A regulatory gene induces trichome formation and embryo lethality in tomato

Changxian Yang[a], Hanxia Li[a], Junhong Zhang[a], Zhidan Luo[a], Pengjuan Gong[a], Chanjuan Zhang[a], Jinhua Li[a], Taotao Wang[a], Yuyang Zhang[b], Yong'en Lu[b], and Zhibiao Ye[a,b,1]

[a]Key Laboratory of Horticultural Plant Biology, Ministry of Education, and [b]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China

Edited* by Qifa Zhang, Huazhong Agricultural University, Wuhan, China, and approved May 20, 2011 (received for review January 13, 2011)

Trichomes are universal biological structures originating from the aerial epidermis, which serve as an excellent model to study plant differentiation at the cell level. Although the pathway regulating trichome formation in the Rosids has been well characterized, only very recently a few genes were identified for trichome initiation in the Asterids. In this study, we cloned *Woolly* (*Wo*), essential for trichome formation in tomato. Transgenic experiments revealed that the woolly phenotype is caused by the mutation in *Wo* which encodes a homeodomain protein containing a bZIP motif and a START domain. We identified three alleles of *Wo* and found that each allele contains a missense mutation, which respectively results in an amino acid substitution at the C terminus. Microarray and expression analysis showed that the expression of a B-type cyclin gene, *SlCycB2*, is possibly regulated by *Wo*, which also participates in trichome formation. Suppression of *Wo* or *SlCycB2* expression by RNAi decreased the number of type I trichomes, and direct protein–protein interaction was detected between them, implying that both proteins may work together in the regulation of this type of trichome formation. Cytological observation and *Wo* transcript analysis in the developing seeds showed that embryo development was also correlated with *Wo*.

cell cycle | multicellular trichome

The development of plants, like all multicellular eukaryotes, lies on the appropriate differentiation of distinct cell types (1). Cell differentiation requires that undifferentiated cells must be chosen before becoming committed to a specified cell (2). In recent years, many scientists have shifted the focus on plant developmental biology from the organization of the whole body or individual organs toward the cell fate determination of the smallest unit of organism, the single cell (3). Plant trichomes, which are found on the aerial epidermal surfaces of nearly all terrestrial plants, provide an excellent model system to study plant development at the single-cell level. Trichomes in the model plant *Arabidopsis thaliana* are single cells that distribute on the epidermis in a regular pattern (4). With numerous *Arabidopsis* mutants exhibiting trichome-related phenotypes, molecular studies have enabled the identification of several key regulators participating in trichome initiation. For example, the key gene *GLABROUS1* (*GL1*) controlling trichome formation in *Arabidopsis* was among the first cloned (5). This gene encodes a protein belonging to the R2R3 MYB family, with two MYB repeats as the DNA binding domain (6). Another important regulator, *TRANSPARENT TESTA GLABRA1* (*TTG1*), also required for trichome formation in *Arabidopsis* (7), encodes a protein containing four conserved WD repeats (8). Combination of these two regulators with another two basic helix–loop–helix proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) forms a regulatory complex (9). This complex activates trichome formation by enhancing the expression of two targeted genes, *GLABRA2* (*GL2*) and *ENHANCER OF GLABRA2* (*EGL2*) (10, 11). Interestingly, the formation of cotton (*Gossypium hirsutum*) fibers resembling trichomes in *Arabidopsis* is controlled by *GaMYB2*, which shows high sequence homology to *GL1* and can restore trichome formation in a *gl1* mutant (12), indicating that these two species share a common pathway controlling trichome formation.

Both *Arabidopsis* and cotton belong to the clades of Rosids (13). Whether there is a similar pathway controlling trichome formation in the clades of Asterids is yet unknown. It was shown that overexpression of MIXTA, an MYB-related regulator controlling conical cell formation in snapdragon (*Antirrhinum majus*), could trigger trichome formation in *A. majus* (14) and tobacco (*Nicotiana tabacum*) (15). Another two MIXTA-like genes also function as activators, inducing trichome differentiation in woody nightshade (*Solanum dulcamara*) (16) and petunia (*Petunia hybrida*) (17). However, overexpression of *GL1* in tobacco or ectopic expression of MIXTA in *Arabidopsis* does not induce trichome formation (15). In addition, ectopic expression of MIXTA in *gl1-1 Arabidopsis* mutant failed to rescue the trichome phenotype (15). These results suggested that trichome formation in the Asterid species such as snapdragon and petunia may engage a different regulatory pathway.

Homeodomain-leucine zipper (HD-Zip) proteins are unique to the plant kingdom, and contain two indispensable conserved domains, HD domain and bZIP motif (18). The former is responsible for DNA binding and the latter for protein–protein interaction (19). Based on gene structures and additional conserved domains, Ariel et al. classified HD-ZIP proteins into four subgroups (HD-Zip I–IV) (20). Each subgroup is involved in different biological processes. Results from *Arabidopsis* studies demonstrated that members of HD-Zip IV (characterized by a START domain) mainly regulated trichome formation and epidermal cell differentiation, such as *GL2* (21) and *PROTODERMAL FACTOR2* (*PDF2*) (22). However, it remains unknown whether trichome formation in tomato is also regulated by the proteins of this subgroup.

