R2R3 MYB-dependent auxin signalling regulates trichome formation, and increased trichome density confers spider mite tolerance on tomato

Yujin Yuan1,2,3,†, Xin Xu1,2,†, Yingqing Luo1,2, Zehao Gong1,2, Xiaowei Hu1,2, Mengbo Wu1,2, Yudong Liu1,2, Fang Yan1,2, Xiaolan Zhang1,2, Wenfa Zhang1,2, Yuwei Tang1,2, Bihong Feng4, Zhengguo Li1,2,19, Cai-Zhong Jiang3,5 and Wei Deng1,2,19

1Key Laboratory of Plant Hormones and Developmental Regulation of Chongqing, School of Life Sciences, Chongqing University, Chongqing, China
2Center of Plant Functional Genomics, Institute of Advanced Interdisciplinary Studies, Chongqing University, Chongqing, China
3Department of Plant Sciences, University of California, Davis, CA, USA
4College of Agriculture, Guangxi University, Nanning, China
5Crops Pathology and Genetics Research Unit, United States Department of Agriculture, Agricultural Research Service, Davis, CA, USA

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*Correspondence (Tel +86 18623127580; fax +86 23 65678902; email dengwei1977@cqu.edu.cn)
†These two authors equally contribute to this work and are considered as co-first authors.

Summary
Unicellular and multicellular tomato trichomes function as mechanical and chemical barriers against herbivores. Auxin treatment increased the formation of II, V and VI type trichomes in tomato leaves. The auxin response factor gene SlARF4, which was highly expressed in II, V and VI type trichomes, positively regulated the auxin-induced formation of II, V and VI type trichomes in the tomato leaves. SlARF4 overexpression plants with high densities of these trichomes exhibited tolerance to spider mites. Two R2R3 MYB genes, SITHM1 and SIMYB52, were directly targeted and inhibited by SIARF4. SITHM1 was specifically expressed in II and VI type trichomes and negatively regulated the auxin-induced formation of II and VI type trichomes in the tomato leaves. SIMYB52 was specifically expressed in V type trichomes and negatively regulated the auxin-induced formation of V type trichomes in the tomato leaves. The regulation of SIARF4 on the formation of II, V and VI type trichomes depended on SITHM1 and SIMYB52, which directly targeted cyclin gene SICycB2 and increased its expression. In conclusion, our data indicates that the R2R3 MYB-dependent auxin signalling pathway regulates the formation of II, V and VI type trichomes in tomato leaves. Our study provides an effective method for improving the tolerance of tomato to spider mites.

Introduction
Pests have threatened crop yield since the commencement of plant domestication. At present, pest invasion ruins approximately 13% of crop production globally (Hamza et al., 2018). Plants tactically developed response mechanisms against pest attacks. They developed mechanical and chemical barriers in the form of trichomes. Differentiated epidermal cells from leaves, stems and floral organs produce plant trichomes, which can be glandular or non-glandular, unicellular or multicellular, and branched or unbranched (Yang and Ye, 2013).

Trichomes are non-glandular, unicellular and branched in Arabidopsis. Trichome formation passes through three stages: cellular determination, specification and morphogenesis of epidermal cell (Yang and Ye, 2013). A trimeric transcription factor (TF) complex formed by R2R3 MYB, WD40 repeat and basic helix–loop–helix (bHLH) proteins positively regulates trichome formation (Ishida et al., 2008). The WD40–bHLH–MYB complex directly induces the expression of GLABRA2 and cell cycle-related SIAMESE (Morohashi and Grotewold, 2009). R3 MYBs compete with R2R3 MYBs and form a repressor complex that inhibits trichome formation (Wang et al., 2007).

Tomato has several types of trichomes: II, III, V and VIII type, which are non-glandular trichomes, and I, IV, VI and VII type, which are multicellular glandular trichomes (Glas et al., 2012). Glandular trichomes produce volatile organic compounds (VOCs) to repel or eradicate pests (Turings et al., 1995). Tomato’s jasmonic acid (JA) signal transduction COI1 regulates VI type glandular trichome formation (Li et al., 2004). Tomato Woolly (Wo), a homeodomain-leucine zipper (HD-Zip) TF, interacts with B-type cyclin SICycB2 protein, which are essential for the formation of type I trichomes in tomato (Yang et al., 2011; Yang and Ye, 2013). The overexpression of Wo in potato and tobacco can promote the production of multicellular trichomes and increase tolerance to aphids (Yang et al., 2015). A single cysteine2–histidine2 (C2H2) zinc-finger TF Hair (H) gene, which interacts with Wo, regulates the formation of type I multicellular trichomes (Chang et al., 2018). SIMY1, a basic bHLH TF, plays important roles in the formation of VI type glandular trichomes and terpene biosynthesis in tomato glandular cells (Xu et al., 2018). The overexpression of the MYB gene GLABROUS1 involved in unicellular trichome formation in Arabidopsis does not affect multicellular trichome formation in Nicotiana, a Solanaceae species (Payne et al., 1999). The formation of
multicellular trichomes in Solanaceae may be controlled by a pathway that is different from that of unicellular trichomes in Arabidopsis (Payne et al., 1999). However, the regulatory mechanism involved in nonglandular and glandular trichome formation in Solanaceae is rarely studied in tomato.

Auxin plays important roles in several physiological processes in plants. Short-lived auxin-indole acetic acid (Aux/IAA) proteins and auxin response factors (ARFs) are involved in auxin-dependent transcriptional regulation. At low auxin levels, Aux/IAA proteins interact with ARFs to repress ARF transcription activity by recruiting the co-repressor TOPLESS (Szemenyei et al., 2008). At high auxin levels, Aux/IAAs bind to the SCF 

result indicated that auxin induced the formation of type II, V and VI trichomes in the tomato leaves.

