

Targeted Editing of *SIMAPK6* Using CRISPR/Cas9 Technology to Promote the Development of Axillary Buds in Tomato Plants

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Abstract

The mitogen-activated protein kinase (MAPK) cascade signaling system has been relatively conserved throughout the evolution of eukaryotes and is involved in the regulation of growth and development and metabolism. In this study, dwarf tomato plants were used as the research material. First, the tissue-specific expression of *SIMAPK6* was measured in wild-type plants by quantitative RT-PCR. The results showed that *SIMAPK6* was highly expressed in the tissues of the stems, leaves and flowers but was expressed at low levels in the tissues of the roots, sepals and fruits. Second, *SIMAPK6*-knockout lines CRISPR-3 and CRISPR-7 were obtained by CRISPR-Cas9 technology and Agrobacterium-mediated transformation. Compared with wild-type, the mutant lines CRISPR-3 and CRISPR-7 showed significant phenotypic characteristics, such as increased numbers of axillary buds and true leaves, thickened stems, and longer leaflets. In addition, to explore the molecular mechanism by which MAPK regulates axillary bud growth, we also showed that *SIMAPK6* positively regulates the strigolactone synthesis genes *SICCD7* and *SICCD8* and the gibberellin (GA) synthesis genes *GA20ox3* and *GA3ox1* and negatively regulates the axillary bud development-related genes *Ls*, *BL* and *BRC1b/TCP8* and the GA synthesis inhibitory gene *GAI*. Therefore, *SIMAPK6* appears to regulate the synthesis of strigolactone and GA to induce the growth and development of tomato axillary buds.

Keywords: axillary buds, gene knockout, *SIMAPK6*, tomato

1. Introduction

Plant morphogenesis has great influence on yield and quality, the number of side branches is very important index to plant architecture. The lateral branches develop from the axillary buds. Many factors, especially hormones, determine the development of axillary buds (Huh et al., 2013; Chen et al., 2016). Strigolactones and gibberellin (GA), two kinds of plant hormones, are reported to regulate shoot branching, stems elongation, plant growth and architecture (Yamaguchi et al., 2008; Vogel et al., 2010; Kohlen et al., 2012). In tomato, CAROTENOID CLEAVAGE DIOXYGENASE8 (*SICCD8*) and CAROTENOID CLEAVAGE DIOXYGENASE7 (*SICCD7*) are two key strigolactone biosynthesis-related genes. GA20oxs are essential

enzymes for GA biosynthesis and control the GA content in different plant species. GA3oxs produce bioactive GAs, such as GA1, GA3, GA4 and GA7, in the final step (Yamaguchi et al., 2008).

The crosstalk between mitogen-activated protein kinases (MAPKs), and hormones, secondary messengers in plant signaling systems facilitates plant to adapt and survive in different environmental, including controlling plant architecture (Smekalova et al. 2014). MAPK cascade signaling system has been relatively conserved throughout the evolution of eukaryotes (Widmann et al., 1999; Group et al., 2002; Xu et al., 2015) and is involved in the regulation of the responses to various biotic and abiotic stresses, growth and development, and metabolism. MAPKs play an important role in the signaling pathway of eukaryotes. The MAPK signaling system is usually composed of tertiary protein kinases, including MAPK, MAPK kinase (MAPKK) and MAPKK kinase. MAPKs acts downstream of the tertiary protein kinase signaling system.

A total of 16 MAPKs have been identified in tomato, which can be further divided into four categories: A, B, C, and D (Kong et al., 2012). Among them, MAPK1, MAPK2 and MAPK3 belong to category A; these MAPKs have been the subject of many studies and are involved in the regulation of various stresses in plants (Stulemeijer et al., 2007; Zhou et al., 2014; Lv et al., 2017). The remaining 13 MAPKs belong to categories B, C, and D, which have been poorly studied. Among them, tomato MAPK6 (*SIMAPK6*) belongs to category B and has a loop containing a MEY motif. However, the function of *SIMAPK6* is still unknown.

Genome-editing technology has been used in *Arabidopsis*, tomato, rice, apple, watermelon, potato, soybean, maize, wheat, sorghum and grape (Feng et al., 2014; Ito et al., 2015; Wang et al., 2016; Reem et al., 2019; Char et al., 2019a; Nishitani et al., 2016; Zhou et al., 2014; Tian et al., 2017; Char et al., 2019b; Di et al., 2019; Lee et al., 2019; Liang et al., 2019; Ren et al., 2019). This study aimed to investigate the function of the *SIMAPK6* gene in the development of axillary buds in tomato plants; the CRISPR/Cas9 system was applied to generate *SIMAPK6* gene knockout mutants. We investigated the development of axillary buds on CRISPR/Cas9-mediated *slmapk3* mutants and wild-type (WT) plants as well as the possible by *SIMAPK6*-mediated regulatory mechanism that influences the development of axillary buds. This study provides insight into the regulatory mechanism of *SIMAPK6*-mediated axillary bud development in tomato plants.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Dwarf tomato plants (*Solanum lycopersicum*) were used in this study as WT. The plants were grown in a pot (18 cm × 22 cm) with matrix (Pere matrix Technology Development Co., LTD, Jiangsu of China), and all plants were growth in artificial climate chamber and watered every three days with Hongland nutrient solution. Transgenic cultures were grown under controlled conditions with a 16-h-day/8-h-night photoperiod, a temperature of 25 °C/18 °C (day/night), a relative humidity of 65-70%, and a light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The first generation (T0) of transgenic tomato originated from tissue culture, and the homozygous *SIMAPK6* knockout lines were screened from the seedlings of the second generation (T1). The third generation (T2) of tomato homozygous lines were used in subsequent experiments.

