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SlMYC1 Regulates Type VI Glandular Trichome Formation and Terpene Biosynthesis in Tomato Glandular Cells[OPEN]

Jiesen Xu,[a] Zeger O. van Herwijnen,[b] Dörthe B. Dräger,[b] Chun Sui,[a,c] Michel A. Haring,[a] and Robert C. Schuurink[a,1]

[a] Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 XH Amsterdam, the Netherlands

[b] Rijk Zwaan Breeding B.V., Burgemeester Crezéelaan 40, 2678 ZG De Lier, The Netherlands

[c] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100193, China

ORCID IDs: 0000-0003-3085-1693 (J.X.); 0000-0003-0415-4827 (Z.O.v.H.); 0000-0001-8125-0658 (D.B.D.); 0000-0002-4678-2218 (C.S.); 0000-0003-3405-6945 (M.A.H.); 0000-0002-9223-9996 (R.C.S.)

Tomato (*Solanum lycopersicum*) glandular trichomes function as biochemical factories that synthesize a diverse array of specialized metabolites. Terpenoids are the most diverse class of plant specialized metabolites, with volatile mono- and sesquiterpenes playing important roles in plant defense. Although the biosynthetic pathways of volatile terpenes in tomato glandular trichomes have been well described, little is known about their regulation. Here, we demonstrate that SlMYC1, a basic helix-loop-helix transcription factor, differentially regulates mono- and sesquiterpene biosynthesis in the type VI glandular trichomes of tomato leaves and stems. SlMYC1 functions as a positive regulator of monoterpene biosynthesis in both leaf and stem trichomes but as a negative regulator of sesquiterpene biosynthesis in stem trichomes. SlMYC1 is also essential for type VI glandular trichome development, as knocking down *SlMYC1* led to the production of smaller type VI glandular trichomes at lower densities, and knocking out this gene led to their absence. Our findings reveal a role for SlMYC1 not only in type VI glandular trichome development but also in the regulation of terpene biosynthesis in tomato.

INTRODUCTION

Trichomes are epidermal outgrowths on the stem and leaf surfaces of plants that serve as physical barriers to herbivorous arthropods. These structures also function as biochemical factories, as they synthesize, store, and secrete a wide range of specialized metabolites, such as terpenoids, phenylpropanoids, flavonoids, alkaloids, and acyl sugars (Schilmiller et al., 2008). Cultivated tomato (*Solanum lycopersicum*) and its wild relatives have eight types of trichomes, including both glandular (types I, IV, VI, and VII) and non-glandular (types II, III, V, and VIII) trichomes (Glas et al., 2012). However, our knowledge of the development of glandular trichomes in tomato is very limited. The *Woolly* (*Wo*) gene, encoding a homeodomain-Leu zipper (HD-ZIP) protein and the *Hair* gene, encoding a Cys2-His2 zinc finger protein that interacts with the *Wo* gene product, are essential for type I trichome development together with the *CyclinB2* gene (Yang et al., 2011; Gao et al., 2017; Chang et al., 2018). The *hairless* gene from tomato, encoding a subunit of a regulatory complex that controls the nucleation of actin filaments, was recently mapped and cloned, indicating that actin plays an important role in the proper development of several types of trichomes (Kang et al., 2010a, 2016). The *dialytic* and *odorless-2* loci, which affect trichome development, await further characterization (Kang et al., 2010b; Chang et al., 2016). Ectopic expression of *SlMIXTA1*, encoding a MYB transcription factor in tomato, led to higher numbers of glandular and non-glandular trichomes (Ewas et al., 2016, 2017). It has become clear that jasmonic acid (JA) also plays an important role in regulating the development of type VI glandular trichomes, as JA-deficient plants have reduced numbers of these trichomes (Yan et al., 2013; Bosch et al., 2014), and there is evidence that the JA-Ile receptor plays a role in this process as well (Li et al., 2004).

Type I and IV glandular trichomes are sources of acyl sugars (McDowell et al., 2011). Type IV trichomes are ubiquitous in some wild tomato relatives (Simmons and Gurr, 2005), but in *S. lycopersicum*, they are present only on embryonic and juvenile leaves (Vendemiatti et al., 2017). Type VI trichomes can produce terpenoids, flavonoids, and methyl ketones (Williams et al., 1980; Fridman et al., 2005; Besser et al., 2009; Schilmiller et al., 2010b; Schmidt et al., 2011; Kang et al., 2014). In the wild tomato *Solanum habrochaites*, type VI glands can produce large amounts of volatile mono- and sesquiterpenes, whereas type VI trichomes in cultivated tomato mainly accumulate monoterpenes (Besser et al., 2009; Sallaud et al., 2009; Schilmiller et al., 2009; Bleeker et al., 2011; Falara et al., 2011). The glandular heads of type VI trichome in these two species have distinct features (Bergau et al., 2015).

Volatile terpenes, representing the largest and most diverse class of plant volatile metabolites, contribute to plant defense responses, as they are repellent or toxic to herbivores or attract predators or parasitoids of the attacking insects (Gershenzon and Dudareva, 2007; Tholl, 2015). In *S. lycopersicum*, 45 terpene synthases (TPSs) have been identified, 30 of which appear to be functional (Falara et al., 2011, 2014). Transcripts for 14 of these genes have been detected in stem trichomes (Spyropoulou et al.,

[1] Address correspondence to r.c.schuurink@uva.nl.
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IN A NUTSHELL

Background: Many plants have hairs (trichomes) on their leaves and stems to defend themselves against insects, as trichomes form physical barriers. Some of these hairs have glandular cells on top that produce special metabolites that can be toxic to insects or, when gaseous (volatile), can attract enemies of these herbivores. The production of volatile metabolites is often induced by herbivores. Tomato has eight types of hairs, but we know relatively little about their development and the regulation of their special metabolite production. Cultivated tomato has glandular trichomes (type VI) that produce volatile terpenes. These molecules are produced by many plants as defense compounds and are often utilized in food and cosmetics as flavors and fragrances.

Question: We wanted to know if, in cultivated tomato, the previously identified regulatory protein MYC1 is involved in the development of type VI glandular trichomes or the regulation of volatile terpene production. We tested this by switching the *MYC1* gene off and by expressing a more stable MYC1 protein.

Findings: We found that cultivated tomato plants can only develop these glandular trichomes when they produce MYC1. When *MYC1* was switched off, type VI glandular trichomes did not develop, although other trichome types did. When MYC1 production was turned down, smaller type VI trichomes developed that produced fewer volatile terpenes. Interestingly, on stems, these smaller trichomes suddenly produced additional volatile terpenes, indicating that MYC1 normally represses their production. Expressing a stable MYC1 specifically in the glandular cells of these trichomes supported these data. Thus, surprisingly, the protein MYC1 has a dual regulatory function in both metabolism and development.

Next steps: Scientists aim to tweak crop plants to produce more special metabolites against herbivores, like their wild relatives quite often do. We would like to find out if MYC1 plays a similar role in the wild relatives of tomato and whether increasing the expression of *MYC1* leads to the formation of more type VI glandular trichomes and/or volatile terpenes.

2014a), and some are induced by JA (van Schie et al., 2007; Bleeker et al., 2011; Falara et al., 2011). *SlTPS5* and *SlTPS9* are specifically expressed in the glandular heads of type VI trichomes (Spyropoulou et al., 2014a; Kortbeek et al., 2016).

In *S. lycopersicum*, only three transcription factors that are potentially involved in regulating volatile terpene biosynthesis in trichomes, SlEOT1 (Expression of Terpenoids 1), SlWRKY73, and SlMYC1, have thus far been identified (Spyropoulou et al., 2014a, 2014b). SlEOT1 and SlWRKY73 transactivate the *SlTPS5* promoter in *Nicotiana benthamiana* leaves, and SlMYC1 transactivates the promoters of several TPS genes (Spyropoulou et al., 2014a, 2014b). SlMYC1 is a basic helix-loop-helix (bHLH) transcription factor. A number of bHLHs from several species have been shown to act as positive regulators of terpenoid biosynthesis: in *Catharanthus roseus* (Madagascar periwinkle) for monoterpenoid indole alkaloid biosynthesis (Zhang et al., 2011; Van Moerkercke et al., 2015, 2016); in *Medicago truncatula* (barrelclover) for triterpene saponin biosynthesis (Mertens et al., 2016); in Arabidopsis (*Arabidopsis thaliana*) for sesquiterpene synthase gene expression (Hong et al., 2012); in *Artemisia annua* (sweet wormwood) for artemisinin biosynthesis (Ji et al., 2014; Shen et al., 2016); in *Oryza sativa* (rice) for diterpenoid phytoalexin biosynthetic gene expression (Yamamura et al., 2015); in *Salvia miltiorrhiza* (red sage) for tanshinone biosynthesis (Zhou et al., 2016); and in *Aquilaria sinensis* (incense tree) for agarwood sesquiterpene biosynthesis (Xu et al., 2017). Two bHLHs from *Betula platyphylla Suk* (Japanese white birch) were recently shown to be involved in regulating triterpenoid biosynthesis (Yin et al., 2017). Interestingly, some bHLH transcription factors appear to act as repressors of terpenoid biosynthesis, including the biosynthesis of the diterpene paclitaxel in *Taxus cuspidata* (Japanese yew; Lenka et al., 2015) and the monoterpenoid indole alkaloid biosynthetic pathway in *C. roseus* (Patra et al., 2018).

The objective of this study was to explore the roles of SlMYC1 in the regulation of volatile terpene biosynthesis in tomato trichomes and trichome development via gene knockdown, knockout, and overexpression. Our results shed light on the roles of SlMYC1 in these two very different processes.

RESULTS

SlMYC1 Knockdown Affects the Expression of Mono- and Sesquiterpene Synthase Genes

To investigate whether SlMYC1 participates in the regulation of volatile terpene biosynthesis, we used an RNA interference strategy to downregulate *SlMYC1* expression in transgenic tomato plants. An *SlMYC1*-inverted-repeat (ir) construct driven by the *Cauliflower mosaic virus* 35S promoter was introduced into *S. lycopersicum* cultivar (cv) Moneymaker. We obtained many independent transgenic lines and selected three lines (ir-*SlMYC1* lines 8, 18, and 25) for further analysis. All three lines had significantly reduced *SlMYC1* transcript levels (by 80 to 95%) in stem trichomes and leaves compared with wild type (Figures 1A and 1B; Supplemental Data Set). Additionally, the transcript levels of monoterpene synthase genes *SlTPS3*, *SlTPS5*, *SlTPS20*, and *SlTPS39* and sesquiterpene synthase genes *SlTPS9*, *SlTPS17*, and *SlTPS31* were reduced in stem trichomes (Figures 1C and 1D). By contrast, *SlTPS12*, encoding a β-caryophyllene/α-humulene sesquiterpene synthase, had significantly higher transcript levels in stem trichomes of ir-*SlMYC1* lines 18 and 25 compared with wild type (Figure 1D). In leaves, the downregulation of *SlMYC1* led to significantly reduced transcript levels of *SlTPS12* and other TPS genes (Figure 1E).

