A single amino acid substitution in the R3 domain of GLABRA1 leads to inhibition of trichome formation in Arabidopsis without affecting its interaction with GLABRA3

Running title: Ser92 in GL1 is critical for its function

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Abstract

GLABRA1 (GL1) is a R2R3 MYB transcription factor that regulates trichome formation in Arabidopsis by interacting with the bHLH transcription factor GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3). The conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature in the R3 domain of MYB proteins has been shown to be required for the interaction of MYBs with R/B-like bHLH transcription factors. By using genetic and molecular analyses, we show that the glabrous phenotype in the *nph4-1* mutant is caused by a single nucleotide mutation in the *GL1* gene, generating a Ser to Phe substitution (S92F) in the conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature of GL1. Activation of the integrated *GL2p:GUS* reporter gene in protoplasts by cotransfection of GL1 and GL3 or EGL3 was abolished by this GL1S92F substitution. However, GL1S92F interacted successfully with GL3 or EGL3 in protoplast transfection assays. Unlike VPGL1GL3, the fusion protein VPGL1-S92FGL3 failed to activate the integrated *GL2p:GUS* reporter gene in transfected protoplasts. These results suggested that the S92 in the conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature of GL1 is not essential for the interaction of GL1 and GL3, but may play a role in the binding of GL1 to the promoters of its target genes.

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Key Words: GLABRA1, GLABRA3, trichome formation, transcription factor, protein-protein interaction, amino acid substitution, Arabidopsis

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INTRODUCTION

Trichomes are hair cells that distributed on the surface of the aerial parts of most land plants, including leaves, stems, and floral organs. Trichomes can act as barriers to protect plants from stresses, UV light irradiation and excessive transpiration (Mauricio and Rausher, 1997; Eisner *et al.*, 1998; Werker, 2000). The MYB transcription factor family is one of the largest transcription factor families in plants (Stracke *et al*., 2001; Dubos *et al*., 2010; Katiyar *et al*., 2012). In Arabidopsis, for example, there are nearly 200 genes encoding MYB transcription factors (Dubos *et al*., 2010). Based on the variation in the number of N-terminal DNA-binding domain repeats (R), the MYB transcription factor family has been divided into four subfamilies, namely 4R-MYB, 3R-MYB, R2R3 MYB, and 1R-MYB subfamilies which contain 4, 3, 2 and 1 DNA-binding repeats, respectively (Dubos *et al*., 2010). GLABRA1 (GL1) is an R2R3 MYB transcription factor that positively regulates trichome formation in Arabidopsis (Oppenheimer *et al*., 1991). Single-repeat R3 MYB transcription factors from the 1R-MYB subfamily, including TRIPTYCHON (TRY) (Schnittger *et al*.,1999; Schellmann *et al*., 2002), CAPRICE (CPC)

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(Wada *et al*., 1997, 2002), ENHANCER OF TRY AND CPC1, 2 and 3 (ETC1, ETC2 and ETC3/CPL3) (Esch *et al*., 2004; Kirik *et al*., 2004a, 2004b; Tominaga *et al*., 2008; Wester *et al*., 2009), TRICHOMELESS1 (TCL1) and TCL2 (Wang *et al*., 2007; Gan *et al*., 2011), are also involved in the regulation of trichome formation in Arabidopsis.

Available evidence suggest that MYB transcription factors control Arabidopsis trichome formation through interactions with several other types of transcription factors, including the basic helix-loop-helix (bHLH) transcription factors GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3) (Payne *et al*., 2000; Zhang *et al*., 2003), the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Galway *et al*., 1994; Walker *et al*., 1999), and the homeodomain protein GLABRA2 (GL2) (Rerie *et al*., 1994; Masucci *et al*. 1996). It was proposed that GL1, GL3 or EGL3 and TTG1 form a GL1-GL3/EGL3-TTG1 activator complex to trigger the expression of *GL2*, thus leading to the promotion of trichome formation (Rerie *et al*., 1994; Schiefelbein, 2003; Pesh and Hülskamp, 2004, 2009; Serna and Martin, 2006, Ishida *et al*., 2008). Interestingly, the same activator complex also induces the expression of some single-repeat R3 MYB genes. Single-repeat R3 MYBs, in turn, move from a trichome precursor cell to its neighboring cells to block the formation of the activator complex by competing with GL1 binding to GL3 or EGL3, thus limiting the formation of the GL1-GL3/EGL3-TTG1 activator complex and inhibiting trichome formation (Hülskamp *et al*., 1994; Schellmann *et al*.,

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2002; Esch *et al*., 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Ishida *et al*., 2008; Wang and Chen, 2014).

Interactions between GL1, GL3 and TTG1 have been demonstrated in both yeast and plant cells (Payne *et al*., 2000; Zimmermann *et al*., 2004; Wang and Chen, 2008; Zhao *et al*. 2008). All the Arabidopsis R3 MYBs have also been shown to interact with GL3 and/or EGL3 in yeast and plant cells (Wang *et al*., 2008; Wester *et al*., 2009; Gan *et al*., 2011). Furthermore, assays in protoplasts showed that interaction of GL1 and GL3 is required for activation of *GL2* and some of the R3 MYB genes including *TRY, CPC, ETC1* and *ETC3* (Wang *et al*., 2008; Wang and Chen, 2008).

Studies in yeast have identified the sequence [D/E]L×2 [R/K]×3L×6L×3R as a conserved amino acid signature required for the interaction of MYB transcription factors with R/B-like bHLH transcription factors (Zimmermann *et al*., 2004). This [D/E]L×2 [R/K]×3L×6L×3R amino acid signature is conserved in GL1 and all the Arabidopsis R3 MYBs (Zimmermann *et al*., 2004; Wang and Chen, 2014). Here we report our analysis of unusual *gl1* mutant allele, which possesses s mutation affecting the Ser92 residue in the conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature of GL1. We show that this residue is critical for GL1 function in regulating trichome formation in Arabidopsis, but is not required for the interaction of GL1 with GL3 or EGL3.

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MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* (Arabidopsis) ecotype Columbia-0 (Col-0) was used for plant transformation and protoplast isolation. The *nph4-1* (Happer *et al*., 2003), *gl1* (Wang *et al*., 2004), *gl2-3* (Wang *et al*., 2010), *try_2970* (Esch *et al*., 2003), and *etc1-1* (Kirik *et al*., 2004a) single

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mutants, and the *35S:HA-TCL1* transgenic plants (Wang *et al*., 2007), are in the Col-0 background. The *ttg1-1* single mutant and *gl3 egl3* double mutant are in the Landsberg *erecta* (L*er*) ecotypic background (Walker *et al*., 1999; Payne *et al*., 2000; Zhang *et al*., 2003). The *cpc-1* mutant and *GL2p:GUS* transgenic plants are in the Wassilewskija (Ws) ecotypic background (Masucci *et al*., 1996; Wada *et al*., 1997).