Woolly (*Wo*) is a spontaneous mutation in tomato (*Solanum lycopersicum*), which is responsible for trichome formation (23) and embryo lethality (24). Here we show that *Wo* encodes an HD-Zip protein containing a START domain. We further found that a B-type cyclin gene, *SlCycB2*, also participates in type I trichome formation in tomato, expression of which may be regulated by *Wo*. In addition, Wo physically interacts with SlCycB2. Our findings not only identify a mechanism for trichome formation in plants, but also shed light on the intrinsic relationship between trichome formation and embryo lethality in tomato.

Results

Map-Based Cloning of *Wo*. We have previously mapped *Wo* to an approximately 200-kb fragment by using an F_2 population from a cross between an introgression line 2–3 and woolly mutant LA3186, in which 19 putative ORFs (ORFs 1–19) were predicted (Fig. S1). The predicted protein corresponding to ORF4 showed 73% amino acid sequence identity to a HD-like gene, *PDF2*, which encodes a transcription factor and regulates the shoot epidermal cell differentiation in *Arabidopsis* (22). As trichomes originate from the aerial epidermal cells, we considered this ORF (ORF4) as a good candidate for *Wo*. The allele of *Wo* in LA3186 is embryo-lethal when homozygous; therefore, this locus remains heterozygous. We amplified and sequenced the genomic and cDNA sequences of this candidate from LA3186, and identified two alleles. One allele is identical to the se-

Author contributions: C.Y. designed research; C.Y., J.Z., Z.L., and J.L. performed research; C.Y., H.L., P.G., C.Z., T.W., Y.Z., Y.L., and Z.Y. analyzed data; and C.Y. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: zbye@mail.hzau.edu.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1100532108/-/DCSupplemental.

quence from the cultivar Ailsa Craig (AC), and the other contains a missense mutation (Fig. S2). This finding further supported the inference that the candidate sequence was the gene responsible for the woolly phenotype.

Because *Wo* allele in LA3186 is a dominant mutation, we transformed nonwoolly plants with the mutant sequence and expected the transformants to be woolly. We prepared overexpression construct for this candidate gene and introduced this construct into nonwoolly segregants by stable transformation. Finally, we obtained seven independent T_0 plants (hereafter referred to as ORF4-NW). Compared with the transgene-negative plants, all these T_0 plants exhibited increased trichome density to various degrees. Two of these plants showed typical woolly phenotype, even with much higher trichome density on leaves than LA3186 (Fig. 1*A*). However, these T_0 plants showed varying degrees of deformity, such as plant dwarfism, leaf curvature, flower bud abnormality (Fig. S3), and no fruit setting. In particular, the two T_0 plants with typical woolly phenotype never grow up and have no lateral root formation. In addition, we also generated RNAi transgenic plants and found that they showed "no trichome" on the epidermis of all aerial parts (Fig. 1*B*), strongly suggesting ORF4 to be the candidate for *Wo*.

As the ORF4-NW transgenic plants produce no fruit, cosegregation analysis cannot be carried out for them. Therefore, we examined just the trichome phenotype of the resulting T_1 family from one RNAi T_0 plants, which contain a single copy transgene. A total of 23 T_1 plants were obtained, 17 of which were positive and the remaining six of which were negative. All the positive segregants showed no trichome, and negative segregants showed normal phenotype. Such perfect cosegregation between transgene and trichome phenotype confirmed that the ORF4 was the right candidate for *Wo*.

Trichome Type(s) Controlled by *Wo*. Previous studies showed that there were seven trichome types (I–VII) on epidermis of tomato plants (25). To investigate which trichome type(s) causes the phenotypic changes, the trichomes on LA3186, nonwoolly segregants, and RNAi-knockdown plants were observed with SEM. Type I trichome density of LA3186 was much higher than that of the nonwoolly segregants, whereas the density of other trichome types was slightly reduced (Fig. S4). Type I trichomes were characterized by a multicellular base, a long multicellular stalk, and a small glandular tip (25). Trichome clusters of this type of trichomes were also observed at some trichome initiation sites on LA3186 (Fig. S4). However, this type of trichomes were nearly absent from the RNAi-knockdown plants (Fig. S4). Thus, we concluded that type I trichomes accounted for the woolly phenotype and that their formation was regulated by *Wo*. Different trichome types on the species that produce more than one trichome types are regulated by distinct pathways, such as tobacco and petunia (15, 16, 26). Our conclusion is consistent with this inference.

Fig. 1. Transgenic analysis of the candidate ORF4. (*A*) Trichome phenotype of transgenic plants overexpressing ORF4 (*Right*, *Middle*) and negative plants (*Left*). (*B*) Trichome phenotype of transgenic plants by RNAi silencing of ORF4 (right two) and negative plants (left two). (Scale bars: 0.15 cm.)