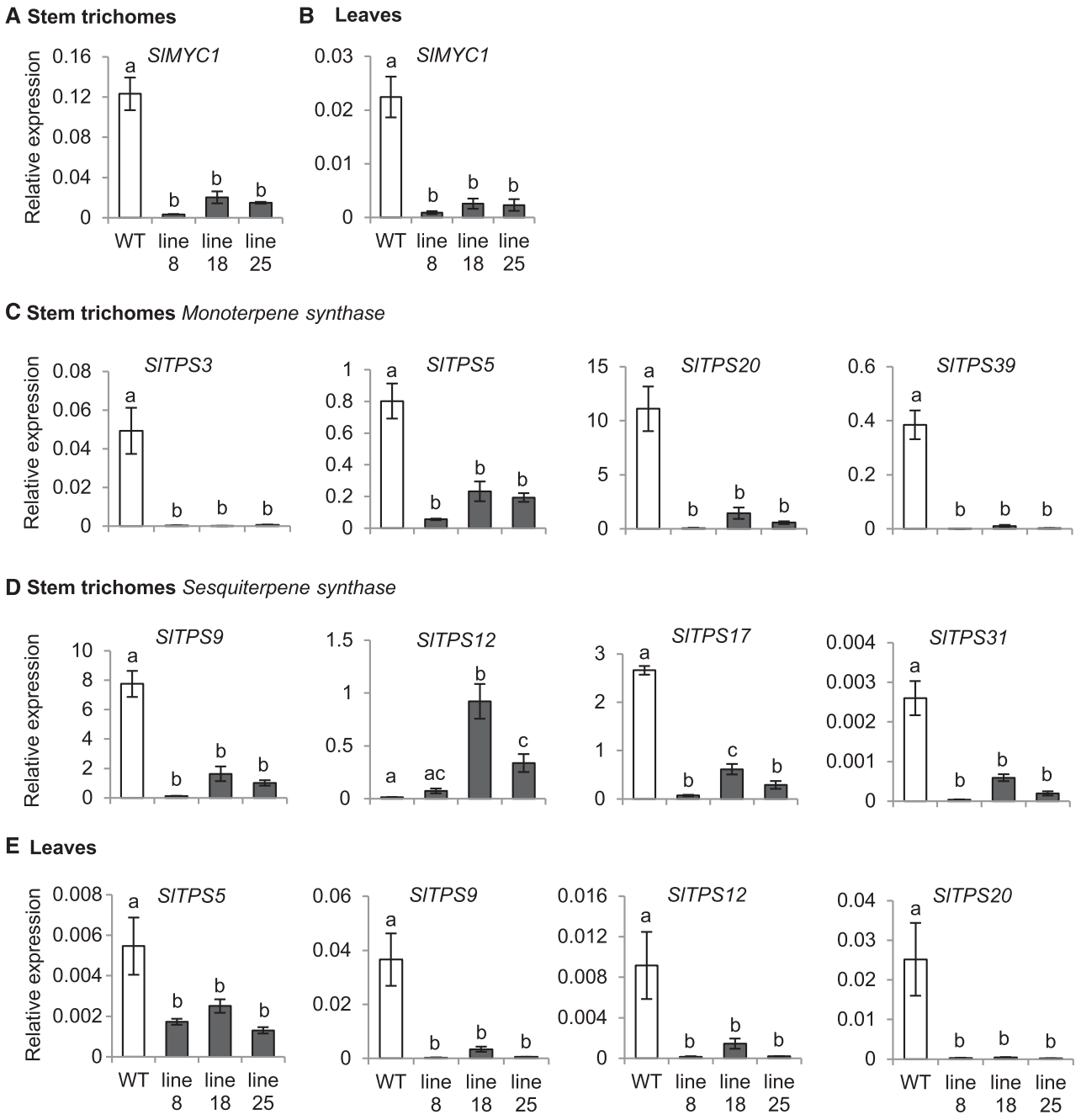

Figure 1. Effect of *SlMYC1* Downregulation on the Expression of Terpene Synthase Genes in Stem Trichomes and Leaves.

Relative transcript levels of *SlMYC1* in **(A)** stem trichomes and **(B)** leaves from wild-type (WT) Moneymaker and ir-*SlMYC1* plants. Relative transcript levels of **(C)** several monoterpene and **(D)** sesquiterpene synthase genes in stem trichomes from wild-type (WT) Moneymaker and ir-*SlMYC1* plants. **(E)** Relative transcript levels of terpene synthase genes in leaves from wild-type (WT) Moneymaker and ir-*SlMYC1* plants. Lines 8, 18, and 25 are three independent transgenic ir-*SlMYC1* lines. All transcript levels were determined by qRT-PCR and normalized to *Actin* transcript levels. Bars represent the mean values (±SE) of three to four biological replicates, each consisting of multiple stem pieces or leaflets from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA, see Supplemental Data Set for details (Brady et al., 2015).

SlMYC1 Knockdown Differentially Affects Mono- and Sesquiterpene Levels in Stem and Leaf Trichomes

Quantification of the volatile terpenes in stem trichomes using gas chromatography-mass spectrometry revealed that the levels of ten monoterpenes identified as α-pinene, 2-carene, α-phellandrene, α-terpinene, *p*-cymene, β-ocimene, γ-terpinene, terpinolene, β-phellandrene, and D-limonene were significantly reduced in the ir-*SlMYC1* lines (Figure 2A). The levels of all of these monoterpenes were lowest in line 8, which had the lowest *SlMYC1* transcript level. Volatile sesquiterpenes were not detected in wild-type Moneymaker stem trichomes. However, considerable amounts of β-caryophyllene and α-humulene were present in the stem trichomes of the ir-*SlMYC1* lines (Figure 2A), which is consistent with the elevated *SlTPS12* transcript levels (Figure 1D). β-caryophyllene and α-humulene are normally produced in leaf trichomes rather than stem trichomes in cultivated tomato plants (Schilmiller et al., 2010a). In leaf trichomes, the ir-*SlMYC1* plants produced significantly less β-caryophyllene and α-humulene than the wild type, and the levels of all monoterpenes were also reduced (Figure 2B). These results indicate that SlMYC1 plays different roles in the regulation of β-caryophyllene and α-humulene biosynthesis in tomato leaf and stem trichomes.

SlMYC1 Is Involved in Type VI Glandular Trichome Development

Type VI trichomes contain four glandular cells on top of an intermediate cell and a stalk, which form the head, where metabolites are stored in a cavity under a waxy cuticle (McDowell et al., 2011; Bergau et al., 2015). Since SlMYC1 regulates terpene biosynthesis in tomato trichomes, we also examined type VI glandular trichome morphology by environmental scanning electron microscopy (ESEM). As shown in Figure 3A, type VI glandular trichomes on the stems of 4-week-old ir-*SlMYC1* plants had shorter stalks and smaller glandular heads compared with wild-type Moneymaker (WT-MM). The gland diameters and stalk lengths of type VI trichomes on ir-*SlMYC1* stems were 60 to 80% and 30 to 45% of those on wild-type stems, respectively (Figure 3C). The glandular cells of type VI trichomes in ir-*SlMYC1* plants occasionally appeared to have undergone an irregular cell division, leading to the formation of five cells (Supplemental

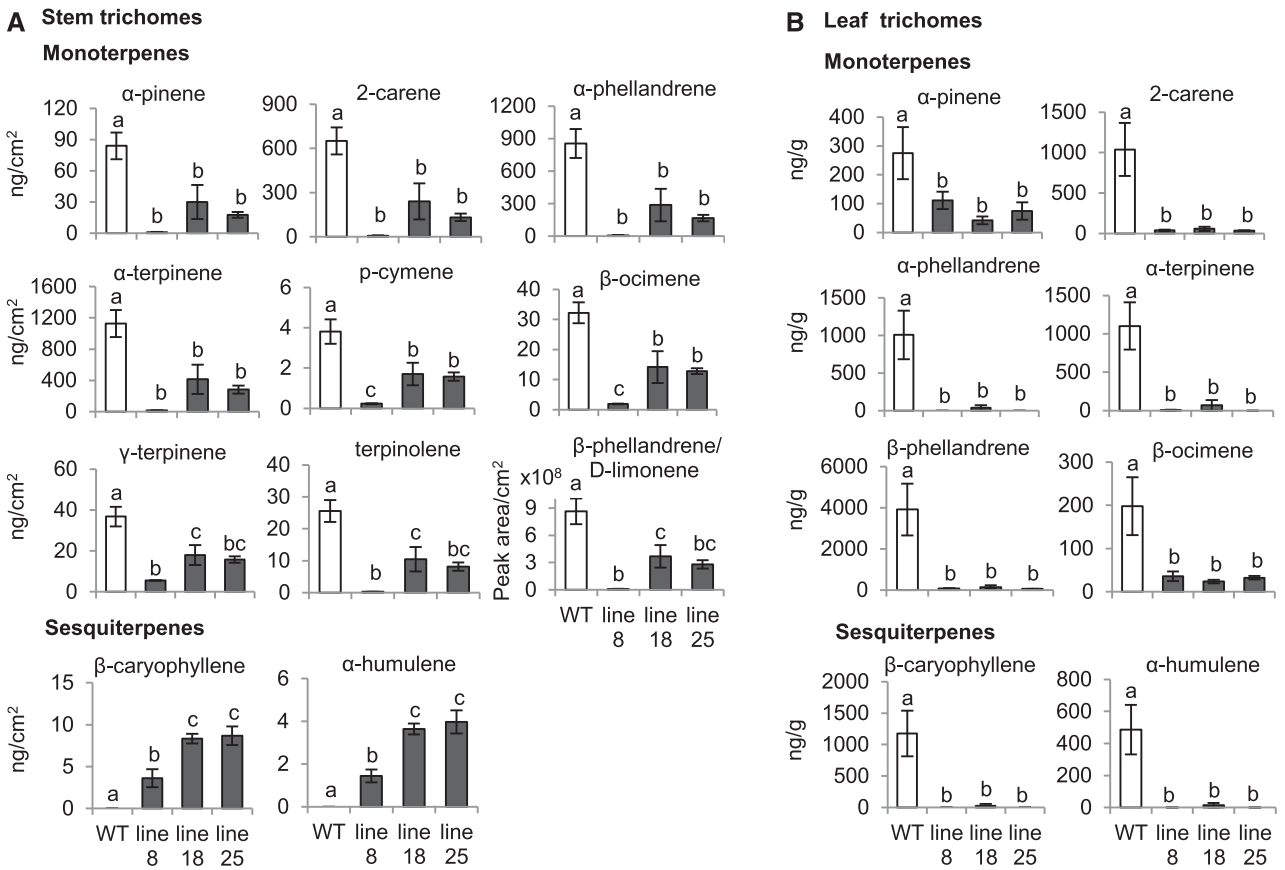

Figure 2. Effect of *SlMYC1* Downregulation on Volatile Terpene Levels in Stem and Leaf Trichomes of Tomato.

