTIN2* provided a control.

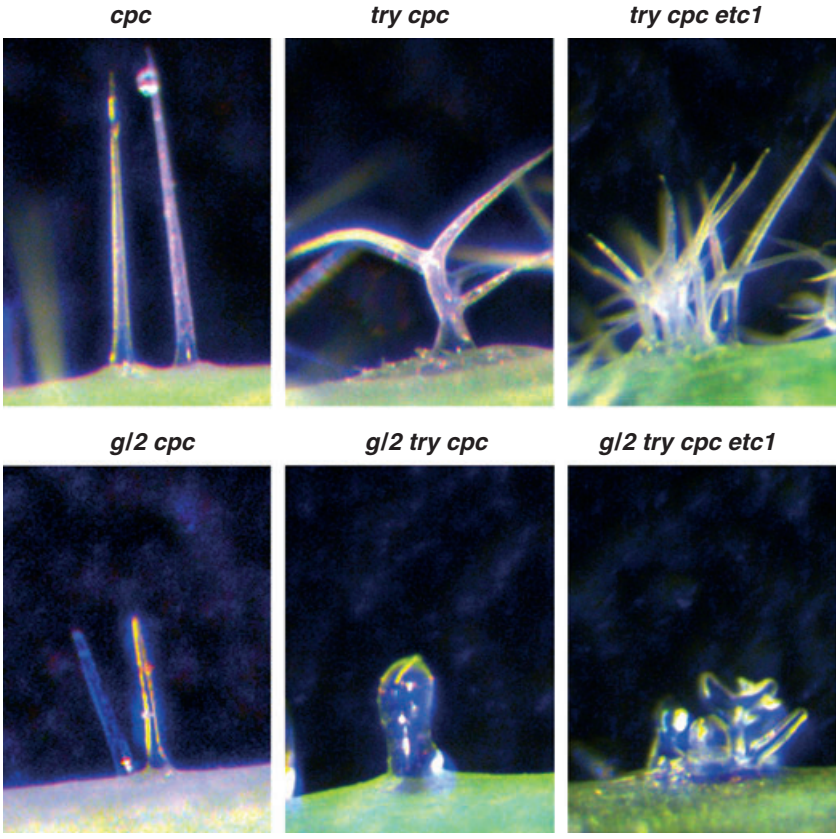

Fig. 7 Both *GL2* and single MYB genes are involved in the regulation of trichome morphology. Photographs were taken from the inflorescence stems of 4-wk-old, soil-grown *Arabidopsis thaliana* plants.

regulation of leaf trichome morphology (Rerie *et al.*, 1994). Therefore, both *GL2* and single *MYB* genes regulate trichome morphology, although their precise relationship in this process remains unclear.

Relationship between *GL2* and single *MYB* genes in root hair formation

Our genetic analyses using double and higher order mutants between *gl2* and single *myb* suggested that single MYBs may not act solely through GL2 to negatively regulate trichome development. We wanted to extend our analysis to root hair formation. *GL2* is required for non-root hair specification and the *gl2* mutant displays ectopic root hair formation (Masucci *et al.*, 1996) (Table 2, Fig. 8). On the other hand, single *MYB* genes are generally considered to be positive regulators of root hair development. In order to investigate the relationship between GL2 and single MYBs in root hair development, we examined root hair development in the *gl2 cpc* double mutant, hypothesizing that a loss of function in *CPC* may suppress the hairy (more root hair) phenotype of the *gl2* mutant. Unexpectedly, no difference in root hair number or hair cell vs non-hair cell specification was observed between the *gl2* single mutant and the *gl2 cpc* double mutant (Fig. 8, Table 2), suggesting that *gl2* is epistatic to *cpc* during root hair formation. We then analyzed the root hair phenotype in the *gl2 try cpc* triple mutant. Although the *try cpc* double mutants were hairless (no root hair formation), when *try* and *cpc* mutations were introduced into the *gl2* mutant background, the *gl2 try cpc* triple mutant still displayed the hairy phenotype, phenocopying the *gl2* single mutant (Fig. 8, Table 2). Similarly, in any other double and higher order mutants between *gl2* and single *myb* examined in this study, loss of function of single *MYB* genes did not significantly modify the root hair phenotype of the *gl2* mutant (Table 2). Taken together, these results suggest that *GL2* is epistatic to single *MYB* genes during root hair development, a relationship that is distinct from that in trichome development.