**SIARF4 positively regulated the auxin-induced formation of II, V and VI type trichomes in the leaves**

qRT-PCR was performed to assess the expression pattern of SIARF4 (Figure S2). SIARF4 is highly expressed in leaf trichomes. The expression pattern of SIARF4 in leaves was further analysed by GUS staining. A transcriptional fusion was generated between SIARF4 promoter and GUS gene (pSIARF4-GUS), and GUS staining was detected in II, V and VI type trichomes (Figures 1b, d and f). No GUS staining was detected in the trichomes of WT plants (Figures 1c, e and g). This result indicated that SIARF4 was expressed in II, V and VI type trichomes in tomato leaves.

The role of SIARF4 in trichome formation was elucidated using transgenic technology. The expression level of SIARF4 decreased in the RNAi-SIARF4 lines and increased in the OE-SIARF4 lines (Figure 1h). Without IAA treatment, the OE-SIARF4 lines displayed increases in the densities of II, V and VI type trichomes in the leaves, whereas the RNAi lines exhibited decreases in the densities of II, V and VI type trichomes compared with WT plants (Figures 1i and j). The OE-SIARF4 lines displayed increase in the density of epidermal pavement cells in the leaves, whereas RNAi lines exhibited decreased density compared with WT plants (Figure S3). Then, the number of trichomes per epidermal cell in the OE-SIARF4 and RNAi-SIARF4 lines was calculated. An apparent increase in the number of trichomes per epidermal cell in the overexpressed lines and decrease in that of the RNAi lines were observed (Figure 1k), indicating that SIARF4 positively regulated the formation of II, V and VI type trichomes. IAA treatment was performed to study the response of SIARF4 transgenic lines to auxin. At 30 mg/L IAA concentration, the densities of II, V and VI type trichomes increased in the leaves of OE-SIARF4, RNAi-SIARF4 and WT plants (Figure 1l). The trichome density ratio between IAA treatment and no-IAA treatment was calculated in studying the effect of auxin treatment on trichome formation. The ratio of OE-SIARF4 plants was similar to that of the WT plants, but RNAi-SIARF4 plants had lower ratio than the OE-SIARF4 and WT plants (Figure 1m). These results indicated that the down-regulation of SIARF4 decreases the effect of IAA treatment on the formation of II, V and VI type trichomes in tomato leaves and that the inducement of auxin on the formation of II, V and VI type trichomes is dependent on SIARF4. Given the fact that SIARF4 was expressed in tomato fruits, the fruit trichome density was analysed. There was no significant difference between WT and SIARF4 transgenic lines (Figure S4).

The function of SIARF4 in trichome formation was validated in Slarf4 CRISPR/Cas9 plants. The trichome density and the number of trichomes per epidermal cell were consistent with those in the RNAi-SIARF4 plants (Figure S5), indicating that SIARF4 positively regulates the formation of II, V and VI type trichomes in tomato leaves.

**Overexpression of SIARF4 conferred spider mite tolerance to tomato**

The response of SIARF4 transgenic plants to spider mite was analysed through spider mite bioassays. Pest preference experiment was used in assessing the relative preference of herbivores for WT, OE-SIARF4 and RNAi-SIARF4 plants. The number of spider mites that preferred the leaves of the OE-SIARF4 plants was lower than that of the spider mites that preferred the WT plants (Figures 2a and b). The number of spider mites that preferred the
leaves from the RNAi-SlARF4 plants was higher than that of the spider mites that preferred the WT plants (Figures 2c and d). Spider mite inoculation assay was performed. The 10- and 25-day trials with adult female mites resulted in the localized collapse of the leaves of the RNAi-SlARF4 lines and in small chlorotic lesions, which are indicative of mite feeding, in the leaves of the OE-SlARF4 lines (Figure 2e). After 45 days of assays, severe damages were observed in the RNAi-SlARF4 and WT plants and some plants died. By contrast, the inoculated leaves of OE-SlARF4 lines remained green and had relatively few signs of macroscopic damage (Figure 2e). The ability of the spider mites to colonize the host was examined by measuring the fecundity of the female mites. The egg number laid on the OE-SlARF4 plants was lower than that on the WT plants, whereas the number of eggs laid on the RNAi-SlARF4 plants was higher than that on the WT plants (Figure 2f). The overexpression of SlARF4 in tomato increased spider mite tolerance, and the down-regulation of SlARF4 increased the sensitivity of the tomato plants to spider mites.

SlARF4 bound to the AuxRE and TGA motifs of the SlTHM1 and SlMYB52 promoters, respectively, and inhibited their expression.

The differentially expressed genes (DEGs) between the WT and SlARF4 RNAi lines were identified through RNA sequencing (RNA-Seq), using the leaves of 45-day-old tomato seedlings. A total of 398 DEGs, including 275 up-regulated and 123 down-regulated...
genes, were identified (Table S1). Gene Ontology (GO) function and pathway enrichment analyses showed that the down-regulation of SIARF4 influenced several metabolic pathways (Table S2, Figure S6 and S7a). Forty-nine DEGs belong to 16 TF families (Table S3). The dominant families included WRKY (nine DEGs), MYB (six DEGs), ERF (six DEGs), MIKC_MADS (five DEGs) and C2H2-type zinc-finger families (five DEGs; Figure S7b). MYBs play an important role in trichome formation (Machado et al., 2009; Wang et al., 2004).

SlTHM1 (Solyc08g081500) and SlMYB52 (Solyc03g093890) contained the AuxRE (TGTCTC) motif and auxin response element (TGA motif, AACGAC), respectively, which are the binding motifs of ARF proteins.