Levels of volatile terpenes in **(A)** stem and **(B)** leaf trichomes from wild-type (WT) and ir-*SlMYC1* line 8, 18, and 25 plants, as quantified by gas chromatography-mass spectrometry (GC-MS). The terpene levels in stem trichomes were normalized by stem surface area and in leaf trichomes by leaf fresh weight. Bars represent the mean values (±SE) of three to four biological replicates, each consisting of multiple stem pieces or leaflets from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

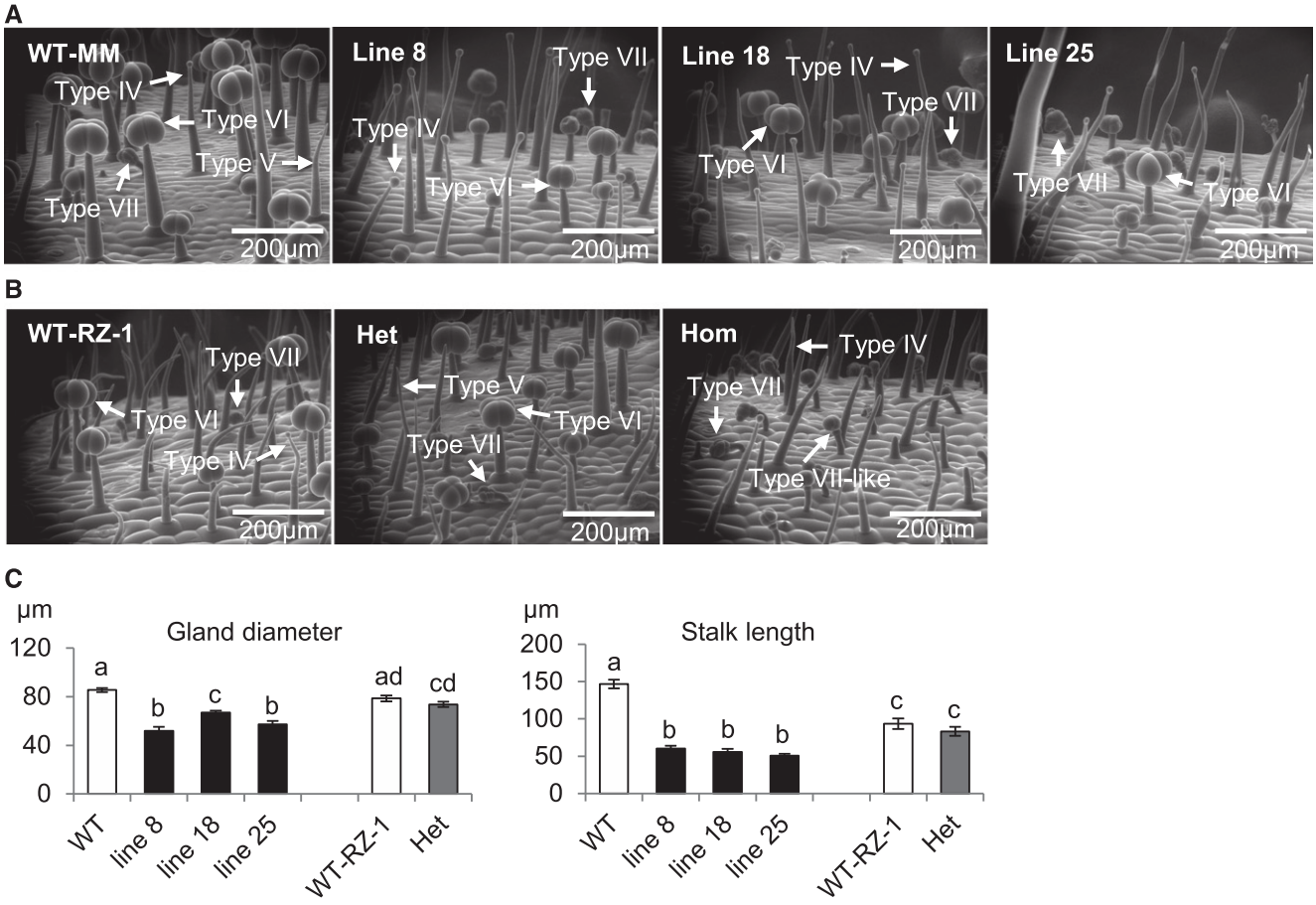

Figure 3. Morphology and Size of Type VI Glandular Trichomes on Stems.

(A) Stem surfaces of wild-type Moneymaker (WT-MM) and ir-*SlMYC1* line 8, 18, and 25 plants.

(B) Stem surfaces of wild-type RZ (WT-RZ-1), heterozygous *MYC1/myc1* (Het), and homozygous *myc1* (Hom) plants. Arrows indicate different types of trichomes.

(C) Gland diameter and stalk length of type VI trichomes on the stem surfaces of wild-type (WT) Moneymaker and ir-*SlMYC1* line 8, 18, and 25 plants and wild-type RZ (WT-RZ-1) and heterozygous *MYC1/myc1* (Het) plants. All plants were 4 weeks old. The bars represent the mean values (±SE), calculated from three to four scanning-electron micrographs of stems from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

Figure 1B). The morphology of type IV, V, and VII trichomes was unaltered in the ir-*SlMYC1* plants (Figure 3A).

To further explore the role of SlMYC1 in the formation of type VI glandular trichomes, we identified a *myc1* mutant from an ethyl methane sulfonate (EMS)-treated *S. lycopersicum* population. The *myc1* mutant has a single nucleotide change of G to T at position 1477 in the coding sequence of SlMYC1, resulting in an early stop codon at amino acid position 493. This truncated SlMYC1 was unable to activate the promoter of *SlTPS5* in *N. benthamiana* leaves (Supplemental Figure 2), indicating that this mutant allele does not provide an active form of SlMYC1. Therefore, we examined the trichomes in homozygous *myc1* and heterozygous *MYC1/myc1* plants. Figure 3B shows that type VI glandular trichomes were absent from the stems of homozygous *myc1* plants. By contrast, the type VI trichomes on the stem surfaces of heterozygous *MYC1/myc1* plants were similar to those of wild-type plants (Figures 3B and 3C). We also occasionally observed some irregular divisions of type VI glandular cells in *MYC1/myc1* plants (Supplemental Figure 1B) similar to those observed in ir-*SlMYC1* plants. The type IV, V, and VII trichomes of *MYC1/myc1* and *myc1* plants were similar to those of wild-type plants (Figure 3B). We also observed some type VII-like glandular trichomes in addition to normal type VII glandular trichomes in homozygous *myc1* plants (Figure 3B). These peculiar type VII-like trichomes contained a single stalk cell with a wide intermediate cell and a berry-shaped glandular head composed of multiple cells, which is similar to the morphology of type VII glandular trichomes. However, the unusual trichomes stood straight up, unlike type VII trichomes, which bent downward at an angle of ~45° (Supplemental Figure 1A). Due to the presence of a wide intermediate cell, we designated these trichomes type VII-like trichomes, but theoretically, they could also have been malformed type VI trichomes. To further confirm the crucial role of SlMYC1 in type VI glandular trichome formation, we constructed *SlMYC1* knockout lines via CRISPR-Cas9 genome

editing (Barrangou et al., 2007; Mali et al., 2013). Two independent transgenic lines with different nucleotide deletions in *SlMYC1* (Supplemental Figure 3) lacked type VI glandular trichomes on the stem surface (Supplemental Figure 4A). Like homozygous *myc1* plants, these two CRISPR-Cas9_*myc1* lines also had type VII-like glandular trichomes (Supplemental Figure 4B).

The effect of reduced *SlMYC1* expression on type VI trichome morphology prompted us to ask whether trichome density was also affected in these plants. In 7-week-old ir-*SlMYC1* plants, the densities of type VI glandular trichomes were lower on both the adaxial and abaxial leaf surfaces but were unchanged on stems compared with wild type (Figure 4A). The type VI trichome densities on the adaxial leaf surface and stem were similar in *MYC1*/*myc1* and wild-type plants but were reduced on the abaxial leaf surface in *MYC1*/*myc1* plants (Figure 4B). The densities of type I, III, and V trichomes on leaves and stems were not affected in any of the lines (Figures 4A and 4B). We also counted type VII-like trichomes together with type VII trichomes, as it was difficult to distinguish between the two. The *myc1* mutant had higher densities of type VII/VII-like trichomes than wild type (Figure 4B), as did ir-*SlMYC1* line 8 and 25 on the adaxial leaf surface (Figure 4A). In these 7-week-old plants, type IV glandular trichomes were absent

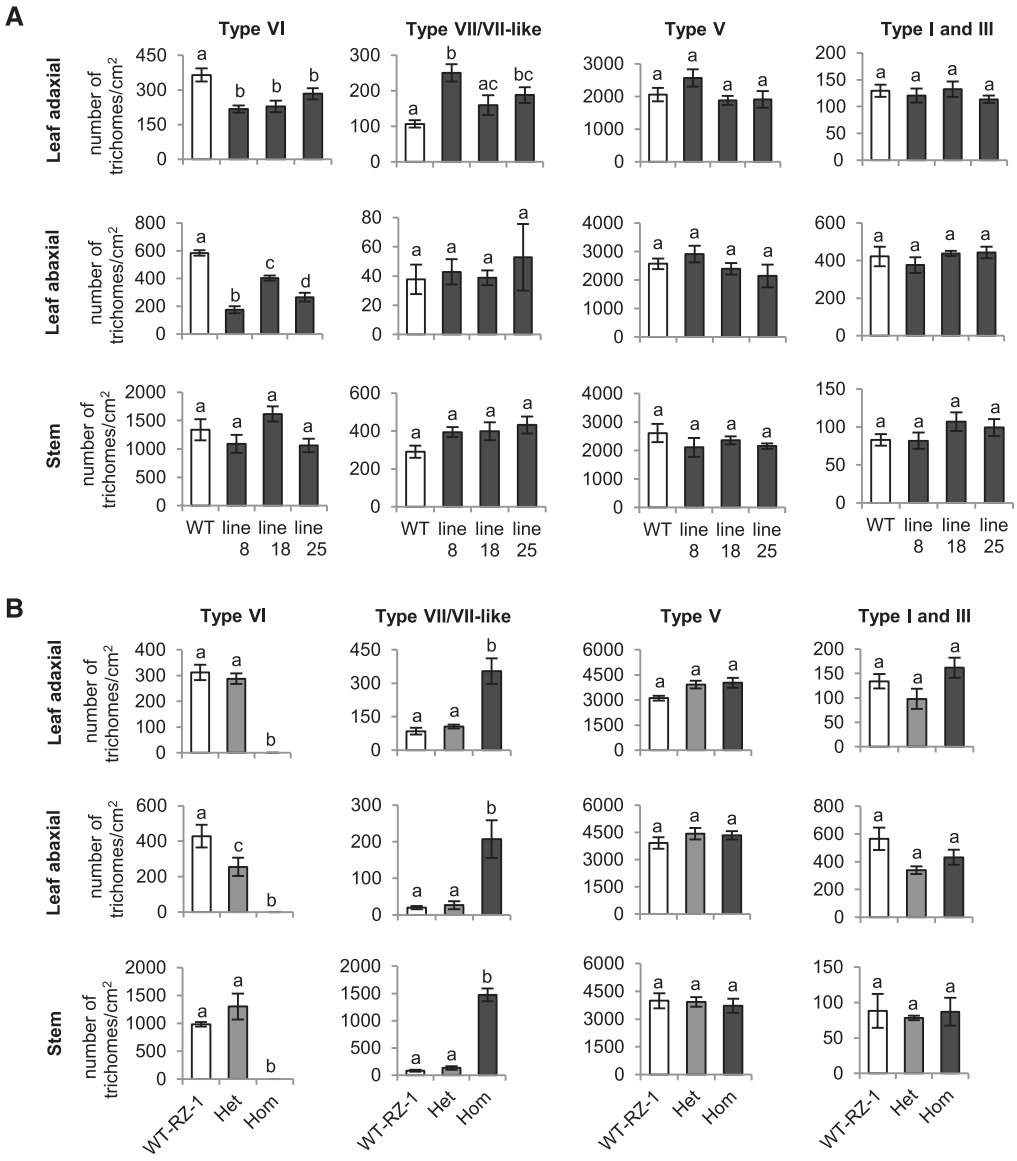

Figure 4. Trichome Density on the Leaf and Stem Surfaces of Tomato.