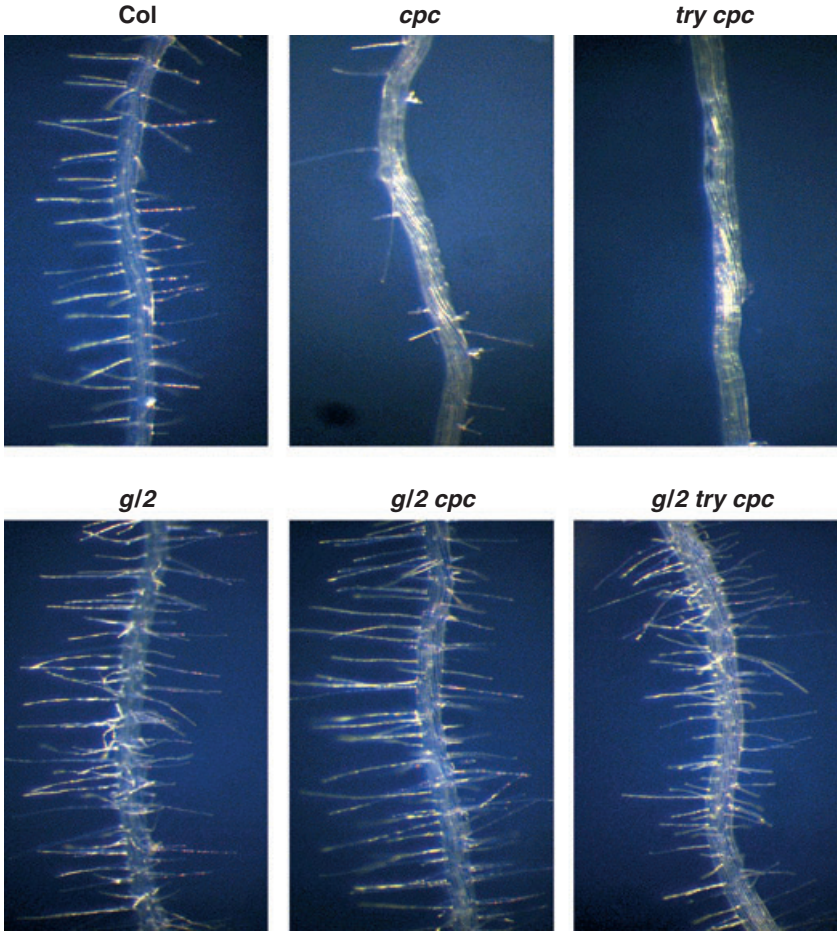

Fig. 8 Loss-of-function mutations in single *MYB* genes do not modify the root hair phenotype of the *gl2* mutant. Photographs were taken from 7-d-old *Arabidopsis thaliana* seedlings grown on vertically oriented ½ MS plates.

The role of *GL2* and single *MYB* genes in the regulation of seed coat mucilage production

Because *GL2* has also been shown to regulate the production of seed coat mucilage (Rerie *et al.*, 1994; Masucci *et al.*, 1996), we extended our analysis of the relationship between *GL2* and single *MYB* genes to mucilage production. As discussed above, a role of single *MYB* genes in trichome and root hair patterning has been well established. Their roles in mucilage production, however, have not been investigated. We found that the single *myb* single, double and triple mutants examined, including *cpc*, *try cpc*, *cpc tcl1*, *try cpc etc1* and *try cpc tcl1*, did not display apparent defects in seed coat mucilage production (Fig. 9). It has been demonstrated that the activation of the transcription of *GL2*, as well as *TTG2*, during mucilage production requires a different transcriptional activator complex, including TTG1, EGL3, TT8, MYB5 and TT2 (Gonzalez *et al.*, 2009). Therefore, it remains unknown whether such an activator complex can also activate the transcription of single *MYB* genes in the seed coat. Not surprisingly, no modification of the *gl2* mucilage phenotype by single *myb* mutations was observed (Fig. 9).