Both SITHM1 and SIMYB52 belong to R2R3MYB transcription factors family that plays regulatory roles in plant development and defence responses as one of the largest transcription factor families in tomato (Li et al., 2016; Zhao et al., 2014). SlTHM1 has an open reading frame (ORF) of 762 bp that encodes a protein containing 253 amino acid residues while SlMYB52 has an open reading frame (ORF) of 996 bp that encodes a protein containing 331 amino acid residues. Phylogenetic analysis of tomato
Figure 2  Tolerance of SIARF4 plants to two-spotted spider mites and SIARF4 targets SITHM1 and SIMYB52. a–d, Preference experiment to analyse the preference of spider mites for WT and SIARF4 plants. Ten adult female spider mites were positioned in an area that was equidistant from WT and SIARF4 leaflets. The number of mites that moved to different leaflets and those that failed to make a choice (nc) were counted 1 h after initiating the assay. The data represent the mean values ± SE from 18 independent experiments. * and ** indicate significant difference between WT and SIARF4 leaves with P < 0.05 and P < 0.01, respectively (t test). g and h, EMSA showing the direct binding of SIARF4 to the promoters of SITHM1 and SIMYB52. Biotin-labelled DNA probes from original promoter or mutants were incubated with GST-SIARF4 protein, and then, DNA–protein complexes were separated on 6% native polyacrylamide gels. + or ++ indicates increasing amounts of unlabelled probes for competition. i and j, ChIP-qPCR assay for the direct binding of SIARF4 to the promoters of SITHM1 and SIMYB52. Values represent the percentage of DNA fragments that co-immunoprecipitated with anti-SIARF4 antibodies or nonspecific antibodies (anti-IgG) comparative to the input DNA. The data are means ± SE from qPCR of four biological replicates. k, Diagrams of the reporter and effector vectors in the dual-luciferase reporter assay. l, SIARF4 suppresses the transcription of SITHM1 and SIMYB52. The activities of firefly LUC and REN in tobacco leaves were measured and LUC/REN ratio was analysed after infiltration with A. tumefaciens carrying the reporter plasmid and different combinations of effector plasmids. Each value represents the mean of six biological replicates, and vertical bars represent the SE.

R2R3MYB family proteins revealed that SITHM1 along with SIMYB56, SIMYB67 and SIMYB62 belong to S15 subgroup; SIMYB52 along with SIMYB51, SIMYB95 and SIMYB105 belong to S6 subgroup (Zhao et al, 2014). Amino acid sequence analyses of SITHM1 and SIMYB52 were conducted with known MYB proteins in the same subgroup, respectively (Figure S10). Both SITHM1 and SIMYB52 proteins contain an R2R3 domain in the N-terminus (Figure S10). In addition, phylogenetic trees of SITHM1 and SIMYB52 with homologous proteins from other species were also generated, respectively (Figure S11). SITHM1 is closely related to SbMYB21 while SIMYB52 is closely related to SpMYB15, indicating possible functional similarity among them. The direct binding of SIARF4 protein to SITHM1 and SIMYB52 genes was verified by electrophoretic mobility shift assay (EMSA). Purified recombinant truncated SIARF4 and glutathione S-transferase (GST) fusion protein (GST-tSIARF4) were successfully obtained (Figure S8). The GST-tSIARF4 fusion protein bound to biotin-labelled probes containing AuxRE and TGA motifs that were derived from SITHM1 and SIMYB52 promoters, respectively, and caused a mobility shift that was effectively abolished in a dose-dependent manner when unlabelled SITHM1 and SIMYB52 promoter fragments were added as competitors (Figures 2g and h). Mobility shift was not observed when biotin-labelled probes were incubated with GST only (Figures 2g and h). This finding indicated that the specific targets of SIARF4 were SITHM1 and SIMYB52. Chromatin immunoprecipitation coupled with quantitative polymerase chain reaction (ChIP-qPCR) was used in confirming the interaction between SIARF4 and the SITHM1 and SIMYB52 genes in vivo. The promoter regions of SITHM1 and SIMYB52 were specifically enriched when FLAG antibodies were used instead of nonspecific antibodies (IgG; Figures 2i and j). SIARF4 possessed transcriptional repression activity and directly targeted the promoters of SITHM1 and SIMYB52. Hence, the transcriptional repression of SITHM1 and SIMYB52 by SIARF4 were determined through transient dual-luciferase assays. The overexpression of SIARF4 remarkably reduced luciferase activity driven by the promoters of SITHM1 and SIMYB52 compared with the vector control (pEAQ; Figures 2k and l). qRT-PCR showed that the expression levels of SITHM1 and SIMYB52 increased in the RNAI-SIARF4 plants but decreased in the OE-SIARF4 plants (Figure S9). The results indicated that SIARF4 targeted SITHM1 and SIMYB52 repressed their transcription.

SITHM1 negatively affected the auxin-induced formation of II and VI type trichomes in the leaves

qRT-PCR was performed to assess the expression pattern of SITHM1 (Figure S12). SITHM1 was expressed in all tissue tested with the highest expression in leaf trichomes. GUS staining showed that SITHM1 was specifically expressed in II and VI type trichomes but not in V type trichome (Figure 3a). SITHM1-green fluorescent protein (GFP) was transiently expressed in the leaf epidermal cells of tobacco for subcellular localization, and fluorescence signals were detected in the nucleus, indicating that the SITHM1 was localized in the nucleus of plant cells (Figure 3b). The transcriptional activity of SITHM1 in tobacco leaves was analysed using a dual-luciferase reporter system. SITHM1 increased the LUC/REN ratio compared with the empty vector pBD, revealing that SITHM1 possesses transcriptional activation activity (Figure S13).