F1 seeds between *nph4-1*/*arf7* and glabrous mutants were obtained by crossing *nph4-1*/*arf7* with *ttg1-1*, *gl1*, *gl2-3*, and *gl3 egl3* mutants, respectively. The *gl1-S92F* mutant was isolated by crossing *nph4-1/arf7* and Col, examining the F2 progeny for the glabrous phenotype and confirming the absence of *ARF7* mutation by genotyping in F2 and subsequent generations. The *try cpc* double and *try cpc etc1* triple mutants have been reported previously (Wang *et al*., 2008). The *gl1 try*, *gl1-S92F try*, *gl1 cpc* and *gl1-S92F cpc* double mutants were generated by crossing single mutants *gl1* or *gl1-S92F* with *try_2970* and *cpc-1*, respectively. The *gl1 try cpc* and *gl1-S92F try cpc* triple mutants were generated by crossing double mutant *gl1 try* or *gl1-S92F try* with *try cpc*. The *gl1 try cpc etc1* and *gl1-S92F try cpc etc1* quadruple mutants were generated by crossing triple mutants *gl1 try cpc* or *gl1-S92F try cpc* and *try cpc etc1*.

Seedlings used for DNA and RNA isolation were obtained by growing surface-sterilized seeds on 0.6% (W/V) phytoagar (Plantmedia)-solidified 1/2 Murashige & Skoog (MS) basal medium with vitamins (plantmedia) and 1% (w/v) sucrose. Plants used for phenotypic analysis,

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plant transformation and protoplast isolation were obtained by directly sowing seeds into soil in pots. Plants were grown at 22℃ with 16/8 hour photoperiod at approximately 120μmol $m^{-2}s^{-1}$.

Identification of the mutation in the *gl1-S92F* mutant

Genomic DNA was isolated from 10-day-old *gl1-S92F* mutant seedlings using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. PCR was used to amplify the full-length genomic sequence of *GL1*, and the PCR products were fully sequenced using *GL1* gene specific primers. The sequences obtained were subjected to alignment analysis with *GL1* genomic sequence (http://blast.ncbi.nlm.nih.gov).

RNA isolation and RT-PCR

Total RNA was isolated from 10-day-old Arabidopsis seedlings using EasyPure™ Plant RNA Kit (Transgene Biotech) by following the manufacturer's instructions. A total of 1μg of total RNA was subjected to cDNA synthesis by Oligo(dT)-primed reverse transcription using EazyScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech) following the manufacturer's instructions. *GL1* specific primers (Wang *et al*., 2007) were used to examine the expression of *VPGL1GL3* and *VPGL1-S92FGL3* in the corresponding transgenic plants. Primers used for amplification of *TRY*, *CPC* and *TCL1* as described previously (Wang *et al*., 2008), were

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used to asses expression of *mTRY*, *mCPC* and *mTCL1*, respectively, in the corresponding transgenic plants. The primers used for examining the expression of *GL2* and *ACT2* have been described previously (Wang and Chen, 2008).

Constructs

The effector constructs *35S:GD*, *35S:GD-TRY*, *35S:GD-CPC*, *35S:GD-TCL1*, *35S:HA-GL1*, *35S:HA-GL3*, *35S:HA-EGL3*, and *35S:VPGL1GL3*, and the reporter construct *Gal4:GUS* have been described previously (Wang *et al*., 2007; 2008; Wang and Chen, 2008).

To generate HA or GD tagged constructs for *GL1-S92F*, the full-length open-reading frame (ORF) of *GL1-S92F* was amplified by RT-PCR using RNA isolated from *gl1-S92F* mutant seedlings, and was cloned in-frame with an N-terminal HA or GD tag into the *pUC19* vector under the control of the double *35S* promoter. The *35S:VPGL1-S92FGL3* construct was generated by replacing *GL1* in the *35S:VPGL1GL3* construct with *GL1-S92F*.

Plasmids of *35S:GD-WER*, *35S:GD-TRY*, *35S:GD-CPC*, and *35S:GD-TCL1* were used as templates to generate the *35S:GD-WER-S94F*, *35S:GD-mTRY*, *35S:GD-mCPC*, and *35S:GD-mTCL1* constructs, respectively, by PCR based mutagenesis using the Fast Mutagenesis System (TransGen Biotech) following the manufacturer's instructions. The primers used to generate *35S:GD-WER-S94F* are 5'-CTTGGTAATAGGTGGTTTTTAATTGCTAAAAG-3' and

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5’-AACCACCTATTACCAAGCAACTTGTGGAG-3’, to generate *35S:GD-mTRY* are 5’-GTCGGTGATAGGTGGTCTTTGATAGCAGGAAG-3’ and 5’-GACCACCTATCACCGACAAGTCTGTACATTC-3’, to generate *35S:GD-mCPC* are 5’-CGTTGGCGACAGGTGGTCGTTGATCGCCGGAAGG-3’ and 5’-GACCACCTGTCGCCAACGAGTTTATACATCC-3’, and to generate *35S:GD-mTCL1* are 5’-TGTTGGCGACAGGTGGTCTTTAATAGCAAGAAG-3’ and 5’-GACCACCTGTCGCCAACAAGTCTGTACATTC-3’.

To generated constructs for plant transformation, corresponding constructs in *pUG19* vector were digested with the proper enzyme, and subcloned into the binary vector *pPZP211* (Hajdukiewicz *et al*., 1994).

Plant transformation and transgenic plant selection

Col wild-type plants of ~5-week-old with several mature flowers on the main inflorescence were transformed with various constructs via *Agrobacterium tumefaciens* GV3101 by using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by growing surface-sterilized T1 seeds on 1/2MS plates containing 50μg/ml kanamycin and 100ug/ml carbenicillin, and about 10-day-old transgenic seedlings were transferred into soil pots. Phenotypes of the transgenic plants were analyzed in the T1 generation, and confirmed in the

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following 2-3 generations. Overexpression of corresponding genes in the transgenic plants was confirmed by RT-PCR. For each construct, more than 60 independent transgenic lines were generated, and at least 10 independent overexpressor lines with similar phenotypes were obtained. Results from representative lines were presented.

Plasmid DNA isolation, protoplast isolation and transfection, and GUS activity assay

Effector and reporter plasmids used for protoplast transfection were isolated using GoldHi EndoFree Plasmid Maxi Kit (Kangwei) following the manufacturer's instructions. The procedures for protoplast isolation and transfection have been described previously (Tiwari *et al.*, 2003; Wang *et al.*, 2005, 2007; Wang and Chen, 2008). Briefly, protoplasts were isolated from rosette leaves of 3-4-week-old Col wild type or *GL2p:GUS* transgenic plants, effector plasmids were transfected into protoplasts isolated from *GL2p:GUS* transgenic plants, whereas effector and reporter plasmids were co-transfected into protoplasts isolated from Col wild type plants. The transfected protoplasts were incubated at room temperature in darkness for 20–22h, and GUS activities were then measured using a Synergy^{TM} HT microplate reader (BioTEK).

Microscopy

Trichomes from the 7th rosette leaf and the cauline leaves of Arabidopsis seedlings were

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analyzed and photographed under a Motic K microscope equipped with a Canon EOS 1100D digital camera. Trichomes from mature plants (including whole rosettes and inflorescences) were directly photographed using a Canon EOS 1100D digital camera.

