Check for updates

# NTL8 Regulates Trichome Formation in Arabidopsis by Directly Activating R3 MYB Genes *TRY* and *TCL1*[1[OPEN]]

**Hainan Tian,[a] Xianling Wang,[a] Hongyan Guo,[a] Yuxin Cheng,[a] Chunjiang Hou,[a] Jin-Gui Chen,[b] and Shucai Wang[a,2]**

[a]Key Laboratory of Molecular Epigenetics of MOE and Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

[b]Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

**ORCID IDs: 0000-0002-1752-4201 (J.-G.C.); 0000-0001-7619-2385 (S.W.).**

The NAM, ATAF1/2, and CUC (NAC) are plant-specific transcription factors that regulate multiple aspects of plant growth and development and plant response to environmental stimuli. We report here the identification of NTM1-LIKE8 (NTL8), a membrane-associated NAC transcription factor, as a novel regulator of trichome formation in Arabidopsis (*Arabidopsis thaliana*). From an activation-tagged Arabidopsis population, we identified a dominant, gain-of-function mutant with glabrous inflorescence stem. By using plasmid rescue and RT-PCR analyses, we found that *NTL8* was tagged; thus, the mutant was named *ntl8-1 Dominant* (*ntl8-1D*). Recapitulation experiment further confirmed that the phenotype observed in the *ntl8-1D* mutant was caused by elevated expression of *NTL8*. Quantitative RT-PCR results showed that the expression level of the single-repeat R3 MYB genes *TRIPTYCHON* (*TRY*) and *TRICHOMELESS1* (*TCL1*) was elevated in the *ntl8-1D* mutant. Genetic analyses demonstrated that NTL8 acts upstream of TRY and TCL1 in the regulation of trichome formation. When recruited to the promoter region of the reporter gene *Gal4:GUS* by a fused GAL4 DNA-binding domain, NTL8 activated the expression of the reporter gene. Chromatin immunoprecipitation results indicated that *TRY* and *TCL1* are direct targets of NTL8. However, NTL8 did not interact with SQUAMOSA PROMOTER BINDING PROTEIN LIKE9, another transcription factor that regulates the expression of *TRY* and *TCL1*, in yeast and plant cells. Taken together, our results suggest that NTL8 negatively regulates trichome formation in Arabidopsis by directly activating the expression of *TRY* and *TCL1*.

Trichomes are specialized epidermal cells (Johnson, 1975). Trichomes are important for plant growth and development in many ways. For example, trichomes can protect plants from ultraviolet irradiation, insect predation, herbivores, and excessive transpiration (Mauricio and Rausher, 1997; Wagner et al., 2004; Schilmiller et al., 2008). The distribution of trichomes is spatially and temporally controlled. During the early vegetative stage, trichomes are only on the adaxial side of the rosette leaves, but in the adult vegetative stage, trichomes appear on both adaxial and abaxial rosette leaves. After entering into the reproductive stage, trichomes gradually decrease on the main inflorescence stem (Telfer et al., 1997).

In Arabidopsis (*Arabidopsis thaliana*), trichome formation has become one of the best models for studying cell fate determination (Schiefelbein, 2003; Pesch and Hülskamp, 2004; Serna, 2005; Schellmann et al., 2007; Wang and Chen, 2014). Studies during the last several decades have identified the key transcription factors that involve in the regulation of trichome formation. Based on their functions, these key transcription factors can be divided into positive regulators and negative regulators of trichome formation. The positive regulators include the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1; Galway et al., 1994; Walker et al., 1999; Bouyer et al., 2008), the R2R3 MYB transcription factor GLABRA1 (GL1; Oppenheimer et al., 1991), the basic helix-loop-helix (bHLH) transcription factors GLABRA3 (GL3), and ENHANCER OF GLABRA3 (EGL3; Payne et al., 2000; Zhang et al., 2003), and the homeodomain protein GLABRA2 (GL2; Rerie et al., 1994; Di Cristina et al., 1996). The negative regulators include a group of seven single-repeat R3 MYB transcription factors; they are TRIPTYCHON (TRY; Schnittger et al., 1999; Schellmann et al., 2002), CAPRICE (CPC; Wada et al., 1997, 2002), TRICHOMELESS1 (TCL1; Wang et al., 2007b), TCL2 (Gan et al., 2011), ENHANCER OF TRY AND CPC1 (ETC1), ETC2, and ETC3 (Kirik et al., 2004a, 2004b; Wester et al., 2009; Wang et al., 2008).

[1] This work was supported by the National Key R&D Program of China (2016YFD0101902), the National Natural Science Foundation of China (31170262), and the Programme for Introducing Talents to Universities (B07017).

[2] Address correspondence to wangshucai@yahoo.com.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Shucai Wang (wangshucai@yahoo.com).

S.W. and J.-G.C. conceived the research; S.W., J.-G.C, and H.T. designed the experiments; H.T., X.W., H.G., Y.C., and C.H. performed the experiments; S.W. and H.T. analyzed the data and drafted the manuscript; and all authors participated in the revision of the manuscript.

[OPEN] Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.17.00510

 © 2017 American Society of Plant Biologists. All Rights Reserved. 

The positive regulators TTG1, GL1, and GL3 or EGL3 can form a MYB-bHLH-WDR (MBW) activator complex to activate the expression of *GL2* (Masucci et al., 1996; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ramsay and Glover, 2005), leading to the promotion of trichome initiation. The same MBW complex can also activate the expression of R3 MYB genes. R3 MYBs, in turn, move to adjacent cells where they can compete with GL1 for the binding site of GL3, thus preventing the formation of activator complex, resulting in inhibition of trichome formation (Schellmann et al., 2002; Schiefelbein, 2003; Digiuni et al., 2008; Lin and Aoyama, 2012; Wang and Chen, 2014). However, loss-of-function of R3 MYB genes resulted in different phenotypes. The *try* mutant produces a trichome-clustering phenotype (Schnittger et al., 1999; Schellmann et al., 2002), trichome number is increased in the *cpc* mutant (Wada et al., 1997, 2002), and ectopic trichomes on the inflorescence stems and pedicels were observed in the *tcl1* mutant (Wang et al., 2007b). On the other hand, it has been reported that the MBW complex can only activate the expression of some of the R3 MYB genes (Wang et al., 2008) and that the conserved motif required the interaction of GL1 and R3 MYBs with GL3/EGL3 may also involve binding of their target genes (Dai et al., 2016). In addition to competing with GL1 for binding GL3 or EGL3, TCL1 can directly activate the expression of *GL1* (Wang et al., 2007b). These results suggest that the regulation of trichome formation is far more complicated than previous thought.

Indeed, additional regulators and regulatory loops of trichome formation have been reported in recent years. The C2H2 transcription factors GLABROUS INFLORESCENCE STEMS (GIS), GIS2, and GIS3, the ZINC FINGER transcription factors ZINC FINGER PROTEIN5 (ZFP5), ZFP6, and ZFP8, and the plant-specific transcription factor SQUAMOSA PROMOTER BINDING PROTEIN LIKE9 (SPL9) have been identified as regulators of trichome formation (Gan et al., 2006, 2007; Yu et al., 2010; Zhou et al., 2011, 2013; An et al., 2012; Sun et al., 2015). Most of these transcription factors regulate trichome formation via regulating, directly or indirectly, the expression of the key transcription factor genes. For example, GIS, GIS3, ZFP5, and ZFP8 can regulate the expression of the MBW complex component genes (Gan et al., 2006, 2007; Zhou et al., 2011, 2013; Sun et al., 2015), whereas SPL9 can directly regulate the expression of the R3 MYB transcription factor genes *TRY* and *TCL1* (Yu et al., 2010).

The NAM, ATAF1/2, and CUC (NAC) transcription factor family is one of the largest plant-specific transcription factor families (Olsen et al., 2005; Yao et al., 2012). NAC transcription factors regulate multiple aspects of plant growth and development, including floral development (Sablowski and Meyerowitz, 1998), apical meristem formation (Hibara et al., 2003), cell cycle control (Kim et al., 2006), and secondary cell wall formation (Dong et al., 2014). NAC transcription factors are also involved in the regulation of plant hormone signaling (Fujita et al., 2004), as well as plant response to biotic and abiotic stresses (Tran et al., 2004; Shao et al., 2015). There are a total of 117 genes in the Arabidopsis genome encoding NAC transcription factors, but none of them had been reported to be involved in the regulation of trichome formation. Fourteen of the NAC transcription factors in Arabidopsis are membrane-bound transcription factors with transmembrane domains (Kim et al., 2007a; Liang et al., 2015). Among them, NAC with Transmembrane Motif1 (NTM1) has been reported to participate in the regulation of cell division (Kim et al., 2006), NTM1-like6 (NTL6) is involved in the regulation of pathogen resistance response (Seo et al., 2010), NTL14/ANAC089 controls ER-stress-induced programmed cell death (Yang et al., 2014), NTL4 participates in heat-stress response (Lee et al., 2014), NTL1/ANAC013 is involved in oxidative stress response (De Clercq et al., 2013), and NTL8 regulates salt-responsive flowering and seed germination (Kim et al., 2007b, 2008).

Here, we report the identification of NTL8 as a novel regulator of trichome formation. We show that NTL8 is a transcription activator and that it negatively regulates trichome formation in Arabidopsis by directly activating the expression of R3 MYB genes *TRY* and *TCL1*.

## RESULTS

### Trichome Formation Is Reduced in the *ntl8-1D/gpa1-2* Mutant

In an attempt to identify novel regulators of trichome formation, we screened an activation-tagged mutagenized Arabidopsis population we had for plants with defects in trichome formation. The population was generated by transforming the activation-tagged *pSKI015* to the *gpa1-2* mutant (Ullah et al., 2001). In addition to the glabrous *trichomeless 1-1 Dominant* (*tcl1-1D*; Wang et al., 2007b), we identified another trichome mutant (Fig. 1). Plasmid rescue and RT-PCR experiments indicated that *NTM1-LIKE8* (*NTL8*), a membrane-associated NAC transcription factor gene (Kim et al., 2007a; Liang et al., 2015), was activated in the mutant (see next section for details), thus the mutant was named *ntl8-1 Dominant* (*ntl8-1D/gpa1-2*).

Unlike the Ws wild-type and *gpa1-2* mutant plants, the *ntl8-1D/gpa1-2* mutant had a glabrous inflorescence stem (Fig. 1A) and reduced trichome formation on rosette leaves (Fig. 1B). Statistical analysis results showed that the trichome number on the first two rosette leaves of the *ntl8-1D/gpa1-2* mutant seedlings was about two-thirds of that in the Ws wild-type and *gpa1-2* mutant seedlings (Fig. 1C).