The SITHM1 function on trichome formation was analysed using RNAi strategy. The homozygous transgenic lines of RNAi-SITHM1 showed a substantially low degree of SITHM1 transcript accumulation (Figure 3c). Without IAA treatment, RNAi-SITHM1 plants exhibited increases in the densities of II and VI type trichomes in leaves and no change in V type trichome compared with WT plants (Figures 3d and e). The density of epidermal pavement cells was analysed, and results showed that the down-regulation of SITHM1 increased the density of the epidermal pavement cells of the tomato leaves (Figure S14). An apparent increase in the number of II and VI type trichomes per epidermal cell in RNAi-SITHM1 lines was observed (Figure 3f). These results indicated that SITHM1 negatively affected the formation of II and VI type trichomes. RNAi-SITHM1 plants with IAA treatment showed obvious increases in the densities of II, V and VI type trichomes in the tomato leaves (Figure 3g). The trichome density ratio between IAA treatment to no-IAA treatment was calculated, showing that the density ratios of II and VI type trichomes on the RNAi-SITHM1 plants were higher than those of the WT plants and that the density ratio of V type trichome was similar to that of the WT plants (Figure 3h). The down-regulation of SITHM1 increased the effect of IAA treatment on the formation of II and VI type trichomes. Our results demonstrated that SITHM1 regulated the auxin-induced formation of II and VI type trichomes in tomato leaves.
Down-regulation of \textit{SlTHM1} conferred spider mite tolerance to tomato

Spider mites were used in analysing the response of the RNAi-SlTHM1 lines to herbivores. Spider mite preference experiment showed that the number of spider mites that preferred RNAi-SlTHM1 leaves was lower than that of the mites that preferred the leaves of WT plants (Figures 4a and b). Spider mite inoculation assay was carried out. The localized collapse of the WT leaves was obvious, but only small chlorotic lesions indicative of mite feeding was apparent on the RNAi-SlTHM1 leaf tissues after 25 days (Figure 4c). After 45 days, spider mites caused severe damage and nearly killed the WT plants, but the RNAi-SlTHM1 plants survived and retained some green leaves (Figure 4c). Furthermore, the number of eggs laid on the RNAi-SlTHM1 leaves was remarkably lower than that on the WT leaves during the 4-day assay (Figure 4d). The results indicated that the down-regulation of \textit{SlTHM1} in tomato improved tolerance to spider mites.

\textit{SlMYB52} negatively regulated the auxin-induced formation of \textit{V} type trichome in tomato leaves

qRT-PCR and GUS staining were used to explore the expression pattern of \textit{SlMYB52} (Figures 5a, S15). GUS staining showed that \textit{SlMYB52} was specifically expressed in the \textit{V} type trichome of leaves (Figure 5a). Subcellular localization assay of \textit{SlMYB52} showed that \textit{SlMYB52} was localized in the nucleus of plant cells (Figure 5b). RNAi-SlMYB52 transgenic lines were generated to...
analyse the functions of \textit{SIMYB52} on trichome formation in tomato. The homozygous transgenic lines of RNAi-SIMYB52 showed a substantially low degree of \textit{SIMYB52} transcript accumulation and obvious increase in the density of V type trichome (Figures 5c–e). The down-regulation of \textit{SIMYB52} increased the density of epidermal pavement cells of tomato leaves (Figure S16). Then, the calculated number of trichomes per epidermal cell of RNAi-SIMYB52 lines indicated an apparent increase in the number of V type trichomes per epidermal cell in the leaves (Figure 5f). Our data indicated that \textit{SIMYB52} negatively affected the formation of V type trichome in the tomato leaves. RNAi-SIMYB52 plants with IAA treatment showed obvious increases in the densities of II, V and VI type trichomes in the leaves (Figure 5g). The calculated trichome density ratio between the IAA treatment and no-IAA treatment of V type trichomes in the RNAi-SIMYB52 plants were higher than that in the WT plants, and the ratios of II and VI type trichomes in the RNAi-SIMYB52 plants were similar with the ratio in the WT plants (Figure 5h). The down-regulation of \textit{SIMYB52} increased the effect of IAA treatment on the formation of V type trichomes in the tomato leaves, and \textit{SIMYB52} was involved in the auxin-induced formation of V type trichome in tomato leaves.

Spider mite inoculation assay, preference assay and fecundity assay were conducted to analyse the response of RNAi-SIMYB52 plants to spider mites. RNAi-SIMYB52 plants showed no change in the response to spider mite compared with WT plants (Figure S17a, b, c).

\textit{SIARF4} depended on \textit{SITHM1} to regulate type II and VI trichome formation

The OE-SIARF4 and RNAi-SIARF4 lines were separately crossed with the RNAi-SITHM1 line to further explore the interaction between SIARF4 and SITHM1 in the formation of type II, V and VI trichomes. The densities of II and VI type trichomes in RNAi-SITHM1 and OE-SIARF4 crossed (OE-SIARF4♂RNAi-SITHM1♀) lines without IAA treatment were similar to those in RNAi-SITHM1 and OE-SIARF4 lines and higher than those in the WT plants (Figure 6a). The densities of V type trichomes on OE-
SIARF4 and RNAi-SITHM1 lines increased compared with those on RNAi-SITHM1 and WT lines and were similar with those in the OE-SIARF4 lines. Moreover, the densities of II and VI type trichomes in RNAi-SIARF4 and RNAi-SIARF4 crossed (RNAi-SIARF4♀ RNAi-SITHM1♂) lines were increased compared with those in the RNAi-SIARF4 and WT lines and were similar to those in the RNAi-SITHM1 and OE-SIARF4 lines. The densities of V type trichome in the RNAi-SIARF4♀ RNAi-SITHM1♂ lines were lower than those in the RNAi-SITHM1 and WT lines and similar to those in the RNAi-SIARF4 lines (Figure 6a). Hybrid experiments showed that the down-regulation of SITHM1 specifically increased II and VI type trichomes in the SIARF4 down-regulation background. SIARF4

