Silencing of the *SL-ZH13* Transcription Factor Gene Decreases the Salt Stress Tolerance of Tomato

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ABSTRACT. Zinc finger-homeodomains (ZF-HDs) are considered transcription factors that are involved in a variety of life activities in plants, but their function in regulating plant salt stress tolerance is unclear. The *SL-ZH13* gene is significantly upregulated under salt stress treatment in tomato (*Solanum lycopersicum*) leaves, per our previous study. In this study, to further understand the role that the *SL-ZH13* gene played in the response process of tomato plants under salt stress, the virus-induced gene silencing (VIGS) method was applied to down-regulate *SL-ZH13* expression in tomato plants, and these plants were treated with salt stress to analyze the changes in salt tolerance. The silencing efficiency of *SL-ZH13* was confirmed by quantitative real-time PCR analysis. *SL-ZH13*-silenced plants wilted faster and sooner than control plants under the same salt stress treatment condition, and the main stem bending angle of *SL-ZH13*-silenced plants was smaller than that of control plants. Physiological analysis showed that the activities of superoxide dismutase, peroxidase, and proline content in *SL-ZH13*-silenced plants were lower than those in control plants at 1.5 and 3 hours after salt stress treatment. The malondialdehyde content of *SL-ZH13*-silenced plants was higher than that in control plants at 1.5 and 3 hours after salt stress treatment; H_2O_2 and O_2^- accumulated much more in leaves of *SL-ZH13*-silenced plants than in leaves of control plants. These results suggested that silencing of the *SL-ZH13* gene affected the response of tomato plants to salt stress and decreased the salt stress tolerance of tomato plants.

Salt stress is a problem for plant growth and agricultural productivity. During different developmental stages, cultivated plants are also exposed to changes in the environment and respond by activating gene expression (Yanez et al., 2009). In studies of stress tolerance in plants, it is found that the transcription factor activates or inhibits the expression of related genes through interactions with other related proteins or itself and then plays an important role in regulating the adaptability of plants to adverse conditions.

The homeobox (HB) gene encodes a highly conserved 60–61 amino acid homeodomain (HD), which confers sequence-specific DNA binding function by folding into a characteristic three alpha helix structure (Ariel et al., 2007; Hanes and Brent, 1989; Zhao et al., 2011). Plants have evolved the specific HD-Zip transcriptional factor family (Ariel et al., 2007), the members of which bear a unique leucine zipper domain at the C-terminus. Zinc fingers, which consist of two pairs of conserved cysteine and/or histidine residues binding a single zinc ion to form a finger-shaped loop (Klug and Schwabe,

1995), are necessary motifs that are found widely in regulatory proteins (Hu et al., 2018; Krishna et al., 2003; Takatsuji, 1999). According to the nature, number, and spacing pattern of zinc-binding residues, zinc fingers could be classified into different types (Englbrecht et al., 2004; Kosarev et al., 2002; Li et al., 2001; Wang et al., 2016; Yanagisawa, 2004). ZF-HD proteins are one of the HD-containing protein families.

The ZF-HD subfamily of homeobox genes has been researched in some model plants. They are considered transcription factors that regulate biotic and abiotic stress, and plant development processes. Functional studies of ZF-HD in abiotic stress responses have been reported in different plant species. AtZHD1 is a transcriptional regulator that binds to the promoter region of ERD1 (early response to dehydration stress 1), and its expression is induced by drought, salinity, and abscisic acid [ABA (Tran et al., 2007)]. The overexpression of NAC and AtZHD1 increases drought tolerance in arabidopsis [*Arabidopsis thaliana* (Wang et al., 2014)]. Thirty-one ZF-HD genes were identified in chinese cabbage (*Brassica rapa* ssp. *pekinensis*), and most of these genes are significantly induced under abiotic stresses (Wang et al., 2016).

Salt stress mainly causes oxidative damage in plants due to the overproduction of reactive oxygen species [ROS (Ali et al., 2017a; Ashraf, 2009)]. Superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) are important ROS that can initiate a series of destructive processes, leading to gradual lipid peroxidation and the inactivation of antioxidant enzymes (Tanou et al., 2009).

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Plants developed an antioxidative defense mechanism for the detoxification of excessively produced ROS. Among various antioxidants, superoxide dismutase (SOD) and peroxidase (POD) are enzymatic ones, while proline (Pro) is a non-enzymatic metabolite. These antioxidants all serve as ROS quenchers, thus protecting cells from oxidative damage (Yadav et al., 2016). The overproduction of ROS results in the accumulation of malondialdehyde (MDA) due to lipid peroxidation. MDA content estimation provides an effective means for assessing the extent of membrane damage (Ali et al., 2017b).