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RESULTS

The glabrous phenotype in *nph4-1* mutant is caused by a single amino acid substitution in GL1

The *nph4-1* (*nonphototropic hypocotyl 4-1*), a null mutant for *ARF7* (*AUXIN RESPONSE FACTOR 7*) (Harper *et al*., 2000; Wang *et al*., 2005), was originally identified from a fast-neutron-mutagenized population by its reduced phototropic response (Lisum and Briggs, 1995). Because the mutagenized seeds were in the Col ecotype background carrying the homozygous recessive *glabrous1* mutation (Koornneef *et al*., 1982; Lisum and Briggs, 1995), the

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nph4-1 mutant has a glabrous phenotype (Fig. 1a). Consistent with the observation that glabrous phenotypes in *glabrous1* was caused by lost of function of R2R3 MYB gene *GL1* (Oppenheimer *et al*., 1991), F1 plants generated by crossing *nhp4-1* and a T-DNA insertion mutant *gl1* (Wang *et al*., 2004) showed glabrous phenotypes, whereas trichome formation in F1 plants generated by crossing *nhp4-1* and other recessive glabrous mutants including *ttg1*, *gl3 egl3* and *gl2* was largely unaffected (Fig. 1a).

However, sequencing results showed that, unlike the *glabrous1* null allele in which the entire locus of *GL1* has been deleted (Oppenheimer *et al*., 1991), the *GL1* gene in *nph4-1* has a cytosine (C) to thymine (T) mutation in its third exon, which resulted in a substitution of the Ser92 residue a Phe residue (S92F) (Fig. 1b). This alteration is identical to the amino acid substitution found in the *gl1-S92F* mutant (Yoshida *et al*., 2009).

As shown in Fig. 1c, the S92F substitution immediately precedes the last Leu (L) in the sequence [D/E]L×2 [R/K]×3L×6L×3R, a conserved amino acid signature that is required for the interaction of MYB transcription factors with R/B-like bHLH transcription factors (Zimmermann *et al*., 2004). Amino acid sequence alignment showed that the Ser residue is conserved in GL1, MYB23, a R2R3 MYB that control trichome branching and trichome formation at leaf margins (Kirik *et al*., 2005), and WEREWOLF (WER), a functionally equivalent protein of GL1 (Lee and Schiefelbein, 2001). In the R3 MYB transcription factors, however, it is replaced by an Asp (D)

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or Glu (E) residue (Fig. 1c).

GL1-S92F can not activate the expression of *GL2*

The observation that F1 plants generated by crossing *nhp4-1* and *gl1* showed glabrous phenotypes indicates that the S92F substitution leads to a loss-of-function mutation of *GL1*. To further examine this, we obtained the *gl1-S92F* mutant by back-crossing the *nph4-1* mutant to Col plants and comparing trichome phenotypes by growing it with the *gl1* mutant side by side. As shown in Fig. 2, both *gl1* and *gl1-S92F* mutants did not produce any trichomes at the seedling stage (Fig. 2a); however, trichome formation was observed at the margins of late development rosette leaves (Fig.2b), and cauline leaves (Fig. 2c). Quantitative analysis shows that *gl1* and *gl1-S92F* mutants produced similar number of trichomes on late developed rosette leaves (Fig. 2b) and on cauline leaves (Fig. 2c).

RT-PCR analysis showed that the expression of *GL2*, a target of GL1, was reduced in the *gl1-S92F* mutant seedlings to a degree that is similar to that in the *gl1* mutant seedlings (Fig. 3a), indicating that GL1-S92F failed to induce the expression of *GL2*. Our previous experiments in protoplasts showed that co-transfection of GL1 and GL3 activated the expression of *GL2* (Wang and Chen, 2008); thus, we decided to further examine whether GL1-S92 can activate *GL2* expression by using protoplast transfection assays. As shown in Fig. 3b, co-transfection of GL1

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and GL3 or EGL3 is able to activate the expression of the integrated reporter gene *GL2p:GUS*, but co-transfection of GL1-S92F and GL3 or EGL3 failed to do so.

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GL1-S92F interacts with GL3 and EGL3

Considering the results above we suspected that GL1-S92F may not be able to interact with GL3 or EGL3. To test if that is the case, a protoplast transient expression system that has been successfully used to test the interaction between GL1 and GL3 (Wang and Chen, 2008), was used to examine the interaction of GL1-S92F and GL3 or EGL3. Plasmids of *Gal4-GUS* reporter, together with the effectors *GL3* or *EGL3* and a Gal4 DNA binding domain (GD) fused to GL1-S92F (*GD-GL1-S92F*) (Fig. 4a), were co-transfected into protoplasts isolated from Col wild-type leaves. As shown in Fig. 4b, GD-GL1-S92F activated the expression of the reporter gene in the presence of GL3 or EGL3, indicating that GL1-S92F interacts with GL3 or EGL3 in plant cells. Similar transfection assays showed that WER with a similar Ser to Phe amino acid residue substitution (WER-S94F) interacted with GL3 and EGL3 (Fig. 4c), indicating that the Ser amino acid residue conserved in GL1 and WER in the conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature may not be required for the interaction of R2R3 MYB proteins with bHLH proteins.

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Asp/Glu to Ser amino acid substitution does not affect interaction of R3 MYB and GL3

Consistent with the proposal that R3 MYB transcription factors regulate trichome formation by competing with GL1 for binding GL3 (Hülskamp *et al*., 1994; Wada *et al.,* 1997; Schellmann *et al*., 2002; Esch *et al*., 2003), both GL1 and R3 MYBs interacted with GL3 in plant cells as examined by protoplasts transfection (Wang *et al*., 2007, 2008; Gan *et al*, 2011). Protoplast transfection assays described above indicate that the Ser92 amino acid in the conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature of GL1 is not required for the interaction of GL1 and GL3 or EGL3 (Fig. 4). Considering that the Ser amino acid is conserved in R2R3 MYB transcription factor GL1 and WER, but in R3 MYBs, where it is replaced by an Asp or Glu residue, we further examined if Ser may affect interaction of MYBs and bHLH transcription factors by taking a reverse mutation approach, i.e. generating R3 MYB constructs with the Asp or Glu to a Ser substitution, and examining if the substitution may affect interaction of R3 MYBs with GL3.

Constructs of *TRY*, *CPC* and *TCL1* with an Asp/Glu to Ser substitution (*mTRY*, *mCPC* and *mTCL1*) were generated by PCR based mutagenesis, and protoplast transfection assays was used to examine interaction of mutated R3 MYBs with GL3. As shown in Fig. 5a, mTRY, mCPC and mTCL1 interacted with GL3, and their affinity to GL3 is largely unaffected as judged by the GUS activities.

