

Article

Genome-Wide Analysis of the *SlSIZ* Gene Family in Tomato and the Role of *SlSIZ1* and *SlSIZ4* in Salt Stress Tolerance

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Abstract

Salinity is an abiotic constraint that limits the growth and productivity of tomato (*Solanum lycopersicum* L.). However, the role of small ubiquitin-like modifier (SUMO) E3 ligases (SIZs) in the salt stress response in tomato remains unknown. Here, a genome-wide analysis identified four SIZ-type genes in tomato, designated *SlSIZ1*–*SlSIZ4*. Gene structure and conserved motif analyses revealed the canonical SAP (SAF-A/B, Acinus, and PIAS) domain and the SP-RING (Siz/PIAS RING finger) domain in all *SlSIZ* proteins. Phylogenetic analysis grouped SIZ proteins from tomato and representative plant species into five major groups (A–E), with *SlSIZ* members distributed across distinct clades, and members within each clade shared conserved gene structures. Online transcriptome data and our qRT-PCR analyses showed that *SlSIZ4* was highly expressed in leaves, flowers, and fruits, whereas *SlSIZ1* showed broad expression, with relatively higher levels in roots, flowers, and multiple fruit stages. Under salt stress, abscisic acid (ABA), and methyl jasmonate (MeJA) treatments, the expression of *SlSIZ1* and *SlSIZ4* was strongly induced, whereas the expression of *SlSIZ2* and *SlSIZ3* was rarely promoted. In addition, virus-induced gene silencing (VIGS) of *SlSIZ1* and *SlSIZ4* markedly reduced salt tolerance of tomato seedlings, as reflected by growth inhibition, chlorophyll loss, increased reactive oxygen species (ROS), reduced proline accumulation, and diminished antioxidant gene expressions. Collectively, these findings identify *SlSIZ1* and *SlSIZ4* as positive regulators of salt stress tolerance in tomato and provide a framework for further dissection of the *SlSIZ* gene family.

Keywords: tomato; gene family; salt stress; virus-induced gene silencing; oxidative damage



Academic Editor: Christina Eynck

Received: 15 December 2025

Revised: 22 January 2026

Accepted: 6 February 2026

Published: 9 February 2026

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1. Introduction

Salinity is among the most widespread abiotic stresses limiting global agricultural productivity. Excess salt accumulation in soil leads to osmotic stress, ion imbalance, and oxidative injury, thereby affecting plant growth and yield formation [1,2]. To adapt to such adverse conditions, plants have evolved complex defense mechanisms that include transcriptional regulation, signal transduction, and post-translational modifications (PTMs), all contributing to cellular homeostasis and stress resilience [1–3]. Among various PTMs, SUMOylation, the covalent attachment of the small ubiquitin-like modifier (SUMO) to lysine residues of target proteins, is a reversible process that regulates protein stability, localization, and activity [3–6].

This cascade involves the sequential actions of an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase. Among them, the E3 ligase determines substrate specificity and thereby defines the physiological consequences of SUMO modification [3,6,7]. Through SUMOylation of transcription factors and signaling proteins, plants can rapidly reprogram responses to various stresses, including salt stress [3,4,8].

SAP and MIZ1 domain-containing (SIZ; scaffold attachment factor A/B–Acinus–PIAS and MIZ1 domain-containing) proteins represent a conserved subfamily of SUMO E3 ligases and are characterized by the SAP (scaffold attachment factor A/B–Acinus–PIAS) domain, the PINIT (Pro–Ile–Asn–Ile–Thr) motif, the SP-RING (SIZ/PIAS-type RING finger) domain, and the SXS (Ser–X–Ser) motif, and have been reported to participate in plant growth, development, and stress responses [4,6,7]. For example, in *A. thaliana*, *AtSIZ1* regulates flowering time [9] and mediates SUMOylation of the transcription factor MYB30, thereby modulating ABA responses [10]. Meanwhile, in pepper (*Capsicum annuum*), *CaSIZ1/CaDSIZ1* enhances drought tolerance and is involved in ABA-related stress signaling, supporting the role of SIZ-type ligases as integrators of hormonal and stress signaling pathways [11,12]. To date, the regulatory roles of SIZ-type ligases have been observed in rice (*O. sativa*), maize (*Z. mays*), soybean (*G. max*), and wheat (*Triticum aestivum*), where SIZ homologs contribute to stress adaptation by enhancing antioxidant defense, osmotic adjustment, and hormone signaling crosstalk [3,4,8,13,14]. In tomato, salt tolerance involves restricting Na⁺ uptake and sequestering it into vacuoles, maintaining K⁺/Na⁺ homeostasis, and achieving osmotic adjustment through compatible solutes. These processes are integrated with ABA-dependent signaling, ROS detoxification, and extensive transcriptional reprogramming mediated by stress-responsive transcription factors. Overall, tomato salt tolerance is shaped by coordinated regulation of ion homeostasis, ABA-dependent signaling, and ROS detoxification, yet the contribution of SUMO-mediated regulation to these pathways remains underexplored [1,2]. In previous studies, *SISIZ1* was the only SIZ-type SUMO E3 ligase identified in tomato, functioning in drought and heat tolerance [15,16]. Nevertheless, the SIZ-type SUMO E3 ligase family has not been comprehensively defined in tomato, and the roles of individual *SISIZ* members in salt stress adaptation remain poorly understood.

Although *SISIZ1* has been implicated in drought and heat tolerance in tomato, it remains unclear whether additional SIZ-type SUMO E3 ligases exist and how the family has diversified in the tomato genome [15,16]. More importantly, the contribution of SIZ-type ligases to tomato salt stress adaptation and the associated physiological basis (e.g., ROS homeostasis and antioxidant capacity) have not been systematically evaluated [3,4,8,13,14]. Therefore, the key knowledge gap is that the SIZ-type SUMO E3 ligase family in tomato has not been comprehensively defined, and the functional roles of individual *SISIZ* members in salt tolerance remain largely unknown. To address this gap, we performed a genome-wide identification and characterization of the *SISIZ* gene family, including phylogenetic relationships, conserved domains/motifs, and promoter features. We further profiled *SISIZ* expression across tissues and under salt and hormone treatments, and functionally validated *SISIZ1* and *SISIZ4* using virus-induced gene silencing [17,18]. In parallel, we assessed ROS-related physiological responses under salt stress by measuring ROS accumulation and antioxidant capacity, thereby linking *SISIZ1/SISIZ4* function to redox homeostasis [17,18]. Importantly, establishing the full set of *SISIZ* genes provides a family-level reference framework and a valuable resource for the tomato research community, enabling consistent gene nomenclature and cross-study comparisons, and offering a rational starting point to prioritize SUMO-E3 candidates for mechanistic dissection and stress tolerance improvement in tomato. This work provides a family-level framework for *SISIZ* genes and provides functional evidence supporting *SISIZ1* and *SISIZ4* as positive regulators of tomato salt tol-

erance. Relative to previous tomato studies that mainly examined *SISIZ1* [15,16], this work places tomato SIZ-type SUMO E3 ligases in a broader, family-level context. By identifying four *SISIZ* candidates in the tomato genome and characterizing their basic features, we provide a reference that may help support more consistent gene nomenclature and cross-study comparisons. This resource also offers a useful basis for selecting SIZ-type SUMO E3 ligase candidates for future functional and mechanistic studies in tomato, including under salinity.

2. Materials and Methods

2.1. Genome-Wide Identification of *SISIZ* Gene Family Members in Tomato

The tomato genome sequence and International Tomato Annotation Group release 4.0 (ITAG4.0) annotation data were retrieved from the Phytozome v13 database (<https://phytozome.jgi.doe.gov/pz/>, accessed on 13 June 2025), whereas the *A. thaliana* AtSIZ1 protein sequence was acquired from the TAIR database (<https://www.arabidopsis.org/index.jsp>, accessed on 13 June 2025) locus: AT5G60410; isoform: AT5G60410.2 [19,20]. Using TBtools software (v2.034), candidate coding sequences were extracted using the “GXF Sequence Extraction” module and translated into protein sequences using the “Batch Translation CDS” module [21]. A one-way BLAST search between tomato candidate proteins and the *A. thaliana* SIZ protein was performed using the “BLAST GUI Wrapper” function [21]. To ensure reliability, reciprocal BLAST verification between tomato and *A. thaliana* was subsequently conducted in the NCBI database (<https://www.ncbi.nlm.nih.gov/>, accessed on 14 June 2025) [22]. Candidate sequences were filtered through the ‘BlastXml to Table’ tool for further analysis [21]. Finally, the tomato *SISIZ* protein sequences were submitted to the SMART database (<http://smart.embl-heidelberg.de/>, accessed on 14 June 2025) to validate conserved structural domains and to screen potential members of the *SISIZ* gene family [23]. The four *SISIZ* gene models identified in tomato correspond to *SISIZ1* (Soly06g010000.3.1), *SISIZ2* (Soly06g010100.2.1), *SISIZ3* (Soly08g008130.4.1), and *SISIZ4* (Soly11g069160.3.1).

2.2. Gene Structure and Chromosomal Localization of *SISIZ* Genes in Tomato

Initially, the ‘visualization of gene structure (from GTF/GFF3 files)’ function in TBtools software (v2.034) was employed to examine the structural characteristics of *SISIZ* family members [21]. Subsequently, the ‘visualization of gene positions from GTF/GFF files’ module within TBtools software (v2.034) was applied to map the chromosomal distribution patterns of these genes [21]. Further positional refinement was achieved through the ‘gene position visualization from GTF/GFF files’ module in TBtools software (v2.034), enabling precise genomic localization [21]. Ultimately, each *SISIZ* member was systematically designated based on its physical chromosomal coordinates within the tomato genome, following standardized nomenclature protocols aligned with their genomic arrangement.

2.3. Secondary and Tertiary Structure Prediction of *SISIZ* Proteins

The online server NPSA (<https://npsa-prabi.ibcp.fr/>, accessed on 15 June 2025) was used to predict the secondary structures of *SISIZ* proteins [24]. The predicted secondary-structure composition, including α -helix, β -turn, random coil, and extended strand, was exported for downstream analysis and visualization. The tertiary structure of each *SISIZ* protein was modeled using SWISS-MODEL (<https://swissmodel.expasy.org/interactive>, accessed on 15 June 2025) [25].