In our previous study, 22 ZF-HD genes (*SL-ZH1–SL-ZH22*) were identified from tomato (Hu et al., 2018), and most of these genes were responsive to abiotic stresses of cold, drought, and salinity. *SL-ZH13* is one of the salinity-responsive genes. This gene is upregulated significantly under salt stress treatment in tomato leaves. In this study, to understand the function of the *SL-ZH13* gene and determine the relationship between *SL-ZH13* expression and salt tolerance ability of tomato plants, we applied virus-induced gene silencing technology to decrease the expression level of *SL-ZH13* in tomato plants and analyzed changes in the salt tolerance ability in these plants.

Materials and Methods

PLANT MATERIAL. Tomato ‘Moneymaker’ was provided by Tomato Research Institute of Northeast Agricultural University (Harbin, China). All plants were grown at the Horticultural Experimental Station of Northeast Agricultural University. At the two- to three-leaf stage, the seedlings were used in a VIGS study.

TARGET FRAGMENTS AMPLIFICATION AND VECTORS CONSTRUCTION. Total RNA was extracted from ‘Moneymaker’ seedling leaves using the Trizol method (Trizol; Invitrogen, Shanghai, China) according to the manufacturer’s instructions. First-strand cDNA was synthesized using an M-MLVRTase cDNA synthesis system (M-MLVRTase cDNA synthesis kit; Takara, Dalian, China) according to the manufacturer’s instructions. Primers (Table 1) of the *SL-ZH13* fragment were designed using the primer design tool of the National Center for Biotechnology Information [NCBI (Ye et al., 2012)], based on the sequence of Solyc03g098060 from Sol Genomics Network [SGN (Fernandez-Pozo et al., 2014)]. The resulting PCR products were analyzed by agarose gel electrophoresis; bands with the correct size were excised from the gel and purified with a PCR purification system (PCR purification kit, Takara). The purified products were cloned into the pMD18-T vector (Takara) and sequenced (Sangon Biotech Co., Shanghai, China).

After the target fragment was identified, re-amplifications were applied using the primers with added restriction site sequences (*EcoRI* and *BamHI*). Target products were excised from the gel, purified and cloned into tobacco rattle virus RNA2 (TRV2), which was digested with restriction endonucleases

EcoRI and *BamHI* (Takara). The cloned TRV2 vectors were transformed into competent cells of *Escherichia coli* DH5 α and incubated at 37 °C overnight. The white clones grown on lysogeny broth (LB) medium containing kanamycin/X-gal/isopropyl β -D-thiogalactoside (IPTG) were picked and cultured in a liquid LB culture with 50 μ g·mL⁻¹ kanamycin, and the plasmids were extracted and verified by sequencing. The identified TRV2-SL-ZH13 strain was cultured in a liquid LB culture with 50 μ g·mL⁻¹ kanamycin and used for plasmid extraction. The TRV constructs were transformed into *Agrobacterium tumefaciens* GV3101 according to the method of Huang et al. (2008).

In this experiment, silencing of *phytoene desaturase* (*PDS*), which causes the plants to photobleach, was used as a silencing experimental efficiency control. This gene was silenced in a separate experimental group parallel to the experimental group with silencing of the target gene *SL-ZH13*. GV3101 carrying the TRV2-PDS vector we used in this experiment was constructed in our previous study (Zhao et al., 2017) and preserved at the Tomato Research Institute of Northeast Agricultural University.

INFILTRATION OF TOMATO SEEDLINGS. Seedlings at the two- to three-leaf stage were used for infiltration experiments. For each target fragment, 20 seedlings were prepared for one experimental repeat, with three experimental repeats in total. All steps were performed as described by Velásquez et al. (2009).

CONFIRMATION OF GENE SILENCING EFFICIENCY BY QUANTITATIVE REAL-TIME PCR. The leaves were collected from the VIGS-treated seedlings and the control seedlings at 25 d after infiltration. RNA extraction and cDNA synthesis were carried out as mentioned above. Quantitative real-time PCR (qRT-PCR) was performed according to a previous study (Zhao et al., 2016). The primers used for target fragment amplification were used as qRT-PCR primers (Table 1). The data were analyzed using the 2^{- $\Delta\Delta$ CT} method (Livak and Schmittgen, 2001) with *EF α 1* as a reference gene for normalization (Rotenberg et al., 2006).