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VPGL1-S92FGL3 fusion proteins failed to activate the expression of *GL2*

By using a GL1GL3 fusion protein, we previous showed that the DNA binding domains in both GL1 and GL3 are required for activation of *GL2*, suggesting that concurrent binding of GL1 and GL3 via their DNA binding domains to the *GL2* promoter may be required for the activation of *GL2* (Wang and Chen, 2008). The fact that co-transfection of GL1-S92F and GL3 or EGL3 failed to activate the expression of *GL2p:GUS* reporter gene (Fig. 3b), yet GL1-S92F interacted with GL3 and EGL3 (Fig. 4b), indicates that the S92F amino acid substitution in GL1 may affect the binding of GL1 to the promoter of *GL2*.

To examine this, we generated a *VPGL1-S92FGL3* construct by replacing *GL1* in the *VPGL1GL3* construct with *GL1-S92F*, and tested its ability to activate *GL2* expression by using protoplast transfection assays. The *VPGL1-S92FGL3*, rather than *GL1-S92FGL3* construct, was generated because the VPGL1GL3 fusion protein has been shown to more efficiently activate the expression of the *GL2p:GUS* reporter gene (Wang and Chen, 2008). When transfected into protoplasts isolated from *GL2p:GUS* transgenic plants, VPGL1S92FGL3 failed to activate the expression of the *GL2* reporter gene, but as a control, VPGL1GL3 (control) was able to activate the reporter gene (Fig. 6a).

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Loss-of-function R3 MYB genes partially restored trichome phenotypes in *gl1-S92F* and *gl1* mutants

In Arabidopsis, R3 MYB transcription factors inhibit trichome formation by competing with GL1 for binding GL3, thus inhibiting the formation of the GL1-GL3/EGL3-TTG1 activator complex (Hülskamp *et al*., 1994; Wada *et al*., 1997; Schellmann *et al*., 2002; Esch *et al*., 2003; Wang *et al*., 2007; Gan *et al*, 2011). Our finding that GL1-S92F interactes with GL3 and EGL3 in transfected protoplasts (Figure 4) and that VPGL1-S92FGL3 fails to activate the expression of the *GL2p:GUS* reporter gene (Figure 6a) implies that GL1-S92F may act in a mianner similar to the R3 MYB proteins.

To test this, we generated double and higher order mutants between *gl1-S92F* and R3 MYB gene mutants, and examined trichome formation in the mutants generated. We discovered that the *gl1-S92F try* and *gl1-S92F cpc* double mutants produce more trichomes than do the *gl1-S92F* single mutants, and trichome clusters were observed in the *gl1-S92F try* double mutants (Fig. 7). More trichomes were produced in the *gl1-S92F try cpc* triple mutant when compared with that in the *gl1-S92F try* and *gl1-S92F cpc* double mutants, and the *gl1-S92F try cpc etc1* quadruple mutant as compared with the *gl1-S92F try cpc* triple mutant (Fig. 7). Moreover, the size of the trichome clusters was also increased in the triple and quadruple mutants when compared with those in the *gl1-S92F try* double mutant (Fig. 7). We also note that, similar to that in *gl1-S92F*

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single mutant, the increased trichomes in the double and higher order mutants between *gl1-S92F* and R3 MYB gene mutants were mainly distributed on the edges of the leaves (Fig. 7).

The double and higher order mutants between *gl1* and the R3 MYB gene mutants were largely indistinguishable from the corresponding double and higher order mutants between *gl1-S92F* and R3 MYB gene mutants (Fig. 7). In addition, RT-PCR results show that expression of *GL2* in both the *gl1-S92F try cpc etc1* and *gl1 try cpc etc1* was restored to a nearly wild type level (Fig. 3a). These results suggest that the R3 MYB genes can still function redundantly to regulate trichome formation in either the *gl1-S92F* or *gl1* mutant background.

Overexpression of *VPGL1GL3* and *VPGL1-S92FGL3* affects trichome formation in Arabidopsis

Our previous results showed that overexpression of *GL1GL3* in Arabidopsis have similar effects as overexpression of both *GL1* and *GL3* or its homologous *R* gene from maize in activating the *GL2p:GUS* reporter gene and ectopic trichome formation (Larkin *et al*., 1994, Szymanski *et al*., 1998, Payne *et al*., 2000, Wang and Chen, 2008). Also, the VPGL1GL3 fusion protein was more efficient than GL1GL3 in activating the *GL2p:GUS* reporter gene (Wang and Chen, 2008). However, the VPGL1-S92FGL3 failed to activate the *GL2p:GUS* reporter gene in transfected protoplasts (Fig. 6a). To further analyze the effects of the S92F amino acid

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substitution on the function of GL1 in trichome formation, we generated transgenic plants overexpressing *VPGL1-S92FGL3* and *VPGL1GL3*, and compared trichome formation in the transgenic plants. We found that overexpression of either *VPGL1-S92FGL3* or *VPGL1GL3* affected trichome formation, but in different ways. Overexpression of *VPGL1GL3* in Arabidopsis resulted in reduced trichome formation on rosette leaves (Fig. 6b), increased trichome formation on flower organs, and ectopic trichome formation on pedicels and siligues (Fig. 6c). On the other hand, overexpression of *VPGL1-S92FGL3* in Arabidopsis resulted in reduced trichome formation in all parts of the plants including rosette leaves, stems, and flower organs (Fig. 6b. 6c).

Overexpression of mutated TCL1 affects trichome formation in Arabidopsis

We next examined whether the amino acid substitution in the R3 MYBs may affect their ability to regulate trichome formation by generating transgenic plants overexpressing the mutated R3 MYB genes. As shown in Fig. 5b, overexpression of *mTRY* or *mCPC* resulted in glabrous phenotypes, similar to the phenotypes observed in *TRY* or *CPC* overexpression plants (Wada *et al*., 1997; Schellmann *et al*., 2002). Overexpression of *mTCL1*, on other hand, inhibited trichome formation (Fig. 5b, c), but none of the transgenic plants obtained showed glabrous phenotypes. RT-PCR results showed that the phenotypes observed in the *mTCL1* transgenic plants were not due to relatively lower expression of the *mTCL1* gene (Fig. 5d). The RT-PCR

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results also showed that expression of *GL1* was reduced in the *mTCL1* transgenic plants, but to a lesser degree than in the *TCL1* transgenic plants (Fig. 5d), suggesting that the Glu to Ser amino acid substitution in TCL1 may affect its function in suppressing *GL1* expression.

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DISCUSSION

Some conserved amino acid residues in the R2 and R3 repeats of MYB transcription factors,

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including the KR residues in the R3 repeat, have been shown to be required for the binding of MYBs to the *CNGTT* core sequence of MYB binding sites (Ogata *et al*., 1994; Solano *et al*., 1997). The conserved [D/E]L×2 [R/K]×3L×6L×3R amino acid signature in the R3 domain of MYB proteins has been shown to be required for their interaction with bHLH transcription factors (Zimmermann *et al*., 2004). We provide evidence here that the Ser92 residue in the [D/E]L×2 [R/K]×3L×6L×3R signature of GL1 is not required for interaction of GL1 with GL3 or EGL3, but it may be required for the binding of GL1 to its target promoters. Our data also suggest that GL1 may use different mechanisms to regulate trichome formation in Arabidopsis, providing new insights into the molecular mechanism of the regulation of trichome formation in Arabidopsis.