2.4. Conserved Domain Analysis

The conserved domains of SIZ-type SUMO E3 ligase proteins from *S. lycopersicum* and *A. thaliana* were identified using multiple databases to ensure annotation accuracy. Protein sequences of the 4 *SISIZ* members were obtained from the Phytozome v13 database (<https://phytozome.jgi.doe.gov/pz/>, accessed on 17 June 2025), based on the *S. lycopersicum* genome sequence and ITAG4.0 annotation data [19,20]. These correspond to *SISIZ1* (Solyc06g010000.3.1), *SISIZ2* (Solyc06g010100.2.1), *SISIZ3* (Solyc08g008130.4.1), and *SISIZ4* (Solyc11g069160.3.1). The protein sequence of *AtSIZ1* was retrieved from the TAIR database (locus: AT5G60410; isoform: AT5G60410.2). Conserved domain prediction was performed using the NCBI Conserved Domain Database (CDD), SMART, and Pfam [23,26,27]. Visualization of domain composition was conducted using TBtools [21]. To reduce bias among databases, we cross-validated the results from the 3 platforms and manually confirmed the domain boundaries using *AtSIZ1* structural data reported previously [6,7].

2.5. Phylogenetic Analysis of *SISIZ*s in Tomato

The SIZ protein sequences of tomato and representative plant species listed in Table S1 were retrieved from the TAIR database for *A. thaliana* (<https://www.arabidopsis.org/index.jsp>, accessed on 18 June 2025) and from Phytozome v13 and/or NCBI RefSeq according to the accession IDs in Table S1 (<https://phytozome.jgi.doe.gov/pz/>, accessed on 18 June 2025). Multiple sequence alignment was performed using MUSCLE implemented in MEGA 11.0 [19,20]. Based on these sequences, a phylogenetic tree comprising 20 SIZ proteins was constructed using the maximum likelihood (ML) method in MEGA 11.0 software, with bootstrap analysis of 1000 replicates [28]. For further refinement of the phylogenetic tree, visualization was performed using the EvolView website (<https://evolgenius.info/evolview-v2/>, accessed on 18 June 2025) [29]. Accession IDs of all sequences used are provided in Table S1.

2.6. Conserved Motifs of Tomato *SISIZ* Proteins

To analyze conserved motifs, tomato *SISIZ* protein sequences were submitted to the MEME online analysis platform (<http://meme-suite.org/tools/meme>, accessed on 19 June 2025) [30]. The conserved motifs of *SISIZ* family members were subsequently visualized using the “Gene Structure View (Advanced)” function in TBtools software (v2.034) [21].

2.7. Interaction Analysis of *SISIZ* Proteins in Tomato

The protein–protein interaction network for the tomato *SISIZ* family members was constructed using the STRING 12.0 database (<https://cn.string-db.org/>). Interactions with a combined score > 0.400 (medium confidence) were retained for downstream analysis.

2.8. Collinearity Analysis of *SISIZ* Genes in Tomato

The genome and annotation files of tomato (*S. lycopersicum*), *A. thaliana*, and soybean (*G. max*) were obtained from the Phytozome v13 online database (<https://phytozome.jgi.doe.gov/pz/>, accessed on 19 June 2025) [19,20]. The collinearity between tomato and soybean was then obtained using the ‘Text Merge for MCScanX’ function in TBtools [21]. Finally, the results were visualized using the Advanced Circos function in TBtools [21].

2.9. Analysis of Cis-Acting Elements in Tomato *SISIZ* Gene Promoters

The 2000 bp promoter sequences upstream of tomato *SISIZ* gene coding sequences were extracted. The sequences were analyzed for *cis*-acting elements using PlantCARE online (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 19 June 2025) and then visualized using TBtools to elucidate the possible transcriptional regulatory network of the *SISIZ* gene family [21,31].

2.10. Tissue-Specific Expression Analysis of *SISIZ* Genes in Tomato

The transcriptome-based tissue expression profiles of tomato *SISIZ* genes were obtained from the Tomato eFP Browser (BAR eFP Browser 2.0; https://bar.utoronto.ca/efp2/Tomato/Tomato_eFPBrowser2.html, accessed on 20 June 2025) [32]. The platform provides pre-processed, normalized expression values; thus, raw RNA-seq reads were not re-mapped or re-quantified in this study. The downloaded expression matrix for *SISIZ* genes was subsequently log2-transformed [$\log_2(x + 1)$] and standardized (Z-score) across tissues for each gene prior to heatmap visualization. Heatmaps were generated using TBtools [21].

2.11. Plant Materials, Cultivation Conditions, and Treatments

The ‘Micro-Tom’ tomato cultivar plants were employed in this study. Seeds of the tomato cultivar ‘Micro-Tom’ were purchased from Totally Tomatoes Co. (Randolph, WI, USA). Seeds were surface-sterilized, germinated, and sown in a substrate composed of peat moss, vermiculite, and perlite (3:1:1, *v/v/v*). Plants were cultivated in a growth chamber under the following controlled environmental conditions: a 14/10 h light/dark photoperiod, day/night temperatures of 22/20 °C, 60% relative humidity, and a photosynthetic photon flux density (PPFD) of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Two-week-old seedlings were transferred to a hydroponic system, and the uniform 21-day-old plants were selected for subsequent stress treatments: salt stress (200 mM NaCl), abscisic acid (ABA, 100 μM), methyl jasmonate (MeJA, 100 μM), and drought stress (simulated with 20% (*w/v*) polyethylene glycol PEG 6000), with all chemical treatments prepared in 1/2 Hoagland nutrient solution. The 21-day seedling stage was chosen because plants are developmentally uniform and well suited to hydroponic Hoagland-based treatments, which improves stress consistency for time-course sampling and downstream comparisons. For UV-C stress, the seedlings were exposed to UV-C irradiation at 253.7 nm using a UV-C lamp (Philips, Warsaw, Poland) under identical growth conditions. The 200 mM NaCl treatment was selected to elicit a robust and reproducible transcriptional induction within the short sampling window (0–24 h), thereby enabling reliable comparison of response amplitudes among *SISIZ* family members. Shoot tissues were sampled at 0 (untreated control), 6, 12, and 24 h after the initiation of each treatment. For each treatment and time point, three biological replicates were collected, with each replicate consisting of eight seedlings. All the harvested samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

2.12. RNA Isolation and qRT-PCR

Total RNA was extracted from samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and its purity and concentration were measured on a Pultton P100+ ultramicrospectrophotometer (Wuzhou Dongfang, Beijing, China) following procedures similar to those described previously for tomato [33,34]. Only RNA samples with A260/A280 ratios between 2.0 and 2.1 were selected for subsequent analysis. cDNA was synthesized from the qualified RNA using the FastQuant First-Strand cDNA Synthesis Kit (Tianen, Beijing, China) under the following thermal conditions: 15 min at 37 °C, 5 s at 85 °C, and a final step at 4 °C. qRT-PCR was performed using the SYBR Green Premix Pro Taq HS kit on a LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). The 20 μL reaction mixture contained 10 μL of 2 $\times$ Premix, 0.4 μL each of forward and reverse primer, 2 μL of cDNA template, and 7.2 μL of ddH₂O. All primers were designed with Primer Premier 5.0 software (Premier Biosoft, San Francisco, CA, USA), and *SIACTIN* (NC015447.3) was used as the internal reference gene based on its stable expression documented previously [35]. The primer sequences are listed in Table 1. Relative gene expression levels were calculated

by the $2^{-\Delta\Delta C_t}$ method, normalizing all data to the 0 h control [36]. All experiments were conducted with 3 independent biological replicates to ensure the reliability of the results.

Table 1. Primers used for qRT-PCR analysis of *SISIZ* family genes in tomato.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
<i>SISIZ1</i>	F: GCACCTTTAGATAGCACAAACCAAATG	R: CACCAAGCCTGAACATCGTATTCC
<i>SISIZ2</i>	F: ATCCATATTCAATCGGGTCACTTCTC	R: CGCACGCCAAGAACCATCAG
<i>SISIZ3</i>	F: CTCACATGTATTGTTACGCCTCCTC	R: CCATTGACACATTAGTCTCTCTCC
<i>SISIZ4</i>	F: GACACTGCGTTGGACTCATTGG	R: CGTTCTCTACTGCTCTTAGTTGATCTG
<i>SIACTIN</i>	F: AATGAACCTTCGTGTGGCTCCAGAG	R: ATGGCAGGGGTGTTGAAGGTTTC

2.13. Salt Tolerance Analysis of VIGS-Silenced Lines

This study utilized ‘Micro-Tom’ tomato as a plant material and employed a tobacco rattle virus (TRV)-based vector for virus-induced gene silencing (VIGS). Gene-specific fragments targeting the coding sequences (CDS) of *SISIZ1* and *SISIZ4* were amplified using primers listed in Table 2 and subsequently cloned into the TRV2 vector. The TRV1 and TRV2 vectors (including the empty TRV2 control, TRV:00) were kindly provided by Prof. Xia Xiaojian, Zhejiang University, China. The sequence-verified recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Following the method described by Liu et al. [37], robust seedlings were selected for agroinfiltration: TRV1 was mixed separately with the empty TRV2 vector (TRV:00) and the recombinant silencing vectors (TRV: *SISIZ1* and TRV: *SISIZ4*) at a 1:1 ratio, and the mixtures were infiltrated into the abaxial side of both cotyledons, ensuring over 80% infiltration coverage [37]. Three days post-infiltration, plants were transferred to a hydroponic system. After a two-day acclimation period with water, the medium was replaced with half-strength Hoagland nutrient solution. Systemic upper (non-infiltrated) newly emerged leaves at a uniform developmental stage were collected 21 days after sowing for qRT-PCR to assess silencing efficiency. Plants exhibiting 60–80% silencing efficiency were selected for salt stress treatment. The qualified plant materials were divided into two groups: one was cultivated in standard Hoagland solution (non-stress control), and the other was treated with Hoagland solution supplemented with 150 mmol L⁻¹ NaCl (salt stress). A moderate salinity level (150 mmol L⁻¹ NaCl) was used for VIGS plants to avoid non-specific, overwhelming injury associated with viral burden and plant-to-plant variability, while still producing stable stress phenotypes that allow gene-dependent differences to be resolved. Both groups contained wild-type, TRV:00 (empty vector), and TRV: *SISIZ1*/TRV: *SISIZ4* (gene-silencing) plants. Phenotypic observations and measurements of relevant physiological indicators were then performed. Under NaCl treatment, the wild-type and TRV:00 plants served as the control groups for salt stress. Phenotypic observations and measurements of relevant physiological indicators were subsequently conducted.

Table 2. Primers used for vector construction in the virus-induced gene-silencing (VIGS) assay.