Figure 5: Expression analysis of SIMYB52, subcellular localization analysis of SIMYB52 protein and trichome density of leaves in SIMYB52 plants. a, Expression pattern of SIMYB52 in trichome tissues explored by GUS reporter gene driven by the SIMYB52 promoter. Scale bars = 0.2 mm. b, Subcellular localization analysis of SIMYB52 in tobacco leaves. 35S: GFP was used as positive control. Scale bars = 15 μm. c, qRT-PCR analysis of the expression of SIMYB52 in RNAi plants. d, Scanning electron micrographs of the leaf surface. Scale bars = 60 mm. e, Density analysis of II, V and VI type trichomes from the leaves of WT and SIMYB52 RNAi plants without IAA treatment. Scale bars = 0.2 mm. f, Number of trichomes per epidermal cell of tomato leaves from WT and RNAi-SIMYB52 plants without IAA treatment. g, Density analysis of II, V and VI type trichomes in the leaves of WT and SIMYB52 lines with IAA treatment. h, Trichome density ratio of IAA treatment to no-IAA treatment. Number of II type trichomes in an area of 0.5 cm² and number of V and VI type trichomes in an area of 2.2 mm² were calculated under a light microscope. WT, wild-type plants; RNAi-1, RNAi-3 and RNAi-9, RNAi-SIMYB52 plants. All experiments were replicated three times. ** represent significant difference between WT and transgenic leaves with P < 0.01 (t-test).
induced the formation of II and VI type trichomes and was dependent on SlTHM1.

The densities of II, V and VI type trichomes in the OE-SlARF4 or RNAi-SlARF4 and RNAi-SlTHM1 plants without IAA treatment were calculated under a light microscope. All experiments were replicated three times. Significant differences (Tukey's multiple range test, P < 0.05) are indicated in lowercase.

Figure 6 Density analysis of II, V and VI type trichomes from the leaves of crossed plants among OE-SlARF4, RNAi-SlARF4, RNAi-SlTHM1 and RNAi-SIMYB52 plants. a, Trichome densities of crossed plants between OE-SlARF4 or RNAi-SlARF4 and RNAi-SlTHM1 plants without IAA treatment. b, Trichome densities of crossed plants between OE-SlARF4 or RNAi-SlARF4 and RNAi-SlTHM1 plants with IAA treatment. c, Trichome density ratio of IAA treatment to no-IAA treatment. d, Trichome densities of crossed plants between OE-SlARF4 or RNAi-SlARF4 and RNAi-SIMYB52 plants without IAA treatment. e, Trichome densities of crossed plants between OE-SlARF4 or RNAi-SlARF4 and RNAi-SIMYB52 plants with IAA treatment. f, Trichome density ratio of IAA treatment to no-IAA treatment. Number of II type trichomes in an area of 0.5 cm² and number of V and VI type trichomes in an area of 2.2 mm² were calculated under a light microscope. All experiments were replicated three times. Significant differences (Tukey's multiple range test, P < 0.05) are indicated in lowercase.

SlARF4 depended on SIMYB52 to regulate V type trichome formation

OE-SlARF4 and RNAi-SlARF4 lines were separately crossed with RNAi-SIMYB52 lines. The densities of V type trichomes on RNAi-SIMYB52 and OE-SlARF4 crossed (OE-SlARF4×RNAi-SIMYB52) lines without IAA treatment were similar to those in the RNAi-SIMYB52 lines and higher than those in the WT plants (Figure 6d). The densities of II and VI type trichomes in the OE-SlARF4×RNAi-SIMYB52 lines increased compared with those in

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the RNAi-SlMYB52 and WT lines and were similar to those in the OE-SlARF4 lines (Figure 6d). Moreover, the densities of V type trichomes in RNAi-SlMYB52 and RNAi-SlARF4 crossed (RNAi-SlARF4♂ RNAi-SlMYB52♀) lines increased compared with those in the RNAi-SlARF4 and WT lines and were similar to those in the RNAi-SlMYB52 and OE-SlARF4 plants (Figure 6d). The densities of II and VI type trichomes in the RNAi-SlARF4♂ RNAi-SlMYB52♀ lines were lower than those in the RNAi-SlMYB52, OE-SlARF4 and WT lines and similar to those in the RNAi-SlARF4♀ plants (Figure 6d). Cross experiments showed that the down-regulation of SlMYB52 gene specifically increased the formation of V type trichome in the SlARF4 down-regulation background. The regulation of SlARF4 in the formation of V type trichomes in tomato was dependent on SlMYB52.

The densities of II, V and VI type trichomes in the OE-SlARF4♂ RNAi-SlMYB52♀ and RNAi-SlARF4♂ RNAi-SlMYB52♀ lines with IAA treatment obviously increased compared with those in plants without IAA treatment (Figure 6e). The trichome density ratios between the IAA treatment and no IAA treatment were calculated. The density ratios of V type trichomes in the OE-SlARF4♂ RNAi-SlMYB52♀ and RNAi-SlARF4♂ RNAi-SlMYB52♀ lines were similar to those in the RNAi-SlMYB52 plants and higher than those in the OE-SlARF4♀, RNAi-SlARF4♂ and WT plants (Figure 6f). The density ratios of II and VI type trichomes in the OE-SlARF4♂ RNAi-SlMYB52♀ plants were similar to those in the OE-SlARF4♀ plants, and the ratios of II and VI type trichomes in the RNAi-SlARF4♂ RNAi-SlMYB52♀ lines were similar to those in the RNAi-SlARF4♀ plants (Figure 6f). The down-regulation of SlMYB52 gene specifically increased the IAA-induced formation of V type trichome in the SlARF4 down-regulation background, and the regulation of SlARF4 in the auxin-induced formation of V type trichomes was dependent on SlMYB52 in tomato.

SITHM1 and SlMYB52 positively regulated the expression of SlCycB2

SlCycB2, a B-type cyclin gene, plays key roles in trichome initiation (Gao et al., 2017). The promoter of SlCycB2 contains an MYB-binding motif (AC-rich). The expression levels of SlCycB2 decreased in the SITHM1 and SlMYB52 RNAi plants (Figure S18). The direct bindings of SITHM1 and SlMYB52 to SlCycB2 promoter were analysed by EMSA. Purified recombinant truncated SITHM1, SlMYB52 and GST fusion proteins (GST-SITHM1 and GST-SlMYB52) were obtained (Figure S19). GST-SITHM1 and GST-SlMYB52 fusion proteins bound to biotin-labelled probes containing the AC-rich motifs of the promoters of SlCycB2 (Figures 7a and b), indicating the specific targets of SITHM1 and SlMYB52 to the promoters of SlCycB2. Transient dual-luciferase assay was used in determining the transcriptional regulation of SlCycB2 by SITHM1 and SlMYB52. The overexpression of SITHM1 and SIMYB52 remarkably increased luciferase activity driven by SlCycB2 promoters compared with the empty control vector (pEAG) (Figures 7c and d), and SITHM1 and SIMYB52 increased the transcription of SlCycB2.