### Identification of the T-DNA Insertion Site and Recapitulation of the *ntl8-1D* Mutant Phenotypes

By using plasmid rescue, a procedure for identifying T-DNA insertion sites in activation-tagged mutants (Weigel et al., 2000), we found that the T-DNA in the *ntl8-1D* mutant was inserted in the chromosome 2 at a position that is 101 bp upstream of the start codon of the *NTL8* gene, and 1898 bp downstream of the stop codon

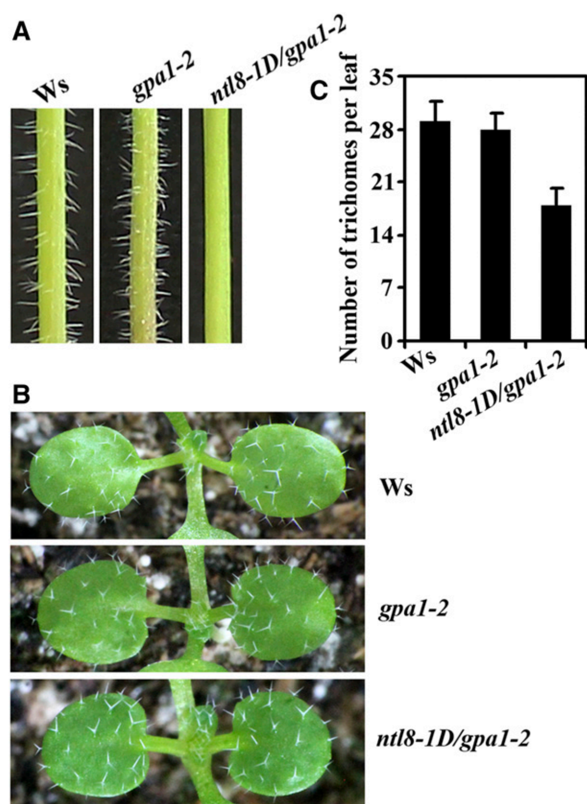


**Figure 1.** *ntl8-1D/gpa1-2* is a gain-of-function, dominant mutant with glabrous inflorescence stems. A, Trichomes on the main inflorescence stems of the Ws wild-type plant (left) and the *gpa1-2* mutant (middle), and glabrous stem of the *ntl8-1D/gpa1-2* dominant mutant (right). Photographs were taken from the first internodes of 1-month-old soil-grown plants. B, Trichomes on the first two rosette leaves of the Ws wild-type plant (top), the *gpa1-2* mutant (middle), and the *ntl8-1D/gpa1-2* dominant mutant (bottom). Photographs were taken from 10-d-old soil-grown seedlings. C, Trichome density on the first two rosette leaves of the Ws wild-type plant, the *gpa1-2* mutant and the *ntl8-1D/gpa1-2* dominant mutant. Data represent the mean ± SD of 18 plants.

of the *At2g27310* gene, with the four outward-facing *35S* enhancers facing the *NTL8* gene (Fig. 2A). RT-PCR results showed that the expression of *NTL8* gene, but not *At2g27310*, was elevated in the mutant (Fig. 2B). Thus, the mutant was named *ntl8-1D/gpa1-2*.

To examine whether the phenotypes observed in *ntl8-1D/gpa1-2* mutant is related to the *gpa1-2* mutant background, we crossed homozygous *ntl8-1D/gpa1-2* mutant with Col wild type and examined the phenotypes in the F1 and F2 populations. We found that all the F1 plants were resistant to Basta treatment and had a trichome phenotype similar to that of the *ntl8-1D/gpa1-2* mutant. The F2 seedlings had a 3:1 segregation ratio of the trichome phenotypes, and all the plants with defect on trichome formation were resistant to Basta treatment. These results suggested that the trichome phenotype observed in the *ntl8-1D/gpa1-2* mutant was caused by a single T-DNA insertion that was independent of the *gpa1-2* mutant background.

To further confirm that the phenotype in the *ntl8-1D/gpa1-2* mutant was caused by elevated expression of *NTL8* and was independent of the *gpa1-2* mutant background, we generated transgenic plants expressing *NTL8* under the control of the *35S* promoter into the Col background. We found that the transgenic plants overexpressing *NTL8* showed a phenotype similar to that of the *ntl8-1D/gpa1-2* mutant (Fig. 2, C–E).

Previously, results have shown that *NTL8* is expressed in all the tissues and organs as examined by GUS staining in the *NTL8p:GUS* transgenic plants (Kim et al., 2007b). Our qRT-PCR results also show *NTL8* is expressed in all the tissues and organs (Supplemental Fig. S1A), and a close view show that *NTL8* is expressed in the basal of trichomes on rosette leaves and stems of the *NTL8p:GUS* transgenic plants (Supplemental Fig. S1, B and C), consistent with the observation that NTL8 plays a role in regulating trichome formation in Arabidopsis.

Because NTL8 has been shown to be a membrane-associated NAC transcription factor, but a nuclear protein if released from the membranes (Kim et al., 2007a; Liang et al., 2015), and relocation of NTL8 to the nucleus was shown to be required for its functions in the regulation of plant growth and development (Kim et al., 2007b, 2008), we examined whether NTL8 without its C-terminal transmembrane domain (NTL8ΔC; Kim et al., 2007b) can regulate trichome formation in Arabidopsis by generating transgenic plants overexpressing *NTL8ΔC*. As shown in Figure 2, C–E, the *35S:HA-NTL8ΔC* plants are similar to the *35S:HA-NTL8* plants in trichome formation, implying the C-terminal transmembrane domain is not required for NTL8's function in regulating trichome formation.

## Trichome Formation in the *ntl8* Mutants Is Largely Unaffected

To further analyze the function of *NTL8*, we took a reverse genetics approach to seek and characterize loss-of-function mutants of *NTL8*. From the T-DNA Express Database (http://signal.salk.edu/cgi-bin/tdnaexpress), we identified two T-DNA insertion alleles of *NTL8* gene, WiscDsLoxHs159_07E and SM_3_16309, with the T-DNA inserted in the third and second exon of the *NTL8*, respectively (Fig. 3A). The presence of the T-DNA at the expected positions was verified by sequencing. Plants homozygous for the T-DNA insertions were isolated by PCR-based screening and named *ntl8-1* and *ntl8-2*, respectively (Fig. 3A). The loss-of-function status of the *ntl8* mutants was then confirmed by RT-PCR using primers amplifying the full-length coding sequence of *NTL8*.

We examined the phenotypes of the *ntl8* mutants in trichome formation by directly comparing them side-by-side with the Col wild-type plants and the *ntl8-1D* mutant plants obtained by crossing homozygous *ntl8-1D/gpa1-2* mutant with the Col wild-type plant. As shown in Figure 3, B to D, no defects on trichome formation in the *ntl8* mutants were observed.

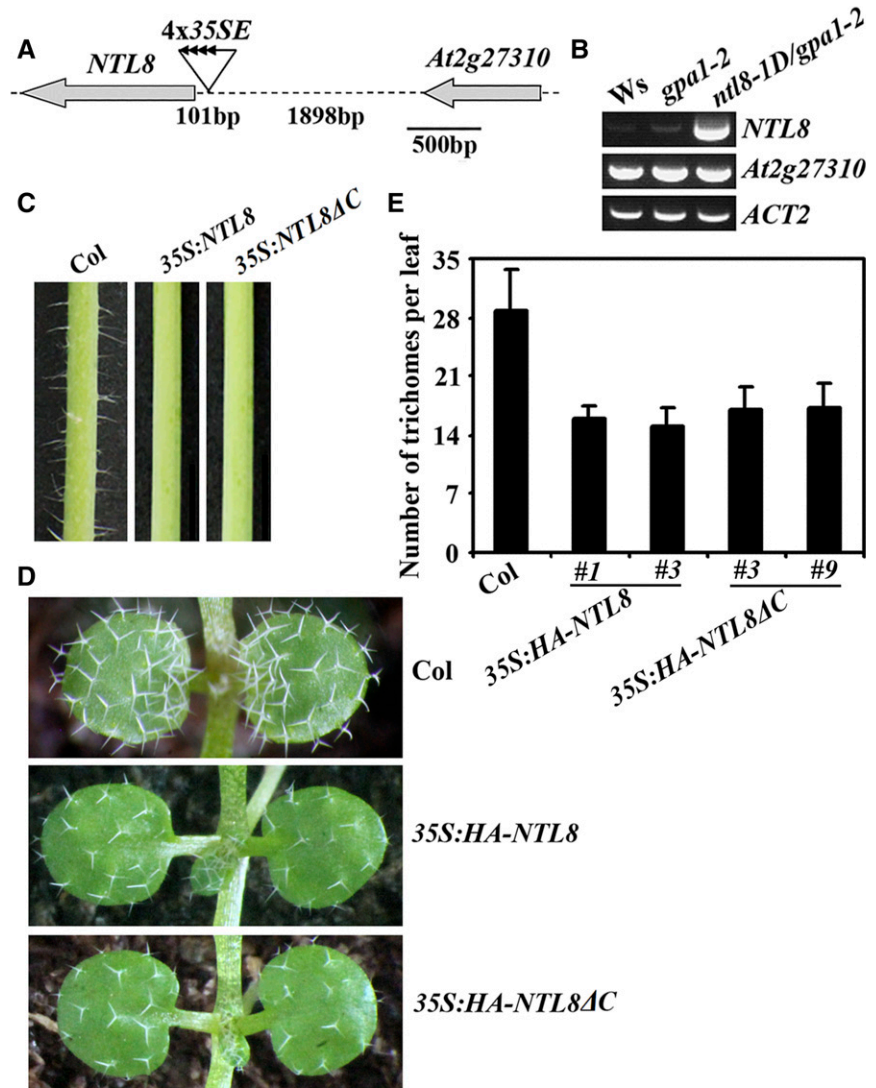


**Figure 2.** Identification of the T-DNA insertion site in the *ntl8-1D/gpa1-2* mutant and recapitulation of the mutant phenotypes. A, Diagram illustrating the activation-tagged T-DNA insertion site in the *ntl8-1D/gpa1-2* mutant. Arrowheads indicate the orientation of the *4X35S* enhancer repeats in the T-DNA situated 101 bp upstream of the start codon of *NTL8*, and 1898 bp downstream of the stop codon of *At2g27310*. B, Expression level of *NTL8* and *At2g27310* in the Ws wild-type, the *gpa1-2* mutant, and the *ntl8-1D/gpa1-2* mutant seedlings. Total RNA was isolated from 10-d-old seedlings grown on 0.5× MS plates, and RT-PCR was used to examine the expression of *NTL8* and *At2g27310*. *ACTIN2* (*ACT2*) was used as a control. C, Trichomes on the main inflorescence stems of the Col wild-type (left), the *35S:NTL8* (middle), and *35S:NTL8ΔC* transgenic plants (right). Photographs were taken from the first internodes of 1-month-old soil-grown plants. D, Trichomes on the first two rosette leaves of the Col wild-type (left), the *35S:NTL8* (middle), and *35S:NTL8ΔC* transgenic plants (right). Photographs were taken from 10-d-old soil-grown seedlings. E, Trichome density on the first two rosette leaves of the Col wild-type plant, the *35S:HA-NTL8* and *35S:HANTL8ΔC* transgenic plants. Data represent the mean ± SD of 18 plants.