2.2 Measurement of Plant Architecture Parameters

To study differences between the WT and *SIMAPK6*-knockout lines, we observed the plant morphological parameters in flower stage, including the stem diameter, number of true leaves, internode length, number of axillary buds and compound leaves from *SIMAPK6*-knockout lines and WT plants.

2.3 Construction of the *SIMAPK6* Knockout Vector

Three target sequences of the *SIMAPK6* gene from the CRISPR-GE web tool (<http://skl.scau.edu.cn/>) were used. The target sequences were introduced into three single-guide RNA (sgRNA) expression cassettes using overlapping PCR. These three sgRNA expression cassettes were then ligated into a pCAMBIA2301 vector. The *SIMAPK6* knockout vector was constructed by the company of RexBiotech.

The diagram of the *SIMAPK6* knockout vector was shown in Supplemental Figure S1.

2.4 Plant Transformation

Via the freeze-thaw method (Jyothishwaran et al., 2007), the confirmed pCAMBIA2301/2x35S:hSpCas9-Tnos/AtU6-26::*SIMAPK6* binary vector was transferred into *Agrobacterium tumefaciens* strain GV3101. Transgenic plants were subsequently obtained through the cotyledon transformation method of *Agrobacterium* mediation. Transgenic lines were screened on the basis of kanamycin resistance. After the regenerated buds rooted, the regenerated shoots were grown for one month and then planted in sterilized soil at 25±2 °C with a 16-h/8-h (light/dark) photoperiod.

2.5 Extraction of RNA and cDNA Synthesis

RNA was extracted from the leaf samples by using Total RNA Extraction Reagent from Novizan Biotechnology Company (Nanjing, China) following the manufacturer's protocol. The RNA quality was observed by nano photometry (Thermo Scientific, China). After extraction, RNA was transcribed into cDNA by using a HiScript II First Strand cDNA Synthesis Kit from Novizan Biotechnology Company (Nanjing, China) following the manufacturer's protocol, and the concentrations of the cDNAs were adjusted to be the same.

2.6 Quantitative Real-Time PCR Analysis

Quantitative RT-PCR (qRT-PCR) was performed using a CFX96™ Real-Time System (C1000™ Thermal Cycler, Bio-Rad, USA). All reactions were performed using a SYBR Premix Ex Taq II kit (Takara, China) in a 20 µl total sample volume (2.0 µl of cDNA, 10.0 µl of 2× SYBR Premix Ex Taq, 1.8 µl of primers, and 6.2 µl of distilled deionized water). The qRT-PCR procedure was as follows: predenaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing at 60 °C for 15 s, for a total of 40 cycles. Standard curves were run simultaneously, and three replications were used for each sample. Tomato *CAC* (*SICAC*) was used as an internal standard as previously described by Exposito-Rodriguez et al. (2008) and Guo et al. (2017). All the primers used for qRT-PCR are shown in Supplemental Table S1.

3. Results

3.1 Transcription Levels of the *SIMAPK6* in WT

As Figure 1 A shows, *SIMAPK6* was expressed in all tissues we examined. Among these transcripts, *SIMAPK6* transcripts were detectable at very high levels in the tissues of the stems, leaves, and flowers, but the transcripts showed relatively low expression in the fruits, compared to the root (Figure 1). The relative expression of sepals had not a significant difference compared to the root. These results indicated that the *SIMAPK6* gene plays a vital role in leaf and stem development.

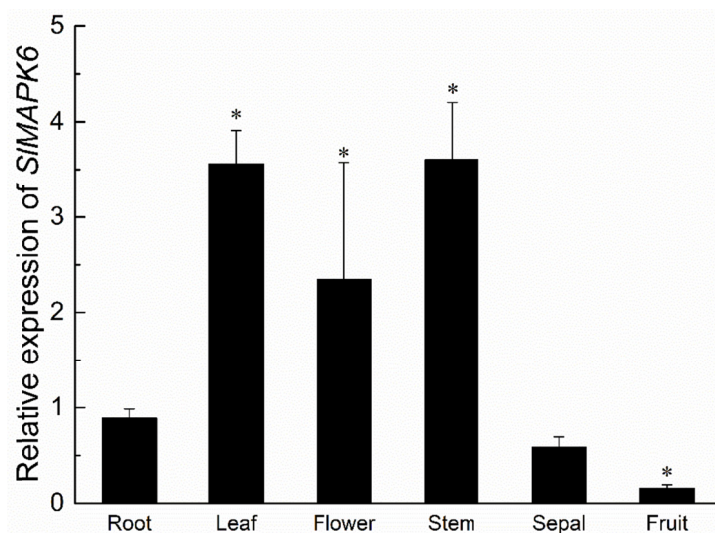


Figure 1. Transcriptional pattern analysis of *SIMAPK6* in WT tomato plants

Note. Relative expression patterns of *SIMAPK6* in different tissues and organs of WT plants. The data represent the means of three replicates with three biological repeats. The error bars indicate the SEs. The single asterisk (*) indicates significant difference.

3.2 CRISPR/Cas9-Mediated Mutagenesis in *SIMAPK6*

The three target sequences, Target1, Target2 and Target3, those we selected were located in the third exon, which encodes the functional domain for the *SIMAPK6* protein (Figure 2A). A schematic diagram of the recombinant vector, which was used to generate transgenic tomato plants, is depicted in Figure 2B. Through *Agrobacterium*-mediated transformation, kanamycin-resistant tomato plants were obtained. The results showed that two plants were edited successfully from 12 transgenic plants (T1), and the editing efficiency was 16.67%. To investigate the functions of *SIMAPK6*, a CRISPR-Cas9 construct targeting *SIMAPK6* (Figure 2B) was

constructed, and several knockout lines were generated, which were confirmed by PCR with Cas9-specific primers. In addition, the target fragments, Target1, Target2 and Target3, were sequenced and compared to the corresponding fragment in WT. Results shown that the target fragments had been changed, such as substitution, deletion, insertion (Figure 2C). So, the *SIMAPK6*-knockout lines, CRISPR-3 and CRISPR-7, had mutated successfully. The primers used for vector construction, positive transgenic plant detection and target fragments are shown in Supplemental Table S2. In addition, we also detected all possible off-target sites, and the results showed that none of the off-target sites changed after sequencing (Supplementary Table S3).