Intra- and Interspecific Allelic Polymorphism at *Wo*. The predicted coding sequence of *Wo* was 2,193 bp based on the annotated genomic sequence of the cultivar Heinz 1706. The protein encoded by this sequence consisted of 730 aa. Through genomic and cDNA sequence comparison of *Wo*, we identified 10 exons and 9 introns. As mentioned earlier, there was one nucleotide substitution in the predicted coding sequence of Wo^- (designated Wo^- in LA3186), which caused one amino acid substitution from Pro-635 to Arg at its C terminus (Fig. S5). Besides LA3186, there are several other woolly mutants maintained in our laboratory, such as LA0053 (Wo^-), LA1531 (Wo^v), MF802 (unknown), LA0258 (Wo^m), and LA1908 (Wo^{mz}). According to the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu), these mutants have different trichome phenotypes. To determine whether there were mutations in the allelic sequences of *Wo* from these mutants, we sequenced and aligned these alleles. By using the cDNA sequence of *wo* as the reference, all of these alleles contain one nucleotide substitution in their 3′ portion of the coding region, respectively (Fig. S6). Alleles of *Wo* in LA0053 and MF802 are identical with Wo^-, including a nucleotide substitution at 1,904 bp downstream of the predicted translation initiation site (TIS). Alleles of *Wo* in LA1531 and LA0258 are identical and homozygous at this locus, including a nucleotide substitution at 1,700 bp downstream of the predicted TIS, which led to an amino acid substitution from Arg-567 to Leu (Fig. S5). Wo^{mz} in LA1908 contains a point mutation at 2,075 bp downstream of the predicted TIS, resulting in an amino acid change from Ile-692 to Thr (Fig. S5). These data indicate that different mutant sites in these alleles result in different trichome phenotypes.

The predicted Wo protein is a member of the HD gene family, which contains a bZIP motif and a START domain (Fig. S5). We further aligned the amino acid sequences encoded by these alleles with 11 homologues from other species, which were all annotated as HD proteins at the National Center for Biotechnology Information. Whereas the homologous gene in *Vitis vinifera* (XP_002266688) had a high amino acid sequence identity with Wo (75%), the one in *Gossypium hirsutum* (AAQ16126) had only a 46% sequence identity with Wo. The remaining nine homologues showed approximately 46% to 75% sequence identity to Wo. Notably, the sites of the mutation within their C terminus described earlier are highly conserved among these homologues from other species (Fig. S5), suggesting that these mutant sites may be functionally important.

Subcellular Localization and Expression Pattern. To investigate the subcellular localization of Wo, we fused the coding sequence of *Wo* with that of EGFP driven by the CaMV 35S promoter (*35S*::*Wo*:*EGFP*) and bombarded the construct into onion epidermal cells. Transient expression of this construct showed that the fusion protein Wo-EGFP localized to the nucleus and membrane (Fig. S7*C*).

To examine the spatial expression pattern of *Wo*, we conducted RT-PCR analysis with total RNA extracted from several tissues of LA3186 and its nonwoolly segregants, including young stems, functional leaves, shoot apexes, flowers, green fruits, and roots. *Wo* is expressed in all tested tissues, being highest in the shoot apexes (Fig. S7*A*). We further carried out RNA in situ hybridization and *Wo* promoter-driven GUS-YFP transformation to analyze sites of *Wo* expression. The RNA in situ hybridization showed that the transcripts of *Wo* were mainly localized in the shoot apical meristem, leaf primordia, and epidermis of the young stem (Fig. S7*B*). There was no obvious difference in its expression pattern between LA3186 and its nonwoolly segregants, indicating that the woolly phenotype may be caused by amino acid substitution in Wo rather than its transcriptional changes. No phenotypic change in transgenic plants overexpressing *wo* cDNA under control of the CaMV 35S promoter also support this inference. GUS was strongly expressed in shoot apexes, axillary meristems, young stems, and adaxial young leaves (Fig. S8*A*). In addition, we also observed high GUS activity in the developing embryos (Fig. 2*B*) and the gland cells of type VII trichomes (Fig. 3*B*), and high YFP activity in the lateral root primordia (Fig. S8*B*). The expression pattern of *Wo* is consistent with its pleiotropic effects, which not only regulate trichome formation, but also participate in some other development processes, such as embryo development, lateral root differentiation, and even possibly synthesis of secondary metabolites in the type VII trichomes.

Identification of *SlCycB2* as a Potential Target of *Wo*. Because *Wo* is a transcription factor and can activate the formation of type I tri-

chomes, each with a multicellular stalk, this suggests that it may promote the formation of this type of trichomes by controlling the expression of cell cycle-related genes. To test this hypothesis, we compared the transcriptomes of LA3186 and its nonwoolly segregants by using tomato TOM2 microarrays. To minimize the possible exaggerated difference caused by subsequent development, we extracted total RNA from the plantlets with only one true leaf for microarray hybridization. From this analysis, we detected approximate 150 genes with elevated transcript levels in LA3186 (Table S1). Many of these up-regulated genes participate in secondary metabolites synthesis, such as flavonol synthase (27), cytochrome P450 (28), and GST (29). As we expected, a unigene SGN-U226950 similar to AT5G06270.1, a hypothetical B-type cyclin (CYCB) in *Arabidopsis*, was found. CYCBs regulate the transition from gap 2 (G2) to mitosis (M) (30). Ectopic expression of *CYCLIN B1;2* in trichomes can induce mitotic divisions and result in the formation of trichome clusters and multicellular trichomes (31). As leaves of LA3186 have obviously increased numbers of trichome clusters and multicellular trichomes (type I), we speculated that SGN-U226950 (referred to as *SlCycB2*) may function downstream of *Wo* and thereby regulate trichome formation in the tomato.