The Ser92 in GL1 is not required for interaction of GL1 and GL3, but may be required for binding of GL1 to the promoter of its target genes

GL1 is an R2R3 MYB transcription factor that regulates trichome formation in Arabidopsis (Oppenheimer *et al*., 1991). Transfection assays in protoplasts have shown that an activator complex formed by GL1 and GL3 is required and sufficient to activate the expression of *GL2* and some of the R3 MYB genes (Wang *et al*., 2008; Wang and Chen, 2008). An amino acid substitution in AtMYC1, a GL3 homologue, abolished its interaction with MYBs and led to

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trichome and root hair patterning defects in Arabidopsis (Zhao *et al*., 2012). These results suggest that the interaction of GL1 and GL3 is required for the activation of downstream target genes and for the regulation of trichome formation.

The *gl1-S92F* mutant is morphological similar to the *gl1* mutant (Fig. 2), and double and higher order mutants of *gl1-S92F* and R3 MYB gene mutants are also indistinguishable from the corresponding mutants of *gl1* and R3 MYB gene mutants (Fig. 7). These findings suggest that *gl1-S92F* is a loss-of-function mutant of *GL1*. Because the S92F mutation in GL1 occurred in the conserved [D/E]L×2[R/K]×3L×6L×3R amino acid signature that is required for the interaction of MYBs and bHLH transcription factor (Zimmermann *et al*., 2004), we initially thought that the Ser92 may be required for interaction of GL1 with GL3. However, several lines of evidence support that the Ser92 residue is required for the binding of GL1 to the promoter of its target genes. First, GL1 with a S92F amino acid substitution (GL1-S92F) interacted with GL3 and EGL3 in protoplasts (Fig. 4). Second, substitution of Asp/Glu for Ser amino acid had no effects on the interaction of R3 MYBs and GL3 (Fig. 5a). Third, GL1-S92F failed to activate the *GL2p:GUS* reporter gene when cotransfected with GL3 or EGL3 (Fig. 3). Fourth, the VPGL1-S92FGL3 fusion failed to activate the *GL2p:GUS* reporter gene in transfected protoplasts (Fig. 6a). Finally, relatively higher expression of *GL1* was observed in transgenic plants overexpressing *mTCL1* when compared with that in TCL1 overexpression plants (Fig. 5d).

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Because *GL1* has been identified as a direct target of TCL1 (Wang *et al*., 2007), this result suggests that the amino acid before the last Leu in the [D/E]L×2[R/K]×3L×6L×3R signature may affect the binding of TCL1 to the promoter of *GL1*. Considering that the Ser92 residue is within the [D/E]L×2[R/K]×3L×6L×3R amino acid signature required for interaction of MYBs and bHLH transcription factors, The S92F substitution in GL1 may lead to a conformational change that does not affect GL1-GL3 interaction, but affects the binding of GL1 to the promoter of *GL2* (Fig. 8). In any case, it will be of great interest to determine how amino acids in the R3 domain may coordinate the interaction of GL1 with GL3 or EGL3 as well as the binding of GL1-GL3/EGL3 complex to promoters of their target genes.

GL1 may use different mechanisms to regulate trichome formation in Arabidopsis

Other types of transcription factors such as C2H2 zinc finger transcription factors and SQUAMOSA PROMOTER BINDING PROTEIN LIKE proteins have also been reported to regulate trichome formation. In most cases, they regulate trichome formation via their direct or indirect effects on the expression of *GL1*, *GL3* or R3 MYB genes (Gan *et al*., 2006, 2007; Yu *et al*., 2010; Zhou *et al*., 2011; Sun *et al*., 2015). Thus GL1, GL3 and EGL3, TTG1, GL2 and R3 MYB transcription factors can be consider as core regulators of trichome formation in Arabidopsis. However, the mechanisms used by these core regulators are still not entirely clear,

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and more and more evidence suggests that they may use different mechanisms to regulate trichome formation. For example, in addition to competing with GL1 for binding GL3, TCLl can also directly suppress the expression of *GL1* (Wang *et al*., 2007). Also, TTG1 is able to compete with GL1 for binding to GL3, thus differentially regulating the expression of R3 MYB gene *TRY* and *CPC* (Pesch *et al*., 2015). In addition, GL2 can activate a positive feedback loop via MYB23 (Khosla *et al*., 2014). On the other hand, expression of *GL1* and a *GL3* homologous maize *R* gene in *ttg1* mutant activated *GL2* expression (Szymanski *et al*. 1998), and overexpression of *GL1* and *GL3* restored trichome formation in *ttg1* (Payne *et al*., 2000), suggesting the TTG1 may not function upstream of GL1 and GL3 to regulate trichome formation. Also, R3 MYBs function redundantly to regulate trichome formation in the *gl2* mutant background (Wang *et al*., 2010), suggesting that GL2 may not be required for trichome formation. In support of this, GL2 has been shown to regulate other processes including seed oil production and anthocyanin biosynthesis in Arabidopsis (Shen *et al*., 2006; Shi *et al*., 2012; Wang *et al*., 2015).

Our data showed that similar to *gl1*, *gl1-S92F* is a loss-of-function mutant (Fig. 2). However, the molecular situations in these two mutants are different. In the *gl1* mutant, transcription and translation of *GL1* was blocked due the T-DNA insertion in *GL1* gene (Wang *et al*., 2004), resulting in the inhibition of the GL1-GL3/EGL3-TTG1 activator complex. In the *gl1-S92F* mutant, formation of the GL1-GL3/EGL3-TTG1 activator complex was unaffected, because the

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interaction of GL1 and GL3 or EGL3 was not affected by the S92F substitution (Fig. 4), but the complex failed to activate its target gene (Fig. 3). Considering that R3 MYBs interact with GL3 and EGL3 in plant cells (Wang *et al*., 2007, 2008; Gan *et al*., 2011), we assume that GL1-S92F may function as a R3 MYB, and therefore the knock out of all R3 MYB genes in *gl1-S92F* may result in recovery of trichome formation. This is indeed the case (Fig. 7), however, the knockout out of R3 MYB genes in the *gl1* mutant also partially recovered the mutant phenotypes (Fig. 7). Because the trichomes in the double and higher order mutant were mainly observed on the edges of the leaves (Fig. 7), and another R2R3 MYB transcription factor, MYB23 has been shown to control trichome formation of leaf margins (Kirik *et al*., 2005), it is likely that the knockout of R3 MYB genes facilitated the formation of MYB23-GL3/EGL3-TTG1 complex, thus leading to the trichome formation on leaf margins. These results suggest that even on the same leaf, trichome formation is likely to be differently regulated. However, it is difficult to explain why double or higher order mutants of *gl1* and *gl1-S92F* mutants have nearly identical phenotypes.