Discussion

Antagonistic role of GL2 and single MYBs in the regulation of trichome formation

Substantial evidence suggests that TTG1, GL1/WER (GL1 for trichome development and WER for root hair development), GL3/EGL3 and GL2 are positive regulators for trichome formation, but negative regulators for root hair formation (Oppenheimer *et al.*, 1991; Galway *et al.*, 1994; Rerie *et al.*, 1994; Masucci *et al.*, 1996; Walker *et al.*, 1999; Payne *et al.*, 2000; Zhang *et al.*, 2003), whereas single MYB transcription factors, including TRY, CPC, TCL1, ETC1, ETC2 and ETC3, are negative regulators for trichome formation, but positive regulators for root hair formation (Wada *et al.*, 1997, 2002; Schnittger *et al.*, 1999; Schellmann *et al.*, 2002; Esch *et al.*, 2004; Kirik *et al.*, 2004a,b; Simon *et al.*, 2007; Wang *et al.*, 2007, 2008; Tominaga *et al.*, 2008; Wester *et al.*, 2009). Current models suggest that a transcriptional activator complex formed by TTG1, GL1/WER and GL3/EGL3 activates the transcription of both *GL2* and single *MYB* genes, and that single MYBs move from trichome precursor cell to its neighboring cell in shoots, or move from an N cell to an H cell in roots, to compete with GL1 or WER for the binding of GL3, thus limiting the activity of the TTG1–GL3/EGL3–GL1/WER transcriptional activator complex (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). Consequently, the expression of *GL2* is reduced in the neighboring cells of trichome precursors in shoots (inhibiting trichome formation) and in H cells in roots (promoting root hair formation). At least five lines of evidence directly or indirectly support this view.

(1) The co-transfection of GL1/WER and GL3/EGL3 activates the transcription of *GL2* and a subset of single *MYB* genes in the Arabidopsis protoplast transient expression system (Wang *et al.*, 2007; Wang & Chen, 2008).