We analyzed the expression of *SlCycB2* in young leaves of LA0258, LA1908, and the *Wo* transgenic plants by using quantitative RT-PCR. *SlCycB2* expression was also up-regulated in these two allelic lines (Fig. S9), and more importantly, it was significantly higher in *Wo*-overexpressing transgenic plants but lower in *Wo*-RNAi plants (Fig. 3*A*). Moreover, we investigated the expression pattern of *SlCycB2* in different tissues of LA3186 by using RT-PCR. The analysis revealed no significant differences in these tissues between *SlCycB2* and *Wo* (Fig. S9). These results further indicated that *SlCycB2* may be regulated by *Wo*. Next, we down-regulated *SlCycB2* by RNAi in LA3186 and found that the RNAi lines showed obvious decrease in trichome density (Fig. 4). SEM observation showed that the type I trichomes nearly disappeared, and the type V-like trichomes increased, most of which have many branches (Fig. 4). We therefore speculated that *Wo* promotes type I trichome formation by up-regulating the expression of *SlCycB2*, which may induce a shift from endoreduplication to mitosis.

Interaction Between Wo and SlCycB2. It has been shown that HD functions in DNA binding and bZIP in protein–protein interaction (19). Therefore, first, we detected the *cis*-element of the *SlCycB2* promoter sequence by using the PLACE program (http://www.dna.affrc.go.jp) and found an 11-bp DNA motif (CTAATTGTTTA) encompassed in the promoter region, which has been demonstrated as a *cis*-element responsible for direct binding with HD-containing proteins (32). To examine whether *SlCycB2* may be a direct target of *Wo*, we tested the binding activity of Wo with this motif by using a yeast one-hybrid assay. The cotransformants could not grow on the SD/-Leu/AbA 50 ng/mL medium, whereas the transformed positive control exhibited normal growth. This result indicated that Wo had no binding activity with this motif, suggesting that *SlCycB2* may not be regulated by promoter binding with Wo.

Subsequently, we used bimolecular fluorescence complementation (BiFC) to determine whether there was protein–protein interaction between Wo and SlCycB2. We transformed the tobacco BY-2 (*N. tabacum* cv. Bright Yellow 2) cells with sets of pUC-SPYNE-*Wo*/pUC-SPYCE-SlCycB2 and pUC-SPYNE-*Wo*/pUC-SPYCE. A strong YFP fluorescence was observed when pUC-SPYNE-*Wo* was coexpressed with pUC-SPYCE-SlCycB2, whereas no signal was detected in the control cells (Fig. 5*A*). This result suggested that Wo interacts with SlCycB2. To further confirm the interaction between Wo and SlCycB2, we performed yeast two-hybrid experiment. The transformants harboring pGBKT7-Wo and pGADT7-SlCycB2 were able to grow on SD/-Ade/-His/-Leu/-Trp medium, whereas the positive and negative controls showed the expected results (Fig. 5*B*). These data, together with the BiFC results, demonstrated that Wo can physically interact with SlCycB2.

Embryo Lethality in *Wo* Homozygous Seeds. Embryo lethality occurs when *Wo* becomes homozygous in LA3186. To detect at which stage the defect is noticeable, we harvested and fixed seeds from the developing fruits of LA3186 from 5 d postanthesis (DPA) to 23 DPA, sequentially stained them with hematoxylin, and sectioned them. No obvious abnormality in embryo was observed until 7 DPA. Embryos at 7 DPA were arrested at the globular stage and stopped further development, and subsequently the cell number increased; on the contrary, normal embryos continued to differentiate into mature embryos (Fig. 2*A*). Our observation was partly consistent with an earlier report that *WoWo* embryos ceased to grow before any organogenesis (24). To detect whether there is a direct cause–consequence relationship between *Wo* and embryo lethality, we analyzed the temporal expression of *Wo* in the developing embryo. GUS activity maintained a high level in the 7-DPA embryo and gradually weakened (Fig. 2*B*), suggesting *Wo* transcription is associated with embryo lethality.

Discussion

Genetic analysis in *Arabidopsis* has established a regulatory pathway that controls trichome initiation. Although trichome structures of different species are convergent, they are regulated by independent networks (13). Exploration of the mechanisms involved in trichome

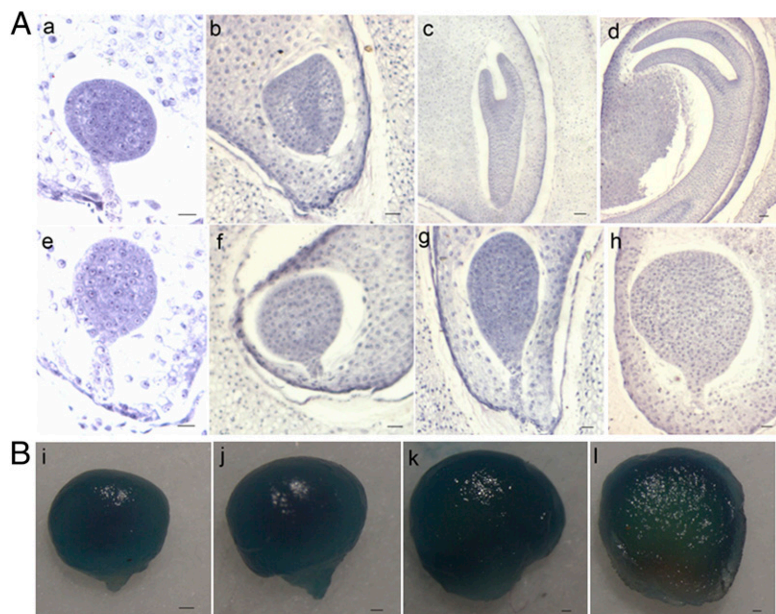

Fig. 2. Observation of the embryo development in LA3186 fruits (*A*) and GUS activity in the developing seeds driven by *Wo* promoter (*B*). *Upper*: Normal embryos. *Lower*: Dying embryos: 7 DPA, *a*, *e* and *i*; 9 DPA, *b*, *f*, and *j*; 15 DPA, *c*, *g*, and *k*; and 23 DPA, *d*, *h*, and *i*. (Scale bars: 0.14 mm.)

