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Full Length Article

Silencing of *SLB3* Transcription Factor Reduces Salt Tolerance in Tomato

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Abstract

Soil salinity is one of the very important factors for plant growth. For now, the study of salt-tolerant plants has always been a cause for concern. BRI1-EMS-suppressor (*BES1*), as an important specific transcription factor in plants, is essential in brassinosteroid (BR) signaling. By regulating the expression of BR-responsive genes, *BES1* is closely related to abiotic stress. In the study of resistance to salinity of tomato, virus-induced gene silencing (VIGS) was performed on the *SLB3* gene, which is from the *BES1* transcription factor family. By reducing the expression of *SLB3*, it was observed whether the salt tolerance of the treated plants would change. The free proline (Pro) content and peroxidase (POD) activity were extremely lower in silenced experimental materials than the control seedlings. The content of active oxygen and malondialdehyde (MDA) and activity of superoxide dismutase (SOD) were higher in silenced experimental materials than the control seedlings. The results showed that the expression level of *SLB3* gene in the successfully silenced plants became lower, and the response of tomato to salt stress was correspondingly affected; the silenced tomato did not live well in the high salt treatment environment. Overall, this article establishes a foundation for further research investigating the salt tolerance mechanism of the *BES1* transcription factor and will help researchers understand the important role of transcription factors plant response to abiotic stress tolerance.

Keywords: *SLB3* gene; Transcription factor; VIGS; Salt stress

Introduction

The problem of soil salinization has been a consistent threat to the limited soil resources depended on by humans and is an increasingly serious environmental and ecological issue (Zhang *et al.* 2004). Studies have shown that salt damage to plant tissues is primarily caused by ionic and osmotic stress (Munns and Tester 2008), thereby inhibiting the normal growth of plants. Among the environmental factors that affect plant growth and development, the effect of soil salinity cannot be ignored (Munns 2005; Rozema and Flowers 2008). Salt stress affects the biochemical processes and metabolic pathways of plants, leading to reduced crop yields. Almost all major physiological processes of plants—such as photosynthesis, protein synthesis, energy and fat metabolism—are affected by salt stress to differential degrees. High salt levels can inhibit the growth of plant tissues and organs, significantly reduce the fresh and dry weight of plants, cause leaves to chloros and senesce, block root growth, and lead to premature aging or even death of plants (Lutts 1996). In recent decades, salt-tolerant plant germplasm resources and plant salt tolerance have been extensively studied (Munns and Tester 2008). The strategies to improve plant salt tolerance have long been a heavily researched issue in plant physiology and ecology research (Sun *et al.* 2014).

Tomato (*Solanum lycopersicum* L.), as one of the most economically important vegetables in the world, plays a great role in vegetable production and agronomic cultivation in China. The open cultivation and greenhouse cultivation of tomato are often affected by salt stress (Zhang and Blumwald 2001). Tomato is a medium salt-tolerant plant (Cuartero and Fernández-Muñoz 1999). Similar to other crops, salinity requirements differ at various stages of tomato growth and development. For example, the period of tomato seed germination and seedling growth is the most sensitive period to salt stress (Johnson *et al.* 1992). At present, studies examining the salt tolerance of tomato have primarily focused on early development (Al-Karaki 2000; Alian *et al.* 2000). The salt tolerance of plants varies with individual developmental stages (Asins *et al.* 1993). Plant hormones that are induced under salt stress also promote salt

tolerance. In salt-sensitive cultivated tomato (*L. esculentum*; Lem), the antioxidant isoenzymes and oxidants induced by salt stress are downregulated, thereby increasing the oxidative damage. Contrarily, in salt tolerant tomato (*L. pennellii*; Lpa), the stress-induced upregulation of antioxidant isoenzymes and oxidants reduced the oxidative stress injury (Mittova *et al.* 2014).

Transcription factors (TFs) have an important effect on plant growth and development and stress response transformation by activating or inhibiting the transcription of target genes (Guo *et al.* 2014; Han *et al.* 2018). When plants are under biotic and abiotic stress, TFs can activate multiple defense mechanisms (Century *et al.* 2008). The *BES1* transcription factor family is a type of plant-specific transcription factor that can regulate the BR signaling pathway (Yin *et al.* 2005). By activating the transcription of downstream genes to regulate the expression of BR target genes, *BES1* ultimately regulates plant growth, development and stress resistance (Yin *et al.* 2002).