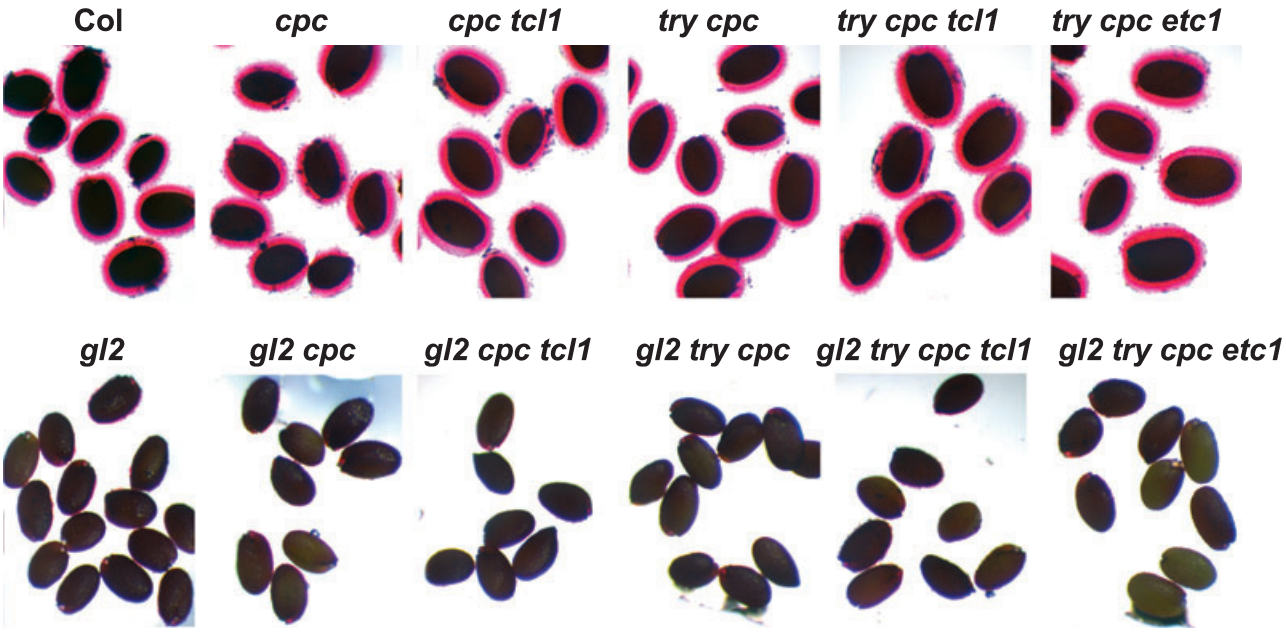

Fig. 9 Loss-of-function mutations in single *MYB* genes do not affect the production of seed coat mucilage. *Arabidopsis thaliana* seeds were stained by shaking in 0.01% (w/v) Ruthenium Red for 2 h and mounted in water before photographing.

This conclusion is further supported by the present study using stable transgenic lines overexpressing the GL1–GL3 fusion protein (Fig. 1). Consistent with these findings, *CPC* has been identified as a direct target gene for WER (Koshino-Kimura *et al.*, 2005; Ryu *et al.*, 2005), and GL1 and GL3 have been shown to be recruited to the promoter region of *CPC* and *ETC1* (Morohashi *et al.*, 2007; Zhao *et al.*, 2008). These findings are also in agreement with the recent finding by ChIP-chip analysis that *GL2* and *TRY*, *CPC*, *ETC1* and *ETC3* are direct targets of GL3/GL1 (Morohashi & Grotewold, 2009). On the other hand, loss-of-function mutations in genes encoding components of the TTG1–GL3/EGL2–GL1/WER complex reduce the expression of some single *MYB* genes. For example, the expression of *TRY*, *CPC* and *ETC1* is nearly abolished in the *ttg1* and *wer* mutant backgrounds (Simon *et al.*, 2007).

(2) Single MYB proteins, at least CPC (Kurata *et al.*, 2005; Digiuni *et al.*, 2008; Zhao *et al.*, 2008), TRY (Digiuni *et al.*, 2008) and ETC3 (Wester *et al.*, 2009), can move from cell to cell. It should be noted that the amino acids within the MYB domain that have been shown to be crucial for the cell-to-cell movement of CPC (Kurata *et al.*, 2005) are entirely conserved in all six single MYBs (Wang *et al.*, 2008).

(3) All single MYBs can physically interact with GL3 or EGL3 (Bernhardt *et al.*, 2003; Esch *et al.*, 2003; Zhang *et al.*, 2003; Kirik *et al.*, 2004a,b; Zimmermann *et al.*, 2004; Tominaga *et al.*, 2008; Wang *et al.*, 2008). Furthermore, it has been shown that single MYBs compete with GL1 in binding to GL3 in yeast cells (Esch *et al.*, 2003, 2004). Consistent with these results, all single MYBs contain the amino acid signature [D/E]Lx2[R/K]x3Lx6Lx3R, which has been shown to be required for interaction with R/B-like bHLH transcription factors (Zimmermann *et al.*, 2004).

(4) Loss-of-function mutations in single *MYB* genes, including *TRY*, *CPC* and *ETC1*, induce ectopic *GL2::GUS*-expressing cells in the H position in roots (Simon *et al.*, 2007). Further, the ectopic non-hair cell specification and excessive *GL2* expression caused by *try* and *cpc* mutations are WER dependent (Simon *et al.*, 2007). In the present study, we showed that the transcript of *GL2* was elevated in single *myb* mutants, including *cpc*, *try cpc*, *try cpc etc1* and *try cpc etc tcl1* single, double, triple and quadruple mutants (Fig. 6b).

(5) Opposite trichome and root hair phenotypes have been observed between *gl2* and single *myb* mutants (in particular, double and higher order mutants of single *MYB*). These models also suggest that single *MYB* genes act through *GL2* (e.g. via the limitation of the transcriptional activity of the TTG1–GL1/WER–GL3/EGL3 complex) to negatively regulate trichome formation and positively regulate root hair formation. Here, we provide genetic evidence that, although this is probably the case for root hair formation, single *MYB* genes may not act solely through *GL2* to execute their inhibitory role in trichome formation.