Fig. 3. Expression of *Wo* and *SlCycB2*. (*A*) *Upper*: Expression of *Wo* in several transgenic lines by using semiquantitative RT-PCR; *Lower*: Expression of *SlCycB2* in these transgenic lines by using quantitative RT-PCR. NW, transgenic negative plants with LA3186 nonwoolly segregants as the recipients; L1, L2, L3, transgenic lines overexpressing *Wo*; L4, L5, L6, transgenic lines suppressing *Wo*. (*B*) *Wo* promoter-GUS expression in trichomes on the leaf epidermis (*Left*) and young stem (*Right*). (Scale bars: 0.45 mm.) (*C*) Expression of *SlCycB2* in the *SlCycB2*-RNAi transgenic plants (*Right*) and negative plants (*Left*) with LA3186 as the recipient by using quantitative RT-PCR. Data are displayed as the ratio of expression to tomato *actin*, given as mean ± SE ($n = 3$).

formation in tomato can provide new insights into understanding the diversity of cell differentiation. In this study, we showed several lines of evidence to suggest that type I trichome formation is controlled by *Wo* in tomato. *GL2*, which was placed in the pathway inducing trichome initiation in *Arabidopsis* (9, 21), showed much lower identities with *Wo* than a shoot epidermal cell differentiation-related gene *PDF2* with it. The closest homologue of *Wo* in *Arabidopsis* (*PDF2*) has not been shown to be involved in trichome formation (22), suggesting functional diversification among *Wo* (*PDF2*) genes in different plant species. This also suggested that trichome formation in tomato may be regulated by a mechanism distinct from that characterized in *Arabidopsis*. Moreover, it would be interesting to see whether the *Wo* homologues in other species of the Asterid division such as petunia, tobacco, and snapdragon are also involved in trichome formation.

Wo contains three conserved domains: a HD domain, a bZIP motif, and a START domain. The HD domain is involved in DNA binding and the bZIP motif is involved in protein homo- or heterodimerization (19). Interestingly, all the amino acid substitutions identified among Wo alleles occurred at their C terminus outside these three domains. Thus, it can be deduced that functional differentiation during these alleles is not caused by any defect in likely protein–DNA and protein–protein interactions. In addition, because *Wo* functions as a transcription factor, it could be inferred that these point mutations might play various roles in the regulation of its downstream genes, showing the functional diversity. It is worth noting that the polymorphic sites in Wo—amino acids 567, 635, and

Fig. 4. Trichome phenotype of *SlCycB2*-RNAi transgenic positive (*Right*) and negative plants (*Left*). Trichome of total plants (*A*), young stems (*B*), old stems (*C*), magnified view of leaflet boxed in *a* (*D*), and SEM observation of leaf trichomes (*E*). (Scale bars: 0.5 cm.)

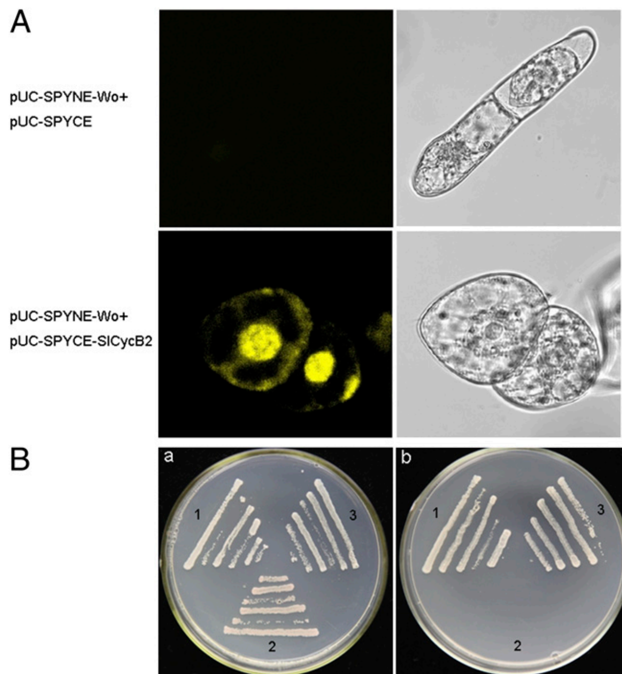

Fig. 5. Wo interacts with SlCycB2 in vivo. (*A*) Interaction between Wo and SlCycB2 in BiFC assays. YFP fluorescence was detected only when pUCSPYNE-Wo was coexpressed with pUCSPYCE-SlCycB2. (*B*) Interaction of Wo and SlCycB2 in transformed AH109 cells grown on SD/-Leu/-Trp (*a*) and SD/-Ade/-His/-Leu/-Trp (*b*). 1, pGBKT7-53+pGADT7-RecT (positive control); 2, pGBKT7-Lam+pGADT7-RecT (negative control); 3, pGBKT7-Wo+pGADT7-SlCycB2.