Wang *et al.* (2002) screened a BR synthesis inhibitory mutant, brassinazole-resistant 1-1D (bzr1-1D), and by map cloning identified the BZR1 gene, which encodes a nuclear protein and is induced by BR. In the same year, Yin *et al.* (2002) screened for the BR receptor inhibitor *BES1*, which was induced by BR and accumulated in the nucleus. It was later confirmed that *BES1* is a BZR1-like protein with high sequence similarity. Yin *et al.* (2005) discovered a new class of transcription factors, *BES1*/*BZR1*, and confirmed that it is unique to plants and the only transcription factor in the BR signal transduction pathway. BRASSINOSTEROID INSENSITIVE2 (BIN2) is a negative regulator of the brassinolide pathway, and *BES1*/*BZR1* can be phosphorylated to lose its original activity (Li *et al.* 2002; Rybel *et al.* 2009; Yan *et al.* 2009). In addition, a BRI1-SUPPRESSOR1 (BSU1) phosphatase upstream of BIN2 and BIN2 can be dephosphorylated to become inactivated, thereby deactivating *BES1*/*BZR1* from *BIN2* inhibition (Kim *et al.* 2009). PP2A is another phosphatase in the downstream pathway of BR, and scientific studies have demonstrated that it is a heterotrimer composed of three different subunits that dephosphorylate the substrate at the threonine and serine positions. Specifically, BZR1 can be dephosphorylated by PP2A, which becomes active and plays a normal role; therefore, it facilitates normalization of the BR signaling pathway (Tang *et al.* 2011). Currently, *Arabidopsis thaliana* has 8 members in the *AtBES1*/*BZR1* gene family (Jiang *et al.* 2015), rice (*Oryza stiva*) has 4 members in the *OsBES1*/*BZR1* gene family (Bai *et al.* 2007) and maize (*Zea mays*) has 11 members in the *ZmBES1*/*BZR1* gene family (Kim *et al.* 2011; Manoli *et al.* 2018). Ye *et al.* (2017) found that *BES1*/*BZR1* is antagonized by RD26 from the NAC transcription factor family. *BES1*/*BZR1* combines with the RD26 gene promoter and inhibits RD26 expression, whereas RD26 protein binds to *BES1*/*BZR1* protein and inhibits RD26 drought response regulation. During drought and starvation stress, SINAT E3 ligase and selective autophagy receptor DSK2 mediate the degradation of dephosphorylated *BES1*, allowing plants to halt growth under adverse conditions (Nolan *et al.* 2017; Yang *et al.* 2017).

In our previous study, nine *BES1* transcription factor family members were identified in tomato, and most of these genes responded to cold, drought and salt stress (Gao *et al.* 2018). *SLB3* is one of the genes that can cope with salt stress. In tomato plants, the gene was significantly upregulated under salt stress treatment. To further determine the function of *SLB3*, we used VIGS to reduce the expression of *SLB3* in tomato plants, observe the phenotypic changes in plants, and analyze the effect of *SLB3* silencing on the salt tolerance of plants.

Materials and Methods

Plant material

The species of tomato used in this experiment was "Moneymaker", which was provided by the lab of genetic breeding in tomato of Northeast Agricultural University (Harbin, China). The tomato seedlings were planted at the Horticultural Experimental Station of Northeast Agricultural University. The soil composition ratio of turfy soil, vermiculite and perlite was 3:1:1.

Target fragment amplification and vector construction

TRIzol (Invitrogen, Shanghai, China) was used to extract total RNA from sample leaves. Following the manufacturer's instructions, we used a cDNA synthesis kit (Thermo Fisher, Beijing, China) to synthesize the first-strand cDNA. We downloaded the mRNA sequence of Solyc02g071990 from the Sol Genomics Network (https://www.solgenomics.org) and then designed the primers for *SLB3*, which contained specific enzyme sites (EcoRI and BamHI) and protective bases, using the online primer design tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The PCR product was analyzed by agarose gel electrophoresis, and the band with the correct was recovered and purified using a PCR purification kit (TaKaRa, Dalian, China).