By examining protein homologs of NTL8 on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), we found NTL5 and NTL14 have higher amino acid similarities with NTL8, followed by NTL2. Phylogenetic analysis further confirmed that NTL8 is closely related to NTL5 and NTL14 (Fig. 3E). However, these three NTLs are more closely related to two membrane-associated NAC transcription factors from soybean (Li et al., 2016) and two from tomato, rather than NTL2 (Fig. 3E). These results suggested that NTL8, NTL5, and NTL14 may have redundant functions.

From the T-DNA Express Database, we identified two T-DNA insertion alleles of *NTL5* gene, SALK-012154 and SAIL_172_A04, with the T-DNA inserted immediately after and before the second exon of the *NTL5*, respectively (Fig. 3A). After confirmed the presence of the T-DNA insertion, isolated homozygous plants for the T-DNA insertions, and confirmed the loss-of-function status of the alleles, we named them *ntl5-1* and *ntl5-2*, respectively (Fig. 3A). Similar to that in the *ntl8* mutants, trichome formation on rosette leaves in *ntl5* single mutants and *ntl5 ntl8* double mutant remained largely unaffected (Fig. 3D). However, more branched trichomes were observed on stems of the *ntl5 ntl8* double mutant (Fig. 3, B and C).

### Expression of R3 MYB Genes *TRY* and *TCL1* Is Elevated in the *ntl8-1D* Mutants

Trichome formation is largely controlled by a MBW transcription activator complex and their target genes (Wang and Chen, 2014). All other identified trichome formation-regulating transcription factors, including SPLs, C2H2, and zinc finger-homeobox transcription factors, have been reported to regulate trichome formation by regulating the expression the MBW complex component genes and/or their target genes (Gan et al.,

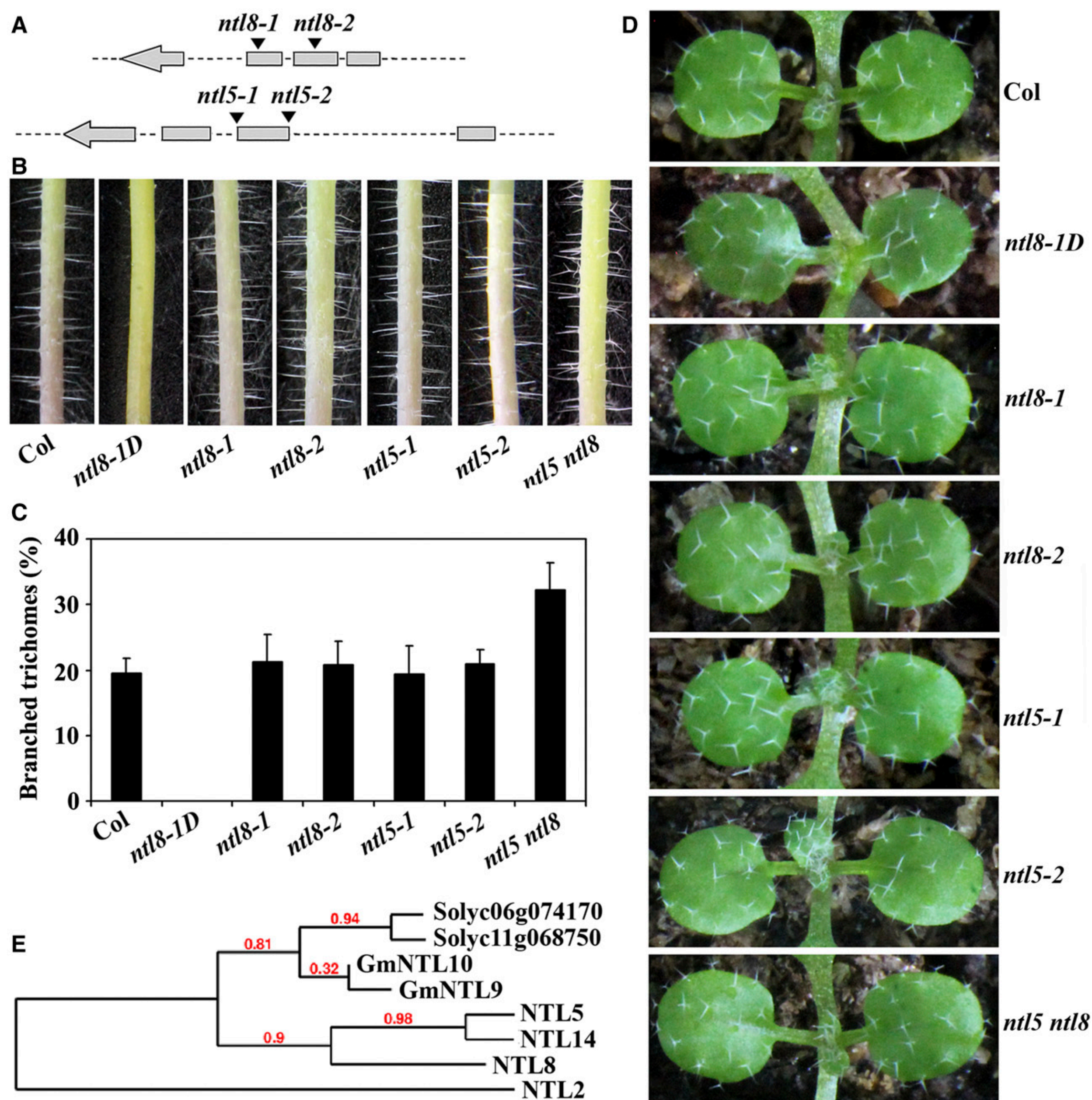


**Figure 3.** Phenotypes of the loss-of-function mutants of *NTL8*. A, Diagram showing the T-DNA insertion sites in *ntl8* and *ntl5* single mutants. The T-DNA is inserted in the third and the second exon of *NTL8*, respectively, for the *ntl8-1* and *ntl8-2* mutants, and immediately after and before the second exon of *NTL5*, respectively, for the *ntl5-1* and *ntl5-2* mutants. B, Trichomes on the main inflorescence stems of the Col wild-type plant, the *ntl8-1D* dominant mutant, the *ntl8* single mutants, the *ntl5* single mutants, and the *ntl5 ntl8* double mutant. Photographs were taken from the first internodes of 1-month-old soil-grown plants. C, Percentage of branched trichomes on the inflorescence stems of the Col wild-type plant, the *ntl8-1D* dominant mutant, the *ntl8* single mutants, the *ntl5* single mutants, and the *ntl5 ntl8* double mutant. Data represent the mean ± SD of 11 or 12 plants. D, Trichomes on first two rosette leaves of the Col wild-type plant, the *ntl8-1D* dominant mutant, the *ntl8* single mutants, the *ntl5* single mutants, and the *ntl5 ntl8* double mutant. Photographs were taken from 10-d-old soil-grown seedlings. E, Phylogenetic analysis of NTL8 and its closely related proteins. The entire amino acid sequences of the proteins were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). "OneClick" mode with default settings on Phylogeny (www.phylogeny.fr) was used to generate the phylogenetic tree. Branch support values are indicated above the branches.

2006, 2007; Yu et al., 2010; Zhou et al., 2011, 2013; An et al., 2012; Sun et al., 2015). To examine if this is the case for NTL8, we tested, by using qRT-PCR, the expression of the MBW complex component genes *TTG1*, *GL1*, *GL3*, and *EGL3*, and their target genes *GL2* and single-repeat R3 MYB genes *TRY*, *CPC*, *ETC1*, *ETC2*, *ETC3*, *TCL1*, and *TCL2* in the *ntl8-1D* mutant seedlings. As shown in Figure 4A, the expression level of R3 MYB

gene *TRY* was elevated about 25-fold, and that of *TCL1* about 10-fold in the *ntl8-1D/gpa1-2* mutant seedlings when compared with that in the Ws wild-type and the *gpa1-2* mutant seedlings, whereas the expression level of all other genes examined had little if any change (Fig. 4A).

We then examined the expression level of *TRY* and *TCL1* in the *35S:HA-NTL8* transgenic plant and *ntl8* mutant seedlings using qRT-PCR. The results show that, when compared with the Col wild-type seedlings, the expression levels of both *TRY* and *TCL1* were elevated in the *35S:HA-NTL8* transgenic plant seedlings (Fig. 4B), similar to that observed in the *ntl8-1D/gpa1-2* mutant seedlings (Fig. 4A). We also noted that the expression of *TRY* was decreased about 2-fold, whereas expression level of *TCL1* remained largely unchanged in the *ntl8* mutant seedlings (Fig. 4B).

Previous studies have shown that the expression level of *TRY* and *TCL1* are increased on successive inflorescence stem internodes (Yu et al., 2010). Our qRT-PCR results showed that the expression level of *NTL8* was also increased on successive inflorescence stem internodes (Fig. 4C).