The observation that transgenic plants overexpressing *VPGL1GL3* inhibited trichome formation on rosette leaves, but promoted trichome formation on flower organs, pedicels and siligues (Fig. 6), provides another piece of evidence that supports differential regulation of trichome formation. On the other hand, overexpression of *VPGL1-S92FGL3* inhibited trichome formation in all parts of the plants (Fig. 6). It is likely that VPGL1-S92FGL3 is able to bind to

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the promoter of *GL2* via the DNA binding domain of GL3, leading to the block of the concurrent binding of GL1 and GL3 to the promoter of *GL2*, thus inhibiting the expression of *GL2*, and resulting in the inhibition of trichome formation (Fig. 8). Considering that the conserved KR residues required for the binding of MYBs to *CNGTT* core sequence of MYB binding sites (Ogata *et al*., 1994; Solano *et al*., 1997) are not affected in GL1-S92F, we could not rule out the possibility that GL1-S92F may still be able to activate other possible target genes. Thus, it is possible that GL1 may also use different mechanisms to regulate trichome formation in Arabidopsis.

In summary, we identified the Ser92 residue in GL1 as a critical amino for GL1's function in regulating trichome formation. We showed that this Ser92 residue is not required for the interaction of GL1 with GL3 or EGL3, but may be required for binding of GL1 to its target genes, and that GL1 may use different mechanisms to regulate trichome formation in Arabidopsis.

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FIGURE LEDGENDS

Fig. 1. The glabrous phenotype in the *nph4-1* mutant is caused by a single amino acid substitution in GL1.

(**a**) Phenotypes of Col wild type, *nph4-1* mutant and F1 plants generated by crossing *nph4-1* with *ttg1*, *gl3egl3*, *gl1* or *gl2* mutants, respectively. Photographs were taken from 2-week-old soil-grown plants. (**b**) Sequence alignment of *GL1* in Col and *nph4-1* mutant indicating the single-base substitution (red) that results in a single amino acid substitution (underline). (**c**) The Ser92 amino acid in GL1 is conserved in GL1, MYB23 and WER, but not single-repeat R3 MYB proteins. Identical amino acids are shaded in black, and similar amino acids in grey. Arrowheads indicate conserved amino acids in the [D/E]L×2[R/K]×3L×6L×3R signature

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required for interacting with R/B-like bHLH transcription factors, asterisks indicate the Ser92 amino acid in GL1 that was substituted in the *nph4-1* mutant, and circles indicate the KN residues in the R3 MYB domain that is required for binding of R2R3 MYB DNA binding domain to the consensus sequence *CNGTT* of Myb.Ph3 binging sites type I.

Fig. 2. The *gl1-S92F* mutant is largely indistinguishable from the *gl1* mutant.

(**a**) Phenotypes of Col wild type, *gl1* and *gl1-S92F* mutant seedlings. Photographs were taken from 10-day-old soil-grown plants. (**b**) Trichome formation on the 7th rosette leaf of Col, *gl1* and *gl1-S92F* mutants. Photographs were taken from 5-week-old soil-grown plants. Data represent mean ± SD of 12-18 plants. (**c**) Trichome formation on cauline leaves of Col, *gl1* and *gl1-S92F* mutants. Photographs were taken from 5-week-old soil-grown plants. Cauline leaves were numbered according to the order from bottom to the top on the inflorescences. Data represent mean ± SD of 10-17 plants.

Fig. 3. GL1-S92F can not activate the expression of *GL2*.

(**a**) Expression of *GL2* in Col wild type, *gl1*, *gl1-S92F*, *gl1 try cpc etc1*, and *gl1-S92F try cpc etc1* mutant seedlings. RNA was isolated from 10-day-old seedling, and RT-PCR was used to examine the expression of *GL2*. The expression of *ACTIN2* (*ACT2)* was used as a control. (**b**)

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Cotransfection of GL1-S92F and GL3 or EGL3 failed to activate *GL2p:GUS* reporter gene. Effector gene *GL1-S92F* and *GL3* or *EGL3* were cotransfected into protoplasts with an integrated *GL2p:GUS* reporter gene. The transfected protoplasts were incubated in darkness for 20-22h before GUS activity was measured. Data represent the mean ± SD of three replicates.

Fig. 4. GL1-S92F interacts with GL3 and EGL3 in plant cells.

(**a**) Diagrams of effector and reporter constructs used in the transfection assays. (**b**) GL1-S92F interacts with GL3 in plant cells. (**c**) WER-S94F interacts with GL3 in plant cells. Reporter gene and effector genes were cotransfected into protoplasts, and the protoplasts were incubated in darkness for 20-22h before GUS activity was measured. Cotransfection of CAT (CHLORAMPHENICOL ACETYLTRANSFERASE) was used as a control (Wang et al., 2005). Data represent the mean ± SD of three replicates.

Fig. 5. Effects of the Asp/Glu to Ser amino acid substitution TRY, CPC and TCL1 on their interaction with GL3, and their functions in regulating trichome formation in plants.

(**a**) The Asp/Glu to Ser amino acid substitution in TRY, CPC and TCL1 does not affect their interaction with GL3 in plant cells. Reporter gene and effector genes were cotransfected into protoplasts, and the transfected protoplasts were incubated in darkness for 20-22h before GUS

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activity was measured. Data represent the mean ± SD of three replicates. (**b**) Phenotypes of transgenic plant seedlings overexpressing *mTRY*, *mCPC* and *mTCL1*. Photographs were taken from 10-day-old soil-grown plants. (**c**) Trichome formation on rosette leaves of Col, and transgenic plants overexpressing *mTLC1*. Data represent mean ± SD of 10-14 plants. (**d**) Expression of *TCL1*, *GL1* and *GL2* in Col wild type, *35S:TCL1* and *35SmTCL1* transgenic plants. RNA was isolated from 7-day-old seedling, and RT-PCR was used to examine the expression of *GL2*. The expression of *ACT2* was used as a control.

Fig. 6. VPGL1-S92FGL3 can not activate the expression of the *GL2p:GUS* reporter gene, but affects trichome formation when onverexpressed in plants.

(**a**) VPGL1-S92FGL3 failed to activate the expression of *GL2p:GUS* when transient expressed in protoplasts. The effector gene *VPGL1-S92FGL3* was transfected into protoplasts with an integrated *GL2p:GUS* reporter gene. The transfected protoplasts were incubated in darkness for 20-22h before GUS activity was measured. Data represent the mean ± SD of three replicates. (**b**) Phenotypes of transgenic plant seedlings overexpressing *VPGL1GL3* and *VPGL1-S92FGL3*. Photographs were taken from 10-day-old soil-grown plants. (**c**) Phenotypes of adult transgenic plants overexpressing *VPGL1GL3* and *VPGL1-S92FGL3*. Photographs were taken from 6-week-old (up and middle panels) and 5-week-old (low panel) soil-grown plants.

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Fig. 7. Loss-of-function mutations in the single *MYB* genes partially restores trichome formation in the *gl1-S92F* and *gl1* mutant backgrounds.

Photographs were taken from 3-week-old, soil-grown plants.

Fig. 8. A model of the S92F amino acid substitution in GL1 on the interaction of GL1 and GL3, and the activation of *GL2* in Arabidopsis.