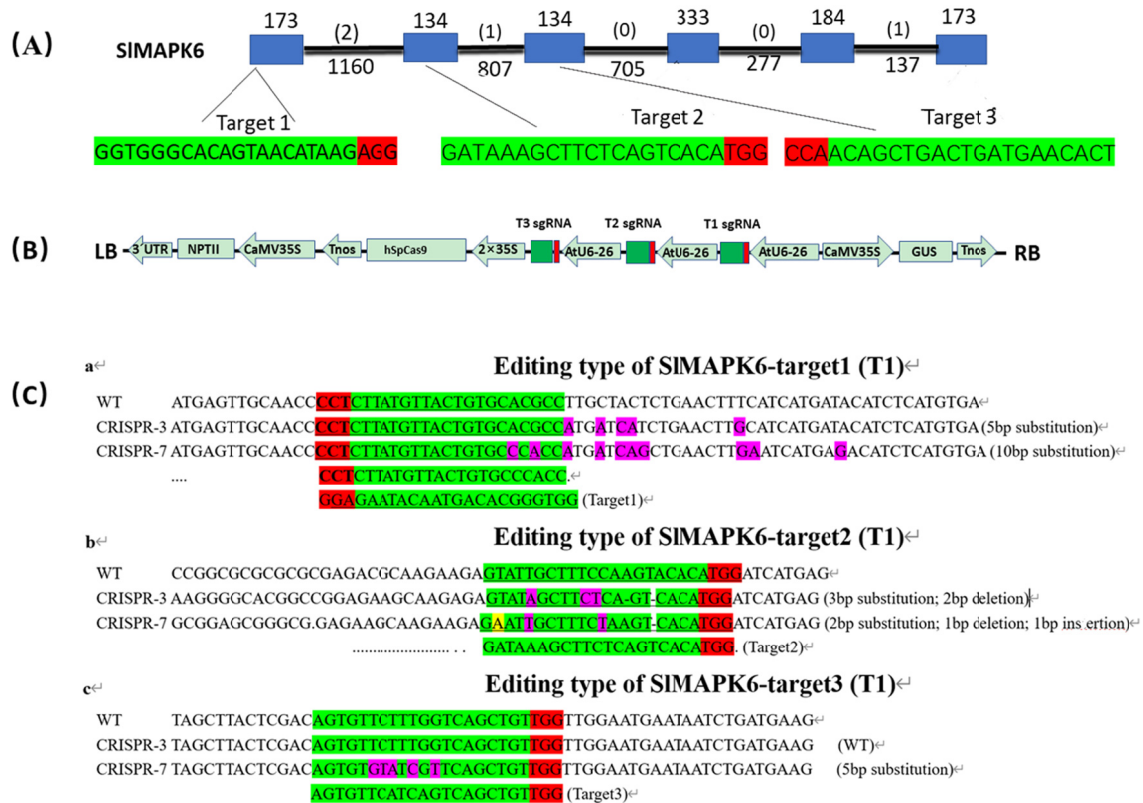


Figure 2. Schematic diagram of the *SIMAPK6* gene structure and vector construction of the CRISPR/Cas9 system

Note. (A) Schematic diagram of the *SIMAPK6* gene structure and target sequences. The black lines indicate introns, and the blue rectangles indicate exons. The protospacer adjacent motif (PAM) sequence is shown with a red background, and the target sequence is marked with a green background. (B) Vector construction of the CRISPR/Cas9 system. NPTII, kanamycin resistance gene; CaMV35S, the promoter of the 35S cauliflower mosaic virus; Tnos, gene terminator; AtU6-26, Arabidopsis U6-26 promoter. (C) CRISPR/Cas9-mediated mutation sequence of the tomato *SIMAPK6* gene. Representative genotypes of *slmapk6* mutants. Target sequences are shown with a green background, with PAM sequences shown with a red background. Deletions are shown by a black dash. Substitutions are shown with a purple background, and insertions are expressed with a yellow background.

3.3 Knockout of *SIMAPK6* Alters Plant Morphogenesis

Two *SIMAPK6*-knockout lines (CRISPR-3 and CRISPR-7) were chosen and used for a more detailed phenotypic and molecular analysis based on their expression level of *SIMAPK6*.

The two *SIMAPK6*-knockout lines (CRISPR-3 and CRISPR-7) were different from the WT plants within a few days after germination (Figure 3A). Compared with the WT lines, the two knockout lines (CRISPR-3 and CRISPR-7) exhibited increased plant height and larger compound leaves (Figure 4B). The increase in adventitious roots in the knockout lines was more prominent than that in the WT (Figure 3G).