### *NTL8* Functions Upstream of *TRY* and *TCL1*

The results described above suggested that NTL8 may inhibit trichome formation in Arabidopsis via activating R3 MYB genes *TRY* and *TCL1*; thus, we hypothesized that loss-of-function mutants of *TRY* and *TCL1* in the *ntl8-1D* mutant background may phenocopy *try* and *tcl1* mutants, respectively. To test this, we generated double and triple mutants between the *ntl8-1D* mutant and the *try* and *tcl1* mutants. By examining the mutants generated, we found that the *try ntl8-1D* double mutant is morphologically indistinguishable from the *try* single mutant. Trichome clusters found in the *try* mutant

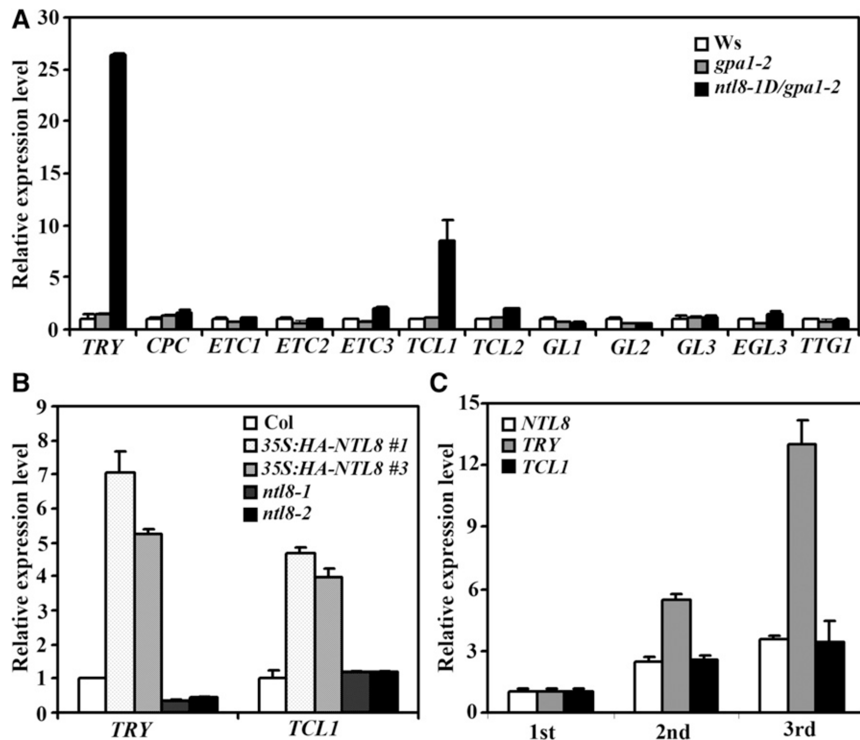


**Figure 4.** Expression of the known key trichome formation-regulating transcription factor genes in the wild type and gain- and loss-of- function mutants of *NTL8*. A, Expression of the known key trichome formation regulating transcription factor genes in the Ws wild-type, the *gpa1-2* mutant, and the *ntl8-1D/gpa1-2* mutant seedlings. Total RNA was isolated from 10-d-old seedlings grown on 0.5× MS plates and qRT-PCR was used to examine the expression of the key transcription factor genes involved in the regulation of trichome formation. *ACT2* was used as reference gene, and expression of each gene in the Ws wild-type seedlings was set as 1. Data represent the mean ± SD of three replicates. B, Expression of *TRY* and *TCL1* in the Col wild type, the *35S:HA-NTL8* transgenic plant, and the *ntl8* loss-of-function mutants. Total RNA was isolated from 10-d-old seedlings grown on 0.5× MS plates, and qRT-PCR was used to examine the expression of *TRY* and *TCL1*. *ACT2* was used as reference gene, and expression of each gene in the Col wild-type seedlings was set as 1. Data represent the mean ± SD of three replicates. C, Expression of *NTL8*, *TRY*, and *TCL1* in the internodes of the main inflorescence stem of Col wild-type plants. Total RNA was isolated from different internodes of soil-grown Col wild-type plants, and qRT-PCR was used to examine the expression of *NTL8*, *TRY*, and TCL1. *ACT2* was used as reference gene, and expression of each gene in first internode was set as 1. Data represent the mean ± SD of three replicates.

(Schnittger et al., 1999; Schellmann et al., 2002) were observed on the rosette leaves and the inflorescence stems in the *try ntl8-1D* double mutant (Fig. 5).

On the other hand, although trichome formation on the inflorescence stems was restored in the *tcl1 ntl8-1D* double mutant, ectopic trichome formation on the upper part of the inflorescence, including pedicels and stem internodes, a phenotype of the *tcl1* mutant (Wang et al., 2007b), was not observed in the *tcl1 ntl8-1D* double mutant (Fig. 5). However, trichome formation in the *tcl1 try ntl8-1D* triple mutant largely phenocopied the *try tcl1* double mutant (Fig. 5).

To further examine the relationship between NTL8 and the single R3 MYB transcription factor TRY and TCL1, we generated double and triple mutants between the *ntl8-2* mutant and the *try* and *tcl1* mutants. Interestingly, we found that loss-of-function of *NTL8* completely restored the ectopic trichome formation phenotypes observed in the *tcl1* mutant and partially restored that observed in the *try tcl1* double mutant (Supplemental Fig. S2). It may seem difficult to judge, based solely on this result, the relationship between NTL8 and R3 MYB protein TRY and TCL1. However, taking the results described above (Fig. 5) into consideration, these genetic analyses suggested that NTL8 acts upstream of TRY and TCL1.

### *TRY* and *TCL1* Are Direct Target Genes of *NTL8*

Having shown that *NTL8* functions upstream of *TRY* and *TCL1* (Fig. 5), and expression of *TRY* and *TCL1* is elevated in the *ntl8-1D* mutant and *35S:HA-NTL8* transgenic plants, we wanted to examine whether *TRY* and *TCL1* are direct target genes of NTL8.

Previous studies have shown that NTL8 is a membrane-associated NAC transcription factor, and that it can be relocated into the nucleus (Kim et al., 2007b). However, it is still unclear if NTL8 can function as a transcriptional activator or repressor. We thus first examined the transcriptional activities of NTL8 by using protoplast transient transfection assays. Plasmids of the reporter gene *Gal4:GUS* and the effector gene *GD-NTL8* or *GD* control were cotransfected into protoplasts, and GUS activities were measured after the transfected protoplasts had been incubated overnight in the darkness. In this system, *GD* and *GD-NTL8* is recruited to the *Gal4* DNA binding site in the *Gal4:GUS* reporter gene. If NTL8 acts as a transcriptional activator, it would activate the expression of the reporter gene. As this was indeed the case (Fig. 6A), we concluded that NTL8 is a transcriptional activator.

We then examined if NTL8 can bind to the promoter regions of the *TRY* and *TCL1* genes by using chromatin immunoprecipitation (ChIP) assays. Previous studies have indicated that the CACG/TACG sequence is a NAC transcription factor binding site (Olsen et al., 2005; Tran et al., 2004); thus, primers spanning the CACG/TACG sequence in the promoter of *TRY* and *TCL1* were used for qRT-PCR in the ChIP assays, and primers amplifying a promoter fragment of the *DFR* gene (Wang et al., 2015) were used as a control. Approximately 20-fold and 5-fold enrichment was detected in the promoter region of *TRY* and *TCL1*, respectively, but no enrichment was observed for the promoter of the *DFR* gene (Fig. 6B).

To further confirm that *TRY* and *TCL1* are target genes of NTL8, we examined if mutation of the CACG/TACG sequence could abolish NTL8's binding to the promoter of *TRY* and *TCL1* genes in transfected protoplasts. As shown in Figure 6C, transfection of NTL8 activated the wild type, but not the mutated *TRY* and *TCL1* reporter genes.

### NTL8 May Function Independently of SPL9 in Regulating Trichome Formation

We have previously showed that SPL9 negatively regulates trichome formation by directly activating the

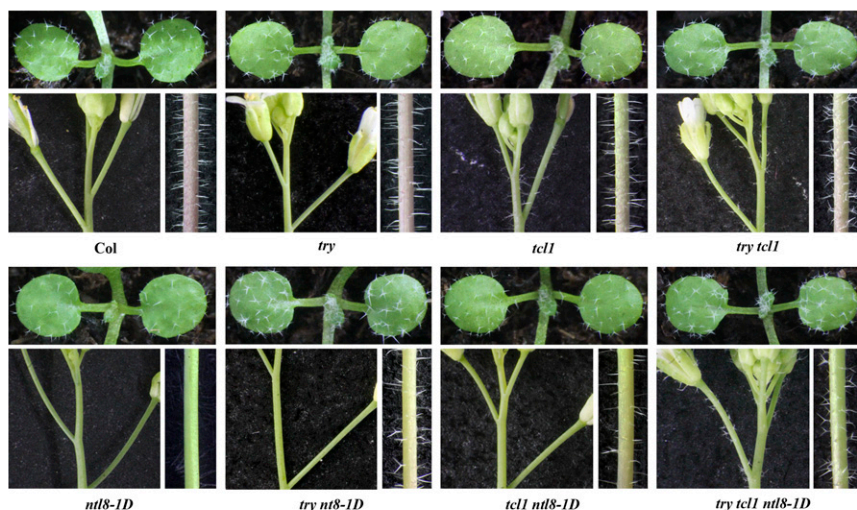


**Figure 5.** *NTL8* functions upstream of TRY and TCL1 in regulating trichome formation. Photographs of first two rosette leaves were taken from 10-d-old soil-grown seedlings. Photographs of flowers and inflorescence stems were taken from 5-week-old soil-grown plants of the Col wild type, *try*, *tcl1*, and *ntl8-1D* single mutants, *try tcl1*, *try ntl8-1D*, and *tcl1 ntl8-1D* double mutants, and *try tcl1 ntl8-1D* triple mutant plants.