Gene	Primer Sequence (5'-3')
<i>SISIZ1</i>	F: aaggttaccgaattctctagaTAAGTCGCAAATTGTCTCGGACA R: aaggttaccgaattctctagaATAATTATCACATCTTCTTCGCCTCC
<i>SISIZ4</i>	F: aaggttaccgaattctctagaAAAGTCGCAAGTTGTCTCCGA R: gagacgcgtgagctcggtaccAACTTTCAGGATACAAAGGGTG

2.14. Plant Phenotypic Analysis

Plant architecture was documented by imaging tomato seedlings positioned uniformly against a dark background. Morphometric parameters, including plant height and stem

diameter, were determined using a digital caliper. For biomass quantification, whole plants in each replicate were blotted dry with filter paper and weighed to determine the fresh weight (FW). The samples were subsequently oven-dried at 80 °C for 2 days until the constant weight was achieved to obtain the dry weight (DW) [38]. Leaf area and root system architecture were assessed using a STD4800 root scanner (Regent Instruments Inc., Quebec, QC, Canada). The fully expanded leaves of each plant were evenly arranged on the scanning platform for image acquisition, and the total leaf area was quantified using the WinRHIZO 5.0 software. The shoots were then excised, and the root systems were imaged and analyzed using the same instrument, following the WinRHIZO (v5.0) workflow and parameter definitions [38]. The experiments above were performed with three biological replicates, with each replicate consisting of eight seedlings.

2.15. Physiological Measurements and Histochemical Staining

Chlorophyll a, b, and total chlorophyll content were extracted and quantified following the method of Dionisio-Sese and Tobita [39]. Briefly, fresh tomato leaf samples were immersed in 10 mL of 80% acetone and incubated in darkness for 48 h until complete decolorization was achieved. Following extraction, the absorbance of the supernatants was measured at 663 nm and 645 nm using a spectrophotometer, with the 80% acetone solution serving as the blank. Hydrogen peroxide and superoxide anion levels were detected by 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively [40]. The malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) assay [41]. The free proline concentration was measured using the acid-ninhydrin method [42]. Leaf cell membrane integrity was assessed by the electrolyte leakage assay [2,43].

2.16. Measurement of Antioxidant Enzyme Activity

Antioxidant enzyme activities were determined using previously described spectrophotometric methods with minor modifications [39,44]. Briefly, fresh tomato leaf samples (0.5 g) were homogenized in 5 mL of ice-cold phosphate buffer (pH 7.8) and centrifuged at $10,000 \times g$ for 15 min at 4 °C. The phosphate buffer was prepared by mixing solution A (23.279 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \text{ L}^{-1}$) and solution B (10.14 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} \text{ L}^{-1}$) at a volume ratio of 917.5:116. The resulting supernatant was used for all enzyme assays. Enzyme activities were quantified using spectrophotometric procedures, as described below. The superoxide dismutase (SOD; EC 1.15.1.1) activity was determined at 560 nm. The assay principle was based on the photochemical inhibition of NBT reduction, with the reaction mixture (0.20 mL supernatant, 1.5 mL phosphate buffer, 0.3 mL methionine, 0.3 mL riboflavin, 0.3 mL EDTA-Na, 0.28 mL distilled water, and 0.3 mL NBT) incubated under 4000 lux illumination for 30 min before measuring absorbance at 560 nm. Peroxidase (POD; EC 1.11.1.7) activity was assayed by the guaiacol method, wherein the reaction was initiated by adding 0.3 mL of 0.6% H_2O_2 to a solution containing 0.1 mL of supernatant and 2.6 mL of 0.3% guaiacol, with the oxidation rate monitored at 470 nm using phosphate-buffered saline as a blank. Catalase (CAT; EC 1.11.1.6) activity was measured following Hu et al. by monitoring the decrease in absorbance at 240 nm over 2 min after adding 2.8 mL of 0.067 mM H_2O_2 to 0.2 mL of supernatant [44]. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined as described by Hu et al. using a reaction system containing 0.2 mL of supernatant, 1.75 mL of phosphate buffer (0.05 M, pH 7.0), 0.3 mL of 1 mM EDTA, 0.3 mL of 5 mM ascorbic acid, and 0.3 mL of distilled water, initiated with 0.3 mL of 1 mM H_2O_2 , with absorbance change at 290 nm recorded for 1 min.

2.17. Statistical Analysis

All statistical analyses were performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA). Data are presented as means $\pm$ standard deviation (SD) of three independent biological

replicates ($n = 3$). Differences among treatments were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at a significance level of $p < 0.05$. In the figures and tables, different lowercase letters indicate statistically significant differences among means.

3. Results

3.1. Genome-Wide Identification of *SISIZ* Genes in Tomato

In our study, four tomato *SISIZ* genes were identified via homology-based searches. Based on the location of the genes on different chromosomes, they were named as *SISIZ1* to *SISIZ4*. As shown in Table 3, the molecular weights of *SISIZ* proteins ranged from 94.50 kDa (*SISIZ1*) to 99.17 kDa (*SISIZ3*), where kDa (kilodalton) is a standard unit of molecular mass used to report protein molecular weight (1 kDa = 1000 Da). The average amino acid number of the tomato *SISIZ* family was 874. *SISIZ2* is a basic protein ($pI > 7$), and the remaining *SISIZ* members are acidic proteins ($pI < 7$). The mean value of the instability index is 46.505, and the mean value of the aliphatic index is 79.0325. The grand average of hydropathicity (GRAVY) values for the *SISIZ* protein members was calculated as -0.419 (*SISIZ1*), -0.293 (*SISIZ2*), -0.426 (*SISIZ3*), and -0.453 (*SISIZ4*). As the positive and negative GRAVY values indicate hydrophobicity and hydrophilicity, respectively, all four proteins are classified as hydrophilic proteins [45]. Notably, *SISIZ2* exhibited the highest GRAVY value among them. In addition, subcellular localization analysis indicated a nuclear distribution for all *SISIZ* proteins [46].

Table 3. Genomic information on the *SISIZ* gene family members in tomato.

Gene	Gene ID	ChrNo.	Protein Length (aa)	Molecular Weight (kDa)	pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity	Subcellular Localization
<i>SISIZ1</i>	Solyc06g010000.3.1.ITAG4.0	Chr06	856	94.50	6.02	48.63	78.69	-0.419	Nucleus
<i>SISIZ2</i>	Solyc06g010100.2.1.ITAG4.0	Chr06	855	97.33	8.49	40.09	87.65	-0.293	Nucleus
<i>SISIZ3</i>	Solyc08g008130.4.1.ITAG4.0	Chr08	907	99.17	6.14	52.85	73.01	-0.426	Nucleus
<i>SISIZ4</i>	Solyc11g069160.3.1.ITAG4.0	Chr11	877	95.56	4.94	44.45	76.78	-0.453	Nucleus

3.2. Gene Structure and Chromosomal Localization of *SISIZ* Genes in Tomato

The exon–intron structures of the four identified *SISIZ* genes in tomato were characterized and presented alongside their phylogenetic relationships (Figure 1A). These genes exhibited variations in their exon count (ranging from 15 to 17) and intron count (ranging from 15 to 17). Specifically, *SISIZ1* possessed 15 exons and 16 introns, *SISIZ2* had 17 exons and 16 introns, *SISIZ3* contained 16 exons and 15 introns, and *SISIZ4* comprised 16 exons and 17 introns. Chromosomal localization analysis revealed that the four *SISIZ* genes were unevenly distributed across three chromosomes (Figure 1B). Two genes, *SISIZ1* and *SISIZ2*, were located on chromosome SL4.0ch06. The remaining two genes, *SISIZ3* and *SISIZ4*, were located on chromosomes SL4.0ch08 and SL4.0ch11, respectively.

3.3. Predictive Analysis of the Secondary and Tertiary Structures of *SISIZ* Proteins in Tomato

The predicted secondary structure composition of the four *SISIZ*-encoded proteins comprised α -helix (14.00–38.71%), random coil (43.86–76.96%), and extended strand (9.04–17.43%; Table 4). In addition, the tertiary structures of *SISIZ* proteins were predicted and modeled (Figure 2). The *SISIZ* proteins contained a relatively high proportion of α -helical and random-coil regions, which was consistent with the secondary structure predictions. Furthermore, the tertiary structures of the four members varied considerably, reflecting differences in amino acid length, polypeptide chain arrangement, and spatial folding.

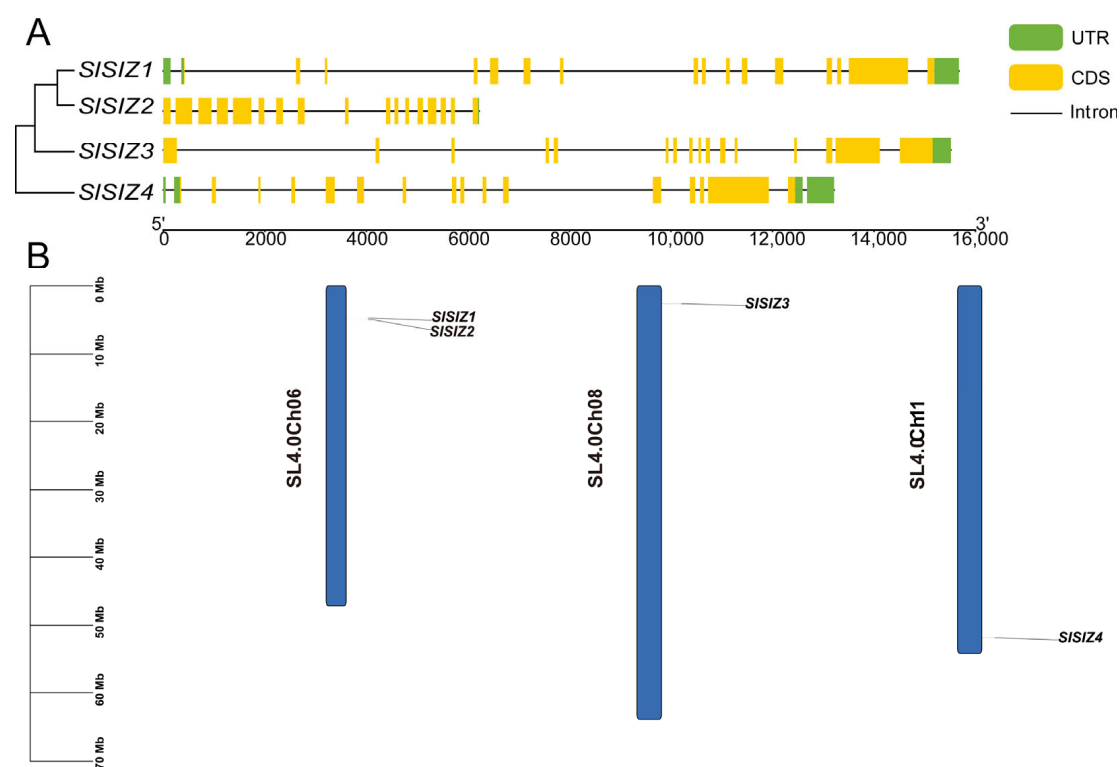


Figure 1. Genomic features of the *SISIZ* gene family in tomato. **(A)** Phylogenetic relationship and gene structure of *SISIZ* genes. The phylogenetic tree (**left**) was inferred from the full-length *SISIZ* protein sequences using the maximum likelihood method in MEGA 11 with 1000 bootstrap replicates. The corresponding gene structures (**right**) were visualized with TBtools, depicting untranslated regions (UTRs) as green boxes, exons as yellow boxes, and introns as black lines. **(B)** Chromosomal distribution of *SISIZ* genes. The physical positions of the four *SISIZ* genes were mapped to their respective chromosomes. Gene names are shown in black, with a scale bar provided on the left.