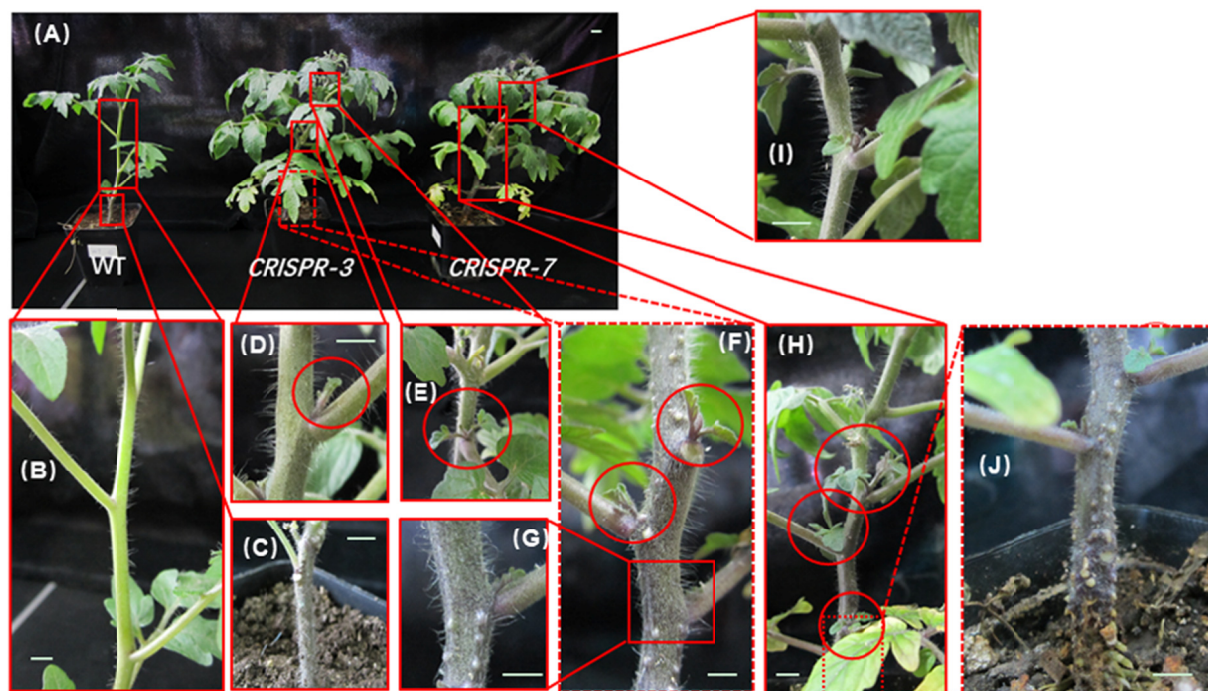


Figure 3. Knockout of *SIMAPK6* alter the plant morphology

Note. (A)-(J) represent the morphology of *SIMAPK6* knockout lines (CRISPR-3 and CRISPR-7) and WT plants.

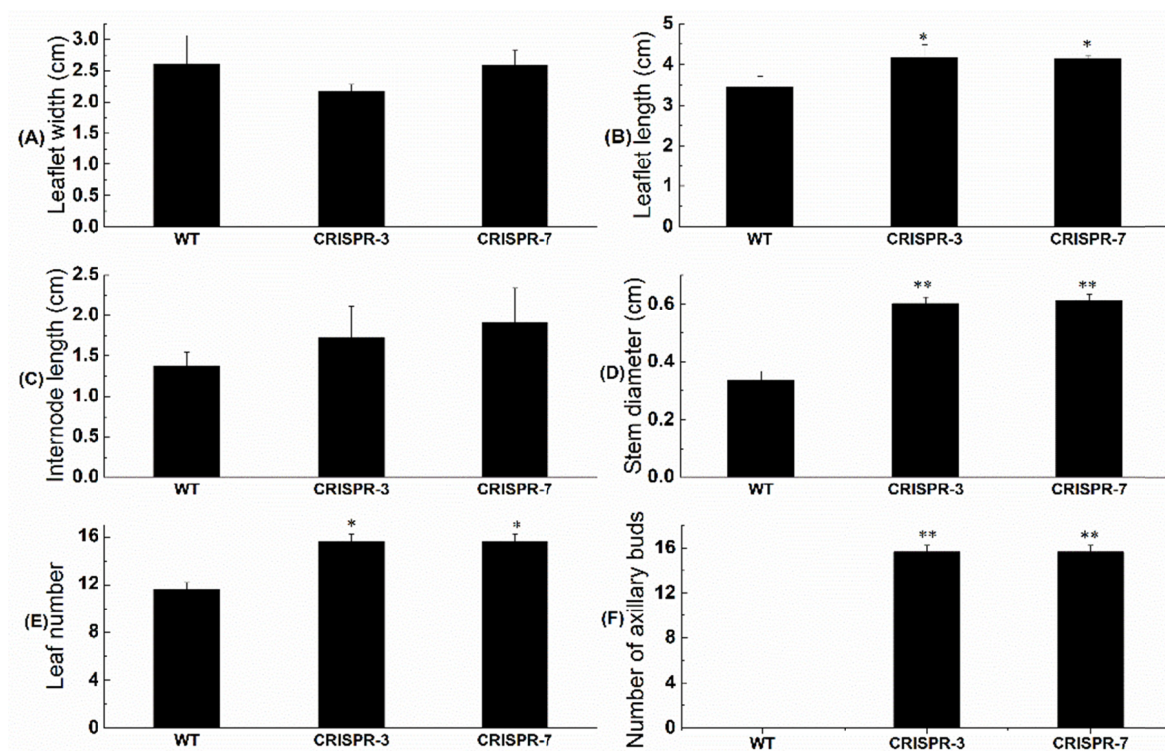


Figure 4. Data related to plant morphology in *SIMAPK6* knockout lines (CRISPR-3 and CRISPR-7) and WT plants

Note. (A)-(F) represent the leaflet width, leaf length, internode length, stem diameter, leaf number and number of axillary buds in *SIMAPK6*-knockout lines and WT. Each value represents the mean±SE of three replicates. The asterisks indicate significant differences ($P < 0.05$) between the WT and *SIMAPK6*-knockout lines.