The plasmid was extracted from bacterial solution containing TRV2. The TRV2 plasmid and the desired fragment were double digested with EcoRI and BamHI (Thermo Fisher, Beijing, China), and the digested plasmid and target fragment were joined with T4 DNA ligase. The successfully ligated TRV2 vector was introduced into *Escherichia coli* DH5α competent cells and incubated overnight at 37°C. We picked white clones from kanamycin-containing lysogeny broth (LB) medium and then cultured them in liquid LB culture amended with 50 μg/mL kanamycin, extracted plasmid from the bacterial solution and verified it by sequencing. The identified TRV2-*SLB3* strain was cultured in liquid LB culture containing

50 μg/mL kanamycin and used for plasmid extraction. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* GV3101. *A. tumefaciens* GV3101 carrying the TRV2-PDS vector and TRV2-TRV1 vector used in this experiment were provided by the lab of genetic breeding in tomato of Northeast Agricultural University (Harbin, China). The phytoene dehydrogenase (PDS) used in this experiment is one of the rate-limiting enzymes affecting the synthesis of carotenoids and participates in linear carotenoid biosynthesis. PDS-silenced plants will turn white from the growing point and can be used as an indicator.

Infection of tomato seedlings

Seedlings with a center leaf and four functional leaves of similar size and good growth were selected for infection experiments. Three replicates were performed for each segment of interest. By using a syringe, the mixed solution was injected into the leaves and tender stems of tomato seedlings. The infected plants were placed in an environment with a temperature of 22°C and humidity of 60%, and cultivated under light intensity conditions of 120 μmol m^{-2} s^{-1} for 16 h followed by dark exposure for 8 h. PDS-silenced seedlings turned white approximately 20 days after infection. The overall whitening phenomenon gradually spread from the top growing point to the bottom and from the petioles to the veins of tomato leaves. This result confirmed that the PDS indicator gene was successfully and stably silenced in the tomato seedlings.

Determination of gene silencing efficiency

Leaf samples were taken from the silencing treatment and control plants. RNA was extracted and cDNA synthesized as described above. Primers were designed for amplification of the target fragment (Table 1). The gene silencing efficiency was determined by RT-qPCR, and the data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Analysis of the gene expression level in silenced plants under salt stress

SLB3-silenced seedlings, with a silencing efficiency of less than 50% compared with the control group, and control seedlings were cultivated in Hoagland's nutrient solution for 24 h. Then, the roots were soaked in 200 mM NaCl for 24 h. Phenotypic changes in the plants were observed at 1.5, 3, 6, 12 and 24 h after treatment, and the tomato leaves were removed and stored at -80°C. The leaves were subjected to RT-qPCR analysis. The experimental method was the same as described above.

Determination of SOD, POD, Pro and MDA contents

The SOD assay kit (SOD-1-Y), POD assay kit (POD-1-Y), Pro content assay kit (Pro-1-Y) and MDA content assay kit (MDA-1-Y) of Suzhou Comin Biotechnology Co., Ltd. (Suzhou, China) were used to measure SOD and POD activities and Pro and MDA contents. The above four kits were used according to the instructions.

Determination of reactive oxygen species (ROS)

In plant tissue, 3,3' diaminobenzidine (DAB) reacts with H_2O_2 to form a brown-red precipitate (Thordal-Christensen *et al.* 1997), and nitro-blue tetrazolium (NBT) reacts with $O_2^{-\bullet}$ to form a deep blue precipitate (Beyer and Fridovich 1987). According to the principle of the chemical reaction, the accumulation of H_2O_2 and $O_2^{-\bullet}$ in tomato leaves was observed by DAB and NBT staining methods. The leaves were stained with the prepared DAB, NBT solution for 24 h, and the dyed leaves were placed in anhydrous ethanol by heating in a boiling water bath for 10 min. The leaves were then placed on a glass slide for observation.

Statistical analysis

The data of this experiment were analyzed and plotted by Excel 2019. All data were mean ± standard error (SE) of three replicates.

Results

Verification of gene silencing efficiency

By comparing the *SLB3* gene expression levels of the silenced, control and empty vector control plants, the *SLB3* gene expression level was obviously decreased in most plants in the silenced group, but three plants showed normal expression levels of the *SLB3* gene. Twelve successfully silenced plants showed a clearly downregulated expression pattern for subsequent experiments (Fig. 1).

Observing the plant phenotype after salt stress

As shown in Fig. 2, there was no obvious difference between the silenced and control plants 1.5 h after salt stress. In both groups, the leaves stretched, and the stems were slightly curved. Six hours after salt stress, compared with the control group, the phenotype was significantly different in the silenced group. Silenced plants had severely curved stems and wilted leaves. In the control group, the stems were slightly curved, and the leaves withered. After 24 h of high-salt treatment, the silenced and control groups suffered from severe water loss and extreme wilting.