692—are highly conserved in Wo homologues from a wide range of organisms. Arg-567 (hydrophilic) is replaced by Leu (hydrophobic) in Wo^{m}, Pro-635 (hydrophobic) is replaced by Arg (hydrophilic) in Wo^{-}, and Ile-692 (hydrophobic) is replaced by Thr (hydrophilic) in Wo^{mz}. These mutations may change the stability and activity of the proteins. However, how these residues are functionally implicated in the trichome formation and embryo development remains to be elucidated.

HD-Zip transcription factors are supposed to act as activators of many downstream target genes controlling specific developmental processes. However, the majority of these potential target genes are still not known. We showed that *SlCycB2*, a B-type cyclin gene, also participates in type I trichome formation. The *SlCycB2* promoter contains an 11-bp motif that has been identified as a *cis*-element recognized by homeodomain proteins (32). However, there was no direct interaction between Wo and this motif, indicating that Wo may not regulate *SlCycB2* expression by binding to this element. Suppression of either of them by using RNAi resulted in an obvious decrease of the number of type I trichomes, and protein–protein interaction was detected by BiFC and yeast two-hybrid assays, demonstrating that they may work together in the regulation of this type of trichome formation. In addition, *SlCycB2* was up-regulated in woolly mutants and *Wo*-overexpressing transgenic plants but down-regulated in *Wo*-RNAi plants, suggesting that *SlCycB2* may be regulated by *Wo*. However, whether and how *SlCycB2* expression was regulated by *Wo* through direct interaction between their encoded proteins remain unknown. This possible regulatory model distinct from that characterized in *Arabidopsis*, may be a unique mechanism underlying trichome formation. It is also conceivable that *Wo* controls other developmental processes through regulation of different targets.

B-type cyclins function at the G2/M transition during mitosis. Enhancement of their expression could induce the shift from endoreduplication to mitosis. Therefore, it is easy to understand that the *Wo* gene results in a cell number increase in dying embryos by activating the expression of *SlCycB2*. However, it is still not known how *SlCycB2* induces the formation of type I trichome. Schnittger el al. found that *CYCLIN B1;2* expression under control of *GL2* promoter can induce mitotic divisions, resulting in trichome cluster and multicellular trichome formation (31). There was no significant similarity between SlCycB2 and CYCLIN B1;2, but there was a 53% sequence identity between SlCycB2 and AT5G06270.1, a hypothetical B-type cyclin. It demonstrated that *SlCycB2* has just a similar function to *CYCLIN B1;2*, but is absolutely a distinct gene. Trichome branch number is correlated with DNA content in *Arabidopsis* (33). Formation of multibranch trichomes in *SlCycB2*-RNAi transgenic plants indicated that suppression of *SlCycB2* expression can activate a shift from mitosis to endoreduplication. Consequently, we inferred that a dramatic increase in type I trichome number may be attributed to the enhancement of mitosis in the epidermal cells destined to become trichomes.

Future studies will be directed to study whether and how *Wo* activates *SlCycB2* expression through protein–protein interaction between their predicted proteins and then type I trichome formation, to determine whether the amino acid polymorphism in *Wo* mutant alleles correlates with functional differentiation, and to investigate how *Wo* may control different developmental processes. As *Wo* expression in homozygous status is associated with embryo lethality, it will be interesting to dissect the likely distinct roles of *Wo* and *SlCycB2* in trichomes formation and embryo development.

Materials and Methods

Plant Materials. Tomato genotypes LA3186, LA0053, LA0258, LA1531, and LA1908 are woolly mutants, and were provided by the Tomato Genetics Resource Center. AC and MF802 are commercial cultivars.

Sequence Alignment. We amplified (with the primers in Table S2) and sequenced the genomic and cDNA sequences of ORF4 from LA3186, LA0053, LA0258, LA1531, LA1908, MF802, and AC. These sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) and edited with GeneDoc.

Transgenic Analysis. Full-length coding sequences of two alleles corresponding to ORF4 (amplified with the primers in Table S2) were inserted into the pMV2 vector, which we rebuilt in our laboratory based on pHellsgate 8 vector under control of the CaMV 35S promoter. Suppression expressions of *Wo* and *SlCycB2* were performed by using RNAi vector pHellsgate 2, which were ligated with the fragment of *Wo* and *SlCycB2* (amplified with the primers in Table S2) by using Clonase BP reaction (Invitrogen). Both constructs of *Wo* were transformed into nonwoolly plants segregated from LA3186 and the construct of *SlCycB2* into LA3186, mediated by *Agrobacterium tumefaciens* strain C58. These empty vectors were also transformed as controls. Transgene copy number was detected by Southern blot hybridization. T_1 populations obtained from the T_0 plants harboring a single-copy transgene were amplified for the neomycin phosphotransferase II gene fragment for segregation analysis (primers in Table S2).