We reasoned that, if single *MYB* genes act solely through *GL2* in trichome formation, *gl2* should be epistatic to the loss-of-function mutations in single *MYB* genes. The *gl2* single mutant shows dramatically reduced trichome formation in shoots, whereas some *myb* single mutants (e.g. *cpc* and *try*), double mutants (e.g. *try cpc*) and higher order mutants (e.g. *try cpc etc1*) show increased trichome formation. This phenotypic difference between *gl2* and single *myb* mutants allowed robust epistatic analysis. By analyzing double and higher order mutants between *gl2* and single *myb* mutants, we found that the glabrous phenotype of the *gl2* mutant could be partially rescued by loss-of-function mutations in single *MYB* genes. Such a rescue was already evident in the *gl2 cpc* double mutant (Fig. 2), but a more dramatic recovery of trichome formation was observed in triple and quadruple mutants, such as *gl2 try cpc* and *gl2 try cpc etc1* (Fig. 2). Furthermore, the restoration of trichome formation in the *gl2* mutant background by single *myb* mutations was not only observed in leaves, but also in inflorescence stems and floral organs, such as sepals (Figs 3–5). Although TCL1 appears to have diverged the most among single MYBs at the protein level (Wang *et al.*, 2007; Wester *et al.*, 2009), we found that a loss-of-function mutation in *TCL1* could also induce ectopic trichome formation on the upper inflorescence stems and pedicels in the *gl2* mutant background (Figs 4 and 5). Therefore, the rescue of trichome formation in the *gl2* mutant background by single *myb* mutations appears to represent a general action of single *MYB* genes, although this has not been tested directly for other single *MYB* genes, including *ETC2* and *ETC3*. Taken together, our genetic studies suggest that single *MYB* genes may not act simply through *GL2* to regulate trichome formation, which may mean that they participate in additional mechanisms of trichome development.

Possible mechanism of the action of GL2 and single MYB transcription factors in the regulation of trichome development

Because single *MYB* genes can still execute their inhibitory roles during trichome formation, even in the absence of a functional *GL2*, the relationship between single *MYB* genes and *GL2* may not simply be linear (e.g. upstream or downstream). From a genetic perspective, an intermediate trichome phenotype (or partially suppressed phenotype) between *gl2* and single *myb* mutants may even suggest an independent role of *GL2* and single *MYB* genes in trichome development. At this point, the precise relationship between *GL2* and single *MYB* genes remains unclear and, with the lack of other evidence, we can only provide speculations. One possibility is that other genes might exist that promote trichome development. This notion is indirectly supported

by the comparison of the expression of *GL2* between organs that normally produce trichomes and organs that normally do not (Fig. 6a). It has been shown that TTG2, a WRKY transcription factor and a positive regulator of trichome development, can also be activated by the TTG1–GL3/EGL3–GL1 complex (Ishida *et al.*, 2007). Therefore, it is possible that the partial suppression of the *gl2* mutant could be a result of enhanced expression of *TTG2* and/or other non-*GL2* target genes. The partial rescue of trichome formation in the *gl2* mutant by loss-of-function mutations in single MYB genes could also be a result of unknown transcriptional regulation of single *MYB* genes by the GL2 protein. For example, it has been shown that, in the root tips, the transcript of *TRY*, but not other single *MYB* genes examined, was reduced in the *gl2* mutant (Simon *et al.*, 2007). In this case, the single MYBs could function both upstream and downstream of GL2. Finally, the partial rescue of trichome formation in the *gl2* mutant background by loss-of-function mutations in single MYB genes could be caused by an additional/alternative role for single MYBs in trichome development. In this case, in addition to inhibiting the TTG1–GL3/EGL3–GL1 complex, the single MYBs may also/instead repress the transcription of trichome-promoting genes in another fashion. Regardless of these possibilities, these results suggest a difference in the relative roles of GL2 and single MYBs in trichome vs root patterning (further discussed below).