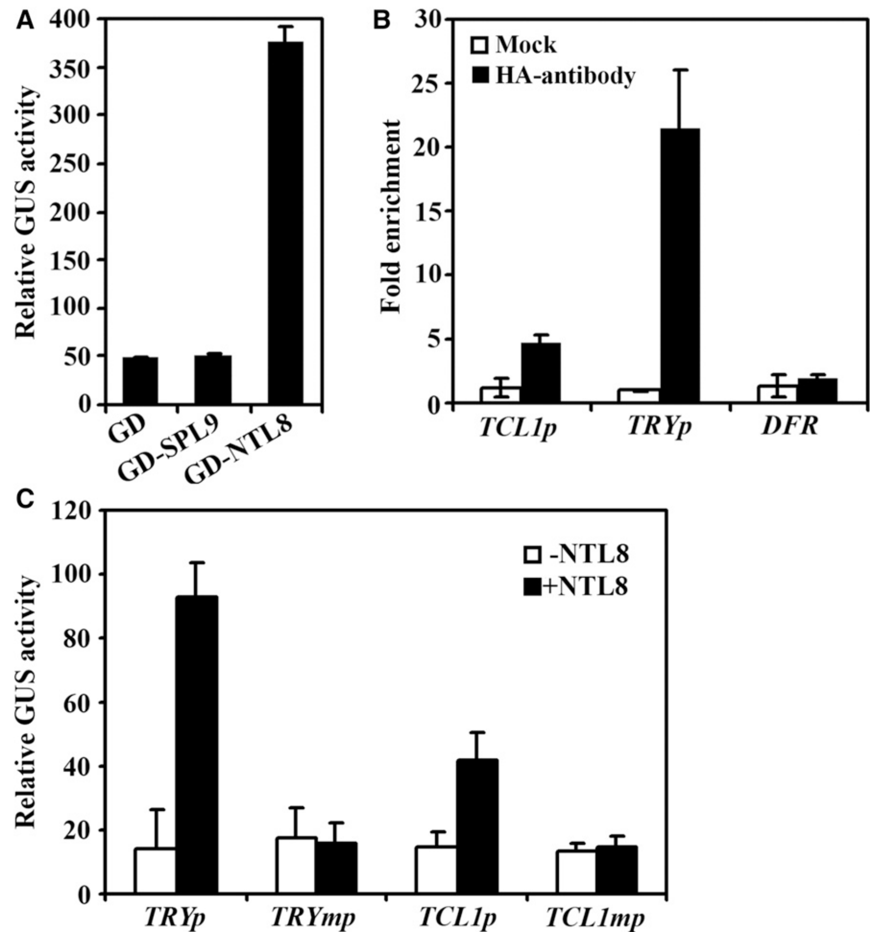


**Figure 6.** *TRY* and *TCL1* are direct target genes of NTL8 transcriptional activator. A, NTL8 is a transcriptional activator. Effectors and the *Gal4:GUS* reporter plasmids were cotransfected into protoplasts isolated from 3- to 4-week-old Col wild-type plants. The protoplasts were incubated in darkness at room temperature for 20 to 22 h, and then GUS activity was measured. Data represent the mean ± SD of three replicates. B, ChIP assay. The *35S:HA-NTL8* transgenic plants were used for ChIP assay with rabbit anti-HA antibodies. Rabbit preimmune serum was used as mock control. Primers spanning putative NAC transcription factor binding sites in the promoters of *TRY* and *TCL1* were used for qRT-PCR analysis. *ACT2* was used as reference gene and DFR as a negative control. Data represent the mean ± SD of three replicates. C, Mutation of the NAC transcription factor binding sites affects the binding of NTL8 to the promoters of *TRY* and *TCL1*. Plasmids of *NTL8* and the wild-type or mutated *TRY* or *TCL1* promoter*:GUS* were cotransfected into protoplasts isolated from 3- to 4-week-old Col wild-type plants. The protoplasts were incubated in darkness at room temperature for 20 to 22 h, and then GUS activity was measured. Cotransfection of *CAT* (*CHLORAMPHENICOL ACETYLTRANSFERASE*) was used as a negative control. Data represent the mean ± SD of three replicates.

expression of *TCL1* and *TRY* (Yu et al., 2010). However, in transfected protoplasts, SPL9 failed to activate the expression of the *Gal4:GUS* reporter gene when recruited to the promoter of the reporter gene by a fused GD domain (Fig. 6A), implying that a coactivator may be required for the activation of *TCL1* and *TRY* by SPL9. The fact that NTL8 functions as a transcriptional activator (Fig. 6A) and that it directly regulates the expression of *TRY* and *TCL1* (Fig. 6B and C) promoted us to examine whether NTL8 may interact with SPL9 to regulate the expression of *TRY* and *TCL1*.

We first used yeast two-hybrid assays to examine the interaction between NTL8 and SPL9. Because nucleus localization of NTL8 is required for its functions in regulating trichome formation (Fig. 2), we used *NTL8ΔC* to examine the interaction of NTL8 and SPL9. As shown in Figure 7A, as a positive control, yeast cells cotransformed with *pNubG-Fe65* and *pOstI-NubI* could grow on the SD-try-leu-his-ade plate supplied with 15 mM 3-AT (3-aminopropyltriethoxysilane; Gou et al., 2011), whereas yeast cells cotransformed with empty bait vector *pDHB1* and empty prey vector *pPR3-N* failed to do so. Yeast cells cotransformed with *pHDB1-SPL9* bait and *pPR3-N* empty prey vector, or *pDHB1* empty bait vector with *pPR3-N-NTL8ΔC* prey, or *pHDB1-SPL9* bait vector *pPR3-N-NTL8ΔC* prey vector all failed to grown on the selective plate (Fig. 7A), suggesting that NTL8 and SPL9 do not interact with each other in yeast cells.

We then used protoplasts transient transfection assays to test the interaction between SPL9 and NTL8. In these assays, effector plasmids *GD-SPL9* and *NTL8*, *NTL8ΔC*, or *CAT* control and reporter plasmids *Gal4:GUS* were cotransfected into protoplasts isolated from rosette leaves of Col wild-type plants. Because NTL8 functions as a transcriptional activator (Fig. 6A), if it interacts with SPL9, cotransfection of effector plasmids *GD-SPL9* and *NTL8* or *NTL8ΔC* will result in the activation of the *Gal4:GUS* reporter gene. However, no activation was observed (Fig. 7B). As a positive control of the transfection assays, cotransfection of effector plasmids GD-VP and *Gal4:GUS* reporter plasmids strongly activated the expression of the reporter gene (Fig. 7B). These results suggested that NTL8 does not interact with SPL9 in plant cells.

To further dissect the relationship between SPL9 and NTL8 in the regulation of trichome formation, we crossed the *rSPL9* transgenic plant (Yu et al., 2010) with the *ntl8-1D* mutants, and obtained the phenotypes of

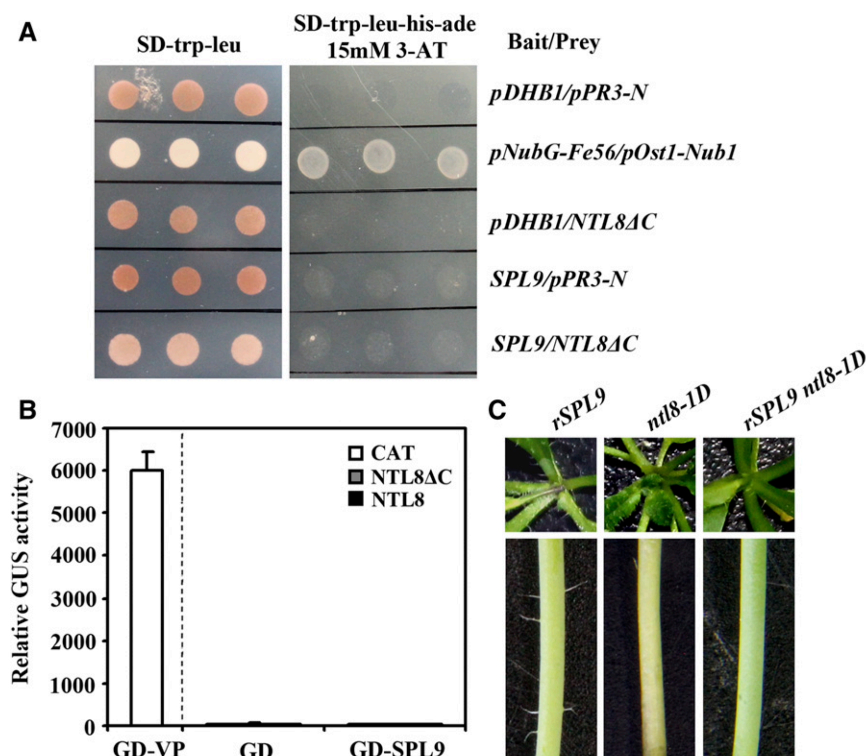


**Figure 7.** NTL8 may function independent of SPL9 in regulating trichome formation. A, NTL8 does not interact with SPL9 in yeast cells. Bait and prey plasmids were cotransformed into NMY51 yeast cells and grown in SD-Trp-Leu plate or SD-Trp-Leu-His-Ade plate containing 15 mM 3-AT for 2 to 4 d before photographs were taken. Cotransformation of empty vector pDHB1 and pPR3-N was used as negative control, and cotransformation of pNubG-Fe56 and pOst1-Nub1 as positive control. B, NTL8 does not interact with SPL9 in Arabidopsis protoplasts. Effectors and the *Gal4:GUS* reporter plasmids were cotransfected into protoplasts isolated from 3- to 4-week-old Col wild-type plants. The protoplasts were incubated in darkness at room temperature for 20 to 22 h, and then GUS activity was measured. Cotransfection of *CAT* was used as a negative control. Data represent the mean ± SD of three replicates. C, Trichomes on the rosettes and main inflorescence stems of the *SPL9p:rSPL9* (left), the *ntl8-1D* (middle) and *SPL9p:rSPL9 ntl8-1D* plants (right). Photographs of rosettes were taken from 3-week-old and stems from the first internodes of 1-month-old soil-grown plants.

the *rSPL9 ntl8-1D* plants. We found that trichome numbers on rosette leaves were further reduced in the *rSPL9 ntl8-1D* plants (Fig. 7C), suggesting that SPL9 and NTL8 may function independently in regulating trichome formation.

## DISCUSSION

Previously, we have shown that only some of the seven R3 MYB genes in Arabidopsis including *TRY*, *CPC*, *ETC1*, and *ETC3* are regulated by the MBW complex, and *ETC1* is the only one whose expression is dramatically reduced in the *gl3 egl3* double mutants (Wang et al., 2008). These results suggested that the expression of *TCL1* and *ETC2* is controlled by unknown mechanisms and that, in addition to the MBW complex, the expression of *TRY*, *CPC*, and *ETC3* may also be controlled by other regulators (Wang et al., 2008). Indeed, we found, later on, that the *miR156*-directed SPL9 is involved in the regulation of trichome formation in Arabidopsis by directly regulating the expression of *TRY* and *TCL1* (Yu et al., 2010). In this study, we identified NTL8 as a novel regulator of trichome formation in Arabidopsis, and found that NTL8 negatively regulates trichome formation by directly activating the expression of *TRY* and *TCL1*.

### NTL8 Is a Novel Negative Regulator of Trichome Formation in Arabidopsis

The NAC transcription factors regulate multiple aspects of plant growth and development (Sablowski and Meyerowitz, 1998; Hibara et al., 2003; Kim et al., 2006; Dong et al., 2014) and plant response to environmental stresses (Tran et al., 2004; Shao et al., 2015). There are more than 100 genes in the Arabidopsis genome encoding NAC transcription factors, and 14 of them are membrane-associated transcription factors (Olsen et al., 2005; Kim et al., 2007a; Liang et al., 2015). NTL8, one of the membrane-associated NAC transcription factors, has been shown to regulate salt-regulated flowering via *FLOWERING LOCUS T* and mediate salt-signaling in seed germination (Kim et al., 2007b, 2008). In this study, we provide evidence that NTL8 is a novel negative regulator of trichome formation in Arabidopsis.