SALT STRESS TREATMENT AND PHENOTYPIC OBSERVATION. According to the method of our previous study (Hu et al., 2018), *SL-ZH13* silenced seedlings (selected based on the qRT-PCR result of gene silencing efficiency confirmation) and control seedlings were grown in nutrient solution for 24 h and then irrigated with 250 mM NaCl for 24 h. Tomato leaf samples were gathered at 1.5, 3, 6, 12, and 24 h after treatment and stored at -80 °C for analysis. Three biological replicates were carried out. The angle between the part of the main stem inside the bottle and the part of the main stem outside the bottle was measured as the plant bending angle. Ten plants of each sample were measured, and the bending angle values were used to obtain the mean bending angle. Bending angle value data were analyzed with analysis of variance (ANOVA) (significance level 0.05) using SAS (version 9.1.3; SAS Institute, Cary, NC).

ASSAY OF SOD, POD, PRO AND MDA. The SOD activity was determined following the method of Giannopolitis and Ries (1977), and the activity of POD was determined following Chance and Maehly (1955). The Pro content in leaf homogenates was estimated by the method of Bates et al. (1973) with the help of the standard curve. The MDA content was estimated following the method of Cakmak and Horst (1991) and determined following the method of Ali et al. (2017a). The activities of SOD and POD and

Table 1. Primers used for *SL-ZH13* fragment amplification and qRT-PCR analysis of *SL-ZH13* expression pattern.

Primer name	(Restriction site sequence) Primer sequence (5′–3′)
VIGS Primer-F	CG (GAATTC) TGCCTGCGGCTGTCACC
VIGS Primer-R	CG (GGATCC)GATCTCCCGTAAACTTCGTCCTAAA
qRT-PCR primer-F	ATGCCACTGATGGCTGTGGA
qRT-PCR primer-R	TGCGGTGGAAGTTACGGTGA

the content of MDA were estimated using the same method of mean bending angle mentioned above.

NITROBLUE TETRAZOLIUM (NBT) AND 3,3' DIAMINOBENZIDINE (DAB) STAINING. Leaves collected from control plants and

SL-ZH13 silenced plants at 0, 1.5, and 3 h after treatment were used for superoxide radical staining. NBT staining was performed according to Rao and Davis (1999), and DAB staining was performed according to Fryer et al. (2002) and Ivan et al. (2009). O_2^- was visualized as a blue color produced by NBT precipitation. H_2O_2 was visualized as a brown color due to DAB polymerization.

GENE EXPRESSION PATTERN ANALYSIS. To further investigate the expression level of *SL-ZH13* in gene-silenced plants during the treatment process, leaves collected at 1.5, 3, 6, 12, and 24 h after treatment were used for qRT-PCR analysis. RNA extraction and cDNA synthesis were carried out as mentioned above. qRT-PCR was performed according to a previous study (Zhao et al., 2016). The primers used for target fragment amplification were used as qRT-PCR primers (Table 1). The data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with *EF α 1* as a reference gene for normalization (Rotenberg et al., 2006).

Fig. 1. Changes in *SL-ZH13* expression. Twenty *SL-ZH13*-silenced tomato plants were analyzed (VIGS-1 to VIGS-20); 15 *SL-ZH13*-silenced plants showed down-regulated expression pattern of the *SL-ZH13* gene. CK = control plant; VIGS-TRV2 = empty vector control plants.

Results

VERIFICATION OF PDS SILENCING EFFICIENCY. Silencing of the *PDS* control gene caused photobleaching in tomato plants. Photobleaching was observed as soon as 12 d after infiltration. Of 45 plants in three experimental repeats in total, 41 (91%)

Fig. 2. Phenotype changes of *SL-ZH13*-silenced (VIGS) tomato plants under salt stress treatment. Plant wilt was very obvious on stems and petioles but not on leaves. Petioles and stems were curved. The mean bending angle of *SL-ZH13*-silenced plants during wilting is smaller than that of control (CK) and empty vector control plants (CK-TRV2) plants. (A) CK plants at 0 h after salt stress treatment; (B) CK plants at 1.5 h after salt stress treatment; (C) CK plants at 3 h after salt stress treatment; (D) mean bending angle of *SL-ZH13*-silenced plants; (E) CK-TRV2 plants at 0 h after salt stress treatment; (F) CK-TRV2 plants at 1.5 h after salt stress treatment; (G) CK-TRV2 plants at 3 h after salt stress treatment; (H) mean bending angle of CK-TRV2 plants; (I) *SL-ZH13*-silenced plants at 0 h after salt stress treatment; (J) *SL-ZH13*-silenced plants at 1.5 h after salt stress treatment; (K) *SL-ZH13*-silenced plants at 3 h after salt stress treatment; and (L) mean bending angle of *SL-ZH13*-silenced plants. Red lines show the bending angle of plants.

showed the characteristic photobleaching phenotype 20 d after infiltration. Empty TRV2 vector-control plants and untreated-control plants grew normally. *SL-ZH13*-silenced plants did not show obvious abnormalities in tomato plant morphology.