Table 4. Secondary structures of *SIZ* family members in tomato.

Protein	Alpha Helix (%)	Extended Strand (%)	Random Coil (%)	Distribution of Secondary Structure Elements
SISIZ1	15.42%	9.81%	74.77%	
SISIZ2	38.71%	17.43%	43.86%	
SISIZ3	14.00%	9.04%	76.96%	
SISIZ4	15.42%	9.81%	74.77%	

3.4. Conserved Domain Architecture of *SIZ* Proteins in Tomato and *A. thaliana*

To characterize the structural features of *SIZ*-type SUMO E3 ligases, conserved domain analysis was performed on the four *SISIZ* proteins in tomato and AtSIZ1 in *A. thaliana*. The results showed that all *SISIZ* proteins contained a conserved SP-RING (MIZ-type) zinc finger domain in the C-terminal region, and an N-terminal SAP domain was present in both tomato and *A. thaliana* proteins (Figure 3). Overall, the domain composition and arrangement patterns of *SISIZ* proteins closely resembled AtSIZ1.

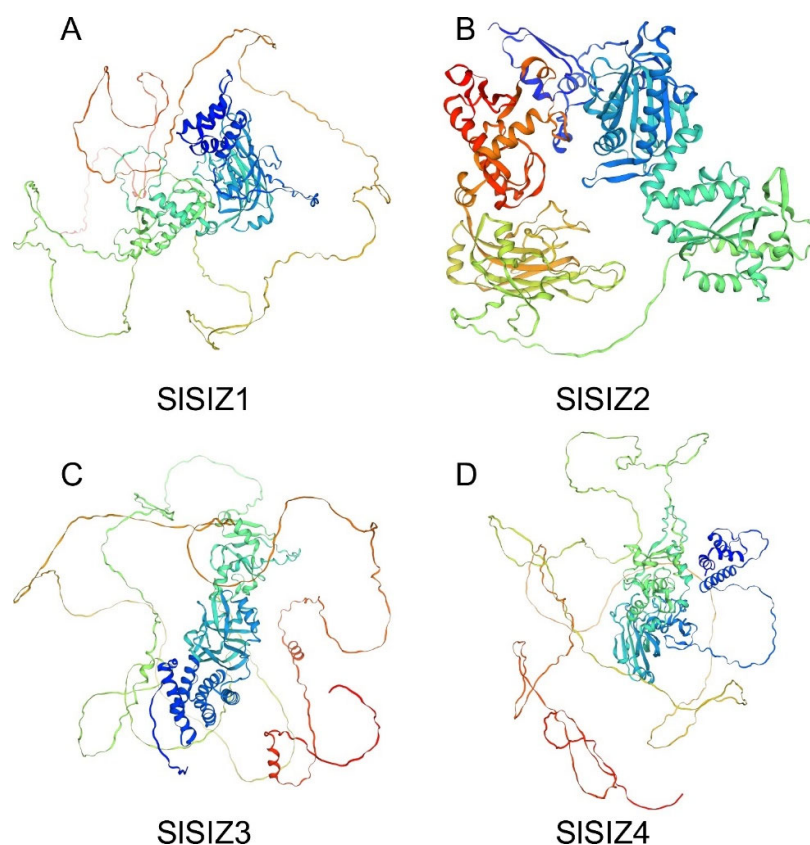


Figure 2. Predicted tertiary structures of SISIZ proteins. (A) Predicted tertiary structure of SISIZ1. (B) Predicted tertiary structure of SISIZ2. (C) Predicted tertiary structure of SISIZ3. (D) Predicted tertiary structure of SISIZ4. The tertiary structure models of the proteins in the figure are all from the N-terminus (blue) to the C-terminus (red) of the protein sequence, with different colors representing the same helix or fold.

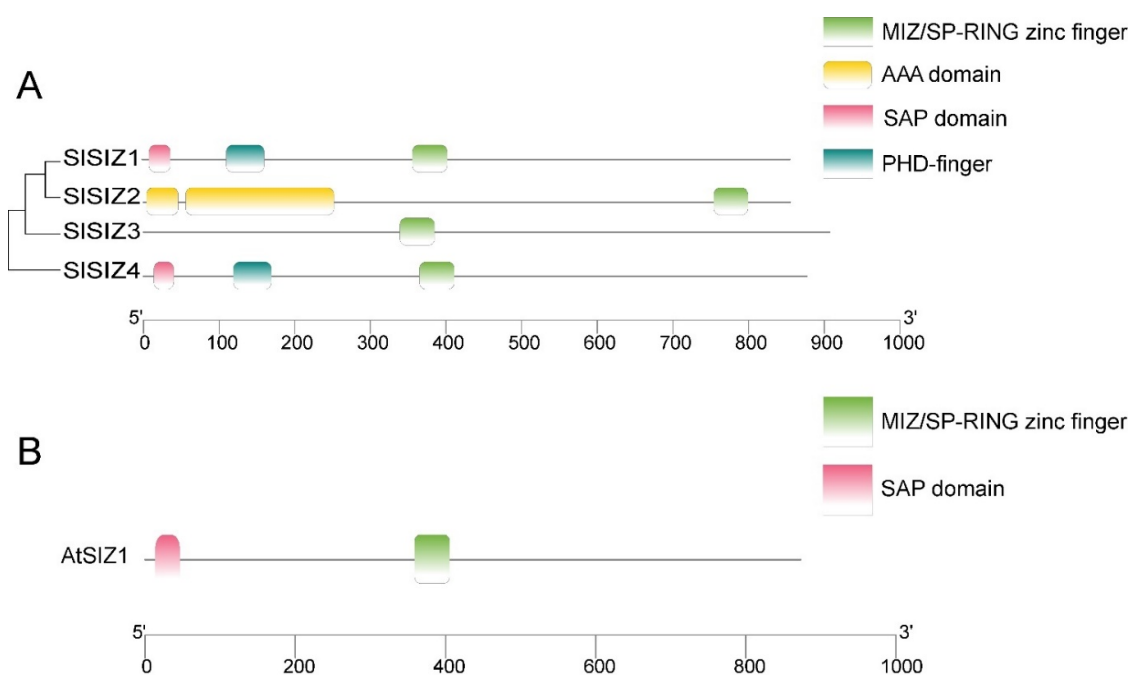


Figure 3. Comparative domain organization of SIZ proteins from tomato and *A. thaliana*. (A) Schematic representation of conserved domains in tomato SISIZ proteins. (B) Schematic representation of conserved domains in *A. thaliana* AtSIZ1.

3.5. Phylogenetic Analysis of SISIZs in Tomato

A maximum likelihood phylogenetic tree was constructed to examine the evolutionary relationships of SISIZ family members in tomato. The analysis was performed using SISZ protein sequences from tomato and representative plant species ($n = 20$; Table S1), with 1000 bootstrap replicates (Figure 4). The resulting tree separated the SISZ proteins into five major groups (A–E). In tomato, *SISIZ1*, *SISIZ2*, and *SISIZ4* were assigned to Group B, whereas *SISIZ3* was placed in Group E. *SISIZ1* and *SISIZ4* formed a sister clade supported by a bootstrap value of 100. Overall, the phylogenetic pattern suggests that *SISIZ3* is more divergent than the *SISIZ1/2/4* subgroup, which may reflect functional differentiation within the tomato SISIZ family.

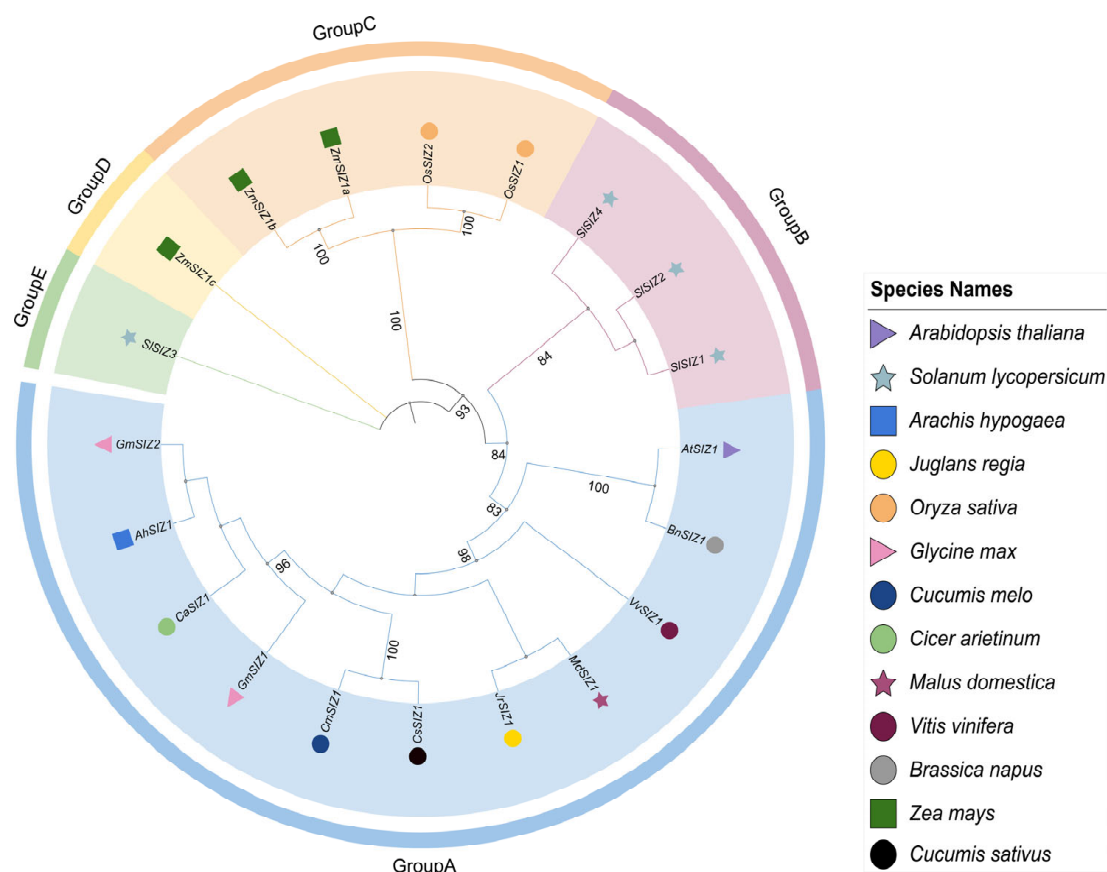


Figure 4. Maximum likelihood phylogenetic tree of SISZ/PIAS-type SUMO E3 ligases from tomato and representative plant species. The tree was inferred from amino acid sequences ($n = 20$) with 1000 bootstrap replicates. Bootstrap values are shown at the nodes. Five major groups (A–E) are indicated. Accession IDs of all sequences included in the analysis are listed in Table S1.