Analysis of gene expression before and after silencing

We directly compared the expression levels of the *SLB3* gene in the control, empty vector group and silenced group after salt stress treatment at the following 6 time points: 0, 1.5, 3, 6, 12 and 24 h, and collected leaves for RT-qPCR.

The results suggested that the *SLB3* gene expression level gradually increased under salt stress in the control and empty vector control group with similar gene expression at the same time (Fig. 3). The expression level in the silenced plants was consistently low.

Analysis of SOD, POD, Pro and MDA

The antioxidant capacity of plants under salt stress can be estimated by measuring SOD and POD activities and Pro and MDA contents. Based on the results, the SOD and POD activities and Pro and MDA contents in each group increased significantly (Fig. 4). After salt stress, POD activity and Pro content increased less and SOD activity and MDA content increased more in the silenced plants than the control and empty vector control plants.

Analysis of ROS content

The results on ROS are shown in Fig. 5. At the beginning of the experiment, the stained areas of control and gene-silenced plants were light, but they became darker after 6 h of salt stress treatment. After the same treatment time, the silenced plants were darker than the control plants.

Discussion

In our experiments, we used VIGS to downregulate the *SLB3* gene. The RT-qPCR analysis results indicated that the success rate of gene silencing was 80% in 15 plants. Under normal circumstances, a lower gene expression level in plants after silencing than half the control plants was considered successful silencing. The average silencing efficiency in the 12 silenced plants was 75%. Li *et al.* (2013) explored the effects of SpMPK1, SpMPK2 and SpMPK3 genes in tomato on acid-mediated drought tolerance. The silencing efficiency was 80% (SpMPK1), 73% (SpMPK2) and 78% (SpMPK3). The efficiency of silencing in our study thus demonstrated an average level. The down-regulation of *SLB3* gene expression caused an obviously different phenotype of the seedlings under salt stress. At 6 h after salt stress treatment, the control group displayed only a slightly curved main stem; the main stem of the *SLB3*-silenced treatment group was severely curved, the petiole was slightly curved, and the leaves were wilted. Under the same salt stress treatment conditions, *SLB3*-silenced plants withered faster and more seriously than control. Other analyses also demonstrated that *SLB3* gene silencing affected plants. In terms of the present silencing efficiency, our experimental results are credible.

During normal physiological metabolism in plants, reactive oxygen species are inevitably produced. When plants are exposed to drought, high temperature, low temperature, salt, pests and other stresses, the production and elimination of ROS are imbalanced; therefore, the level of intracellular ROS exceeds the range that cells can tolerate and may ultimately cause cell death (Mittler 2002; Sharma *et al.* 2012). These ROS, including superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals, can seriously damage plants (Shi *et al.* 2010). Antioxidant enzymes in plants, such as SOD, POD, catalase (CAT) and glutathione reductase (GR), work as ROS quenchers to protect cells from oxidative damage. Changes in antioxidant enzyme levels have been used to assess the impact of different abiotic stresses.

Free proline is a non-enzymatic metabolite. Under stress conditions, Pro acts as a stabilizer of subcellular structures, and a scavenger of free radicals (Nanjoa *et al.* 1999). Increases in Pro are beneficial to prevent cell dehydration and can alleviate damage to the membrane system. Kishor *et al.* (1995) have indicated a positive correlation between the accumulation of Pro and stress tolerance in plants. The main function of SOD and POD is to eliminate the intracellular ROS induced by stress, inhibit the accumulation of unsaturated fatty acids in the membrane and of MDA, maintain the stability and integrity of the plasma membrane, and enhance the plant body. The level of these enzyme activities and MDA content can reflect the strength of plant tolerance to some extent (Greenway and Munns 1980).

In this study, the activities of SOD and POD and the contents of Pro and MDA were measured to assess the antioxidant capacity of plants during salinity stress. After 3 h of salt treatment, Pro and MDA contents and POD activity were increased in all experimental groups. The content of Pro and activity of POD were extremely lower in silenced experimental materials than the control seedlings.