Transcript Test. Total RNA was extracted with TRIzol reagent (Invitrogen) and converted into first-strand cDNA by using PrimeScript reverse transcriptase (TaKaRa). RT-PCR was carried out to amplify an approximately 400 bp fragment of *Wo* with 31 cycles using the first-strand cDNA as a template. In addition, *actin* was amplified with 24 cycles as an internal control. Quantitative RT-PCR was performed in a total volume of 20 μL containing 8 μL of the first-strand cDNA, 8 μL SYBR Green I Master Mix (Roche), and 1 μL gene-specific primers (10 μM/μL) with the Roche LightCycler 480 system by an initial incubation of 10 min at 50 °C, followed by 45 cycles of 10 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C. Relative gene expression was calculated with Microsoft Excel. All primers used in this analysis are listed in Table S2.

Subcellular Localization and Tissue Distribution. To analyze the cellular localization of *Wo* (allele in LA3186), we created a CaMV 35S::*Wo-EGFP* fusion construct, and bombarded it into onion epidermal cells using Biolistic PDS-1000 (Bio-Rad). Samples were observed with a Leica TCSST2 confocal laser microscope. We cloned a 3.5-kb *Wo* endogenous promoter region upstream of the start codon from LA3186 genomic DNA (amplified with the primers in Table S2) to construct the promoter *Wo*:GUS-YFP fusion vector. Transformants carrying this construct were obtained as described earlier. These transformants were submerged in a solution containing 25 mM phosphate buffer (NaH_2PO_4 and Na_2HPO_4), 1.25 mM $K_3[Fe(CN)_6]$, 1.25 mM $K_4[Fe(CN)_6]$, 0.25% Triton X-100, 0.5 mM EDTA, and 1.25 mg/mL X-Gluc. After vacuum-infiltrating for 3 to 5 min, the transformants were incubated at 37 °C overnight. Chlorophyll was removed after staining by incubation in 75% ethanol, and YFP fluorescence was observed by confocal microscopy.

RNA in Situ Hybridization. We amplified a 453-bp fragment from the cDNA of woolly plants with the primers in Table S2 for an RNA probe and cloned it into the pEASY-T3 cloning vector (TransGen). Sense and antisense RNA probes were synthesized from linearized pEASY-T3 plasmid by using T7 and SP6 RNA polymerase with a DIG RNA Labeling Kit (SP6/T7; Roche). Probe sensitivity was checked with a comparison of labeled probes and labeled control RNA with the dot blot method. Paraffin sections of shoot apexes and young stems of LA3186 and its nonwoolly segregants were prepared by using the methods described by Narváez-Vásquez and Ryan (34).

SEM Observation. Young leaves and stems were fixed with 2% glutaraldehyde for approximately 24 h. After washing in a 0.1 M cacodylate buffer, these samples were dehydrated in a graded ethanol series, dried in a desiccator (HCP-2; Hitachi), and coated with a film of gold. Observations were carried out on a JSM-6390/LV scanning electron microscope.

Microarray Analysis. To compare the transcriptomes between LA3186 and its nonwoolly segregants, triplicate RNA samples with five seedlings of each line were isolated from the young tomato shoots with only one true leaf and sent for microarray hybridization by tomato TOM2 oligo microarray (CapitalBio). Unigenes with a false discovery rate (35) less than 0.05 and a fold change greater than or equal to two were identified as differentially expressed genes. We convert these unigenes to corresponding probe ID and then carry out probe annotation at the Tomato Expression Database (http://ted.bti.cornell.edu/). Some unannotated unigenes were further analyzed at the National Center for Biotechnology Information.

Yeast One-Hybrid Assay. Yeast one-hybrid assay was carried out by using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech). Oligonucleotides (33 bp) with three tandem repeats of the 11-bp DNA motif CTAATTGTTTA from the *SlCycB2* promoter was cloned into pAbAi. Plasmid was linearized and transformed into Y1HGold. Positive yeast cells were then transformed with pGADT7-AD, which contains *Wo*. The DNA–protein interaction was determined based on the growth ability of the cotransformants on SD/-Leu medium with 0 to 100 ng/mL aureobasidin A.

BiFC Assay. The full-length coding sequence of *Wo* and *SlCycB2* (without their stop codons) were cloned into pUC-SPYNE and pUC-SPYCE, respectively. Plasmids were bombarded into tobacco BY-2 cells, and YFP fluorescence was imaged after incubation at 24 °C for 18 h as described earlier. The tobacco BY-2 cells containing pUC-SPYNE-*Wo* and pUC-SPYCE were also analyzed as control.

Yeast Two-Hybrid Assay. For yeast two-hybrid analysis, we created the constructs pGBKT7-Wo and pGADT7-SlCycB2 by inserting *Wo* and *SlCycB2* into pGBKT7 and pGADT7 vectors separately. The two plasmids were cotransformed into *Saccharomyces cerevisiae* strain AH109. Simultaneously, we also transformed pGBKT7-53 and pGADT7-RecT as positive control, and pGBKT7-Lam and pGADT7-RecT as negative control, which were provided with the BD Matchmaker library construction and screening kits. The transformants were then tested on SD/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp mediums.

Paraffin Section. All seeds in the developing fruits (7 to 23 DPA) of LA3186 were harvested and fixed in formalin/acetic acid/alcohol for 24 h, and then stained in hematoxylin, dehydrated with each grade of ethanol, infiltrated with paraffin with chloroform used as the solvent, and gradually embedded with paraffin. Then, the specimens were cut into 8- to 10-μm sections and mounted on microscope slides. For removal of paraffin, slides were immersed in xylene (twice for 20 min) and enveloped with neutral resin. The finished slides were cured in a 42 °C oven until dry. Photomicrographs were taken by using an Olympus microscope.