3.6. Conserved Motifs of Tomato SISIZ Proteins

By analyzing the tomato SISIZ protein family, 10 conserved motifs were identified with lengths ranging from 15 to 50 amino acids (Figure 5A; Table 5). All members contained the core conserved motifs 1, 2, and 9. In addition, *SISIZ4* and *SISIZ1* possessed all 10 motifs, *SISIZ3* lacked motif 10, and *SISIZ2* uniquely contained two copies of motif 2.

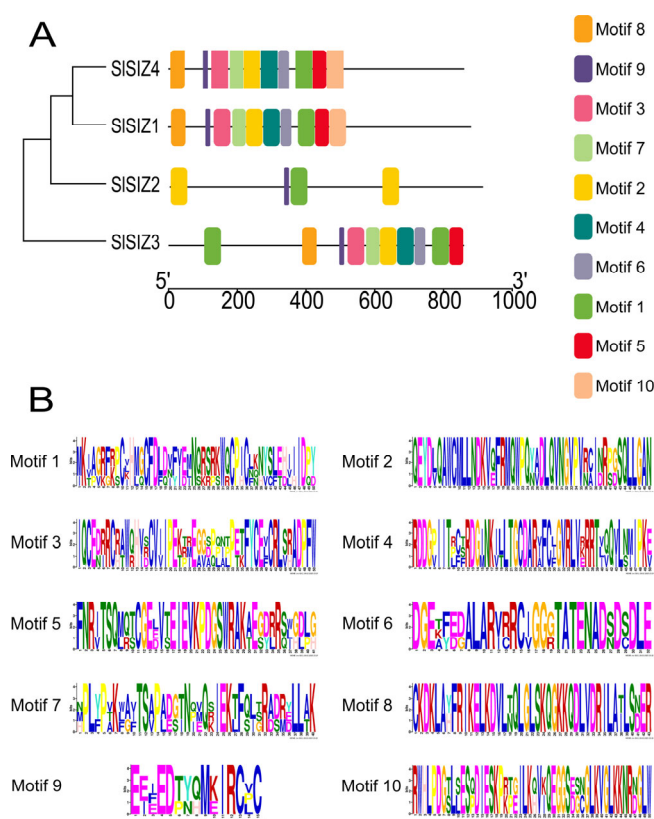


Figure 5. Motif composition and distribution of tomato SISIZ proteins. **(A)** Colored boxes represent different conserved motifs, and a phylogenetic tree is shown on the left to illustrate the evolutionary relationships among SISIZ proteins. **(B)** Motifs 1–10 are shown. Amino acid sequences of different conserved motifs, represented by stacked letters at each position. The total height of the stack represents the information content of the relative amino acid in the position of each letter in the motif in bits. The height of the individual letter in a stack was calculated by the probability of the letter at that position times the total information content of the stack. The X- and Y-axes represent the width and the bits of each letter, respectively.

Table 5. Detailed information on the 10 conserved motifs of the tomato SIZ proteins. E-values and sites (occurrence numbers) were obtained from the MEME analysis.

Motif	Width (aa)	Motif Sequence	E-Value	Sites (n)
Motif 1	50	MKIAGRFPKCIHMGCFDLDFVIEMNQSRKWWQCPICLKNYSLEHIIDPY	7.2×10^{-65}	4
Motif 2	49	QEYDLQAWCMLLNDKVQFRMQWPQYADLQVNGVPVRCINRPGSQLLGAN	1.8×10^{-51}	3
Motif 3	50	IQCEDRRRCRAWQHVDVVIPEKPIEGGAPPAPPETFYCELCRLSRADPFW	4.6×10^{-41}	3
Motif 4	50	RDDGPIITPCSRDGINKILLTGCDARVFCLGVRLVKRRTIQQVLNMIPKE	2.5×10^{-33}	3
Motif 5	41	FNRITSQMQTCGEDVTEIEVKPDGWSWRAKAEGDRRSLGDLG	7.5×10^{-27}	3
Motif 6	32	DGEKFEDALARVRCIGGGTATENADSDSDLE	1.8×10^{-18}	3
Motif 7	40	HPLYPVKWAITSAPADGTNPVQKIEKTFQLSRADRDLLAK	5.7×10^{-13}	3
Motif 8	43	CKDKLAHFRIKELKDVLNQLGLSKQGKKQDLVDRILAILSDER	9.0×10^{-10}	2
Motif 9	15	EEFEDTYQMKIRCLC	1.6×10^{-9}	3
Motif 10	50	RWHLPDGSLLESPDIESKPKPEILKQVKQEGGSEGCGLKVLGKKNRDGLW	3.3×10^{-8}	2

3.7. Interaction Analysis of SISIZ Proteins in Tomato

The predicted protein–protein interaction network of tomato SISIZ proteins comprised nine proteins, including four SISIZ members and five non-SISIZ proteins (A0A3Q7HDM5, A0A3Q7HEK2, A0A3Q7FE42, A0A3Q7JL9, and A0A3Q7JME2; Figure 6). Within this network, SISIZ3 interacted with six other proteins, whereas SISIZ1 and SISIZ2 each interacted with five proteins. In contrast, SISIZ4 was predicted to have only two interactions. SISIZ1, SISIZ2, and SISIZ3 formed a tightly interconnected cluster, suggesting a potential func-

tional module. The five non-SISIZ proteins also exhibited predicted interactions with SISIZ members in the network.

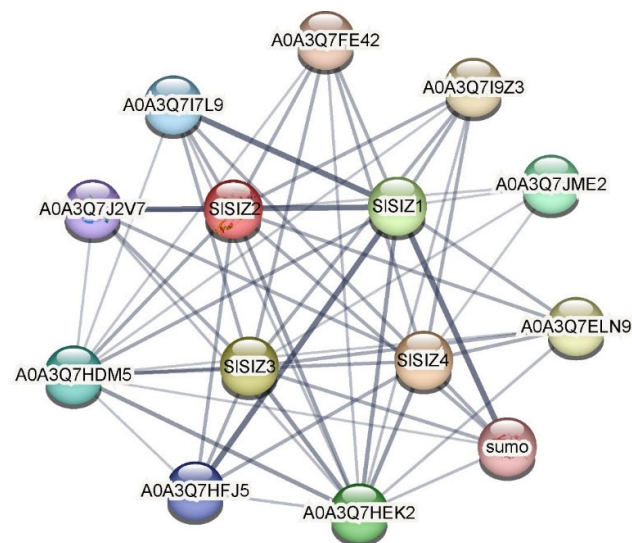


Figure 6. Interaction network of tomato SISIZ proteins. Lines indicate protein–protein interactions; thicker lines represent higher interaction confidence.

3.8. Collinearity Analysis of SISIZ Genes in Tomato

To investigate the evolutionary expansion of the *SIZ* gene family, a comparative analysis of *SIZ* members was conducted in tomato (*S. lycopersicum*), *A. thaliana*, and soybean (*G. max*). The analysis identified two homologous gene pairs between tomato and *A. thaliana* and eight pairs between tomato and soybean (Figure 7).

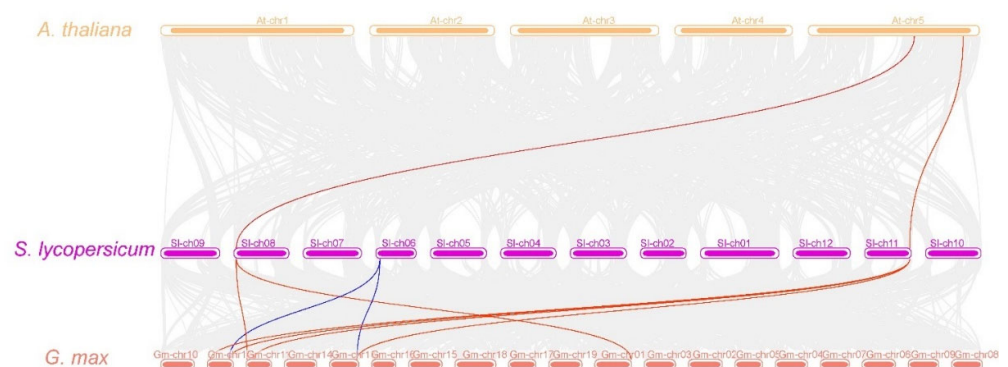


Figure 7. Collinearity analysis of *SIZ* genes. Colored curves indicate collinear *SIZ* gene pairs between tomato and *A. thaliana* and between tomato and soybean, while grey lines in the background represent other collinear blocks within and between the three genomes.

3.9. Analysis of Cis-Acting Elements in Tomato SISIZ Gene Promoters

To elucidate the regulatory mechanisms of *SISIZ* genes, we analyzed *cis*-acting elements in their 2000 bp promoter regions. A total of 25 types of *cis*-acting elements were identified in the tomato *SISIZ* genes (Table 6). These elements were classified into four major functional categories (Figure 8A). Within them, light-responsive elements were ubiquitous, presented in all the *SISIZ* promoters. Hormone-related elements, particularly gibberellin-responsive motifs, were detected in *SISIZ1*, *SISIZ3*, and *SISIZ4*, but were absent in *SISIZ2*. Additionally, ABA-responsive elements were identified in both *SISIZ1* and *SISIZ4*.

Table 6. Each member of the tomato *SIZ* gene family has the original function of *cis*-acting elements.