First, the phenotype observed in the gain-of-function mutant *ntl8-1D*, including glabrous inflorescence stem and reduced trichomes on the rosette leaves, was caused by elevated expression of *NTL8* (Fig. 1). Second, overexpression of *NTL8* in the Col wild-type plants resulted in the inhibition of trichome formation on inflorescence stem and reduction in trichome formation on the rosette leaves (Fig. 2). However, loss-of-function mutants of *NTL8* were morphologically similar to the Col wild-type plants (Fig. 3). It is well known that functional redundancy of transcription factor genes in plant is a common phenomenon. For example, all single mutants, and double or triple mutants of closely related *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes exhibit wild-type phenotypes (Overvoorde et al., 2005), and this is also the case for *Arabidopsis thaliana Ovate Family Proteins* (*AtOFPs*; Wang et al., 2007a, 2011). Considering the fact that there are only 29 genes in

Arabidopsis encoding Aux/IAA proteins, and 19 genes encoding AtOFPs, whereas more than 100 genes encoding NAC transcription factors (Olsen et al., 2005; Kim et al., 2007a; Liang et al., 2015), it is likely that NTL8 may function redundantly with other NAC transcription factors to regulate trichome formation in Arabidopsis. The observation that more branched trichomes were produced on stems of *ntl5 ntl8* double mutants (Fig. 3) suggests that this maybe the case. Considering that NTL14 is also closely related to NTL8 (Fig. 3), it will be of great interest to examine if NTL14 and any of the other NAC transcription factors, especially the membrane-associated ones, is involved in the regulation of trichome formation in Arabidopsis.

### Mechanism for the Action of NTL8 in the Regulation of Trichome Formation in Arabidopsis

So far, all the other identified trichome formation-regulating transcription factors, including the C2H2 transcription factors GIS, GIS2, and GIS3, the ZINC FINGER transcription factors ZFP5, ZFP6, and ZFP8, and the miR156-directed transcription factor SPL9, are reported to function through the regulation of, directly or indirectly, the expression of the key transcription factor genes in the trichome formation pathway (Gan et al., 2006, 2007; Yu et al., 2010; Zhou et al., 2011, 2013; An et al., 2012; Sun et al., 2015). Our experimental data suggest that this is also the case with NTL8.

First, transgenic Arabidopsis plants overexpressing *NTL8ΔC* showed a phenotype similar to the plants overexpressing *NTL8* (Fig. 2), indicating that the nucleus localization, rather than the membrane-binding function of NTL8, is critical for its function in regulating trichome formation. Second, expression of R3 MYB genes *TRY* and *TCL1* was elevated in the *ntl8-1D* and the *35S:HA-NTL8* transgenic plants, the expression of *TRY* was reduced in the *ntl8* mutants, and the expression pattern of *NTL8* in inflorescence stem internodes was similar to that of *TRY* and *TCL1* (Fig. 4), suggesting that NTL8 may regulate the expression of *TRY* and *TCL1*. Third, genetic analysis showed that trichome formation in *tcl1 try ntl8-1D* triple mutant was similar to that in the *try tcl1* double mutant (Fig. 5), and more branched trichomes was observed on the stems of the *ntl5 ntl8* double mutants (Fig. 3), a phenotype similar to the *try tcl1* double mutant (Supplemental Fig. S2), implying that NTL8 functioned upstream of TRY and TCL1 in regulating trichome formation. Finally, protoplast transient transfection results showed that NTL8 is a transcriptional activator, and ChIP assays and protoplast transient transfection with mutated *TRY* and *TCL1* promoters indicated that NTL8 can bind to the promoter region of *TRY* and *TCL1* (Fig. 6). Collectively, these results suggested that NTL8 can directly activate the expression of *TRY* and *TCL1*. However, because NTL8 also regulates other aspects of plant growth and development (Kim et al., 2007b, 2008), it is likely that NTL8 may regulate the expression of other genes.

Because SPL9 has been reported to regulate trichome formation in Arabidopsis by directly activating the expression of *TRY* and *TCL1* (Yu et al., 2010) and SPL9 does not function as a transcriptional activator in the protoplast transfection assays (Fig. 6), we examined whether SPL9 may interact with NTL8. Results from two different interaction assays indicated that NTL8 and SPL9 do not directly interact with each other (Fig. 7). These results suggest that NTL8 may function in parallel with SPL9 in regulating the expression of *TRY* and *TCL1*. Crossing between *rSPL9* transgenic plants and *ntl8-1D* mutant suggested a syngenetic effect of SPL9 and NTL8 on trichome formation. However, we still could not rule out the possibility that NTL8, SPL9, and other unknown transcription factors may form an activator complex to regulate the expression of *TRY* and *TCL1*.

As a membrane-associated NAC transcription factor, relocation of NTL8 to the nucleus has been shown to be required for its functions in the regulation of plant growth and development (Kim et al., 2007b, 2008). We show that NTL8 is involved in the regulation of trichome formation in Arabidopsis (Figs. 1–3 and 5), that NTL8ΔC have similar function as NTL8 in regulating trichome formation (Fig. 2), and that NTL8 directly regulates the expression of *TRY* and *TCL1* (Figs. 4 and 6), suggesting that relocation of NTL8 to the nucleus is required for its functions in the regulation of trichome formation. However, it is unclear how NTL8 can be released from membrane and relocated to the nucleus. It is possible that some inner and/or environmental cues may be involved in this process.

## CONCLUSION

We identified NTL8 as a novel regulator of trichome formation. We showed that NTL8 is a transcriptional activator and that it negatively regulates trichome formation in Arabidopsis by directly activating the expression of *TRY* and *TCL1*.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used for plant transformation and protoplast isolation. The *ntl8-1* (WiscDsLoxHs159_07E), *ntl8-2* (SM_3_16309), *ntl5-1* (SALK-012154), and *ntl5-2* (SAIL_172_A04) mutants were obtained from the Arabidopsis Biological Resource Center and are in the Col background. The *try* (*try_2970*), *tcl1* (*tcl1-1*), *try tcl1* (*try_2970 tcl1-1*) mutants, and the *rSPL9* transgenic plant are in the Col background (Esch et al., 2003; Wang et al., 2007b, 2008; Yu et al., 2010). The *gpa1-2* mutant is in the Wassilewskija (Ws) ecotype background (Ullah et al., 2001).

The *ntl8-1D/gpa1-2* mutant was identified from an activation-tagged mutagenized population of Arabidopsis plants in the *gpa1-2* mutant background. The *ntl8-1D* mutant was obtained by crossing the *ntl8-1D/gpa1-2* with the Col wild-type plant, examining the mutant phenotype in the F2 generation, and confirming the homozygous status in the F3 to F4 generations. The *ntl5 ntl8* double mutant was obtained by crossing *ntl5-1* and *ntl8-2*. The *try ntl8-1D* and *tcl1 ntl8-1D* double mutants were generated by crossing *ntl8-1D* with *try* and *tcl1*, respectively. The *try tcl1 ntl8-1D* triple mutant was generated by crossing double mutant *try ntl8-1D* with *tcl1 ntl8-1D*. The *try ntl8-2* and *tcl1 ntl8-2* double

mutants were generated by crossing *ntl8-2* with *try* and *tcl1*, respectively. The *try tcl1 ntl8-2* triple mutant was generated by crossing double mutant *try ntl8-2* with *tcl1 ntl8-2*. The *rSPL9 ntl8-1D* plants were generated by crossing *ntl8-1D* with *rSPL9* transgenic plant.

To obtain plants for plant transformation, protoplast isolation, and phenotypic analysis, Arabidopsis seeds were directly sown into soil in pots. To obtain seedlings for DNA and RNA isolation, Arabidopsis seeds were sterilized and sown on 0.5× Murashige and Skoog (MS) plates with vitamins (PlantMedia) and 1% (w/v) Suc, solidified with 0.6% (w/v) phytoagar (PlantMedia). The plates were kept in dark at 4°C for 2 d before transferred to a growth room. All plants were grown in a growth room with a temperature at 22°C in a long day (16 h light/8 h dark) conditions at approximately 120 $\mu$mol m$^{-2}$ s$^{-1}$.

## Isolation of the *ntl8-1D/gpa1-2* Mutant and Identification of the T-DNA Insertion Site

The *ntl8-1D/gpa1-2* dominant mutant was isolated from an activation-tagged mutant population containing approximately 10,000 plants. The mutant population was generated by transforming *gpa1-2* mutant plants with the activation tagging vector *pSKI015*.

The *NTL8* gene locus was cloned by using plasmid rescue (Weigel et al., 2000). Briefly, genomic DNA (~20 $\mu$g) was isolated from the *ntl8-1D/gpa1-2* mutant and digested with *Pst*I. The digested DNA was purified, ligated, and transformed into *E. coli DH5α*, and the transformants were selected on LB plates containing amplicillin. T-DNA left-boarder primer 5′-TTGACAGTGACGACAAATCG-3′ was used to sequence plasmid DNA isolated from colonies obtained, and to confirm the T-DNA insertion site in the *ntl8-1D/gpa1-2* mutant.

## Plasmid Constructs

The effector constructs *35S:GD*, *35S:GD-VP*, *35S:CAT*, and reporter construct *Gal4-GUS* used for protoplast transfection have been described previously (Tiwari et al., 2001, 2004; Wang et al., 2007a, 2007b).

To generate *NTL8* and *NTL8ΔC* constructs for plant transformation and/or protoplast transfection assays, the full-length open-reading frame (ORF) or ORF encoding amino acid 1-309 of *NTL8* was amplified by RT-PCR and cloned in frame with an N-terminal HA or GD tag into the *pUC19* vector under the control of the double *35S* promoter (Tiwari et al., 2001; Wang et al., 2005). HA-tagged *NTL8* and *NTL8ΔC* constructs in *pUC19* were digested with proper enzymes and subcloned into the binary vector *pPZP211* (Hajdukiewicz et al., 1994). To generate *SPL9* construct for protoplast transfection assays, the full-length ORF of *SPL9* was amplified by RT-PCR and cloned in frame with an N-terminal GD tag into the *pUC19* vector under the control of the double *35S* promoter.

To generate *pDHB1-SPL9* and pPR3-*NTL8ΔC* constructs for yeast two-hybrid assays. The full-length ORF of *SPL9* and ORF of *NTL8ΔC* was amplified and cloned into the *pDHB1*and the *pPR3-N* vector, respectively.

To generate the *NTL8p:GUS*, *TRYp:GUS*, and *TCL1p:GUS* constructs, the 1951-bp *NTL8*, 2138-bp *TRY*, and 1941-bp *TCL1* promoters (Kim et al., 2007b; Yu et al., 2010), were amplified and used to replace the *OFP1* promoter in the *OFP1p:GUS* construct in *pUC19*. The *NTL8p:GUS* construct in *pUC19* was digested with proper enzymes and subcloned into the binary vector *pPZP211* for plant transformation. The *TRYmp:GUS* and *TCL1mp:GUS* constructs were generated by fast mutagenesis using *TRYp:GUS* and *TCL1p:GUS* plasmid DNA as template. The primers used to generate *TRYmp*:GUS are 5′-ACATGACTCGCCTCAAGAAATGTCTTTCG-3′ and 5′-TTCTTGAGGCGAGTCATGTGATGTATATC-3′, and for *TCL1mp:GUS* are 5′-AAATCAAATCGAGGGAACCAAAAAAAAGT-3′ and 5′-GGTTCCCTCGATTTGATTTCTCAAACCT-3′.