Discussion

Increased trichome density confers spider mite tolerance

The two-spotted spider mite is a ruthless pest that damages more than 140 plant families and 1100 plant species, including tomato (Dermaww, et al., 2013). Spraying synthetic acaricides is primary performed to inhibit spider mite infestation. The drawback to the use of synthetic acaricides is that spider mites have the ability to upsurge resistance to acaricides (Dermauw, et al., 2013; Van Leeuwen et al., 2004). An imperative substitute approach to beating spider mites is the breeding of tomato cultivars with resistance to spider mites (Johnson, 1992).

Elevated trichome density hampers pest feeding and migration and diminishes herbivore populations (Handley et al., 2005; Horgan et al., 2009). VOCs by glandular trichomes fend off or destroy pests (Schilmiller et al., 2010). In this study, the overexpression of SlARF4 amplified the densities of II, V and VI type trichomes and the tolerance of tomato to spider mites (Figures 1 and 2). Furthermore, the down-regulation of SITHM1, a repressor of trichome formation, increased the densities of II and VI type trichomes and tolerance to spider mites (Figures 3 and 4). However, the RNAi-SlMYB52 plants with increased density of V type trichome did not exhibit increase in tolerance to spider mite compared with the WT plants (Figure S17). Our study confirmed the positive correlation between trichome density and spider mite tolerance and demonstrated that the regulation of trichome densities by genetic engineering is an effective strategy for increasing plant resistance to herbivores. In addition, the major agricultural traits of the transgenic plants were also analysed. No significant difference was detected between transgenic and WT plants (Figures S20, S21 and S22), indicating that the R2R3 MYB-dependent auxin signalling may not be involved in the regulation of some other agricultural traits.

Auxin triggers the formation of unicellular and multicellular trichomes via SlARF4

Hormones regulate trichome formation, and different hormones stimulate different type of trichomes in tomato (Maes and Goossens, 2010). The utilization of JA application promotes the creation of multicellular I and VI type trichomes and unicellular V type trichome (Maes et al., 2010), whereas cytokinin and gibberellin cause the creation of I type trichomes in tomato (Maes et al., 2010). Given the fact that both auxin and JA play key roles in trichome formation, the expression levels of SlARF3, SlARF4, SITHM1, SlMYB52 and SlCycB2 in response to auxin and JA treatments were analysed by qRT-PCR (Figures S23, S24). All five genes showed response to IAA treatment, with SlARF4 showing the highest up-regulation whereas SlARF3 displayed slight up-regulation (Figure S23). On the other hand, SITHM1, SlMYB52 and SlCycB2 were down-regulated in response to IAA treatment (Figure S23). Moreover, SlARF3, SlARF4, SITHM1 and SlMYB52 were barely responsive to JA treatment while SlCycB2 manifested mild down-regulation (Figure S24). In this study, auxin promoted the formation of unicellular V type trichome and multicellular II and VI type trichomes. SlARF4 positively modulates the formation of II, V and VI type trichomes, and the down-regulation of SlARF4 decreases the effect of IAA treatment on the formation of II, V and VI type trichomes (Figure 1). Our results demonstrated that auxin induced the formation of II, V and VI type trichomes in tomato leaves by promoting SlARF4 expression. In addition, IAA treatment also increased the density of trichomes in RNAi-SlARF4 and Slarf4 plants, which suggested that, other than SlARF4, there might be another regulatory pathway in controlling trichome formation. Further research will be needed to examine trichome formation in response to IAA treatment.

It is noteworthy that another auxin-responsive gene, SlARF3, is involved in the formation of epidermal cells and trichomes (Zhang et al., 2015). The relation between SlARF3 and SlARF4 in regulating trichome formation was explored by multiple
The expression level of SlARF3 was first assessed in SlARF4 transgenic lines (Figure S25). qRT-PCR results revealed that there was no significant change of the expression level of SlARF3 in SlARF4 transgenic lines, suggesting that SlARF3 and SlARF4 could function independently in regulating trichome formation. Next, further examination of both proteins reveals that SlARF4 protein has typical domains (B3, ARF and Aux/IAA) while SlARF3 only contains two conserved domains, B3 and ARF (Zhang et al., 2015). Furthermore, we generated SlARF3/4 double knockdown tomato plants by crossing RNAi-SlARF3 and RNAi-SlARF4 transgenic lines. These plants exhibited significant lower trichome density, epidermal cell density and number of trichomes per epidermal cells compared with the parent lines (Figures S26, S27 and S28). Finally, RNA-Seq analyses of RNAi-SlARF3 and RNAi-SlARF4 plants revealed that SlARF3 and SlARF4 regulate the different set of down-stream genes (data not shown). These

**Figure 7** SITHM1 and SIMYB52 target SlCycB2 and working model for R2R3 MYB-dependent auxin signalling pathway. a and b, EMSA showing the direct binding of SITHM1 and SIMYB52 to SlCycB2 promoters. + or +++ indicates increasing amounts of unlabelled probes for competition. c, Diagrams of reporter and effector vectors in the dual-luciferase reporter assay. d, SITHM1 increases the transcription of SlCycB2. e, SIMYB52 increases the transcription of SlCycB2. Each value represents the mean of six biological replicates, and vertical bars represent the SE. f, Working model. Auxin induces the expression of SlARF4 gene, and SlARF4 protein inhibits the expressions of SlTHM1 and SlMYB52 genes. The inhibited SlTHM1 levels reduce the SlCycB2 expression, which promotes the formation of II and VI type trichomes. The inhibited SlMYB52 levels reduce the SlCycB2 expression, which promotes the formation of V type trichome.
results demonstrate that SiARF3 and SiARF4 play different regulatory roles in regulating trichome formation.