ACKNOWLEDGMENTS. We thank Drs. H. Kuang, S. Xiao, and L. Peng for critical reading of this manuscript and suggestions. We thank Dr. C. Chen for technical assistance. We are grateful to the Tomato Genetic Resource Center for supplying the tomato seeds of the woolly mutants. This work was supported by 973 Project Grant 2011CB100600, National Natural Science Foundation Grant 30971997, and China Agricultural Research System Grant CARS-25-A-02.

1. 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.
2. Larkin JC, Marks MD, Nadeau J, Sack F (1997) Epidermal cell fate and patterning in leaves. *Plant Cell* 9:1109–1120.
3. Hülskamp M (2004) Plant trichomes: A model for cell differentiation. *Nat Rev Mol Cell Biol* 5:471–480.
4. 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.
5. Marks MD, Feldmann KA (1989) Trichome development in Arabidopsis thaliana. I. T-DNA tagging of the GLABROUS1 gene. *Plant Cell* 1:1043–1050.
6. 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.
7. Koornneef M (1981) The complex syndrome of *ttg* mutants. *Arabidopsis Inf Serv* 18: 45–51.
8. Walker AR, et al. (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.
9. Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A (2008) The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. *Development* 135:1991–1999.
10. Larkin JC, Brown ML, Schiefelbein J (2003) How do cells know what they want to be when they grow up? Lessons from epidermal patterning in Arabidopsis. *Annu Rev Plant Biol* 54:403–430.
11. Serna L (2004) A network of interacting factors triggering different cell fates. *Plant Cell* 16:2258–2263.
12. Wang S, et al. (2004) Control of plant trichome development by a cotton fiber MYB gene. *Plant Cell* 16:2323–2334.
13. Serna L, Martin C (2006) Trichomes: different regulatory networks lead to convergent structures. *Trends Plant Sci* 11:274–280.
14. Glover BJ, Perez-Rodriguez M, Martin C (1998) Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor. *Development* 125:3497–3508.
15. Payne T, Clement J, Arnold D, Lloyd A (1999) Heterologous myb genes distinct from *GL1* enhance trichome production when overexpressed in *Nicotiana tabacum*. *Development* 126:671–682.
16. Glover BJ, Bunnewell S, Martin C (2004) Convergent evolution within the genus *Solanum*: the specialised anther cone develops through alternative pathways. *Gene* 331: 1–7.
17. Avila J, Nieto C, Cañas L, Benito MJ, Paz-Ares J (1993) Petunia hybrida genes related to the maize regulatory C1 gene and to animal myb proto-oncogenes. *Plant J* 3:553–562.
18. Schena M, Davis RW (1992) HD-Zip proteins: members of an Arabidopsis homeodomain protein superfamily. *Proc Natl Acad Sci USA* 89:3894–3898.
19. Lee YH, Chun JY (1998) A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment. *Plant Mol Biol* 37: 377–384.
20. Ariel FD, Manavella PA, Dezar CA, Chan RL (2007) The true story of the HD-Zip family. *Trends Plant Sci* 12:419–426.
21. 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.
22. Abe M, Katsumata H, Komeda Y, Takahashi T (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*. *Development* 130:635–643.
23. Shilling PR (1959) An investigation of the hereditary character, woolly in the tomato. *Ohio J Sci* 59:289–302.
24. Huang PC, Paddock EF (1962) The time and site of the semidominant lethal action of 'Wo' in *Lycopersicon esculentum*. *Am J Bot* 49:388–393.
25. Luckwill LC (1943) The genus Lycopersicon: A historical, biological and taxonomic survey of the wild and cultivated tomatoes. PhD thesis (Aberdeen Univ, Aberdeen, UK).
26. Goodspeed TH (1954) The genus *Nicotiana*. *Chronica Botanica*, ed Verdoorn F (Chronica Botanica, Waltham, MA), pp 102–131.
27. Owens DK, et al. (2008) Functional analysis of a predicted flavonol synthase gene family in *Arabidopsis*. *Plant Physiol* 147:1046–1061.
28. Wang E, et al. (2001) Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product-based aphid resistance. *Nat Biotechnol* 19:371–374.
29. Vanhaelen N, Haubruge E, Lognay G, Francis F (2001) Hoverfly glutathione S-transferases and effect of brassicaceae secondary metabolites. *Pestic Biochem Physiol* 71:170–177.
30. Ishida T, Kurata T, Okada K, Wada T (2008) A genetic regulatory network in the development of trichomes and root hairs. *Annu Rev Plant Biol* 59:365–386.
31. Schnittger A, Schöbinger U, Stierhof YD, Hülskamp M (2002) Ectopic B-type cyclin expression induces mitotic cycles in endoreduplicating *Arabidopsis* trichomes. *Curr Biol* 12:415–420.
32. Korfhage U, Trezzini GF, Meier I, Hahlbrock K, Somssich IE (1994) Plant homeodomain protein involved in transcriptional regulation of a pathogen defense-related gene. *Plant Cell* 6:695–708.
33. Perazza D, et al. (1999) Trichome cell growth in *Arabidopsis thaliana* can be derepressed by mutations in at least five genes. *Genetics* 152:461–476.
34. Narváez-Vásquez J, Ryan CA (2004) The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. *Planta* 218:360–369.
35. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *JR Stat Soc* 57:289–300.