## Plant Transformation and Transgenic Plant Selection

About 5-week-old soil-grown Col wild type plants with several mature flowers on the main inflorescence were used for plant transformation. The transgenic plants were generated with the *35S:HA-NTL8* or *35S:HA-NTL8ΔC* construct via *Agrobacterium tumefaciens* GV3101 by using the floral-dip method (Clough and Bent, 1998). Surface-sterilized T1 seeds were grown on 0.5× MS medium containing 50 $\mu$g/mL Kanamycin and 100 $\mu$g/mL carbenicillin to select transgenic plants, and ~10-d-old seedlings of the transgenic plants were transferred into soil pots. Phenotypes of the transgenic plants were observed in the T1 generation, and overexpression of *NTL8* or *NTL8ΔC* in the transgenic plants was confirmed by RT-PCR. For each construct, at least five transgenic lines with similar phenotypes were observed, and two of them were used for detailed analysis. *NTL8p:GUS* transgenic plants were generated as described by Kim et al. (2007b).

## DNA and RNA Isolation, RT-PCR, and qRT-PCR

DNA was isolated from 2-week-old seedlings using NuClean PlantGen DNA Kit (CWBIO) according to the manufacturer's instructions. Total RNA was isolated from 10-d-old seedlings or different tissues and organs using EasyPure Plant RNA Kit (TransGen Biotech) according to the manufacturer's instructions, and 2 $\mu$g RNA was used for cDNA synthesized by oligo(dT)-primed reverse transcription using the EasyScript First-Strand DNA Synthesis Super Mix (TransGene Biotech). The primers used for RT-PCR examination of *NTL8* are 5′-ATGTCTAAAGAAGCTGAGATG-3′ and 5′-TTAGTTCCTAGCTATTAATACAGTCC-3′, for *NTL5* 5′-are GCAGCTGCACCACCGATCGAG-3′ and 5′-TCACACTAAATAAAACACCAACAC-3′, and for *At2g27310* are 5′-ATGGCAACCG TCGATGTC-3′ and 5′-TTACCTTAACAAAATAAAAC-3′. Arabidopsis gene *ACTIN2* (*ACT2*) were used as controls for RT-PCR and qRT-PCR. The primers used for examining the expression of the MBW complex component gene and their target genes have been described previously (Wang et al., 2007b, 2008; Wang and Chen, 2008; Zheng et al., 2016).

## Plasmid DNA Isolation, Protoplast Isolation, Transfection, and GUS Activity Assays

Reporter and effector plasmid DNA were isolated using the GoldHi Endo Free Plasmid MaxiKit (CWBIO) according to the manufacturer's instructions. Protoplast isolation, transfection, and GUS activity assay were performed as described previously (Tiwari et al., 2003; Wang et al., 2005, 2007a, 2007b, 2015; Wang and Chen, 2008; Tian et al., 2015; Dai et al.,2016; Zheng et al.,2016). In brief, protoplasts were isolated from rosette leaves of 3- to 4-week-old soil-grown Col wild-type plants, cotransfected with effector and reporter plasmids, incubated under darkness at room temperature for 20 to 22 h before GUS activities were measured by using a Synergy HT microplate reader (BioTEK).

## Microscopy

Photographs were taken under a Motic K microscope equipped with an EOS 1100D digital camera. The first two leaves of soil-grown plants were used for observing leaf trichomes, and soil-grown adult plants were used for observing of stem and pedicel trichomes.

## ChIP Assay

ChIP assay was performed as described previously (Wang et al., 2007a, 2007b, 2015). In brief, ~3-week-old *35S:HA-NTL8* transgenic seedlings were cross linked using 1% formaldehyde solution, ground to powder with liquid nitrogen, and sonificated using a sonifier. Soluble chromatin was precipitated using anti-HA antibodies or rabbit preimmune sera, and collected by using salmon sperm DNA/protein A-agarose. DNA-protein cross-links were reversed, and the DNA was purified and used in qRT-PCR reactions. The primers used for amplifying the promoter region of DRF have been described previously (Wang et al., 2015). The primers used for the promoter region of *TRY* are 5′-GCAATGAGGATGATGCGATATAC-3′ and 5′-GATTAAACGGAAGAAATCTGTAGTC-3′, and for the promoter region of *TCL1* are 5′-GAAATCAAATCGAGGGCGTAAA-3′ and 5′-CCCAATGAACGGTACATGATTC-3′.

## Yeast Two-Hybrid Assays

The split-ubiquitin system (Stagljar et al., 1998) was used to examine the interaction between SPL9 and NTL8. *SPL9* was cloned into the bait vector *pDHB1* and *NTL8ΔC* into the prey vector *pPR3-N*. Bait and prey constructs were cotransformed into yeast strain *NMY51*. Cotransformation of empty bait and prey vectors was used as a negative control, and cotransformation of *pNubG-Fe65* and *pOst1-NubI* constructs were used as a positive control. The yeast transformants containing both prey and bait were able to grow on minimum synthetic dextrose dropout medium lacking both Trp and Leu (SD-Trp-Leu). A positive interaction between two proteins was indicated by the growth of yeast colony on the minimum synthetic dextrose medium lacking Leu, Trp, His, and Ade (SD-Trp-Leu-His-Ade) supplied with 15 mM 3-AT and a white color due to the activation of Ade synthesis.

## Phylogenetic Analysis

Proteins with higher similarities with NTL8 were identified, and their entire amino acid sequences were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). "OneClick" mode with default settings on Phylogeny (www.phylogeny.fr) was used to generate phylogenetic tree.

## Histochemical Staining for GUS Activity

Histochemical staining was used to examine the GUS activity using substrate 5-bromo-4-chloro-3-indolyl-$\beta$-D-glucuronide (Kim et al., 2007b; Wang et al., 2007a).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Expression pattern of *NTL8*.

**Supplemental Figure S2.** Loss of function of *NTL8* affects ectopic trichome formation in *tcl1* and *try tcl1* mutant.

## ACKNOWLEDGMENTS

We thank Dr. Xiao-Ya Chen (Chinese Academy of Sciences) for providing seeds of the *SPL9p:rSPL9* transgenic plants, Drs. Tom Guilfoyle and Gretchen Hagen (University of Missouri-Columbia) for providing the effector construct *GD* and the reporter construct *Gal4:GUS* for protoplast transfection assays, Dr. Brian Ellis (University of British Columbia) for providing vectors for yeast two-hybrid analysis, and all the lab members for their helpful discussion.

Received April 14, 2017; accepted June 19, 2017; published June 25, 2017.

## LITERATURE CITED

**An L, Zhou Z, Sun L, Yan A, Xi W, Yu N, Cai W, Chen X, Yu H, Schiefelbein J, et al** (2012) A zinc finger protein gene ZFP5 integrates phytohormone signaling to control root hair development in Arabidopsis. Plant J **72:** 474–490

**Bouyer D, Geier F, Kragler F, Schnittger A, Pesch M, Wester K, Balkunde R, Timmer J, Fleck C, Hülskamp M** (2008) Two-dimensional patterning by a trapping/depletion mechanism: The role of TTG1 and GL3 in Arabidopsis trichome formation. PLoS Biol **6:** e141

**Clough SJ, Bent AF** (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

**Dai X, Zhou L, Zhang W, Cai L, Guo H, Tian H, Schiefelbein J, Wang S** (2016) 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. Plant Cell Environ **39:** 897–907

**De Clercq I, Vermeirssen V, Van Aken O, Vandepoele K, Murcha MW, Law SR, Inzé A, Ng S, Ivanova A, Rombaut D, et al** (2013) The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in Arabidopsis. Plant Cell **25:** 3472–3490

**Di Cristina M, Sessa G, Dolan L, Linstead P, Baima S, Ruberti I, Morelli G** (1996) The Arabidopsis Athb-10 (GLABRA2) is an HD-Zip protein required for regulation of root hair development. Plant J **10:** 393–402

**Digiuni S, Schellmann S, Geier F, Greese B, Pesch M, Wester K, Dartan B, Mach V, Srinivas BP, Timmer J, et al** (2008) A competitive complex formation mechanism underlies trichome patterning on Arabidopsis leaves. Mol Syst Biol **4:** 217

**Dong Y, Yang X, Liu J, Wang BH, Liu BL, Wang YZ** (2014) Pod shattering resistance associated with domestication is mediated by a NAC gene in soybean. Nat Commun **5:** 3352

**Esch JJ, Chen M, Sanders M, Hillestad M, Ndkium S, Idelkope B, Neizer J, Marks MD** (2003) A contradictory GLABRA3 allele helps define gene interactions controlling trichome development in Arabidopsis. Development **130:** 5885–5894

**Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K** (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J **39:** 863–876

**Galway ME, Masucci JD, Lloyd AM, Walbot V, Davis RW, Schiefelbein JW** (1994) The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. Dev Biol **166:** 740–754

**Gan Y, Kumimoto R, Liu C, Ratcliffe O, Yu H, Broun P** (2006) GLABROUS INFLORESCENCE STEMS modulates the regulation by gibberellins of epidermal differentiation and shoot maturation in Arabidopsis. Plant Cell **18:** 1383–1395

**Gan Y, Liu C, Yu H, Broun P** (2007) Integration of cytokinin and gibberellin signalling by Arabidopsis transcription factors GIS, ZFP8 and GIS2 in the regulation of epidermal cell fate. Development **134:** 2073–2081

**Gan L, Xia K, Chen JG, Wang S** (2011) Functional characterization of TRICHOMELESS2, a new single-repeat R3 MYB transcription factor in the regulation of trichome patterning in Arabidopsis. BMC Plant Biol **11:** 176

**Gou JY, Felippes FF, Liu CJ, Weigel D, Wang JW** (2011) Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. Plant Cell **23:** 1512–1522

**Hajdukiewicz P, Svab Z, Maliga P** (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol **25:** 989–994

**Hibara K, Takada S, Tasaka M** (2003) CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. Plant J **36:** 687–696

**Johnson HB** (1975) Plant pubescence: An ecological perspective. Bot Rev **41:** 233–258

**Kim SY, Kim SG, Kim YS, Seo PJ, Bae M, Yoon HK, Park CM** (2007a) Exploring membrane-associated NAC transcription factors in Arabidopsis: implications for membrane biology in genome regulation. Nucleic Acids Res **35:** 203–213