Trichome density on the adaxial and abaxial leaf and stem surfaces of **(A)** wild-type (WT) Moneymaker and ir-*SlMYC1* line 8, 18, and 25 plants and **(B)** wild-type RZ (WT-RZ-1), heterozygous *MYC1*/*myc1* (Het) and homozygous *myc1* (Hom) plants. All plants were 7 weeks old. Bars represent the mean values (±SE) of three to four leaf or stem samples from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

from the leaf surfaces and occasionally present on stems. However, these trichomes were present on the stems of 4-week-old plants and, interestingly, the densities of these type IV trichomes were higher in ir-*SlMYC1* line 8 and 25 than in wild-type plants (Supplemental Figure 5). These results indicate that the downregulation or absence of *SlMYC1* led to an increase in the numbers of other trichomes.

Regulation of Terpene Biosynthesis by SlMYC1 Occurs in Type VI Glandular Trichomes

In cultivated tomato plants, volatile mono- and sesquiterpenes are mainly synthesized by the glandular cells of type VI trichomes (Schilmiller et al., 2009; Kang et al., 2010a; Widhalm et al., 2015). As predicted, homozygous *myc1* plants produced very few monoterpenes and sesquiterpenes in the remaining stem trichomes. The heterozygous *MYC1/myc1* plants contained similar amounts of most monoterpenes to wild-type plants (Figure 5A) but interestingly, the levels of β-caryophyllene and α-humulene were higher in these plants than in wild type (Figure 5A), which is similar to what we observed in ir-*SlMYC1* plants (Figure 2A). The terpene levels coincided well with the transcript levels of TPS genes. In stem trichomes, *MYC1/myc1* plants had significantly higher transcript levels of β-caryophyllene and α-humulene synthase (*SlTPS12*) genes than wild type, whereas the *myc1* plants had extremely low transcript levels of all TPS genes (Figure 6A). The leaf trichomes of the *myc1* plants had lower levels of most terpenes (Figure 5B), which is consistent with the low expression levels of TPS genes (Figure 6B). *SlTPS5* was still expressed in *myc1* leaf trichomes but not stem trichomes, and these leaf trichomes produced tiny amounts of terpenes. *MYC1/myc1* plants had reduced levels of monoterpenes and similar levels of sesquiterpenes in their leaf trichomes compared with wild-type plants (Figure 5B), although *SlTPS12* expression levels were higher (Figure 6B).

To further explore the role of SlMYC1 in regulating terpene levels, we examined the terpene composition of isolated type VI glandular cells. For each type of analysis, we collected 200 individual type VI glands from the stems or leaves of each plant with a stretched glass pipette. We could not collect type VI trichomes

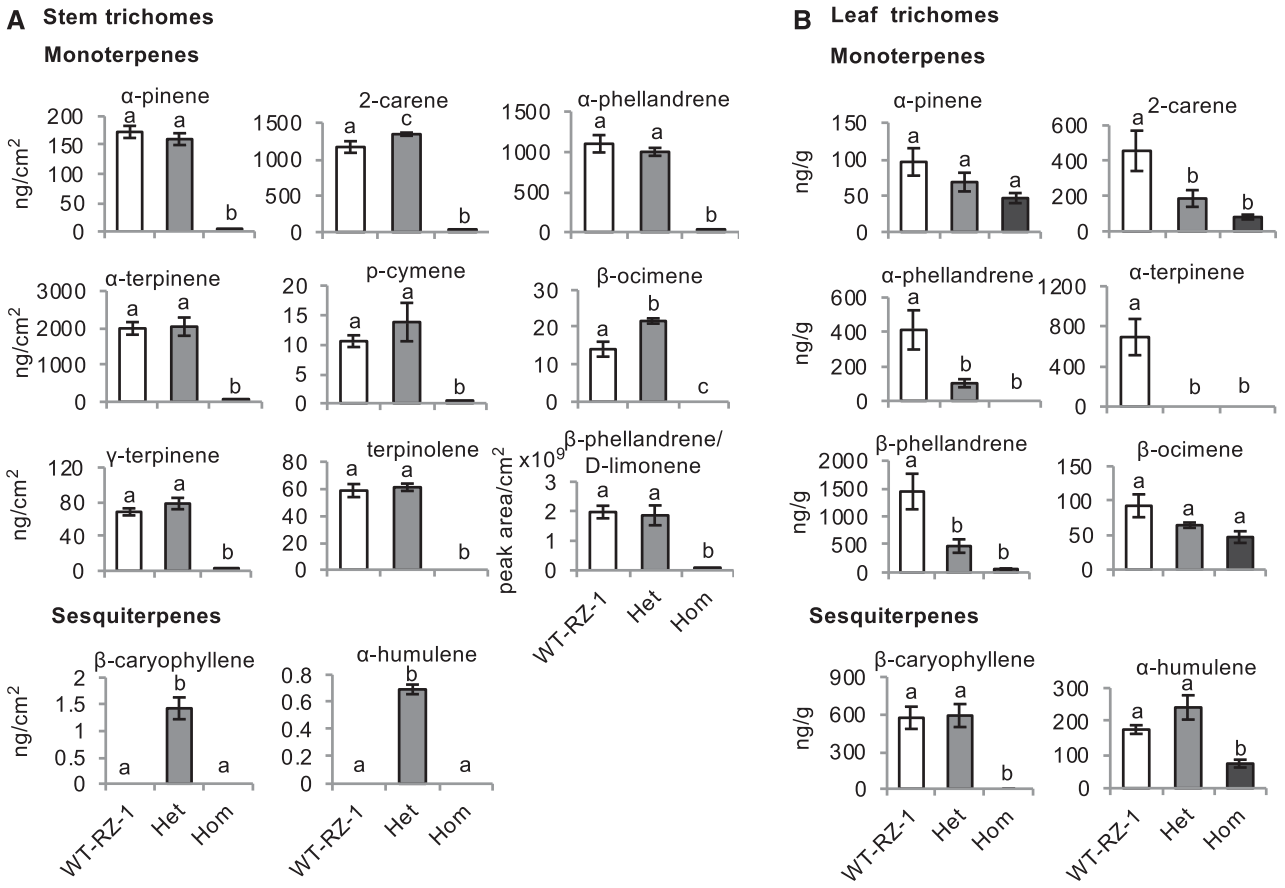

Figure 5. Volatile Terpene Levels in Trichomes of Hetero- and Homozygous *myc1* Mutants.

Volatile terpene levels in **(A)** stem and **(B)** leaf trichomes from wild-type RZ (WT-RZ-1), heterozygous *MYC1/myc1* (Het), and homozygous *myc1* (Hom) plants, as quantified by gas chromatography-mass spectrometry (GC-MS). Terpene levels in stem trichomes were normalized by stem surface area and in leaf trichomes by leaf fresh weight. Bars represent the mean values (±SE) of three to four biological replicates, each consisting of multiple stem pieces or leaflets from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

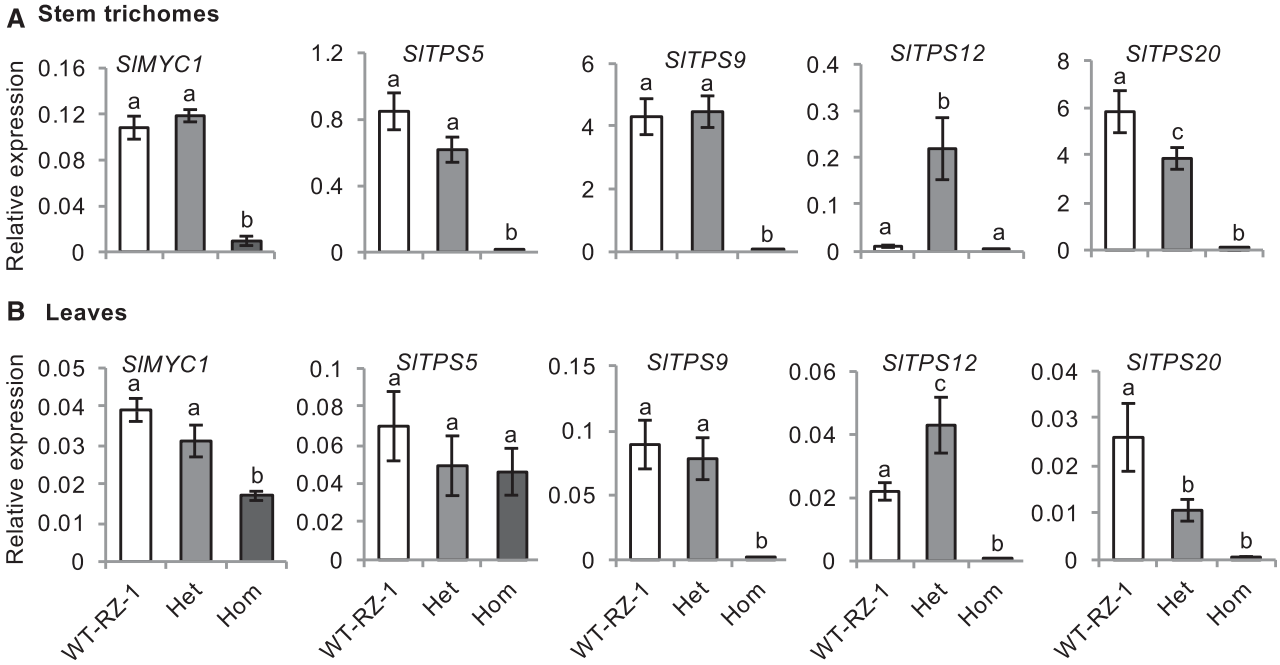

Figure 6. Expression of Terpene Synthase Genes in Hetero- and Homozygous *myc1* Mutants.

Relative transcript levels in **(A)** stem trichomes and **(B)** leaves from wild-type RZ (WT-RZ-1), heterozygous *MYC1/myc1* (Het), and homozygous *myc1* (Hom) plants. All transcript levels were determined by qRT-PCR and normalized to *Actin* transcript levels. Bars represent the mean values (±SE) of three to four biological replicates, each consisting of multiple stem pieces or leaflets from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

from homozygous *myc1* plants, as these were absent. As shown in Figure 7A, the monoterpene levels were the most strongly reduced in line 8, in which the expression level of *SlMYC1* was lowest (Figure 1A). The *MYC1/myc1* plants had the same monoterpene levels as wild-type plants in type VI stem trichomes (Figure 7A). Conversely, the *MYC1/myc1* and ir-*SlMYC1* plants exhibited elevated levels of β-caryophyllene and α-humulene in these trichomes (Figure 7A). Among type VI leaf trichomes, monoterpene levels were reduced in ir-*SlMYC1* and *MYC1/myc1* plants compared with wild type (Figure 7B). Also, β-caryophyllene and α-humulene levels were reduced in type VI trichomes on the leaves of ir-*SlMYC1* plants but were similar to wild type in *MYC1/myc1* (Figure 7B). These results indicate that SlMYC1 differentially regulates of mono- and sesquiterpene biosynthesis in type VI glandular trichomes.