<i>cis</i> -Element	Sequence of <i>cis</i> -Element	Functions of <i>cis</i> -Elements
ABRE	AACCCGG	<i>cis</i> -acting element involved in the abscisic acid responsiveness
CGTCA-motif	CGTCA	<i>cis</i> -acting regulatory element involved in the MeJA responsiveness
TGACG-motif	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA responsiveness
P-box	CCTTTTG	gibberellin-responsive element
TCA-element	TCAGAAGAGG	<i>cis</i> -acting element involved in salicylic acid responsiveness
TGA-element	AACGAC	auxin-responsive element
LTR	CCGAAA	<i>cis</i> -acting element involved in low-temperature responsiveness
MBS	CAACTG	MYB binding site involved in drought inducibility
TC-rich repeats	ATTCTCTAAC	<i>cis</i> -acting element involved in defense and stress responsiveness
ARE	AAACCA	<i>cis</i> -acting regulatory element essential for the anaerobic induction
ACE	GACACGTATG	<i>cis</i> -acting element involved in light responsiveness
ATC-motif	AGTAATCT	part of a conserved DNA module involved in light responsiveness
ATCT-motif	AATCTAATCC	part of a conserved DNA module involved in light responsiveness
Box 4	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box II	TGGTAATAA	part of a light-responsive element
chs-CMA1a	TTACTTAA	part of a light-responsive element
chs-CMA2a	TCACTTGA	part of a light-responsive element
GA-motif	ATAGATAA	part of a light-responsive element
Gap-box	CAAATGAA(A/G)A	part of a light-responsive element
GATA-motif	AAGATAAGATT	part of a light-responsive element
G-box	CACGTC	<i>cis</i> -acting regulatory element involved in light responsiveness
GT1-motif	GGTTAA	light-responsive element
I-box	TGATAATGT	part of a light-responsive element
MRE	AACCTAA	MYB binding site involved in light responsiveness
TCT-motif	TCTTAC	part of a light-responsive element

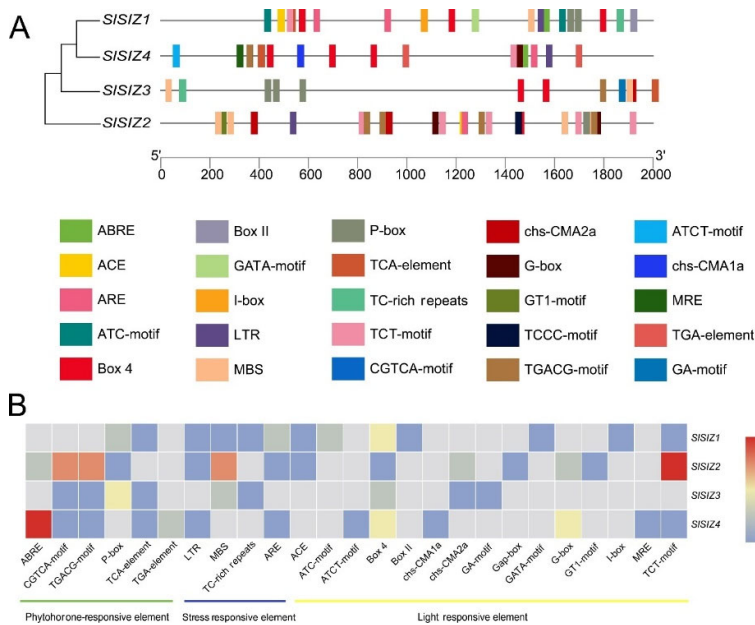


Figure 8. Analysis of *cis*-acting elements in the tomato *SIZ* gene family. (A) Distribution of *cis*-acting elements in the 2000 bp upstream promoter regions of *SIZ* genes—different *cis*-elements are represented by colored wedges. Promoter lengths are drawn to scale, and the scale bar indicates DNA sequence length (bp). (B) Heatmap showing the quantitative profile of *cis*-acting elements in *SIZ* genes, where the color and numerical value in each grid cell represent the number of a given regulatory element in each promoter.

In addition, stress-responsive elements exhibited distinct member-specific distribution patterns. Drought-responsive elements were found in *SIZ1*, *SIZ2*, and *SIZ3*, while low-temperature-responsive elements were specifically identified in *SIZ1* and *SIZ4*. Further analysis revealed distinct *cis*-element distribution patterns among the genes (Figure 8B). Among them, *SIZ2* was enriched with the TCT-motif and a cluster of CGTCA-motif, TGACG-motif, and MBS elements. In contrast, Box-4 elements were particularly abundant in *SIZ1* and *SIZ4*, with ABRE being most prominent in *SIZ4*.

3.10. Tissue-Specific Expression of Tomato *SISIZ* Genes

To investigate the roles of *SISIZ* genes in different tomato tissues during various developmental stages, we analyzed the expression levels of *SISIZ* genes across 14 tomato tissues using expression data from the eFP database, including unopened flower buds, fully opened flowers, leaves, roots, 1 cm fruit, 2 cm fruit, 3 cm fruit, mature green fruit, breaker fruit, breaker fruit + 10, pimpinellifolium immature green fruit, pimpinellifolium breaker fruit, pimpinellifolium breaker + 5 fruit, and pimpinellifolium leaf (Figure 9). *SISIZ4* was highly expressed in all the examined tissues. *SISIZ1* exhibited moderate expression levels in fully opened flowers, roots, 3 cm fruit, pimpinellifolium immature green fruit, and pimpinellifolium breaker + 5 fruit, while showing relatively low expression in other tissues. *SISIZ2* was expressed at uniformly low levels across all examined tissues, whereas *SISIZ3* exhibited pronounced expression in roots and pimpinellifolium immature green fruit, with diminished expression levels observed in other tissues.

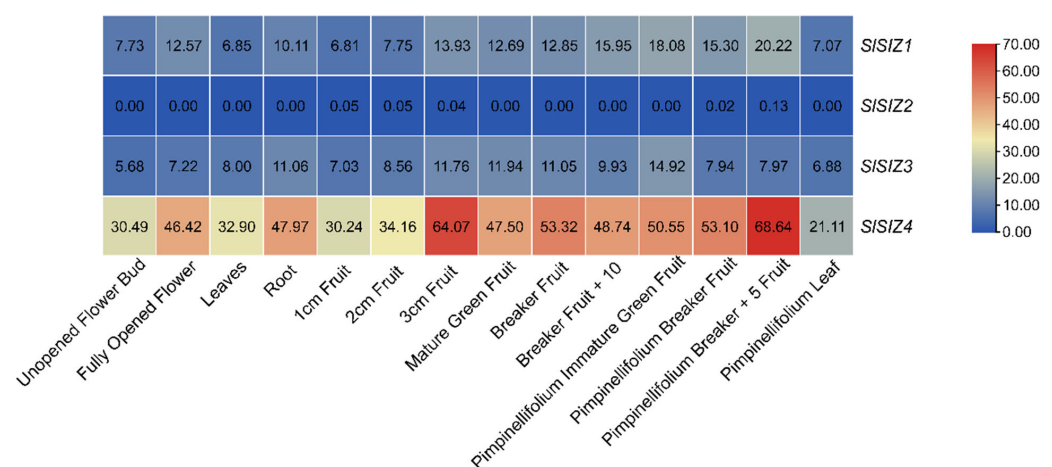


Figure 9. Heatmap of *SISIZ* gene expression across diverse tomato tissues. Expression profiles in 14 tissues, including flower buds, fully opened flowers, leaves, roots, and fruits at different developmental stages, were obtained from the eFP database and visualized using TBtools. Values shown are expression values downloaded directly from the eFP database (as provided), and the color scale (0–70) indicates relative expression levels (blue to red: low to high).

3.11. Expression Analysis of *SISIZ* Genes Under Different Treatments

To investigate the expression patterns of the *SISIZ* gene family under various abiotic stresses and phytohormone treatments, we quantified the expression levels of four *SISIZ* genes in tomato following exposure to NaCl, PEG, ABA, MeJA, and UV. Under NaCl stress, the expression of all genes was significantly upregulated, peaking at 12 h before declining, with *SISIZ1* and *SISIZ4* showing the most pronounced induction (Figure 10A). PEG treatment induced sustained upregulation of *SISIZ1*, *SISIZ2*, and *SISIZ4*, whereas *SISIZ3* expression was transiently suppressed at 6 h (Figure 10B). Under ABA treatment, the transcripts of *SISIZ1* and *SISIZ4* peaked at 24 h, while *SISIZ2* and *SISIZ3* were highly expressed at 6 h (Figure 10C). MeJA treatment resulted in generally low expression levels of *SISIZ* genes, with all genes being downregulated at 6 h and 12 h; however, the transcriptional levels of *SISIZ1* and *SISIZ2* were upregulated after 24 h of MeJA treatment (Figure 10D). Following UV exposure, the transcripts of *SISIZ1*, *SISIZ3*, and *SISIZ4* were significantly suppressed at 6 h. *SISIZ1* and *SISIZ3* gradually recovered thereafter, whereas the expression of *SISIZ4* remained suppressed throughout the UV treatment time course (Figure 10E). Based on these findings, *SISIZ1* and *SISIZ4* exhibited the most pronounced transcriptional activation under salt stress and were, therefore, selected for subsequent investigation of salt stress response mechanisms.

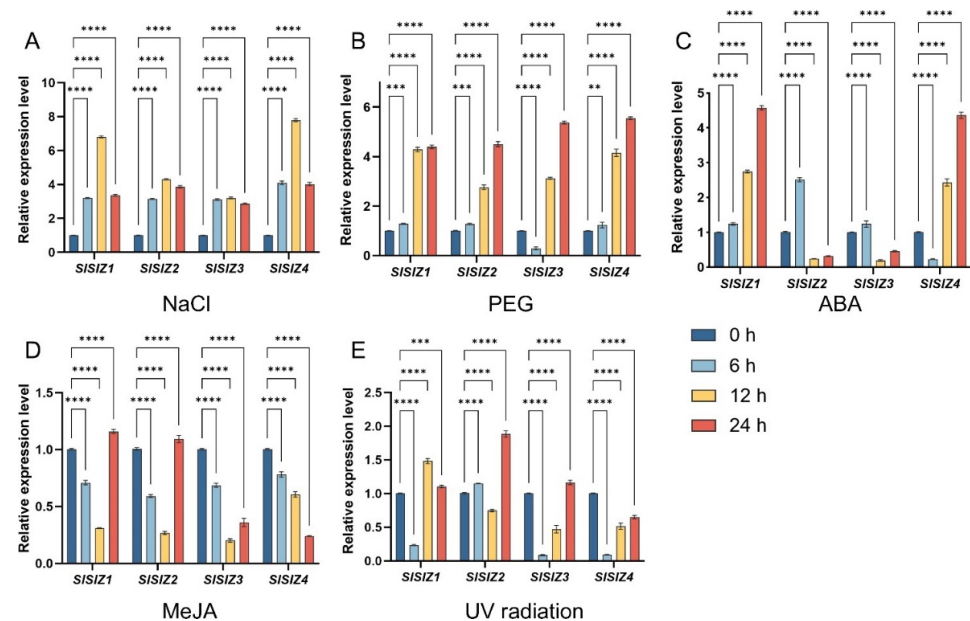


Figure 10. Expression levels of the *SISIZ* gene family in response to stress and hormonal treatments: (A) NaCl, (B) PEG, (C) ABA, (D) MeJA, and (E) UV radiation. The asterisk indicates the corresponding gene that was significantly up- or down-regulated compared with the 0 h status (** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, one-way ANOVA followed by Duncan's multiple range test). The samples in the 0 h treatment were used as controls.