**Kim SG, Kim SY, Park CM** (2007b) A membrane-associated NAC transcription factor regulates salt-responsive flowering via FLOWERING LOCUS T in Arabidopsis. Planta **226:** 647–654

**Kim YS, Kim SG, Park JE, Park HY, Lim MH, Chua NH, Park CM** (2006) A membrane-bound NAC transcription factor regulates cell division in Arabidopsis. Plant Cell **18:** 3132–3144

**Kim SG, Lee AK, Yoon HK, Park CM** (2008) A membrane-bound NAC transcription factor NTL8 regulates gibberellic acid-mediated salt signaling in Arabidopsis seed germination. Plant J **55:** 77–88

**Kirik V, Simon M, Huelskamp M, Schiefelbein J** (2004a) The ENHANCER OF TRY AND CPC1 gene acts redundantly with TRIPTYCHON and CAPRICE in trichome and root hair cell patterning in Arabidopsis. Dev Biol **268:** 506–513

**Kirik V, Simon M, Wester K, Schiefelbein J, Hulskamp M** (2004b) ENHANCER of TRY and CPC 2 (ETC2) reveals redundancy in the region-specific control of trichome development of Arabidopsis. Plant Mol Biol **55:** 389–398

**Lee S, Lee HJ, Huh SU, Paek KH, Ha JH, Park CM** (2014) The Arabidopsis NAC transcription factor NTL4 participates in a positive feedback loop that induces programmed cell death under heat stress conditions. Plant Sci **227:** 76–83

**Li S, Wang N, Ji DD, Xue ZY, Yu YC, Jiang YP, Liu JL, Liu ZH, Xiang FN** (2016) Evolutionary and function analysis of membrane-bound NAC transcription factor genes in soybean. Plant Physiol **172:** 1804–1820

**Liang M, Li H, Zhou F, Li H, Liu J, Hao Y, Wang Y, Zhao H, Han S** (2015) Subcellular distribution of NTL transcription factors in *Arabidopsis thaliana*. Traffic **16:** 1062–1074

**Lin Q, Aoyama T** (2012) Pathways for epidermal cell differentiation via the homeobox gene GLABRA2: Update on the roles of the classic regulator. J Integr Plant Biol **54:** 729–737

**Mauricio R, Rausher MD** (1997) Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. Evolution **51:** 1435–1444

**Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD, Schiefelbein JW** (1996) The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. Development **122:** 1253–1260

**Olsen AN, Ernst HA, Leggio LL, Skriver K** (2005) NAC transcription factors: Structurally distinct, functionally diverse. Trends Plant Sci **10:** 79–87

**Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD** (1991) A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. Cell **67:** 483–493

**Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Liu A, Onodera C, Quach H, et al** (2005) Functional genomic

analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in *Arabidopsis thaliana*. Plant Cell **17:** 3282–3300

**Payne CT, Zhang F, Lloyd AM** (2000) GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. Genetics **156:** 1349–1362

**Pesch M, Hülskamp M** (2004) Creating a two-dimensional pattern de novo during Arabidopsis trichome and root hair initiation. Curr Opin Genet Dev **14:** 422–427

**Ramsay NA, Glover BJ** (2005) MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci **10:** 63–70

**Rerie WG, Feldmann KA, Marks MD** (1994) The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. Genes Dev **8:** 1388–1399

**Sablowski RW, Meyerowitz EM** (1998) A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell **92:** 93–103

**Schellmann S, Hülskamp M, Uhrig J** (2007) Epidermal pattern formation in the root and shoot of Arabidopsis. Biochem Soc Trans **35:** 146–148

**Schellmann S, Schnittger A, Kirik V, Wada T, Okada K, Beermann A, Thumfahrt J, Jürgens G, Hülskamp M** (2002) TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in Arabidopsis. EMBO J **21:** 5036–5046

**Schiefelbein J** (2003) Cell-fate specification in the epidermis: A common patterning mechanism in the root and shoot. Curr Opin Plant Biol **6:** 74–78

**Schilmiller AL, Last RL, Pichersky E** (2008) Harnessing plant trichome biochemistry for the production of useful compounds. Plant J **54:** 702–711

**Schnittger A, Folkers U, Schwab B, Jürgens G, Hülskamp M** (1999) Generation of a spacing pattern: The role of triptychon in trichome patterning in Arabidopsis. Plant Cell **11:** 1105–1116

**Seo PJ, Kim MJ, Park JY, Kim SY, Jeon J, Lee YH, Kim J, Park CM** (2010) Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. Plant J **61:** 661–671

**Serna L** (2005) Epidermal cell patterning and differentiation throughout the apical-basal axis of the seedling. J Exp Bot **56:** 1983–1989

**Shao H, Wang H, Tang X** (2015) NAC transcription factors in plant multiple abiotic stress responses: Progress and prospects. Front Plant Sci **6:** 902

**Stagljar I, Korostensky C, Johnsson N, te Heesen S** (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc Natl Acad Sci USA **95:** 5187–5192

**Sun L, Zhang A, Zhou Z, Zhao Y, Yan A, Bao S, Yu H, Gan Y** (2015) GLABROUS INFLORESCENCE STEMS3 (GIS3) regulates trichome initiation and development in Arabidopsis. New Phytol **206:** 220–230

**Telfer A, Bollman KM, Poethig RS** (1997) Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. Development **124:** 645–654

**Tian H, Guo H, Dai X, Cheng Y, Zheng K, Wang X, Wang S** (2015) An ABA down-regulated bHLH transcription repressor gene, bHLH129 regulates root elongation and ABA response when overexpressed in Arabidopsis. Sci Rep **5:** 17587

**Tiwari SB, Hagen G, Guilfoyle T** (2003) The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell **15:** 533–543

**Tiwari SB, Hagen G, Guilfoyle TJ** (2004) Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell **16:** 533–543

**Tiwari SB, Wang XJ, Hagen G, Guilfoyle TJ** (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell **13:** 2809–2822

**Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K** (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell **16:** 2481–2498

**Ullah H, Chen JG, Young JC, Im KH, Sussman MR, Jones AM** (2001) Modulation of cell proliferation by heterotrimeric G protein in Arabidopsis. Science **292:** 2066–2069

**Wada T, Kurata T, Tominaga R, Koshino-Kimura Y, Tachibana T, Goto K, Marks MD, Shimura Y, Okada K** (2002) Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. Development **129:** 5409–5419

**Wada T, Tachibana T, Shimura Y, Okada K** (1997) Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. Science **277:** 1113–1116

**Wagner GJ, Wang E, Shepherd RW** (2004) New approaches for studying and exploiting an old protuberance, the plant trichome. Ann Bot (Lond) **93:** 3–11

**Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC** (1999) The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. Plant Cell **11:** 1337–1350

**Wang S, Chang Y, Guo J, Chen JG** (2007a) Arabidopsis Ovate Family Protein 1 is a transcriptional repressor that suppresses cell elongation. Plant J **50:** 858–872

**Wang S, Chang Y, Guo J, Zeng Q, Ellis BE, Chen JG** (2011) Arabidopsis ovate family proteins, a novel transcriptional repressor family, control multiple aspects of plant growth and development. PLoS One **6:** e23896

**Wang S, Chen JG** (2008) Arabidopsis transient expression analysis reveals that activation of GLABRA2 may require concurrent binding of GLABRA1 and GLABRA3 to the promoter of GLABRA2. Plant Cell Physiol **49:** 1792–1804

**Wang S, Chen JG** (2014) Regulation of cell fate determination by single-repeat R3 MYB transcription factors in Arabidopsis. Front Plant Sci **5:** 133

**Wang S, Hubbard L, Chang Y, Guo J, Schiefelbein J, Chen JG** (2008) Comprehensive analysis of single-repeat R3 MYB proteins in epidermal cell patterning and their transcriptional regulation in Arabidopsis. BMC Plant Biol **8:** 81

**Wang S, Kwak SH, Zeng Q, Ellis BE, Chen XY, Schiefelbein J, Chen JG** (2007b) TRICHOMELESS1 regulates trichome patterning by suppressing GLABRA1 in Arabidopsis. Development **134:** 3873–3882

**Wang S, Tiwari SB, Hagen G, Guilfoyle TJ** (2005) AUXIN RESPONSE FACTOR7 restores the expression of auxin-responsive genes in mutant Arabidopsis leaf mesophyll protoplasts. Plant Cell **17:** 1979–1993

**Wang X, Wang X, Hu Q, Dai X, Tian H, Zheng K, Wang X, Mao T, Chen JG, Wang S** (2015) Characterization of an activation-tagged mutant uncovers a role of GLABRA2 in anthocyanin biosynthesis in Arabidopsis. Plant J **83:** 300–311

**Weigel D, Ahn JH, Blázquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrándiz C, Kardailsky I, Malancharuvil EJ, Neff MM, et al** (2000) Activation tagging in Arabidopsis. Plant Physiol **122:** 1003–1013

**Wester K, Digiuni S, Geier F, Timmer J, Fleck C, Hülskamp M** (2009) Functional diversity of R3 single-repeat genes in trichome development. Development **136:** 1487–1496

**Yang ZT, Wang MJ, Sun L, Lu SJ, Bi DL, Sun L, Song ZT, Zhang SS, Zhou SF, Liu JX** (2014) The membrane-associated transcription factor NAC089 controls ER-stress-induced programmed cell death in plants. PLoS Genet **10:** e1004243

**Yao D, Wei Q, Xu W, Syrenne RD, Yuan JS, Su Z** (2012) Comparative genomic analysis of NAC transcriptional factors to dissect the regulatory mechanisms for cell wall biosynthesis. BMC Bioinformatics (Suppl) **13:** S10

**Yu N, Cai WJ, Wang S, Shan CM, Wang LJ, Chen XY** (2010) Temporal control of trichome distribution by microRNA156-targeted SPL genes in *Arabidopsis thaliana*. Plant Cell **22:** 2322–2335

**Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A** (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development **130:** 4859–4869

**Zheng K, Tian H, Hu Q, Guo H, Yang L, Cai L, Wang X, Liu B, Wang S** (2016) Ectopic expression of R3 MYB transcription factor gene OsTCL1 in Arabidopsis, but not rice, affects trichome and root hair formation. Sci Rep **6:** 19254

**Zhou Z, An L, Sun L, Zhu S, Xi W, Broun P, Yu H, Gan Y** (2011) Zinc finger protein5 is required for the control of trichome initiation by acting upstream of zinc finger protein8 in Arabidopsis. Plant Physiol **157:** 673–682

**Zhou Z, Sun L, Zhao Y, An L, Yan A, Meng X, Gan Y** (2013) Zinc Finger Protein 6 (ZFP6) regulates trichome initiation by integrating gibberellin and cytokinin signaling in *Arabidopsis thaliana*. New Phytol **198:** 699–708