(**a**) GL1 interacts with GL3, binds concurrently to the promoter of *GL2* via their own DNA binding domains, and together they activate the expression of *GL2*. (**b**) S92F amino acid substitution in GL1 resulted in conformational changes, which does not affect the interaction of GL1 and GL3, however, GL1S92F cannot bind to the promoter of *GL2*, thus failed to activate the expression of *GL2*.

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Brief summary statement

The R2R3 MYB transcription factor GL1 is a key regulator of trichome formation in Arabidopsis. The conserved [D/E]L×2[R/K]×3L×6L×3R amino acid signature in the R3 domain is required for the interaction of MYBs with R/B-like bHLH transcription factors. S92F amino acid substantiation in the conserved [D/E]L×2[R/K]×3L×6L×3R signature in GL1 lead to loss-of-function mutation of GL1. However, our results indicate that Ser92 residue is not required for the interaction of GL1 with bHLH transcription factor GL3 or EGL3, but may required for binding of GL1 to its target genes.

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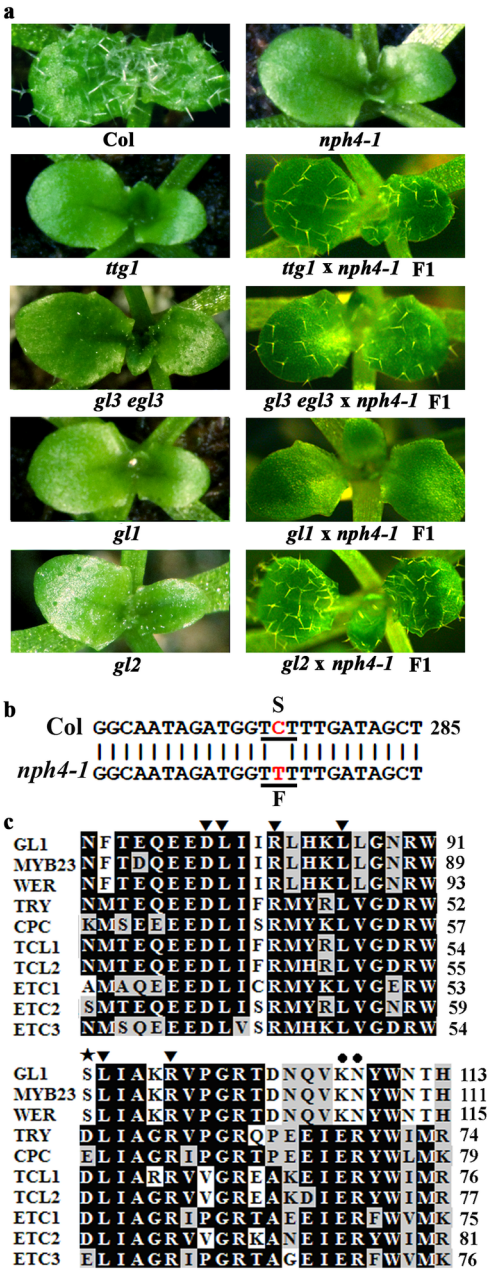

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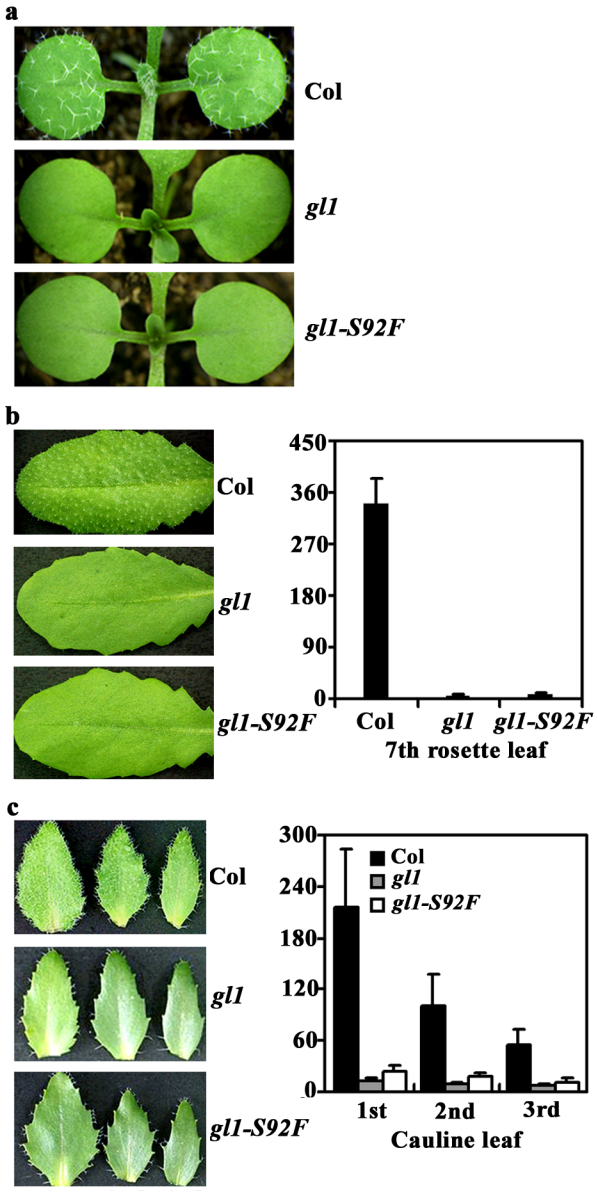

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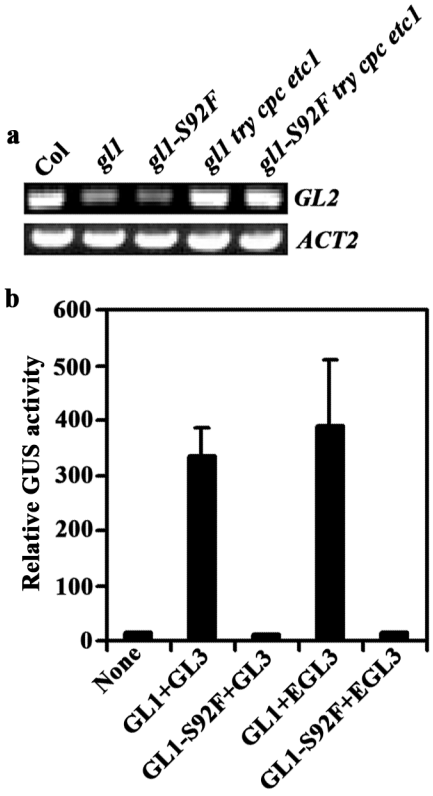