**SIARF4 regulates the auxin-induced formation of unicellular and multicellular trichomes through the transcriptional inhibition of SiMYB52 and SITHM1**

Numerous overexpressed genes linked to unicellular trichomes in Arabidopsis negate the same result for Solanaceae species with multicellular trichome formation (Payne et al., 1999). In Arabidopsis, the overexpression of MiXTA and AmMYBML1, which are multicellular trichome-related genes, did not prompt the formation of unicellular trichomes (Glover et al., 1998), whereas the interaction between Wo and SlCycB2 regulated trichome formation in tomato (Yang et al., 2011). The promotion of trichome formation was not observed in Arabidopsis with overexpressed Wo gene (Yang and Ye, 2013). These reported data implied tomato and tobacco multicellular trichomes and Arabidopsis unicellular trichomes employ different regulatory pathways (Yang and Ye, 2013).

**SIARF4** regulated the formation of unicellular and multicellular trichomes. **SIARF4** negatively regulated **SITHM1** and **SiMYB52** expression by binding to AuxRE and TGA motifs, respectively (Figure 2). **SITHM1** functioned as a repressor in the regulation of the formation of II and VI type trichomes, and **SiMYB52** acted as a repressor during V type unicellular trichome formation. qRT-PCR showed that the expression levels of **SITHM1** and **SiMYB52** increased in the RNAi-SIARF4 plants and decreased in the OE-SIARF4 plants (Figure S9). Hybrid experiments showed down-regulation of **SITHM1** specifically increased the formation of II and VI type trichomes, and **SiMYB52** specifically increased the formation of V type trichome in the SIARF4 down-regulation background (Figure 6). **SIARF4** modulated the formation of II, V and VI type trichomes depending on SITHM1 and SiMYB52. II and V type trichomes are nonglandular, whereas V type is glandular (Deng et al., 2012). **SIARF4** positively regulated the formation of unicellular and multicellular trichomes through the direct transcriptional inhibition of **SiMYB52** and **SITHM1**. Our study demonstrated the presence of consistent but different characteristics in the regulation of unicellular and multicellular trichome formation in tomato leaves.

**SITHM1** and **SiMYB52** regulate trichome formation by directly binding to **SlCycB2**

Cyclins are involved in the transition between the phases of the cell cycle in eukaryotes and function as the positive regulators of cell proliferation (Meijer and Murray, 2001). B-type cyclins play important roles in G2/M transition (Fobert et al., 1994; Hirt et al., 1992). In Arabidopsis, the specific expression of B-type cyclin B1:2 induces the formation of multicellular trichomes; hence, B-type cyclins play important roles in unicellular and multicellular trichome formation (Schnittger et al., 2002). In tomato, Wo protein interacts with SiCycB2, which is essential for type I trichome formation (Yang et al., 2011). The overexpression of SiCycB2 decreases the levels of glandular I and VI type trichomes and all nonglandular trichomes. On the other hand, the suppression of SiCycB2 promotes the formation of nonglandular III and V type trichomes on Ailsa Craig tomato (Gao et al., 2017). Hence, SiCycB2 may function as an inhibitor in the formation of glandular and nonglandular trichomes (Gao et al., 2017). In the present study, **SITHM1** and **SiMYB52** functioned as repressors in the formation of multicellular and unicellular trichomes (Figures 3 and 5). SITHM1 and SiMYB52 directly targeted the SiCycB2 and activated its expression (Figure 7). We anticipated that **SITHM1** and **SiMYB52** regulate trichome formation through the activation of **SiCycB2** that may act as a repressor in the formation of unicellular and multicellular trichomes in ‘Micro-Tom’ tomato cultivar. Future study could focus on functional analysis of SiCycB2 in ‘Micro-Tom’ using CRISPR/Cas9 technology.

We proposed a model of how auxin induces the formation of II, V and VI type trichomes in tomato leaves. Auxin induces the expression of **SIARF4**, and **SIARF4** protein inhibits the expressions of **SITHM1** and **SiMYB52**. Decreased SITHM1 levels reduce SiCycB2 expression, which promotes the formation of II and VI type trichomes. Decreased SiMYB52 levels inhibit SiCycB2 expression, resulting in the promotion of the formation of V type trichomes (Figure 7). The **SITHM1**/**SiMYB52**-dependent auxin signalling pathway modulates the formation of unicellular and multicellular trichomes in tomato, and increasing trichome density is an effective method to improve the tolerance of tomato to spider mites.

**Methods**

**Plant material and growth conditions**

Tomato (*Solanum lycopersicum* ‘Micro-Tom’) plants were used. ‘Micro-Tom’ is a typical laboratorial tomato cultivar because of its short life cycle and efficient genetic transformation system. Standard greenhouse conditions are 14-h day/10-h night cycle, 25°C/20°C day/night temperature, 80% relative humidity (RH) and 250 mol/m²/s intense luminosity. Major agricultural traits were measured as previously described by Lovelli et al., 2012.

**Sequence analysis**

Sequence analysis was performed according to Zhang et al. (2015). GenBank accession numbers for the alignment as well as phylogenetic analysis are presented in supplementary materials.

**Trichome counts and phenotyping**

Forty-five-day-old tomato seedlings were used for trichome counts. Fully expanded leaves were collected from the fifth internode counted from the shoot tip. Samples were dissected from midway between the margin and midrib in 10 mm × 4 mm
strips covering the whole leaf blade (avoiding the primary veins). II, V and VI type trichomes on the adaxial leaf surfaces were analysed under a JNOEC JSZ5B stereo microscope and a HITACHI TM400 plus scanning electron microscope. The numbers of type II trichomes in an area of 0.5 cm² and the numbers of V and VI type trichomes in an area of 2.2 mm² were calculated. Adaxial epidermal pavement cells were analysed through colourless nail polish printing mark method.