SlMYC1^{E161K} Expression Leads to the Downregulation of *SlTPS12* in Stem Trichomes

The Arabidopsis *myc2-322B* mutant is a gain-of-function *AtMYC2* mutant in which a glutamate to lysine (E165K) substitution in the transcriptional activation domain causes enhanced transcriptional activity and the partial release of AtMYC2 from repression by jasmonate ZIM-domain proteins (Gasperini et al., 2015). We investigated whether the corresponding E161K mutation in SlMYC1 would affect the expression of TPS genes. *SlMYC1^{E161K}* driven by the glandular trichome-specific *SlTPS5* promoter (Spyropoulou et al., 2014b) was transformed into *S. lycopersicum* cv Moneymaker. Accordingly, *SlMYC1^{E161K}* was expressed in the stem trichomes of two independent *SlTPS5p:SlMYC1^{E161K}* lines (Figure 8A). The total *SlMYC1* transcript levels (*SlMYC1/SlMYC1^{E161K}*) differed between the two lines (Figure 8A). *SlMYC1^{E161K}* expression led to the upregulation of most monoterpene synthase genes (Figure 8B). *SlMYC1^{E161K}* expression had several effects on sesquiterpene synthase genes: (1) *SlTPS12* expression was almost abolished in *SlTPS5p:SlMYC1^{E161K}* plants; (2) *SlTPS17* expression was reduced; (3) *SlTPS31* expression did not change; and (4) *SlTPS9* expression increased (Figure 8C). These experiments using a "stabilized" SlMYC1^{E161K} help confirm the finding that SlMYC1 is involved in regulating the expression of TPS genes. Additionally, we examined type VI trichome morphology in *SlTPS5p:SlMYC1^{E161K}* plants. The type VI stem trichomes in these plants were similar to those of wild-type plants (Supplemental Figures 4A and 4B).

JA Application Fails to Restore the Phenotype of Type VI Trichomes in *SlMYC1* Knockdown Plants

Consecutive applications of methyl jasmonate increase the density of type VI glandular trichomes on newly formed tomato leaves (Boughton et al., 2005). Therefore, we treated ir-*SlMYC1* and *myc1* plants with JA to determine whether type VI trichome morphology could be restored. After multiple applications of JA, the gland size and stalk length of type VI trichomes on newly expanded leaves were similar in untreated versus treated plants. As shown in Figure 9, the stalks of type VI trichomes on newly

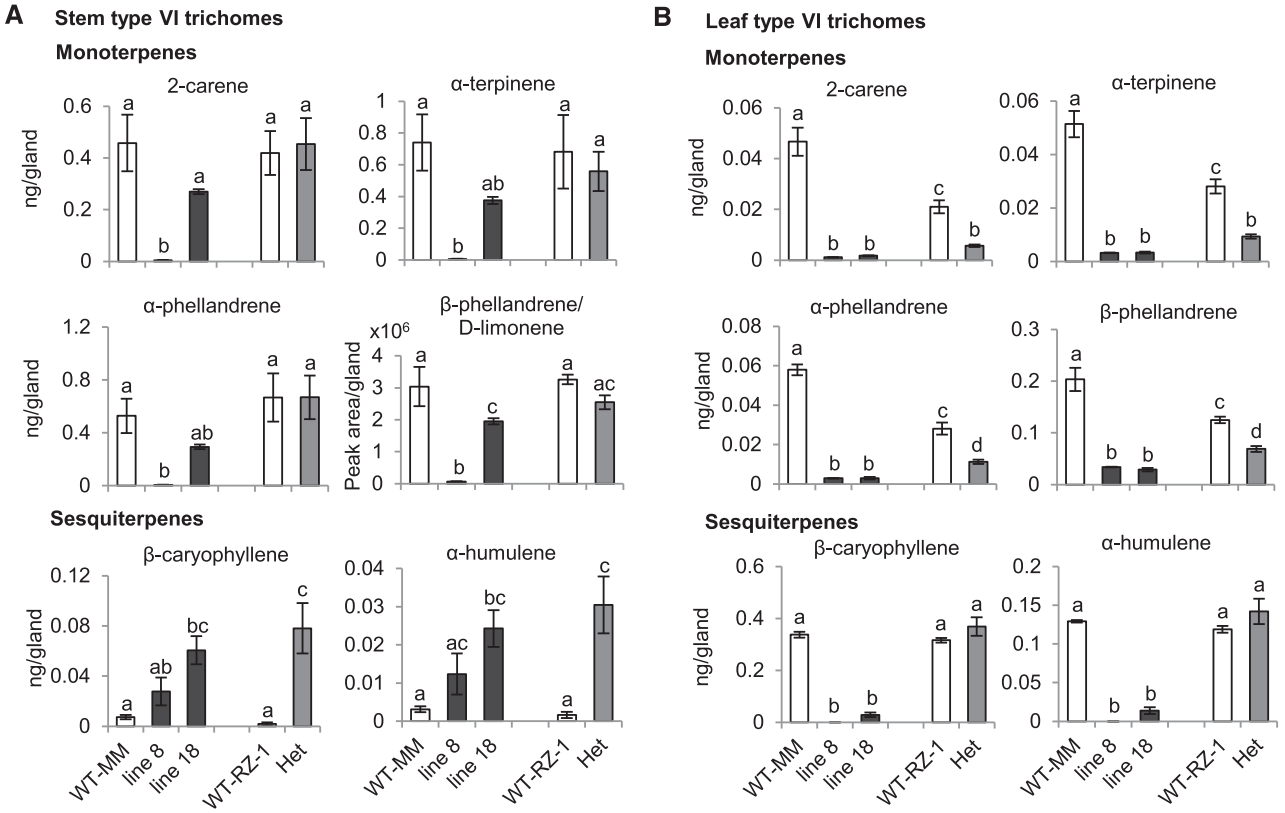

Figure 7. Effect of *SlMYC1* Downregulation on Volatile Terpene Levels in Type VI Glandular Trichomes.

Volatile terpene levels in **(A)** stem and **(B)** leaf type VI glandular trichomes from wild-type Moneymaker (WT-MM) and ir-*SlMYC1* line 8 and 18 plants and wild-type RZ (WT-RZ-1) and heterozygous *MYC1*/*myc1* (Het) plants. For each sample, 200 type VI glandular trichomes were collected with a glass capillary to quantify volatile terpene levels by gas chromatography-mass spectrometry (GC-MS). Bars represent the mean values (±SE) of three to four biological replicates, each consisting of 200 trichomes collected from multiple leaves from different plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

developed stems were shorter in both untreated and JA-treated ir-*SlMYC1* plants compared with wild type. Therefore, consecutive treatments with JA failed to restore the changes in type VI trichome morphology caused by the reduced expression of *SlMYC1*.

We also investigated type VI trichome density after exogenous JA application. JA treatment did not rescue the loss of type VI trichomes in homozygous *myc1* plants (Figure 9C). Treatment with JA resulted in an increase in type VI trichome density on the adaxial leaf surface in wild type, ir-*SlMYC1*, and *MYC1*/*myc1* plants (Figures 9B and 9C). The increase was significantly higher in wild-type plants and *ir-SlMYC1* line 18 than in the two other *ir-SlMYC1* lines (Figure 9B). The increases in type VI trichome density on the adaxial leaf surfaces of JA-treated *MYC1*/*myc1* and wild-type plants were similar (Figure 9C). On the abaxial leaf surface, however, JA treatment led to significantly increased type VI trichome density only in ir-*SlMYC1* plants (Figures 9B and 9C). These results indicate that the reduced expression of *SlMYC1* did not impair the induction of type VI trichome formation by JA. Interestingly, JA application led to a reduction in trichome density on the stems of wild type (Figure 9B) and *MYC1*/*myc1* plants (Figure 9C).

SlMYC1 Is Required for the Induction of *SlTPS3* by JA

JA induces the expression of several TPS genes in *S. lycopersicum* (Falara et al., 2011). For instance, *SlTPS3* and *SlTPS5* are induced by JA treatment (Falara et al., 2011), and both of their promoters can be transactivated by SlMYC1 (Spyropoulou et al., 2014a). Thus, we investigated whether SlMYC1 also controls the JA-induced expression of *TPS* genes. After 24 h of JA treatment, the JA-responsive marker gene *Jasmonate-inducible protein-21* was significantly induced by JA treatment in the stem trichomes and leaves of all genotypes (Figures 10A and 10B). In stem trichomes of the *myc1* mutant, *SlTPS5* and *SlTPS3* were not induced by JA (Figures 10A and 10B), but *SlTPS5* was induced in ir-*SlMYC1* and heterozygous *MYC1*/*myc1* plants, in contrast to *SlTPS3* (Figures 10A and 10B). *SlTPS3* was not expressed in leaves, but interestingly, JA still induced *SlTPS5* expression in the leaves of all genotypes (Figures 10A and 10B). *SlMYC1* itself was not induced by JA in stem trichomes (Supplemental Figure 6A), as was shown previously (Spyropoulou et al., 2014a), but it was induced by JA in leaves, albeit not in ir-*SlMYC1* plants (Supplemental Figure 6B). These results demonstrate that SlMYC1 is essential for the induction of *SlTPS3*, but not of *SlTPS5*, in response to JA.

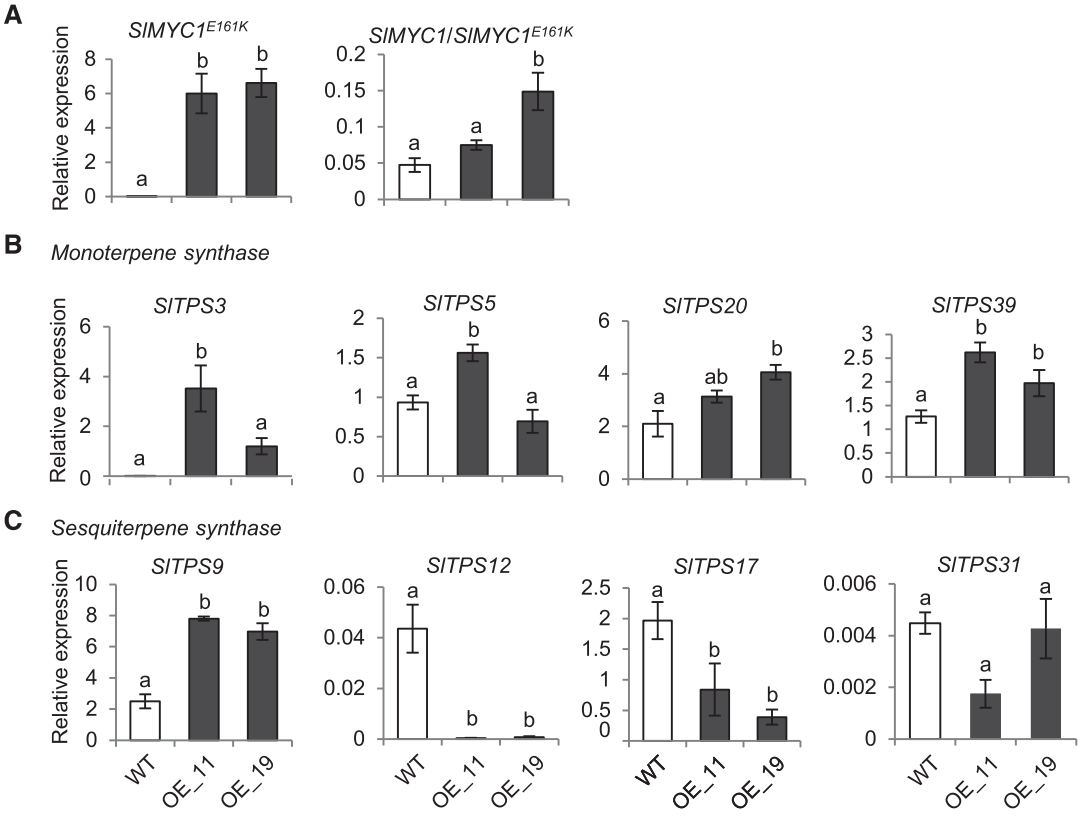

Figure 8. Transcript Levels of *SlMYC1* and Terpene Synthase Genes in the Stem Trichomes of *SlTPS5p:SlMYC1*^{E161K} Plants.