Table 1: Primers used for target fragment amplification and qRT-PCR analysis

Primer Name	(Restriction Site Sequence) Primer sequence (5′–3′)
VIGS Primer-F	GC(TCTAGA)ACACTTGGGAACTCCAGCAC
VIGS Primer-R	CG(GGATCC)ACTAACTGCTGCTCCTACC
qRT-PCR primer-F	CACGAGGCTACCGACATGGA
qRT-PCR primer-R	TCTTCAACAATCCAACCAGCCTCT
Actin Primer-F	ATTGGTGCTGAGAGGTTCCG
Actin Primer-R	CGGGAAACAGACAGGACACT

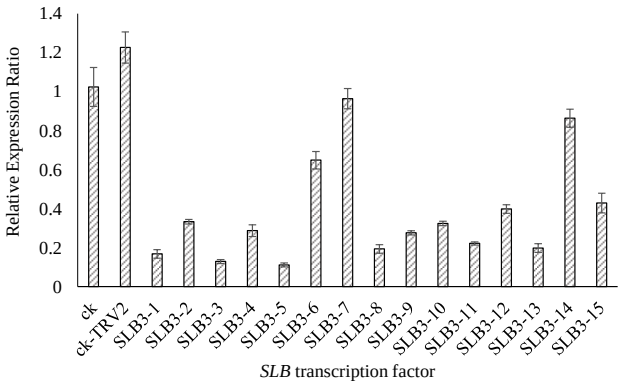

Fig. 1: Changes in *SLB3* expression
SLB3-1~ *SLB3*-15: 15 *SLB3*-silenced plants

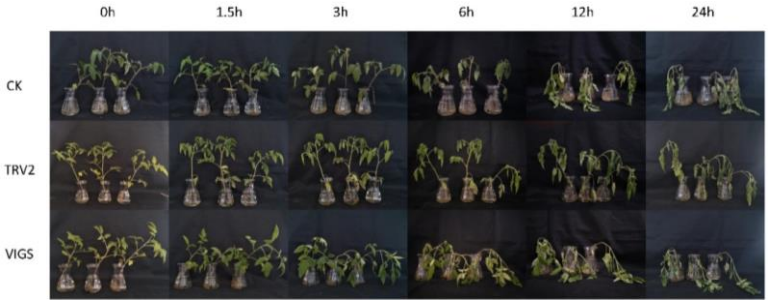

Fig. 2: Changes of phenotypic in control plants and *SLB3*-silenced plants under salt stress

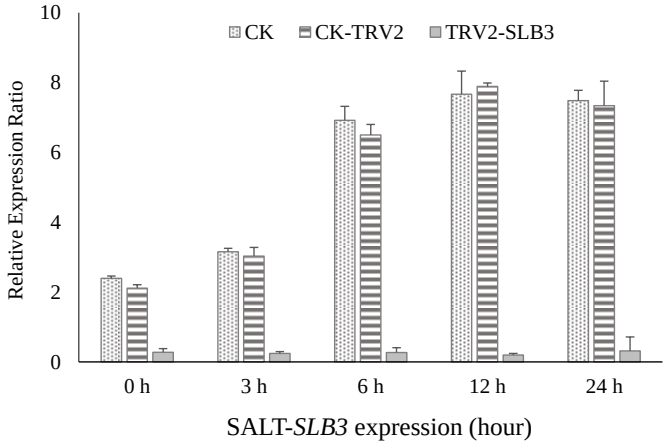

Fig. 3: Time course expression of *SLB3* gene in different plant under salt stress

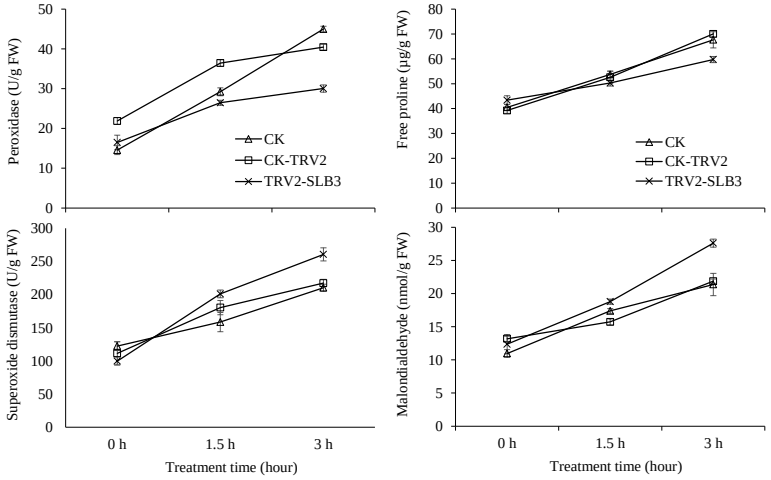

Fig. 4: The change of SOD and POD activity and Pro and MDA content in tomato plants under salt stress treatment