**Auxin and JA treatment**

Indole acetic acid (IAA) and jasmonate acid (JA) were purchased from Sigma company. Fifteen-day-old tomato seedlings for auxin treatment were sprayed with IAA solution (0, 1, 10, 30, 50 mg/L) every 2 days for 1 month. Four tomato seedlings were used for each group, and three groups were used for each treatment. Trichome analysis was conducted 1 month after the first spray. IAA solution (30 mg/L) was used in subsequent experiments in this study. For qRT-PCR analysis, JA solution (100 μM) and IAA solution (30 mg/L) were used. Chemical induction of 3-week-old tomato plants was conducted by dipping the tomato leaf in a solution containing either IAA or JA. Leaves from four WT were collected at three time points (0 h, 2 h and 6 h) during IAA and JA treatments, and frozen immediately in liquid nitrogen for RNA extraction.

**Subcellular localization of SITHM1 and SIMYB52**

Subcellular localization assays were conducted in tobacco (Nicotiana benthamiana) leaves. The coding regions of SITHM1 and SIMYB52 without a stop codon were cloned into the pCX-DG vector in frame with the GFP sequence and cauliflower mosaic virus (CaMV) 35S promoter. *Agrobacterium tumefaciens* strain GV3101, which carried the fusion constructs and the control GFP vector (pCX-DG frame), were infiltrated into the abaxial air space of 4- to 6-week-old tobacco plants, using a needleless 2-mL syringe. GFP fluorescence was observed using a laser scanning confocal microscope. All transient expression assays were repeated at least three times. The primers used are listed in Table S4.

**Generation of transgenic plants**

The open reading frame sequence of SIARF4 was amplified and cloned into plant binary vector pLP100 to obtain SIARF4 overexpression vector. RNAi vector was constructed by cloning the target sequences of SIARF4, SITHM1 and SIMYB52 into pCAMBIA2301. GUS staining vector was constructed by cloning the 2 kb promoter sequences of pCAMBIA2301. GUS staining vector was conducted according to Yuan et al., (2019).
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Conflicts of interest statement
The authors declare no conflicts of interest.

Author contributions
W.D. and Z.G.L. conceived the research. Y.J.Y., X.X., Y.Q.L., Z.G.L. and W.D. performed experiments. W.F.Z. analysed the data. W.D. and Y.J.Y. wrote the manuscript. C.Z.J. revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Density analysis of epidermal pavement cells in the leaves of IAA-treated tomato plants.

Figure S2 qRT-PCR analysis of SIARF4 expression levels

Figure S3 Density analysis of epidermal pavement cells in the leaves of OE-SIARF4 and RNAi-SIARF4 plants.

Figure S4 Trichomes densities of fruits in SIARF4 transgenic plants.

Figure S5 Trichomes densities of leaves in Slarf4 CRISPR-Cas9 mutants.

Figure S6 RNA-Seq analysis of RNAi-SIARF4 plants.

Figure S7 RNA-Seq analysis of RNAi-SIARF4 plants.

Figure S8 SDS-PAGE gel stained with Coomassie brilliant blue demonstrating the affinity purification of recombinant GST-SIARF4 protein used for EMSA.

Figure S9 qRT-PCR analysis of the expression levels of SITHM1 and SIMYB52 genes in RNAI-SIARF4 and OE-SIARF4 plants.

Figure S10 Sequence alignments of SIMYB52 and SITHM1 with homologus proteins from other species.

Figure S11 Phylogenetic trees of SITHM1 and SIMYB52 with homologous proteins from other species

Figure S12 qRT-PCR analysis of SITHM1 expression levels.

Figure S13 Diagrams of the reporter and effector vectors and transcriptional activation activity assay of SITHM1.

Figure S14 Density of epidermal pavement cells in RNAI-SITHM1 plants.

Figure S15 qRT-PCR analysis of SIMYB52 expression levels.

Figure S16 Density of epidermal pavement cells in RNAI-SIMYB52 plants.

Figure S17 Spider mite bioassay of SIMYB52 RNAI plants

Figure S18 qRT-PCR analysis of the expression of SLCycB2 in leaves of SITHM1 and SIMYB52 transgenic lines.

Figure S19. SDS-PAGE gel stained with coomassie brilliant blue demonstrating affinity purification of the recombinant GST-SITHM1 and GST-TMYB52 used for the EMSA assay

Figure S20 Agricultural trait analysis of the SIARF4 transgenic plants.

Figure S21 Agricultural trait analysis of the SITHM1 transgenic plants.

Figure S22 Agricultural trait analysis of the SIMYB52 transgenic plants.

Figure S23 Expression patterns of SIARF3, SIARF4, SITHM1, SIMYB52 and SLCycB2 genes in response to IAA treatments.

Figure S24 Expression patterns of SIARF3, SIARF4, SITHM1, SIMYB52 and SLCycB2 genes in response to JA treatments.

Figure S25 qRT-PCR analysis of the expression of SIARF3 in leaves of SIARF4 transgenic lines.

Figure S26 Density analysis of II, VI and VI type trichomes of RNAi-SIARF3 and RNAi-SIARF4 crossed plants.

Figure S27 Number of trichomes per epidermal cell of RNAi-SIARF3 and RNAi-SIARF4 crossed plants.

Figure S28 Density of epidermal pavement cells of RNAI-SIARF3 and RNAI-SIARF4 crossed plants.

Table S1 All DEGs in RNAi-SIARF4 plants

Table S2 GO function and pathway enrichment analyses of DEGs in RNAI-SIARF4 plants

Table S3 DEGs encoding TFs in RNAi-SIARF4 plants.

Table S4 Primers used in this study.