The expression levels of target gene *SL-ZH13*-silenced plants were compared with the expression levels in wild-type control plants and empty vector control plants. The results showed that the expression level of the *SL-ZH13* gene decreased significantly after *SL-ZH13* silencing in most plants, but there were five plants showing normal expression levels of the *SL-ZH13* gene. Fifteen successfully silenced plants that showed obviously down-regulated expression patterns were used for subsequent experiments (Fig. 1).

Fig. 3. *SL-ZH13* expression patterns of different tomato plants under salt stress treatment. The expression level of the *SL-ZH13* gene was very low and stable in *SL-ZH13*-silenced plants (VIGS). CK = control plants; CK-TRV2 = empty vector control plants.

Fig. 4. Activities of superoxide dismutase (SOD) and peroxidase (POD) and contents of proline (Pro) and malondialdehyde (MDA) in tomato plants under salt stress treatment. The SOD and POD activities and Pro content in *SL-ZH13*-silenced (VIGS) plants were lower than those in the control (CK) and empty vector control (CK-TRV2) plants at 1.5 and 3 h after salt stress treatment. The MDA content in *SL-ZH13*-silenced plants was higher than that in the control plants CK and CK-TRV2 at 1.5 and 3 h after salt stress treatment.

PLANT PHENOTYPE OBSERVATION UNDER SALT STRESS TREATMENT. As shown in Fig. 2, all plants under salt stress treatment were wilted. This phenomenon was observed at 1.5 h after treatment and was even worse at 3 h after treatment. Plant wilt was very obvious on stems and petioles but not on leaves. Petioles and stems were curved. The mean bending angle of *SL-ZH13*-silenced plants during wilting is smaller than that of CK and CK-TRV2 plants.

GENE EXPRESSION PATTERN ANALYSIS. To further confirm the effect of gene silencing throughout the whole treatment process and to compare expression levels between control plants and *SL-ZH13*-silenced plants directly, qRT-PCR analysis was applied to leaves gathered from control plants and *SL-ZH13*-silenced plants at 0, 1.5, 3, 6, 12, and 24 h after salt stress treatment. The results (Fig. 3) showed that the *SL-ZH13* gene expression level increased after salt stress treatment in control plants and peaked at 3 h after salt stress treatment. In *SL-ZH13*-silenced plants, the expression levels were very low and stable.

ASSAY OF SOD, POD, Pro, AND MDA. SOD and POD activities and Pro content were increased significantly in all studied plants due to the imposition of salt stress (Fig. 4). Salt-induced increases in SOD and POD activities and Pro content were larger in the control plants CK and CK-TRV2 compared with the *SL-ZH13*-silenced plants. Similar to the SOD and POD activities and Pro content, the leaf MDA content also increased significantly due to the imposition of salt stress, while a smaller increase was found in control plants compared with *SL-ZH13*-silenced plants.

NBT AND DAB STAINING. Superoxide radical detection and quantification were performed using the NBT staining method. As shown in Fig. 5, the levels of superoxide radical staining before salt treatment were quite similar between control plants and *SL-ZH13*-silenced plants. A blue coloration appeared in all studied plants treated with salt. The blue coloration in *SL-ZH13*-silenced plants was stronger than that in the control plants CK and CK-TRV2 at the same treatment time. A brown precipitate in the presence of H₂O₂ was detected in all DAB stained leaves. Similar to the NBT staining result, the brown precipitate in *SL-ZH13*-silenced plants was stronger than that in the control plants CK and CK-TRV2.

Discussion

In this study, VIGS technology was used to down-regulate the *SL-ZH13* gene. qRT-PCR analysis results showed that the gene silencing success rate of twenty plants was 75% (plants with a *SL-ZH13* gene expression level reduction of more than 50% were classified as successfully silenced plants); the average silencing efficiency of 15 successfully silenced plants was 54%. This

Fig. 5. Nitroblue tetrazolium (NBT) and 3,3' diaminobenzidine (DAB) staining of tomato leaves. The blue coloration in *SL-ZH13*-silenced (VIGS) plants was stronger than that in the control (CK) and empty vector control (CK-TRV2) plants at the same treatment time; the brown precipitate in *SL-ZH13*-silenced plants was stronger than that in the control plants CK and CK-TRV2. (A) CK samples at 0 h after salt stress treatment; (B) CK samples at 1.5 h after salt stress treatment; (C) CK samples at 3 h after salt stress treatment; (D) CK-TRV2 samples at 0 h after salt stress treatment; (E) CK-TRV2 samples at 1.5 h after salt stress treatment; (F) CK-TRV2 samples at 3 h after salt stress treatment; (G) *SL-ZH13*-silenced plants at 0 h after salt stress treatment; (H) *SL-ZH13*-silenced plants at 1.5 h after salt stress treatment; and (I) *SL-ZH13*-silenced plants at 3 h after salt stress treatment.