3.4 Expression Analysis of the Genes Involved in GA Biosynthesis, the GA Response and Cell Elongation in WT and SIMAPK6-Knockout Lines

In *SIMAPK6*-knockout lines, the relative expression levels of *GA20ox3* and *GA3ox1* were significantly low (Figures 5A and 5B), which might lead to the reduction in GA content in the *SIMAPK6*-knockout lines, CRISPR-3 and CRISPR-7. Furthermore, *GAI*, a repressor of GA responses, was assessed at the transcriptional level. It was found that the expression level of *GAI* was significantly higher in the *SIMAPK6*-knockout lines (Figure 5C).

Members of the *PRE* (*Paclobutrazol Resistance*) family positively regulate cell elongation, and they encode protein structures that have a small helix-loop-helix (HLH) motif (Lee et al., 2006; Zhang et al., 2009). Three tomato *PREs*, *PRE1*, *PRE2* and *PRE3*, were identified, and their transcription was examined. The results showed that the expression levels of *PRE1*, *PRE2* and *PRE3* were significantly lower in the knockout lines (Figures 5C-5E).

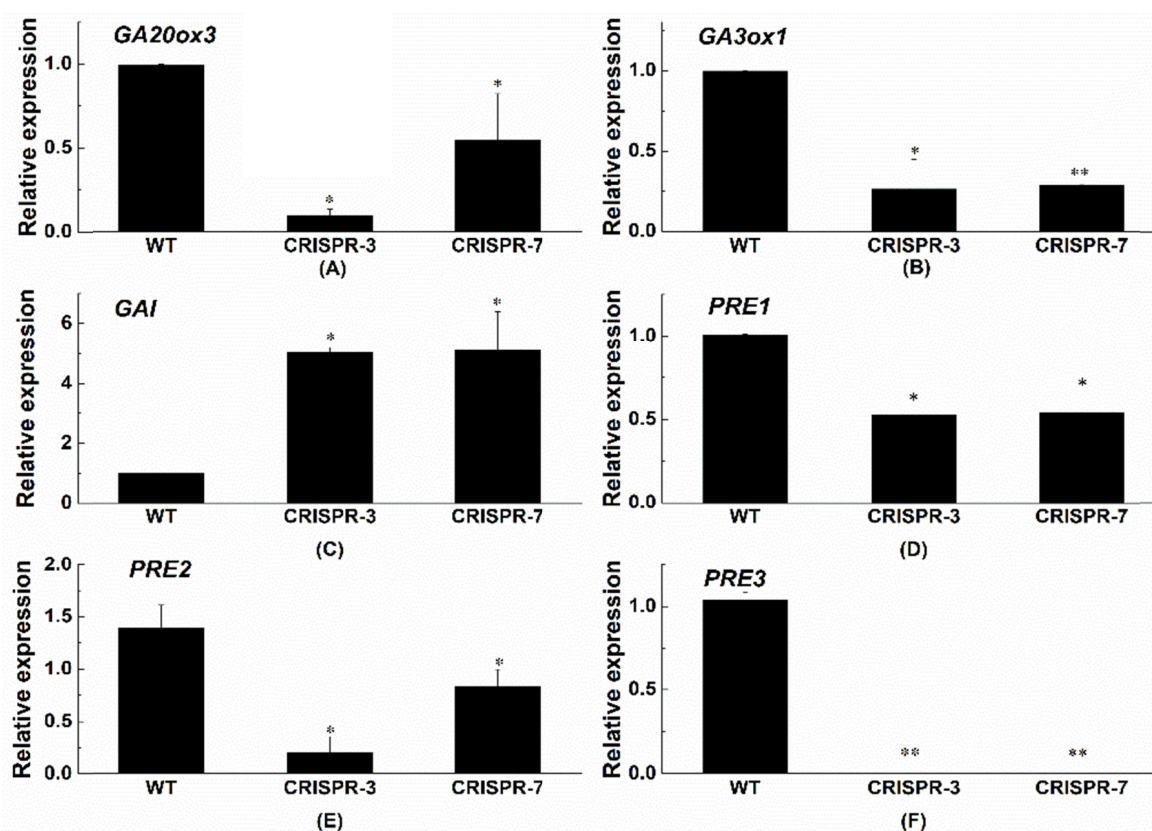


Figure 5. Expression levels of genes involved in the GA response, GA biosynthesis and cell elongation in WT and *SIMAPK6*-knockout lines

Note. (A)-(C) show the relative transcript levels of *GA3ox1*, *GA20ox3* and *GAI*, respectively, in WT and *SIMAPK6*-knockout lines. (D)-(F) show the transcript levels of *PRE1*, *PRE2* and *PRE3*, respectively, in WT and *SIMAPK6*-knockout lines. Each data from three biological and technological replicates and represents the mean \pm SE. The single asterisks (*) indicate significant differences ($P < 0.05$) and double asterisks (**) indicate highly significant differences ($P < 0.01$).

3.5 Expression of Tomato Strigolactone Biosynthesis- and Lateral Bud Development-Related Genes in *SIMAPK6*-Knockout Lines

Our results showed that *SICCD7* and *SICCD8* were significantly upregulated in the lateral buds of the knockout plants (Figures 6A and 6B). In addition, the expression of three lateral bud development-related genes, *Lateral suppressor* (*Ls*) (Schumacher et al., 1999), *Blind* (*BL*) (Schmitz et al., 2002) and *BRC1b/TCP8* (Martín - Trillo et al., 2011), was examined in WT and two *SIMAPK6*-knockout lines. The transcript level of tomato *Ls*, encoding a new member of the VHIID protein, did not differ between the knockout lines and the WT (Figure 6C).