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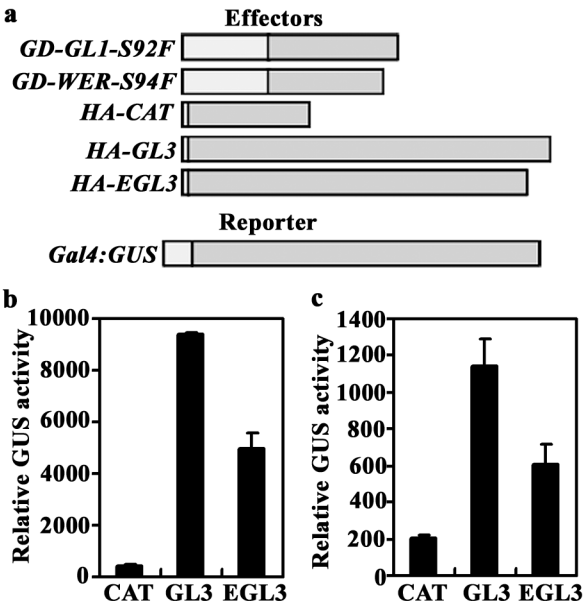

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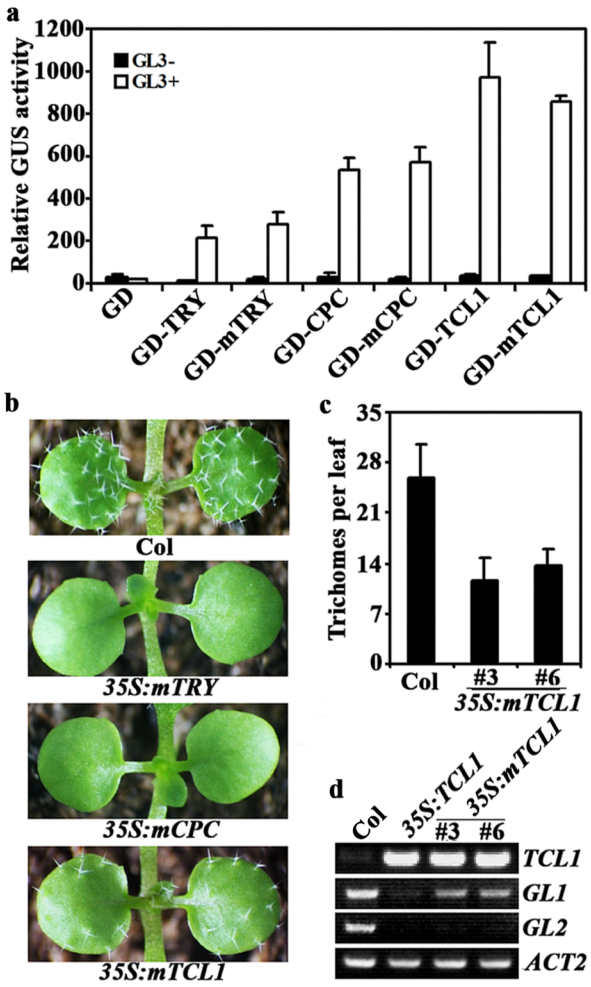

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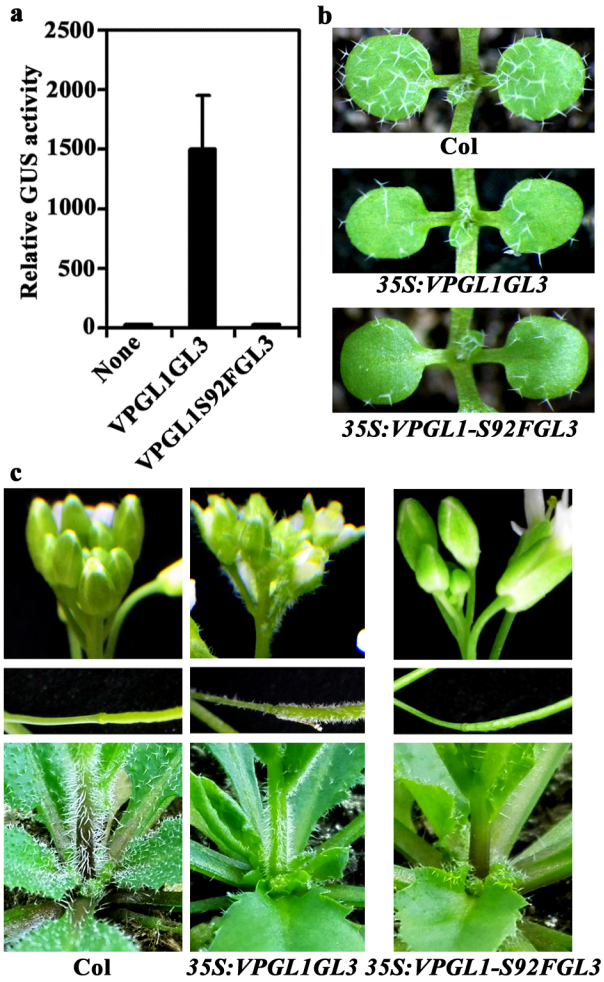

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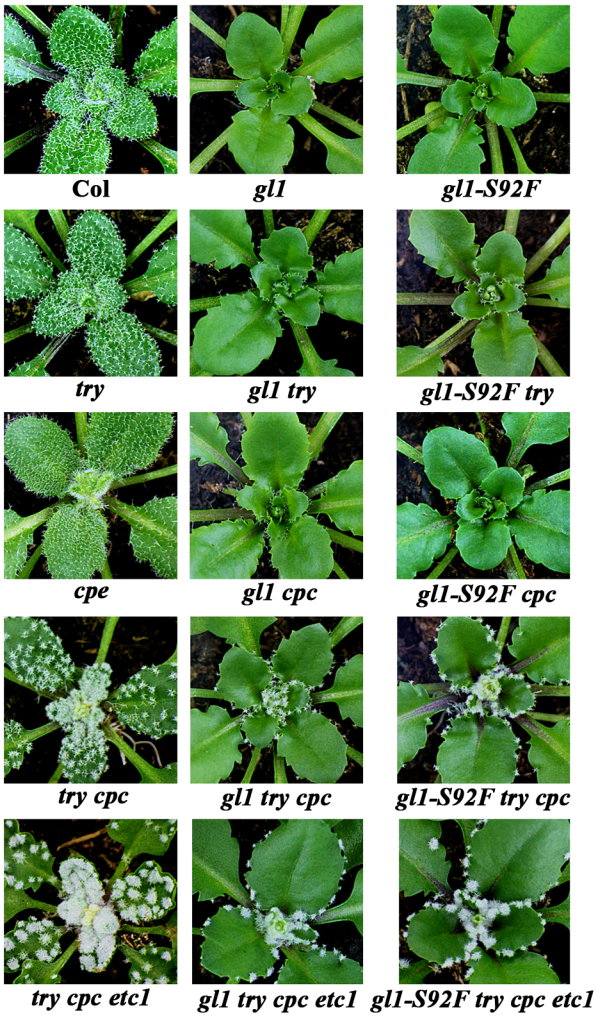

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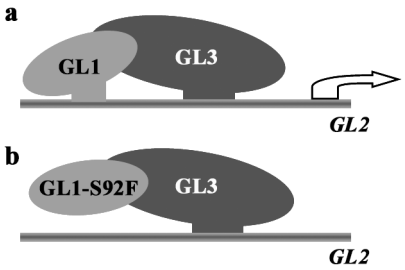

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