silencing efficiency is relatively low compared with our previous study (Zhao et al., 2017). The phenotypic observation indicated that the down-regulation of *SL-ZH13* gene expression in this study caused a change in the plant phenotype under salt stress treatment. At 1.5 and 3 h after salt stress treatment, *SL-ZH13*-silenced plants showed more serious wilting than control plants and stems drooped more obviously. Another analysis also showed changes caused by *SL-ZH13* silencing. These results indicated that the influence of gene function analysis in this study caused by the relatively low silencing efficiency is limited.

Under stressful biotic and abiotic conditions, enhanced formation of ROS attacks nucleic acids, photosynthetic pigments, proteins and lipids, as well as induces both cellular damage and protective responses (Caverzan et al., 2016; Lin and Kao, 2000). Plant cells contain a wide range of antioxidant enzymes, such as SOD, POD, CAT, and glutathione-S-transferase (GST), and nonenzymatic metabolites (e.g., Pro), which serve as ROS quenchers, thus protecting cells from

oxidative damage (Yadav et al., 2016). MDA is a by-product of lipid peroxidation and an indicator of abiotic stress-induced oxidative damage in plants. Damage to lipids further results in the generation of ROS and subsequent oxidative stress. In this study, SOD and POD activities as well as Pro and MDA contents were measured to evaluate plant antioxidant ability under salt stress. According to our results, SOD and POD activities as well as Pro and MDA contents all increased in plants treated with salt stress, while the activities of SOD and POD and the content of Pro in *SL-ZH13*-silenced plants were all lower than those in control plants at the same treated time. The MDA content of *SL-ZH13*-silenced plants was higher than that of control plants at the same treated time. This result indicated that silencing of the *SL-ZH13* gene reduced the salt stress tolerance of tomato plants. Similar results were also found in plant salt stress tolerance ability studies in arabidopsis (Zhang et al., 2016). The study indicated that arabidopsis overexpressing the *Tamarix hispida* zinc finger protein ThZFP1 showed more tolerance to salt and osmotic stress. The POD and SOD activities, Pro content and expression levels of relative genes, such as delta-pyrroline-5-carboxylate synthetase (P5CS), all increased (Sun et al., 2007; Zang et al., 2015).

DAB and NBT staining methods have been used in many abiotic stress tolerance-related gene functional studies (Fryer et al., 2002). DAB reacts with H_2O_2 in the presence of peroxidases to produce a brown polymerization product, making the production of H_2O_2 visible in leaves infiltrated with DAB. O_2^- was visualized as a blue color produced by NBT precipitation (Fryer et al., 2002). These analyses could intuitively show the damage situation of the study plants. In this study, DAB and NBT staining results showed that H_2O_2 and O_2^- accumulated in tomato leaves treated with salt stress, and this accumulation increased with treatment time. At 1.5 and 3 h after salt stress treatment, brown and blue colors of leaves from *SL-ZH13*-silenced plants were all deeper than colors of control plant leaves. The results showed that salt stress caused more damage to plants with *SL-ZH13* gene silencing and coincided with SOD and POD activity as well as Pro and MDA content changes.

In all analyses of this study, there were no remarkable differences between control plants and *SL-ZH13*-silenced plants before salt stress treatment. This finding may suggest that the down-regulation of the *SL-ZH13* gene did not cause remarkable damage to the plants.

In conclusion, we obtained tomato plants with *SL-ZH13* gene silencing using the VIGS method. The silencing efficiency was confirmed by the down-regulation of *SL-ZH13* gene expression. *SL-ZH13*-silenced plants wilted faster and sooner than control plants under the same salt stress treatment conditions. Physiological analysis showed that the SOD and POD activities and Pro content in *SL-ZH13*-silenced plants were lower than those in control plants at 1.5 and 3 h after salt stress treatment. The MDA content of *SL-ZH13*-silenced plants was higher than that in control plants at 1.5 and 3 h after salt stress treatment. H_2O_2 and O_2^- accumulated much more in leaves from *SL-ZH13*-silenced plants than those from control plants. These results suggested that silencing of the *SL-ZH13* gene affected the response of tomato plants under salt stress and decreased the salt stress tolerance of tomato plants.

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