Relative transcript levels of **(A)** *SlMYC1*^{E161K} and *SlMYC1* (*SlMYC1*/*SlMYC1*^{E161K}) and **(B)** several monoterpene and **(C)** sesquiterpene synthase genes in stem trichomes from wild-type (WT) Moneymaker and *SlTPS5p:SlMYC1*^{E161K} plants determined by qRT-PCR. Line OE_11 and OE_19 are two independent transgenic *SlTPS5p:SlMYC1*^{E161K} lines of the T1 generation. Bars represent the mean values (±SE) of three to four biological replicates, each consisting of multiple stem pieces from different plants normalized to *Actin* transcript levels. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

SlMYC2 Is Not Involved in Regulating Type VI Glandular Trichome Development or Volatile Terpene Biosynthesis in Trichomes

SlMYC1 and SlMYC2 are homologous proteins belonging to the bHLH IIIe subclade in *S. lycopersicum* (Goossens et al., 2017). The key role of SlMYC2 in various JA-mediated processes (Du et al., 2017) prompted us to investigate whether this protein also participates in the regulation of volatile terpene biosynthesis and type VI glandular trichome development. We used an RNA interference strategy to downregulate *SlMYC2* in transgenic tomato plants. We obtained three independent transgenic lines with a 90% reduction in *SlMYC2* transcript levels but no consistent effect on *SlMYC1* expression (Supplemental Figures 7A and 7B). However, downregulation of *SlMYC2* did not lead to significant changes in the transcript levels of the *TPS* genes, which are regulated by SlMYC1 (Supplemental Figures 7C and 7D), nor were type VI glandular trichomes affected in these lines (Supplemental Figures 8A and 8B). We also investigated whether SlMYC2 is required for the induction of TPS genes by JA. In wild-type tomato plants, *SlMYC2* was induced by JA in both stem trichomes and leaves (Supplemental Figures 7E and 7F). Although *SlMYC2* was not induced by JA in 35S:ir-*SlMYC2* plants, the JA-regulated induction of *SlTPS3* and *SlTPS5* was not affected in these plants (Supplemental Figures 7E and 7F). These results indicate that unlike SlMYC1, SlMYC2 is not involved in regulating TPS genes or the formation of type VI trichomes.

DISCUSSION

Our study demonstrates that the bHLH transcription factor SlMYC1 is involved in the formation of type VI glandular trichomes and positively regulates monoterpene biosynthesis in tomato leaf and stem trichomes. In addition, SlMYC1 plays differential roles in regulating sesquiterpene biosynthesis, as it is necessary for β-caryophyllene and α-humulene biosynthesis in leaf trichomes, whereas it inhibits their biosynthesis in stem trichomes.

SlMYC1 Regulates Glandular Trichome Formation

The current model for trichome differentiation is based the development of nonglandular trichomes in Arabidopsis, in which an R2R3 repeat MYB protein (GLABRA1), a bHLH IIIf subfamily protein (GL3 or ENHANCER OF GLABRA3), and a WD40 repeat

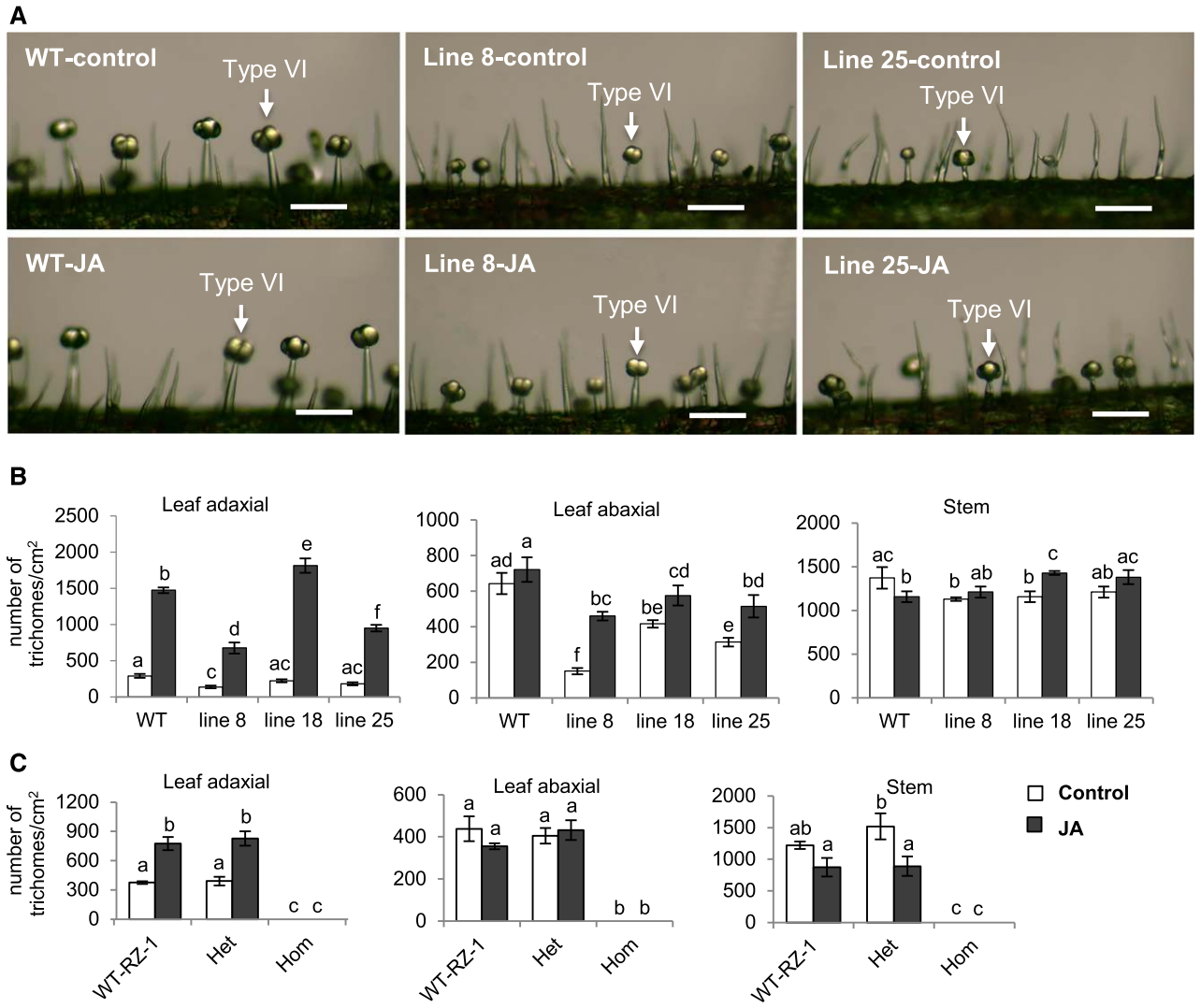

Figure 9. Type VI Glandular Trichome Morphology and Density after JA Treatment.

(A) Morphology of type VI trichomes on the stem surfaces of control and JA-sprayed wild-type (WT) Moneymaker, and ir-*SlMYC1* line 8 and 25 plants (scale bars, 200 μm), 28 days after JA treatment.

(B) and **(C)** Type VI trichome density in 7-week-old control plants and plants sprayed with jasmonic acid (JA) on newly formed stems and leaves 28 days after treatment; **(B)** wild-type Moneymaker (WT) and ir-*SlMYC1* line 8, 18, and 25 plants; **(C)** wild-type RZ (WT-RZ-1), heterozygous *MYC1*/*myc1* (Het), and homozygous *myc1* (Hom) plants. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA. Mean values (±SE) of three to four biological replicates are shown.

protein (TRANSPARENT TESTA GLABRA1) constitute an active MYB/bHLH/WD40 complex that triggers the expression of *GLABRA2,* a class IV HD-ZIP (HD-ZIP IV) transcription factor to induce trichome formation (Rerie et al., 1994; Schiefelbein, 2003; Pesch and Hülskamp, 2004, 2009; Serna and Martin, 2006; Ishida et al., 2008). In addition, several R3 MYBs (e.g., AtTRY) move from early trichome cells to neighboring cells, where they inhibit trichome initiation by competing with R2R3-repeat MYB proteins for interaction with bHLH proteins (Esch et al., 2004). Whether the initiation and differentiation of glandular trichomes involves analogous MYB/bHLH/WD40 complexes and downstream HD-ZIP and R3 MYB transcription factors has been unclear. MYB and HD-ZIP IV transcription factors have been implicated in the development of glandular trichomes in some species (Glover et al., 1998; Yang et al., 2011; Matías-Hernández et al., 2017; Yan et al., 2017; Shi et al., 2018).