3.12. Verification of *SISIZ1* and *SISIZ4* Silencing Efficiency

Quantitative real-time PCR (qRT-PCR) was used to verify the silencing efficiency of VIGS in *SISIZ1* and *SISIZ4* knockdown lines (Figure 11). In non-silenced plants, the relative transcript levels of *SISIZ1* and *SISIZ4* did not differ significantly between WT and TRV:00 plants, indicating that the empty vector itself had no obvious effect on gene expression. By contrast, expression of *SISIZ1* and *SISIZ4* was dramatically reduced in the TRV: *SISIZ1* and TRV: *SISIZ4* lines (third and fourth bars), where transcript levels were only about 10–20% of those in TRV:00 controls. These results demonstrate that the VIGS constructs efficiently suppressed *SISIZ1* and *SISIZ4* expression, providing reliable *SISIZ1*/*SISIZ4*-deficient materials for subsequent analyses of salt stress responses.

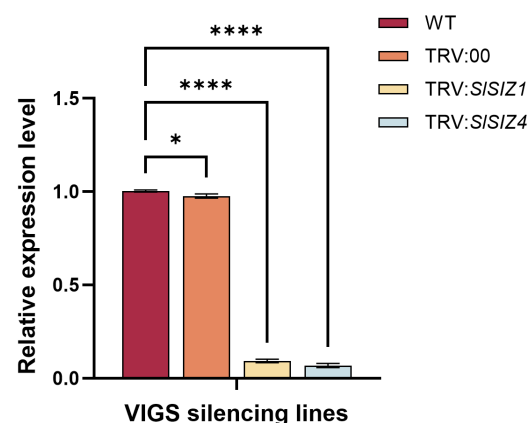


Figure 11. Expression analysis of *SISIZ1* and *SISIZ4* after VIGS treatment. Relative expression levels of *SISIZ1* and *SISIZ4* in WT, TRV:00, TRV: *SISIZ1*, and TRV: *SISIZ4* plants were determined by qRT-PCR. Data are presented as means $\pm$ SD ($n = 3$). Asterisks indicate significant differences compared with WT (* $p < 0.05$; **** $p < 0.0001$).

3.13. Growth and Physiological Indicators Analysis

To investigate the roles of *SISIZ1* and *SISIZ4* in salt tolerance, we generated corresponding gene-silencing tomato lines using VIGS. *SISIZ1*- and *SISIZ4*-silenced lines showed no visible differences from wild-type (WT) and empty vector (TRV:00) controls under normal conditions, whereas salt stress severely impaired their growth when compared with WT and TRV:00 lines. In detail, the TRV: *SISIZ1* and TRV: *SISIZ4* plants exhibited leaf yellowing and root-stunted phenotypes (Figure 12A–C). Specifically, TRV: *SISIZ1* and TRV: *SISIZ4* lines displayed decreases in plant height (22.65%; 29.25%) and stem diameter (46.24%; 48.94%) compared with the control, respectively (Figure 12D,E). Reductions in fresh weight (28.98%; 37.50%) and dry weight (30.53%; 39.47%) were also observed in the TRV: *SISIZ1* and TRV: *SISIZ4* plants under salt stress compared to their respective controls without stress (Figure 12F,G), alongside pronounced declines in leaf area (63.98%; 60.39%) and root length (55.84%; 58.39%; Figure 12H,I). In addition, the chlorophyll content analysis revealed that salt stress triggered significant reductions in the silenced lines (Figure 12J–L). In TRV: *SISIZ1* plants, chlorophyll a, b, and total chlorophyll dropped by 36.75%, 24.32%, and 32.41% compared with the control, respectively. Similarly, the different chlorophyll contents of TRV: *SISIZ4* plants showed decreases of 44.49%, 37.88%, and 42.19% in comparison with the control, respectively.

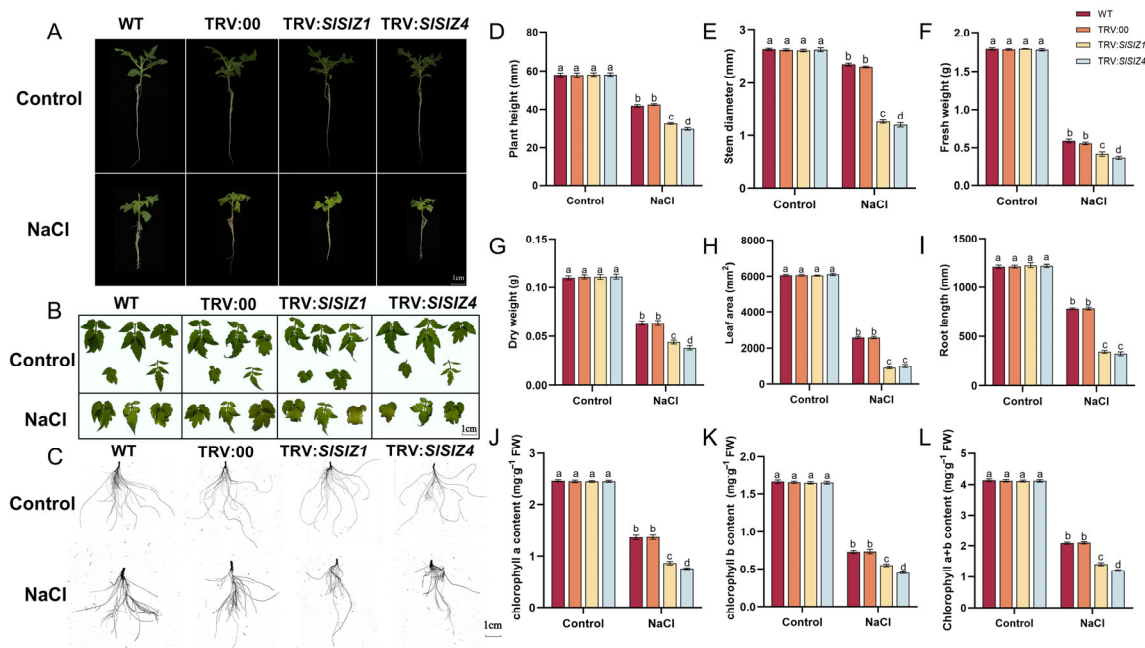


Figure 12. Effects of *SISIZ1* and *SISIZ4* silencing on growth traits and chlorophyll content of tomato seedlings under salt stress. (A) Phenotypic comparison between wild-type and transiently silenced plants. (B) Leaf phenotype comparison between wild-type and transiently silenced plants. (C) Root phenotype comparison between wild-type and transiently silenced plants. (D) Plant height, (E) stem diameter, (F) fresh weight, (G) dry weight, (H) leaf area, (I) root length, (J) chlorophyll a, (K) chlorophyll b, and (L) total chlorophyll content. Values are means $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($p < 0.05$, Duncan's test).

To assess the physiological impact of *SISIZ* gene silencing on salt stress tolerance, we measured malondialdehyde (MDA) and proline contents and ion leakage (Figure 13). No genotypic differences were observed under control conditions. Under salt stress, however, the TRV: *SISIZ1* and TRV: *SISIZ4* lines accumulated significantly higher levels of MDA (increases of 23.15% and 21.41%, respectively) and exhibited increased ion leakage (rises of 19.69% and 24.68%, respectively) compared to WT and TRV:00 controls (Figure 13A,C),

indicating heightened oxidative damage and membrane instability. Consistent with these signs of cellular damage, proline analysis revealed a compromised stress response in the silenced lines. Although salt stress induced proline accumulation across all genotypes, this response was significantly attenuated in TRV: *SISIZ1* and TRV: *SISIZ4* plants, which were 20.37% and 15.97% lower than those of the controls, respectively (Figure 13B).

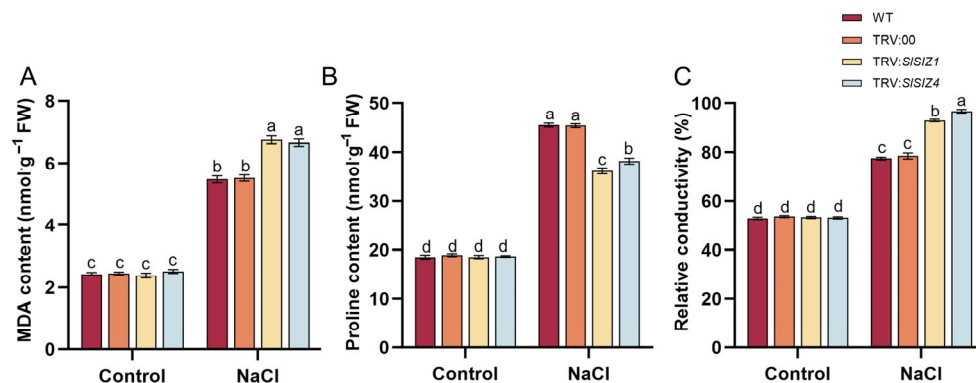


Figure 13. Analysis of osmotic regulation in transiently silenced *SISIZ1* and *SISIZ4* lines. (A) MDA content, (B) proline content, and (C) ion leakage rate. Values are means $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.

3.14. Effects of *SISIZ1* and *SISIZ4* Silencing on ROS Accumulation and Antioxidant Enzyme Activity Under Salt Stress

To elucidate the physiological mechanisms of oxidative stress in TRV: *SISIZ1* and TRV: *SISIZ4* lines under salt stress, we first employed histochemical staining. DAB staining revealed light brown deposits in WT leaves, whereas both silenced lines exhibited intense, widespread coloration, indicating substantial H₂O₂ accumulation (Figure 14A). This pattern was corroborated by NBT staining, which showed faint blue in WT leaves but dense blue formazan deposits in the silenced lines, signifying O₂⁻ overaccumulation (Figure 14B). The corresponding quantitative assays further confirmed these observations (Figure 14C,D). Under salt stress, TRV: *SISIZ1* accumulated 28.62% more H₂O₂ and 25.88% more superoxide anion than WT, while TRV: *SISIZ4* showed even greater increases of 58.60% and 63.25% compared with WT, respectively. To determine the cause of this ROS burst, we profiled key antioxidant enzyme activities (Figure 14E–H). Under salt stress, both silenced lines exhibited significantly reduced activities of SOD, POD, CAT, and APX. Specifically, these enzyme activities showed decreases of 27.24%, 44.88%, 24.80%, and 40.50% in the TRV: *SISIZ1* line, while reductions of 23.14%, 51.95%, 28.60%, and 44.33% in TRV: *SISIZ4* plants, respectively.