The *BL* gene can regulate the formation of lateral meristems by encoding a MYB transcription factor (Schmitz et al., 2002). The *BRC1b* gene is involved in the control of cell growth and proliferation in lateral organs and meristems by encoding a transcription factor of the TCP family (*Proliferating Cell Factors, Cycloidea, Teosinte Branched 1*) (Cubas et al., 1999; Martín-Trillo et al., 2010). Significantly decreased expression levels of these two genes were found in the lateral buds of transgenic plants (Figures 6D and 6E).

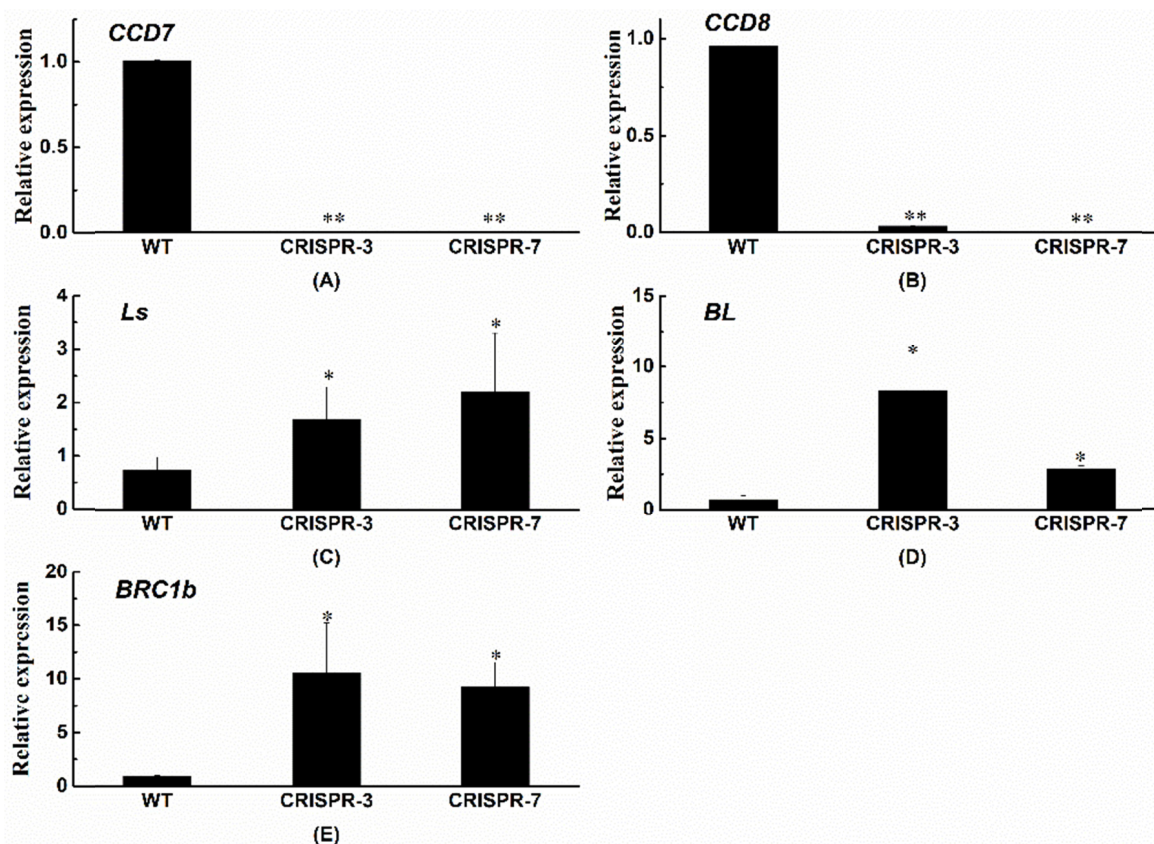


Figure 6. Relative expression levels of tomato lateral bud development- and strigolactone biosynthesis-related genes in WT and *SIMAPK6*-knockout lines

Note. (A) and (B) show the relative expression levels of strigolactone biosynthesis genes *CCD7* and *CCD8*, respectively, in lateral buds of the WT and the *SIMAPK6*-knockout lines. (C), (D) and (E) show the relative expression of the lateral bud development-related genes *Ls*, *BL* and *BRC1b*, respectively, in the lateral buds of the WT and *SIMAPK6*-knockout lines. Each data from three biological and technological replicates and represents the mean \pm SE of. The single asterisks (*) indicate significant differences ($P < 0.05$) and the double asterisks (**) indicate highly significant differences ($P < 0.01$).

4. Discussion

In this study, a gene-editing technology was used to knockout the gene of *SIMAPK6* to explore its function. It was found that the *SIMAPK6*-knockout line, CRISPR-3 and CRISPR-7 have special phenotype, for example, an increased number of axillary buds and an enhancement in the length of leaflets, stem diameter, number of axillary buds and number of compound leaves. These results suggest that *SIMAPK6* plays a key role in maintaining plant growth and morphogenesis.