Here, we show that one partner of this potential complex, the bHLH transcription factor SlMYC1, functions in glandular trichome development in tomato. In addition, tomato appears to contain a functional ortholog of the R3 MYB gene *AtTRY*, as heterologous expression of tomato *SlTRY* in Arabidopsis inhibits trichome formation (Tominaga-Wada et al., 2013). Furthermore, tomato *Wo*, which encodes an HD-ZIP IV transcription factor, specifically controls the initiation and development of type I

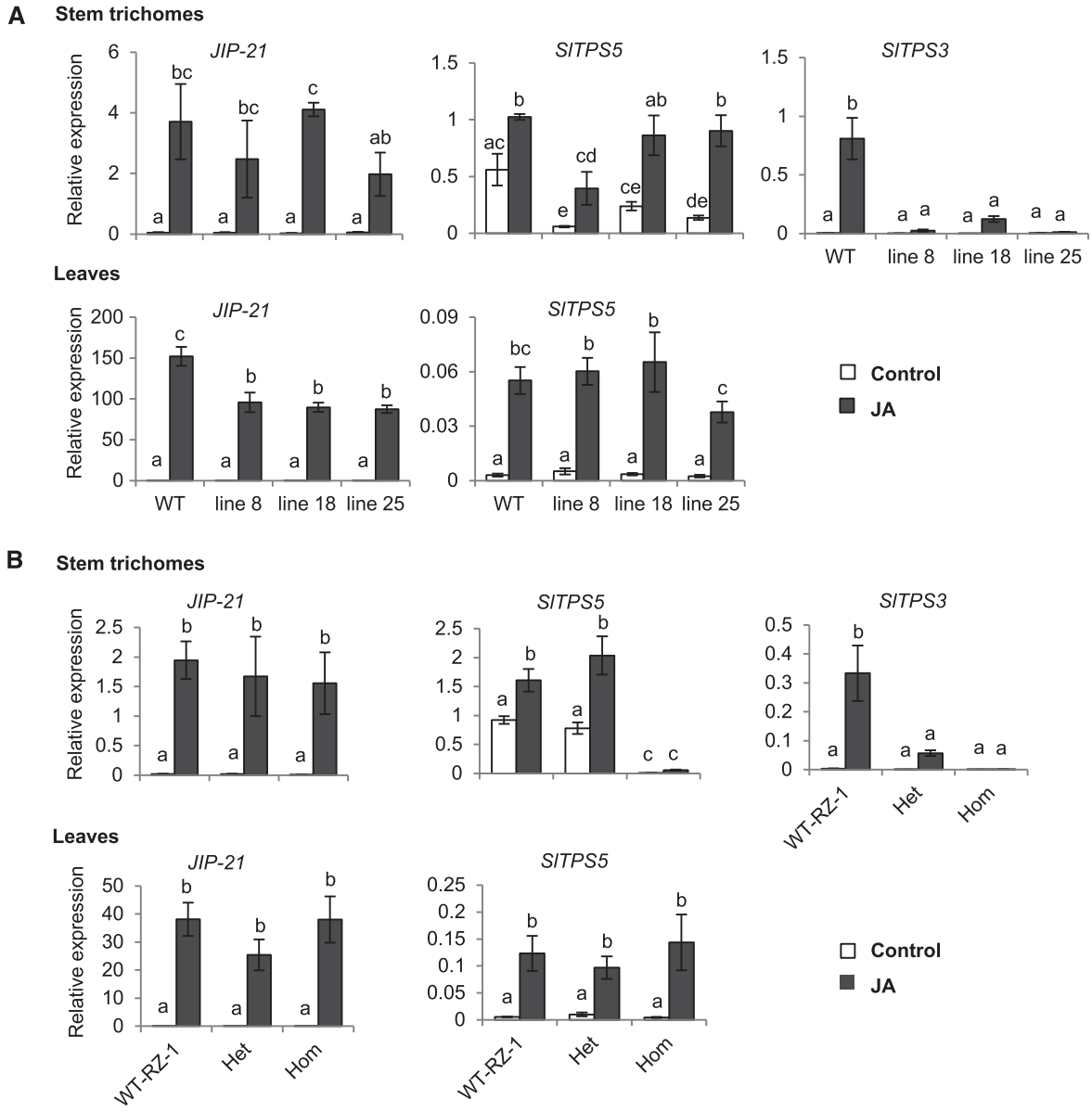

Figure 10. Effect of *SlMYC1* Downregulation on JA-Induced Expression of Terpene Synthase Genes.

Relative transcript levels of *Jasmonate-inducible protein-21* (*JIP-21*) and terpene synthase genes in stem trichomes and leaves of **(A)** wild-type (WT) Moneymaker and ir-*SlMYC1* line 8, 18, and 25 and **(B)** wild-type RZ (WT-RZ-1), heterozygous *MYC1*/*myc1* (Het), and homozygous *myc1* (Hom) plants. The plants were sprayed with JA solution (1 mM JA and 0.05% SilwetL-77 in tap water) or control solution (0.05% SilwetL-77 in tap water) and samples were collected 24 h later. Transcript levels were determined by qRT-PCR. Bars represent mean values (±SE) of three to four biological replicates normalized to *Actin* transcript levels. Bars annotated with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$) after ANOVA.

trichomes (Yang et al., 2011). Together, these data suggest that MYB/bHLH/WD40 complexes and downstream HD-ZIP IV and TRY transcription factors may indeed function in glandular trichome development.

We found that knocking down *SlMYC1* (in the ir-*SlMYC1* lines) led to the production of fewer type VI trichomes on both sides of the leaf but also to more type IV glandular trichomes on stems (Supplemental Figure 5), likely due to reduced inhibition of the formation of other types of trichomes. In addition, an aberrant type of glandular trichome formed in these knockdown lines and in the homozygous *myc1* mutant. Since the intermediate cell in this type of trichome was wider than the stalk, as is the case for type VII glandular trichomes, we considered these to be type VII-like glandular trichomes (Supplemental Figure 1A). However, since these stalks did not bend but were instead straight, like type VI glandular trichomes, one could argue that they were type VI-like glandular trichomes, a notion that requires further investigation. The total numbers of glandular trichomes on the abaxial leaf

surfaces of these knockdown and knockout lines were lower than in wild-type plants (Figure 4), indicating that SlMYC1 plays a role in trichome initiation. In addition, the observation that trichomes of the ir-*SlMYC1* lines had shorter stalks and smaller glandular heads than the wild type indicates that SlMYC1 is involved in glandular trichome development. We thus conclude that SlMYC1 is involved in both the initiation and maturation of type VI glandular trichomes.

The Role of SlMYC1 in Regulating Terpene Biosynthesis

The downregulation of *SlMYC1* resulted in reduced terpene levels as well as altered trichome densities. The changes in the overall terpene profiles of total trichomes (Figures 2 and 5) were similar to those of individual type VI glands (Figure 7), demonstrating that this was not due to the presence of fewer type VI glandular trichomes. Stem trichomes of the knockdown plants and *MYC1*/*myc1* plants produced more sesquiterpenes than the wild type, while the leaf trichomes produced reduced amounts of monoterpenes. The sizes of type VI trichomes in *MYC1*/*myc1* plants were similar to those of the wild type, indicating that the altered terpene production was not simply due to altered type VI trichome development. Our findings thus indicate that SlMYC1 itself is a regulator of TPS genes. Consistent with this conclusion, the stem trichomes of transgenic tomato plants expressing *SlMYC1*^{E161K}, encoding a (putatively) more stable SlMYC1 (Gasperini et al., 2015), under the control of the type VI-specific *SlTPS5* promoter (Spyropoulou et al., 2014b) had higher *SlTPS3* and lower *SlTPS12* transcript levels than the wild type, indicating that SlMYC1 regulates these genes.

One glandular head of a type VI stem trichome produced more than 10 times the amount of monoterpenes found in the leaf but almost no sesquiterpenes (Figure 7). In cultivated tomato, most sesquiterpenes are synthesized from precursors provided by the mevalonate pathway, which functions in the cytosol, endoplasmic reticulum, and peroxisomes, whereas the plastidial methylerythritol-4-phosphate pathway is mainly responsible for producing precursors for monoterpene biosynthesis (Simkin et al., 2011; Pulido et al., 2012). Although they are physically separated, metabolic crosstalk occurs between these pathways (Gutensohn et al., 2013; Henry et al., 2018), and we thought that the decrease in monoterpene biosynthesis could have caused an increase in sesquiterpene biosynthesis. However, in the stem trichomes of *MYC1*/*myc1* plants, monoterpene levels were not reduced, although sesquiterpene levels were elevated. This observation indicates that SlMYC1, and not metabolic crosstalk, modulates the expression of mono- and sesquiterpene synthase genes in tomato leaf and stem trichomes.

Although bHLH transcription factors are involved in regulating terpenoid biosynthesis in other species, most function as positive regulators of biosynthesis genes. In Arabidopsis, for instance, AtMYC2 forms homo- or heterodimers with bHLH transcription factors AtMYC3 and AtMYC4 to regulate the expression of target genes (Fernández-Calvo et al., 2011). Perhaps the differential regulation of sesquiterpene biosynthesis in tomato trichomes could be explained by a model in which another bHLH transcription factor expressed in stem but not leaf trichomes regulates *SlTPS12* expression. Such a process would lead to the formation of a bHLH complex in which SlMYC1 inhibits the expression of terpene biosynthesis genes in stem trichomes, and this inhibition would be alleviated under reduced SlMYC1 levels.

The Role of SlMYC1 in JA Responses

In tomato, the leaves of both JA receptor and JA biosynthesis mutants have reduced numbers of type VI glandular trichomes (Li et al., 2004; Boughton et al., 2005; Yan et al., 2013; Bosch et al., 2014). In addition, reduced levels of JA lead to lower levels of volatile terpenes in individual type VI trichomes (Yan et al., 2013). The components that act downstream of JA remain to be identified. Our data show that SlMYC1 is an essential downstream component, as JA failed to restore the formation of type VI trichomes in the *myc1* mutant and failed to increase the size of the smaller type VI trichomes in the ir-*SlMYC1* plants. Interestingly, our data also demonstrate that the induction of TPS genes by JA is not entirely dependent on SlMYC1, and thus, other transcription factors, such as SlEOT1, might be involved in this process (Spyropoulou et al., 2014b). Further research using the tools that we have developed should allow these regulators to be identified. A challenge for future studies will be to identify other molecular players involved in type VI trichome development in tomato that might act in a network with the bHLH transcription factor SlMYC1.

METHODS

Plant Growth Conditions and Sampling

Tomato (*Solanum lycopersicum* cv Moneymaker) and *Nicotiana benthamiana* plants were grown in soil in a greenhouse with day/night temperatures of 23/18°C and a 16/8 h light/dark regime with supplemental light when necessary (150 μE $m^{-2}s^{-1}$; Philips Master Green Power; www.usa.lighting.philips.com). Four-week-old tomato plants and 4- to 5-week-old *N. benthamiana* plants were used for all experiments unless otherwise indicated. For all experiments, multiple stem pieces or leaflets were harvested from multiple plants for one biological replicate.

Constructs and Generation of Transgenic Plants

To create the 35S:ir-*SlMYC1* and 35S:ir-*SlMYC2* constructs, Gateway technology (Invitrogen; www.thermofisher.com) was used. Primer pairs ir-MYC1_attB1 and ir-MYC1_attB2 (targeting bases 1953 to 2174 of *SlMYC1* mRNA) and ir-MYC2_attB1 and ir-MYC2_attB2 (targeting bases 2071 to 2361 of *SlMYC2* mRNA), containing attB sites, were used to amplify the *SlMYC1* and *SlMYC2* fragments, respectively (Supplemental Table). The fragments were cloned into the pDONR207 vector (Invitrogen) and subsequently recombined in destination vector pK7GWIWG2(1) (Karimi et al., 2002). To create the *SlTPS5p:SlMYC1*^{E161K} construct, primer pairs MYC1_F and MYC1^{E161K}_R and MYC1^{E161K}_F and MYC1_R were used to amplify two halves of the fragments (nucleotides 1 to 491 and 471 to 1833) for *SlMYC1*^{E161K} with a single nucleotide mutation introduced into position 481 of the coding sequence. The open reading frame of *SlMYC1*^{E161K} was amplified with a mixture of the two fragments using primer pair MYC1_F and MYC1_R. The *SlMYC1*^{E161K} sequence was verified and cloned into the pDONR207 vector with primer pair MYC1_attB1 and MYC1_attB2, creating SlMYC1^{E161K}-pENTR. The *SlTPS5* promoter (comprising 1254 bp of the genomic sequence upstream of the start codon of *SlTPS5*) was amplified with primer pair SlTPS5p_attB4 and SlTPS5p_attB1r and cloned into the pGEM BOX1-P4P1R vector, creating SlTPS5p-pENTR. SlTPS5p-pENTR and SlMYC1^{E161K}-pENTR were recombined in destination vector pK7m24GW(3) (Karimi et al., 2002). To create the truncated SlMYC1

construct 35S:SlMYC1tru493, primer pair MYC1_attB1 and MYC1tru493_attB2 with a single nucleotide mutation introduced (from G to T at position 1477 of the SlMYC1 coding sequence, resulting in an early stop codon at amino acid position 493) was used to amplify the fragment. The fragment was cloned into pDONR207 and subsequently recombined in destination vector pK2GW7(0) (Karimi et al., 2002). To create the CRISPR-Cas9_myc1 construct, three guide RNAs (gRNAs), designed to target the open reading frame of *SlMYC1* (583 to 603 bp, 1825 to 1844 bp, and 1092 1111 bp) were synthesized (www.eurofinsgenomics) and cloned into vector pYPQ131, pYPQ132, and pYPQ133, respectively (Addgene; www.addgene.org; Lowder et al., 2015), creating pYPQ131-gRNA1, pYPQ132-gRNA2, and pYPQ133-gRNA3. The three guide RNAs were then assembled into pYPQ143 using Golden Gate technology to create the gRNA entry vector pYPQ143-gRNAs. The Cas9 entry vector pYPQ150 (Addgene) and pYPQ143-gRNAs were recombined in destination vector pK2GW7(0) using Gateway technology. All constructs were verified by sequencing and introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90). Stably transformed transgenic tomato plants were created using explants derived from the cotyledons of sterile *S. lycopersicum* cv Moneymaker seedlings as previously described (Cortina and Culiáñez-Macià, 2004). The kanamycin-resistant transformants were screened for the presence of the nptII gene.