Both the establishment of trichome cell fate and trichome morphological development and maturation are required for normal trichome development. Because trichome morphology was altered in both the *gl2* mutant (Rerie *et al.*, 1994) and the single *myb* mutants (especially in double and higher order mutants of singe *MYB* genes), and the double and higher order mutants between them (Figs 3 and 7), these results suggest that both *GL2* and single *MYB* genes regulate trichome morphology. Collectively, our genetic analysis suggests that single *MYB* genes play roles in both the establishment of trichome cell fate and in trichome morphological development, and may not act solely through GL2 via an unidentified mechanism.

The role of GL2 and single MYB transcription factors in the regulation of root hair formation

It is generally believed that root hair patterning is largely controlled by the same transcriptional complex as used for trichome patterning, except that GL1 is replaced by another R2R3 MYB-type transcription factor, WER (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). Positive regulators for trichome formation inhibit root hair formation, whereas negative regulators for trichome formation promote root hair formation. As discussed above, loss-of-function mutations in single *MYB* genes could partially restore trichome formation in the *gl2* mutant background, suggesting that single MYBs may not solely depend on GL2 in normal trichome development. During root hair patterning, we observed a different scenario. Double and higher order mutants between *gl2* and single *myb* phenocopied the root hair phenotype of the *gl2* single mutant (Fig. 8, Table 2). These results are consistent with the models proposed previously (reviewed by Larkin *et al.*, 2003; Schiefelbein, 2003; Pesch & Hülskamp, 2004; Ishida *et al.*, 2008; Schiefelbein *et al.*, 2009). Therefore, the relationship between GL2 and single MYBs appears to be different during trichome and root hair development. This probably represents an organ-specific regulation of epidermal cell patterning, which is discussed further below.

How could a common machinery (WD–bHLH–MYB three-component complex) used for trichome and root hair patterning result in such distinct relationships between GL2 and single MYB transcription factors? In both cases, the underlying mechanism requires the regulation and interaction between positive regulators and negative regulators. For example, in trichome development, the positive regulators (e.g. TTG1, GL3/EGL3 and GL1) activate the negative regulators (e.g. single MYBs), and the negative regulators, which can move between cells, inhibit the activators (e.g. GL2). As discussed above, two components of the three-component transcriptional activator complex, WD-repeat protein (TTG1) and bHLH-type transcription factor (GL3/EGL3), are required for both trichome and root hair patterning. In this three-component complex, the MYB-type transcription factor has more specialized roles in different organs. WER specifically functions in root hair patterning, whereas GL1 specifically functions in trichome patterning. Other MYB-type transcription factors, such as MYB23 (Kirik *et al.*, 2005; Kang *et al.*, 2009), function in both trichome and root hair patterning. There are also differences in spatial regulation between trichome and root hair patterning. Specifically, although trichome formation in shoots does not depend on a specific position except on other trichomes, root hair formation normally only occurs in epidermal cells overlying a cleft between two underlying cortex cells. It has been found that the position-dependent specification of root epidermal cells requires SCM, a receptor-like kinase (Kwak *et al.*, 2005). Therefore, it is possible that the difference in MYB-type transcription factors and the difference in spatial regulation between trichome and root hair patterning may have contributed to the distinct relationships between GL2 and single MYBs in different organs (e.g. shoots and hairs), although GL1 or WER, together with GL3/EGL3, activated the transcription of single *MYB* genes in a similar manner (Wang *et al.*, 2008).

In summary, we have provided genetic evidence that trichome formation in the *gl2* mutant background can be partially restored by loss-of-function mutations in single *MYB*

genes, whereas *gl2* is epistatic to single *myb* mutations during root hair patterning. Although the transcription of both *GL2* and single *MYB* genes can be activated by the same transcriptional activator complex, the genetic interaction between *GL2* and single *MYB* genes appears to be organ specific. We propose that single MYBs may not act solely through GL2 to regulate trichome cell specification. This work provides new insights into the molecular mechanism of epidermal patterning and the interactions between transcription factors.

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