To determine the molecular mechanism underlying axillary buds in the *SIMAPK6*-knockout lines, cell elongation, GA biosynthesis and GA response-related genes in tomato, including *PRE1*, *PRE2*, *PRE3*, *GA20ox3*, *GA3ox1* and *GAI*, were observed. Among them, the expression levels of *GA20ox3* and *GA3ox1* were slightly downregulated in knockout plant leaves, which may be involved in the production of a minimum amount of GAs in *SIMAPK6*-knockout lines. However, *GAI*, a repressor of GA responses, was highly upregulated in the *SIMAPK6*-knockout lines. These results suggest that the knockout of *SIMAPK6* affects the biosynthesis and

catabolism of GA. Our results are in accordance with those of a previous study of Martín-Trillo et al. (2010), where it was shown that the expression level of *PRE1* was regulated by GA. Moreover, the expression levels of *PRE1*, *PRE2* and *PRE3* were significantly reduced in *SIMAPK6*-knockout lines (Martín-Trillo et al., 2010). However, our results suggest that *SIMAPK6* positively regulates GA-related genes. Our results are in agreement with the development of tomato axillary buds being regulated by members of several transcription factor families, such as *MYBs* (e.g., the *BL* gene) (Schmitz et al., 2002), *TCPs* (e.g., the *BRC1b* gene) (Cubas et al., 1999; Martín-Trillo et al., 2010) and *GRASs* (e.g., the *Ls* gene) (Schumacher et al., 1999).

During the vegetative growth of the plants whose *SIMAPK6* gene was knocked out, there were a greater number of compound leaves and more vigorous plants. These results showed an increase in the vegetative growth of the *SIMAPK6*-knockout lines. These results are in agreement with those of a previous study in which *SICCD7* and *SICCD8* were reported to inhibit the growth of branches of tomato plants through the methods of expressing antisense constructs or RNAi-mediated silencing (Kohlen et al., 2012; Vogel et al., 2010). The expression levels of the *SICCD7* and *SICCD8* genes were also examined in the *SIMAPK6*-knockout lines. It was observed that the expression levels of both genes were downregulated in *SIMAPK6*-knockout plants, indicating that with the abundant production of axillary buds, strigolactone biosynthesis might be decreased.

In this study, the increase in the number of axillary buds was examined due to the loss of function of *SIMAPK6* in the knockout lines. The level of expression was observed in lateral buds for related developmental genes, such as *Ls*, *BL* and *BRC1b*, in both WT and knockout plants. The highly expressed *BL* genes in the *SIMAPK6* knockout lines indicated that *SIMAPK6* negatively regulates *BL* genes. Moreover, the *BRC1b* gene were highly upregulated in the *SIMAPK6* knockout lines, which contradicts the increased axillary bud phenotype of the knockout plants. Shoot branching of angiosperms determines the aspects of plant morphological structure, such as visibility to pollinators, plant height and nutrient allocation. Thus, the vigorous vegetative growth of the *SIMAPK6* knockout plant probably accounts for the low fruit set percentage of the transgenic lines.

These results were also in agreement with those of a previous study in which RNA interference was applied to the *BL* control of the development of lateral axes. After interference with *BL* in tomato, the number of lateral axes decreased because the initiation of lateral meristems was stopped (Schmitz et al., 2002). *SIBRC1b* was found to suppress the growth of shoot branches because increased branch outgrowth was shown in *SIBRC1b*-loss-of-function tomato plants (Martín-Trillo et al., 2011).

5. Conclusions

The function of *SIMAPK6* was studied through CRISPR-Cas9 technology. First, it was observed that *SIMAPK6* was ubiquitously expressed in the different tissues we examined, and *SIMAPK6* expression levels were higher in the tissues of the leaves, stems and flowers than in the tissues of the roots, fruits and sepals. Second, the *SIMAPK6*-knockout lines CRISPR-3 and CRISPR-7 were obtained using CRISPR-Cas9 technology. It was found that, as a negative regulator, *SIMAPK6* plays a key role in regulating the development of axillary buds and architecture of tomato plants. In addition, a potential molecular mechanism for increasing the number of axillary buds was studied in *SIMAPK6*-knockout plants. It was possible that *SIMAPK6* negatively regulate strigolactone biosynthesis via *SICCD7* and *SICCD8* and positively regulated GA biosynthesis via *PRE1*, *PRE2*, *PRE3*, *GA20ox3*, *GA3ox1* and *GAI*, which led to the phenotype of increased numbers of axillary buds.

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Supplementary

Supplemental Table S1. PCR primers used in this study

Primers	Primer sequences (5'→3')
<i>SIMAPK6</i> -F	CGTTCAGTTCAAAAGTTCTCACATGATCA
<i>SIMAPK6</i> -R	CCACCATATTTATTCAGTCATCTTCACTGGG
Target1-F	CAAGATTCTTCAACCCCTTTTCT
Target1-R	CACGACCAATAGGTCTGATAGGA
Target2-F	GAAGATTGGGAATGCATTTGATA
Target2-R	AACAAGGCAAAGACATTTGAAGA
Target3-F	ATGTTATACGGCCTCCTCAAAAG
Target3-R	CCTGAAAAGTGTTTACCGCTATG
Kan-F	GGCGATACCGTAAAGCACGA
Kan-R	ATGGATTGCACGCAGGTTCT
<i>SIMAPK6</i> -Q-F	TGGAGATTTTGGGCTAGCAA
<i>SIMAPK6</i> -Q-R	CCAGGAAAAAGGGGTTGTCT
<i>SICAC</i> -F	CCTCCGTTGTGATGTAAGTGG
<i>SICAC</i> -R	ATTGGTGAAAAGTAACATCATCG
<i>GA20ox3</i> -Q-F	AGCCAAATTATGCTAGTGTTAC
<i>GA20ox3</i> -Q-R	TTTTATGAGATTTGTGTCAACC
<i>GA3ox1</i> -Q-F	ATAGGCACCCACCCTTGATA
<i>GA3ox1</i> -Q-R	GGATGAAAGTGCCTTGTCAAAAT
<i>GAI</i> -Q-F	CCAGCACTTGTCATTCTTACCC
<i>GAI</i> -Q-R	AAAGCTCATCCATCCAGCA
<i>PRE1</i> -Q-F	CGAAAGAACGAAAAGAGAGACATT
<i>PRE1</i> -Q-R	GCTAGAGCGACGATTGCGAA
<i>PRE2</i> -Q-F	TATGTCTGGGAGAAGGTCAAGGA
<i>PRE2</i> -Q-R	CGACGATTACGAATTCAGGAAG
<i>PRE3</i> -Q-F	TTCACACTCTCCATAGCAACACAT
<i>PRE3</i> -Q-R	TCCTCACTTATCCTTGATCCTCC
<i>CCD7</i> -Q-F	AGCCAAGAATTCGAGATCCC
<i>CCD7</i> -Q-R	GGAGAAAGCCCACATACTGC
<i>CCD8</i> -Q-F	CCAATTGCCTGTAATAGTTCC
<i>CCD8</i> -Q-R	GCCTTCAACGACGAGTTCTC
<i>Ls</i> -Q-F	TGTTTTCTACCTCCACCGCCT
<i>Ls</i> -Q-R	ATGATTTGCTTCCTTCTCCGC
<i>BL</i> -Q-F	GGAAGATTTGATAGTTGTGTTG
<i>BL</i> -Q-R	CAAAAATAGAGCTACACAAAACC
<i>BRC1b</i> -Q-F	AGAAGGCCGAGGCGAAAA
<i>BRC1b</i> -Q-R	GACCACGAGCGGTGTTGATC
<i>RbcS3B</i> -Q-F	TGCTCAGCGAAATTGAGTACCTAT
<i>RbcS3B</i> -Q-R	AACTCCACATGGTCCAGTATCTG