Identification of the *myc1* Mutant

Seeds of *S. lycopersicum* breeding line Rijk Zwaan (RZ)-1 were treated with EMS by submerging ~10,000 seeds in an aerated solution of 0.5% (w/v) EMS for 24 h at room temperature. The treated seeds were germinated, and the resulting plants were grown in a greenhouse to produce M2 seeds. M2 seeds were harvested from mature plants and bulked in one pool. The resulting pool of M2 seeds was used as starting material to screen 8000 M2 plants to identify individual M2 plants that showed an aberrant type VI glandular trichome phenotype under a stereomicroscope. Using quantitative trait loci-mapping of an F2 population of the mutant and the parent, the recessive trait was localized to chromosome 8. The mutant had a single nucleotide change, G to T at position 1477, in the coding sequence of SlMYC1, resulting in an early stop codon at amino acid position 493. This mutation was predictive for the phenotype in an F4 population.

RNA Isolation and Quantitative RT-PCR

For RNA isolation, stems from whole plants and the second pair of leaflets from the fourth leaf were collected and frozen in liquid nitrogen. Trichomes were isolated from the frozen stems by shaking in liquid nitrogen in 50-mL tubes with a vortex mixer. For RNA extraction, total RNA was isolated using TRIzol (Invitrogen) and treated with TURBO DNase (Ambion; www.thermofisher.com) to remove contaminating DNA. RNA quantity was determined with a NanoDrop spectrophotometer (Thermo Fisher; www.thermofisher.com), and complementary DNA was synthesized from 1.5 μg RNA using RevertAid M-MuLV Reverse Transcriptase (Fermentas; www.thermofisher.com). For quantitative RT-PCR (qRT-PCR), cDNA equivalent to 5 ng total RNA was used as a template, and PCR was performed with SYBR Green qPCR SuperMix (Solis Biodyne; www.sbd.ee) and 300 nM of each primer (Supplemental Table) in a total volume of 10 μL in an ABI 7500 Real-Time PCR System (Applied Biosystems; www.appliedbiosystems.com) with the following cycling program: 2 min 50°C, 15 min 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C. Primer pair efficiencies were calculated by analyzing amplification curves from a standard cDNA dilution range. Expression levels were normalized to the transcript level of *ACTIN*. To analyze the *SlTPS5p:SlMYC1^{E161K}* transgenic lines, SlMYC1^{E161K}-QF (bases 1791 to 1810 of *SlMYC1* mRNA) and SlMYC1^{E161K}-QR (16 to 36 bp of primer MYC1_attB2) were used to check *SlMYC1^{E161K}* expression, and SlMYC1-QF (731 to 750 bp of *SlMYC1* mRNA) and SlMYC1-QR (877 to 858 bp of *SlMYC1* mRNA) were used to measure total *SlMYC1* transcript levels (*SlMYC1*/*SlMYC1^{E161K}*).

Analysis of Volatile Terpenes

To measure volatile terpenes, stem pieces from the third internodes of plants were collected and frozen in liquid nitrogen. Trichomes were isolated from the frozen stems by shaking in liquid nitrogen in 15-mL tubes with a vortex mixer. Terpene extraction was performed using 1 mL of hexane plus 0.5 ng μL^{-1} benzyl acetate (Sigma-Aldrich; www.sigmaaldrich.com) as an internal standard. Na_2CO_3 (Sigma-Aldrich) was used to remove any water from the hexane. For leaf trichomes, the second pair of leaflets from the fourth leaf was washed with 1 mL of hexane plus 0.5 ng μL^{-1} benzyl acetate. For terpenes in type VI glandular trichomes, 200 individual type VI glands were collected from stems or leaves with a pulled Pasteur pipette and dissolved in 100 μL of the same solvent. The volatiles were analyzed using an Agilent (www.agilent.com) 7890A gas chromatograph, coupled to an Agilent 7200 accurate-mass quadrupole time-of-flight mass spectrometer operating in electron-impact mode. Liquid injection of a 1-μL sample was performed at 50°C, with the injector port directly heated to 275°C at a rate of 240°C min^{-1}. The oven temperature was maintained at 40°C for 3 min and increased by 5°C per min until it reached 140°C. The temperature was then increased by 10°C per min until it reached 250°C and maintained at 250°C for 5 min. Compounds were separated on a capillary HP-5ms column (30 m × 250 μm, 0.25 μm film thickness; Agilent) with helium as the carrier gas at a flow rate of 1 mL min^{-1}. Terpene standards were used for compound identification and quantification.

Trichome Density and Morphology

Scanning electron micrographs of fresh, unfixed plant material were produced with an environmental scanning electron microscope (ESEM) type XL-30 FEG (FEI/Philips; www.fei.com) operating in wet mode. The gas pressure in the ESEM chamber (1.5 mBar) was regulated by introducing water vapor. A gaseous secondary electron detector was used for imaging. Stem trichomes from the third fully grown internode of each plant were scanned to determine their size. An average of four to five images were taken per line. Light microscopy images of trichomes were obtained under a Leica MZFLIII microscope (www.leica-microsystems.com), which was also used to count to the trichomes. Images were captured with a Nikon DS-Fi2 5-megapixel CCD camera (www.nikon.com) using NIS-Elements (version F4.30.00). To measure trichome density on leaves, the second pair of leaflets of the seventh leaf of each 7-week-old tomato plant was used. To measure trichome density on stems, stem pieces from the third internodes of 4-week-old plants and the sixth internodes of 7-week-old plants were used. The morphology of stem trichomes submerged in water was also examined under an EVOSfl (www.thermofisher.com) inverted microscope.

JA Treatment

For JA treatment, 4-week-old tomato plants were sprayed with 1 mM JA (Duchefa; www.duchefa-biochemie.com) in tap water plus 0.05% SilwetL-77; the control plants were sprayed with tap water plus 0.05% SilwetL-77. Stem pieces and leaflets were collected for RNA isolation and volatile terpene measurements 24 h later. To induce trichome formation on newly formed leaves, the plants were sprayed weekly with JA or control solution. On day 28 after the first treatment, newly developed stems and leaflets were used for trichome counting and to determine the morphology of type VI glandular trichomes.

Transient Transactivation Assay in *N. benthamiana* Leaves

The β-glucuronidase (*GUS*) gene driven by the *SlTPS5* promoter (Spyropoulou et al., 2014b) was used as a reporter to verify the transactivation of the effectors. The 35S:SlMYC1 and 35S:RFP effector constructs were used as positive and negative controls, respectively (Spyropoulou et al., 2014a). *A. tumefaciens* cultures were grown overnight

from a single colony until an OD_{600} between 1.0 and 1.5 was reached. After centrifugation, the bacterial pellets were resuspended in infiltration buffer (50 mM MES pH 5.8, 0.5% glucose, 2 mM Na_2HPO_4, 100 μM acetosyringone; Sigma-Aldrich) to OD_{600} of 0.3 and incubated for 1 h at room temperature. A construct containing the luciferase (*LUC*) gene driven by the *Cauliflower mosaic virus* 35S promoter (Spyropoulou et al., 2014b) was used to normalize for transformation efficiency and protein extraction efficiency. Leaves from 4- to 5-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* cultures carrying various MYC1 or RFP constructs, the SlTPS5p:GUS reporter construct, and a 35S:LUC construct at a 5:5:2 ratio. Two leaves each from three plants were infiltrated for each combination. Two days later, leaf disks from the infiltrated areas were collected, frozen in liquid nitrogen, and used to prepare crude extracts in extraction buffer containing 25 mM TRIS phosphate pH 7.8, 2 mM dithiothreitol, 2 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid pH 7.8, 10% glycerol, and 1% Triton X-100 (Sigma-Aldrich). 4-methylumbelliferyl-β-d-glucuronid and LUC activities were measured using a Fluorocount SYNERGY H1 microplate reader (BioTek; www.biotek.com). Enzymatic GUS activity was determined by normalizing 4-methylumbelliferyl-β-d-glucuronide activity with the LUC activity of each sample.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL/Solgenomics databases under the following accession numbers: SlMYC1, KF430611; SlMYC2, Solyc08g076930; SlTPS3, Solyc01g105870; SlTPS5, Solyc01g105890; SlTPS9, Solyc06g059930; SlTPS12, Solyc06g059910; SlTPS17, Solyc12g006570; SlTPS20, Solyc08g005670; SlTPS31, Solyc01g101170; SlTPS39, Solyc10g005390; Jasmonate-inducible protein-21, Solyc03g098790; ACT, Solyc03g078400.

Supplemental Data

Supplemental Figure 1. Environmental scanning electron micrographs of stem trichomes.

Supplemental Figure 2. Truncated SlMYC1 does not transactivate the *SlTPS5* promoter in *Nicotiana benthamiana* leaves.

Supplemental Figure 3. Nucleotide sequences of *SlMYC1* in the CRISPR-Cas9 knockout lines.

Supplemental Figure 4. Morphology of type VI glandular trichomes on stems.

Supplemental Figure 5. Type IV glandular trichome density on the tomato stem surface.

Supplemental Figure 6. *SlMYC1* expression in tomato stem trichomes and leaves treated with JA.

Supplemental Figure 7. Effect of *SlMYC2* downregulation on the expression of TPS genes in stem trichomes and leaves.

Supplemental Figure 8. Morphology of type VI glandular trichomes on the stem surface in ir-*MYC2* plants.

Supplemental Table. List of primers used in this study.

Supplemental Data Set. ANOVA tables.

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AUTHOR CONTRIBUTIONS

J.X. performed most of the experiments and analyzed the data; R.C.S. designed the study; R.C.S. and M.A.H. supervised the research; Z.O.v.H. and D.B.B. generated the *myc1* mutant; C.S. created the CRISPR-Cas9_myc1 construct; J.X. and R.C.S. wrote the manuscript; and M.A.H. revised the manuscript.

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