The MDA content was markedly higher in the silenced than the control plants under the same conditions. This result indicates that adversity leads to an increase in superoxide radicals. To resist peroxidation of cell membranes by stress, the intracellular self-regulatory mechanism plays an important role, and antioxidant enzyme activity is enhanced. The degree of oxidation of lipids on the cell membrane is increased, which increases the membrane permeability and destroys the cell membrane system. The SOD content was increased in all plants after 3 h of salt stress treatment, and the MDA content was extremely elevated in *SLB3*-silened plants compared with the control seedlings. It is speculated that *SLB3* gene silencing may be involved in the negative regulation of SOD. The above conclusions indicate that *SLB3* gene silencing can reduce salt tolerance. Similar results were found in other studies on salt tolerance in

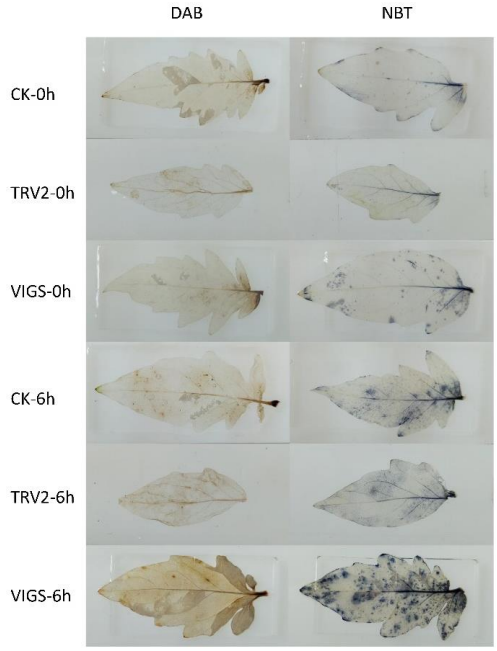

Fig. 5: NBT and DAB staining

tomato. In stress assays, *SlbZIP1-RNAi* transgenic plants showed reduced tolerance to salt stress, decreases in CAT activity and elevated MDA content (Zhu *et al.* 2018).

In abiotic stress tolerance-related gene function studies, at present, DAB and NBT staining methods are commonly used. In the presence of peroxidase, DAB reacts with H_2O_2 to produce a brown polymer, which makes it possible to observe the production of H_2O_2 in tomato leaves after DAB staining. The production of $O_2^{-\bullet}$ appears blue after the leaves are stained with NBT. The dyeing situation can visually show the damage in the plant under study. In the present research, by observing the stained area of the leaves, we concluded that the content of H_2O_2 and $O_2^{-\bullet}$ in tomato leaves increased with extension of the salt stress treatment time. Six hours after salt stress treatment, the size and color of the blue and brown areas were larger and darker on the leaves of the *SLB3*-silenced plants than the control group. The results revealed that silencing of the *SLB3* gene resulted in greater damage to the plants by salt, which was consistent with the changes in POD activity and Pro and MDA contents.

In this experiment, there was no significant difference in phenotype between the control, empty vector control and *SLB3*-silenced group before salt stress treatment. This finding suggested that down-regulation of the *SLB3* gene had little effect on plant growth. The *SLB3* gene may play a vital part in the salt stress process.

Conclusion

The VIGS method was used to silence the *SLB3* gene in tomato plants. Under the same salt stress conditions, the *SLB3*-silenced plants wilted faster and to a greater extent than the control plants. Physiological analysis indicted that after 3 h of salt stress, POD activity and Pro content were both increased in *SLB3*-silenced plants but to a lesser extent than in the control plants. SOD activity and MDA content increased to a greater extent in *SLB3*-silenced than control plants. Compared with the control, H_2O_2 and $O_2^{-\bullet}$ accumulated to a greater extent in leaves of *SLB3*-silenced plants. These results confirmed that *SLB3* gene silencing affected tomato seedlings in the presence of salt treatment. Downregulation of the *SLB3* gene reduced salt tolerance in tomato plants.

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Author Contributions

Yufang Bao planned and executed experiment, completed the data analysis, and wrote the first draft of the paper. Ziyu Wang, Yingmei Gao, Huanhuan Yang, He Zhang, Jingbin Jiang and Jingfu Li participated in experimental design and analyzed experimental data. Tingting Zhao and Xiangyang Xu contributed to the central idea, guided experimental design, data analysis, dissertation writing and revision. All authors read and agreed with the final draft.

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