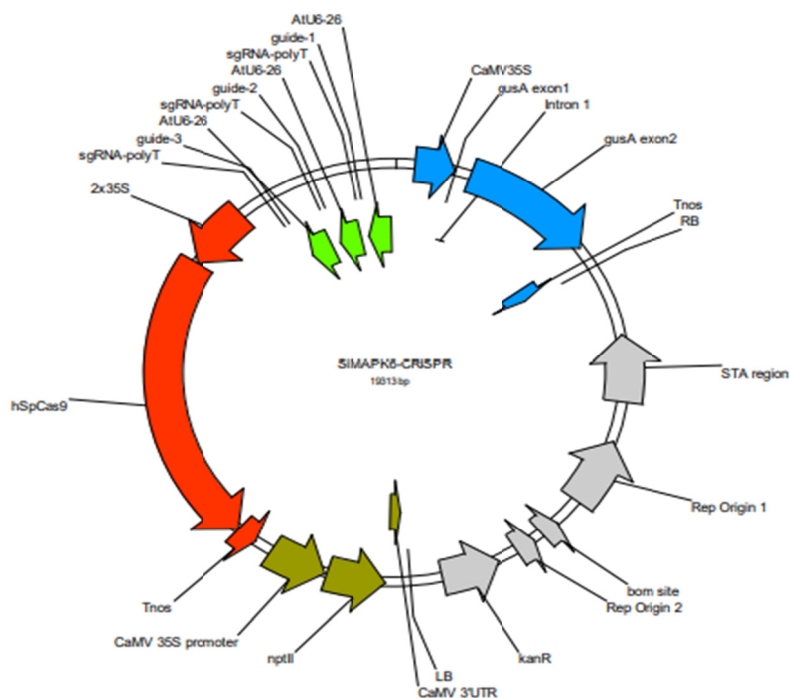
Supplemental Table S2. Primers used for vector construction

Aim	Primer name	Primer sequence	Product size (bp)
PCR of potential off-target 1 for target sequence 1	Off-target1.1-F	TCACTCCTTGGCCTTTTCTT	240
	Off-target1.1-R	GGACATGCATGCTTATCTTGAA	
PCR of potential off-target 2 for target sequence 1	Off-target1.2-F	CCATGATGTGTGGGATCTTCT	238
	Off-target1.2-R	CAGGACAGTGACCATCCCTAA	
PCR of potential off-target 1 for target sequence 2	Off-target2.1-F	GCTCGAGTTTGTCCGAATC	222
	Off-target2.1-R	CACTTCCTCCTGAACCCTGA	
PCR of potential off-target 2 for target sequence 2	Off-target2.2-F	TTTTTCTTCAGTGCCGCTTT	196
	Off-target2.2-R	TGCACAAGGTACAAGGCAGT	
PCR of potential off-target 1 for target sequence 3	Off-target3.1-F	GGCCACCACAAACTGAGAAT	227
	Off-target3.1-R	TGGCTAGTTCAGGCGACATA	
PCR of potential off-target 2 for target sequence 3	Off-target3.2-F	TGCTTTCACATTGCCAGGTA	241
	Off-target3.2-R	CAGGCACTGCATTACCATCA	

Supplementary Table S3. Mutations of potential miss sequences

Target	Name of potential off-target sites	Potential off-target sequence	No. mismatch bases	No. plants tested	No. mutations
T1	T1-O1	GGTGTAACAGTAACATGAGGAG	3	30	0
	T1-O2	GGTGAAAACAGTAACATAAAAGG	4	30	0
T2	T2-O1	CAAATAGCTTATCAGTCACAGGG	4	30	0
	T2-O2	GATAAAGCTTCTTCGTCACATGG	2	30	0
T3	T3-O1	AGTGATCATCAGTCAACTGTTGG	2	30	0
	T3-O2	AGGTCATAAAGTCAGCTGTTGG	4	30	0
	T3-O3	AGTGATCATCAGTCAACTGTTGG	2	30	0

Note. The PAM sequence (NGG) is underlined, and the NAG analog was also used for testing. Mismatch bases are marked in red.

Supplemental Figure S1. The diagram of SIMAPK6-knockout vector**Copyrights**

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