To investigate the roles of *SISIZ1* and *SISIZ4* in response to salt stress, the expression levels of antioxidant-enzyme-related genes, including *SISOD*, *SIPOD*, *SICAT*, and *SIAPX*, were analyzed under different treatments (Figure 15). Under control conditions, no significant differences in gene expression were detected among WT, TRV:00, TRV: *SISIZ1*, and TRV: *SISIZ4* plants. After salt treatment, all four genes showed a marked increase in expression. The highest transcript levels were observed in WT and TRV:00 plants, while *SISOD*, *SIPOD*, *SICAT*, and *SIAPX* expression was significantly reduced in *SISIZ1*- and *SISIZ4*-silenced plants ($p < 0.05$).

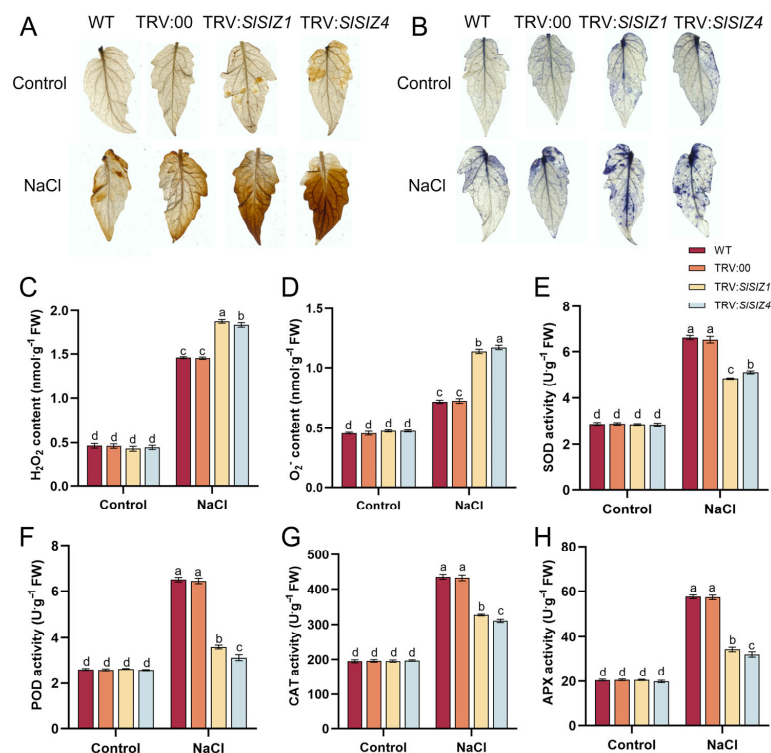


Figure 14. Oxidative stress and antioxidant defense in *SIZ1*- and *SIZ4*-silenced plants under salt stress. (A) DAB staining, (B) NBT staining, (C) H₂O₂ content, (D) O₂⁻ content, (E) SOD activity, (F) POD activity, (G) CAT activity, and (H) APX activity. Data are presented as means ± SD (n = 3). Different lowercase letters indicate significant differences (p < 0.05, Duncan's test).

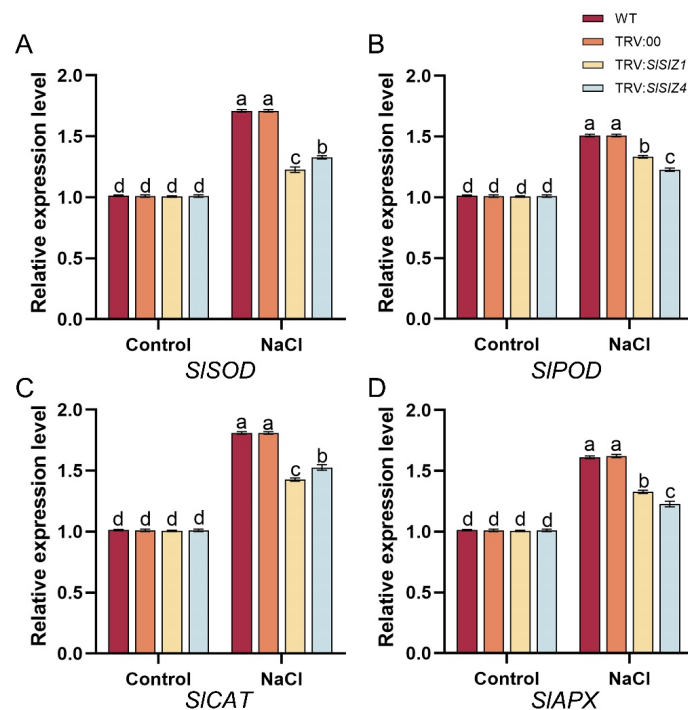


Figure 15. Expression analysis of antioxidant enzyme genes: (A) *SISOD*, (B) *SIPOD*, (C) *SICAT*, and (D) *SIAPX*. Data are presented as means ± SD (n = 3). Different lowercase letters indicate significant differences (p < 0.05, Duncan's test).

4. Discussion

4.1. Identification and Evolutionary Analysis of the *SISIZ* Gene Family

In the present study, four *SIZ*-type SUMO E3 ligase genes were identified in the tomato genome (Figure 1A). In comparison, previous studies have reported one *SIZ* gene in *A. thaliana*, three in maize, and two in rice; additionally, the *SIZ1* homolog *MdSIZ1* has been functionally characterized in apple [13,47]. Such marked variation in *SIZ* gene copy number among species is generally attributed to differences in genome size, the frequency of whole-genome duplication (WGD) events, and the subsequent retention or loss of duplicated genes [48]. These lineage-specific differences are associated with the functional diversification and regulatory complexity that enable plants to adapt to heterogeneous environmental conditions by preserving duplicated genes with adaptive value.

The phylogenetic tree constructed in this study (Figure 4) provides an integrated view of the evolutionary relationships among *SIZ* proteins from tomato and representative monocot and dicot species (Table S1). The proteins clustered into five major groups (A–E), with tomato *SISIZ* members grouping together with homologs from monocot and dicot species. This pattern suggests that the *SIZ* gene family likely originated prior to the divergence of these major plant lineages and has remained relatively conserved throughout subsequent evolutionary history. The similar clade structures have been described for *AtSIZ1* in *A. thaliana* and *OsSIZ1* in rice, further supporting the view that *SIZ* proteins share a stable evolutionary framework across angiosperms [4,8].

Chromosomal localization (Figure 1B) revealed that the *SISIZ* family members are unevenly distributed across the genome. *SISIZ1* and *SISIZ2* are positioned adjacently on chromosome 6, whereas *SISIZ3* and *SISIZ4* occur individually on chromosomes 8 and 11. In numerous plant genomes, gene family members arranged consecutively on the same chromosome are indicative of tandem duplication events, which contribute substantially to family expansion [48,49]. Tandemly duplicated clusters are often dynamic regions of plant genomes, contributing to phenotypic plasticity and environmental adaptability through the retention or divergence of duplicated genes [48,49]. Thus, the adjacency of *SISIZ1* and *SISIZ2* suggests a potential tandem duplication origin within them, which requires more detailed analysis of genomic spacing and duplication history. By contrast, the isolated positioning of *SISIZ3* and *SISIZ4* across different chromosomes, together with their syntenic relationships, suggests that these genes likely arose through segmental duplication rather than tandem duplication (Figure 7). Segmental duplication is often associated with large-scale genome rearrangements and polyploidization, which enables duplicated genes to disperse across the genome, reduces the risk of redundant gene clusters, and provides a broader evolutionary context for functional divergence [48,49]. This pattern parallels that observed in *A. thaliana* and rice, indicating that segmental duplication may have played a predominant role in shaping the expansion of the *SISIZ* family.

Within the phylogenetic topology, *AtSIZ1* was placed in Group A, whereas *SISIZ1*/*SISIZ2*/*SISIZ4* clustered in Group B (Figure 4), highlighting a conserved relationship between tomato and *A. thaliana*. *AtSIZ1* encodes a SUMO E3 ligase known to regulate diverse biological processes, including flowering time, leaf senescence, and freezing tolerance, through SUMOylation of downstream transcription factors [4,9,50]. The strongly supported sister relationship between *SISIZ1* and *SISIZ4* (bootstrap = 100), therefore, implies that *SISIZ1* and *SISIZ4* may perform similar regulatory roles in tomato. In contrast, *SISIZ3* was placed in Group E and was clearly separated from the *SISIZ1*/*SISIZ2*/*SISIZ4* cluster (Group B), indicating a more divergent evolutionary trajectory [3,4]. In addition, protein domain analysis demonstrated that all *SISIZ* proteins possess the conserved MIZ/SP-RING catalytic domain, which is characteristic of SUMO E3 ligases, while auxiliary domains such as SAP, PINIT, and SXS vary among members (Figure 3). The high conservation of the

core catalytic region combined with divergence in auxiliary domains suggests that the essential enzymatic activity of the family has been strictly maintained, whereas regulatory or functional specialization may have occurred among members [4,6,7]. Domain arrangements similar to those in *SISIZ* proteins have been documented in other species. The PINIT region of *AtSIZ1* mediates substrate recognition, while functional analyses in rice indicate that differences in auxiliary domains do not abolish ligase activity or stress-related functions [8,51]. Although automated prediction tools (Pfam, SMART, and CDD) detect only SAP and MIZ/SP-RING domains in *AtSIZ1*, failing to identify PINIT or SXS, likely due to low conservation and short lengths, biochemical studies unequivocally confirm their presence [6,7]. These findings emphasize the limitations of relying solely on sequence-based prediction and highlight the need to integrate structural and biochemical evidence when examining *SISIZ* protein function.

In addition, motif analysis (Figure 5) showed that all *SISIZ* proteins share several conserved motifs, with the SP-RING-associated motif being critical for SUMO E3 activity [4,6]. Additional member-specific motifs likely contribute to differences in substrate recognition, protein interactions, or intracellular targeting, providing further evidence of fine-scale functional divergence. Synteny analysis revealed conserved genomic blocks containing *SIZ* genes among tomato, *A. thaliana*, and soybean (Figure 7), reflecting historical genome duplication and rearrangement events [48,49]. Although these species have undergone substantial genomic divergence, the preservation of certain syntenic relationships suggests an evolutionarily stable backbone shared by *SIZ* genes, as reported for other gene families [48,49]. Secondary and tertiary structure predictions revealed high proportions of α -helices and random coils (Table 4; Figure 2), features associated with structural flexibility required for protein–protein interactions. Moreover, predicted protein–protein interaction networks indicate that *SISIZ1* and *SISIZ4* may interact with core SUMOylation pathway components, consistent with observations in pepper where *CaSIZ1* participates in stress signaling by mediating SUMOylation of transcription factors [11,12].

Recent crop studies further suggest that diversification of SUMO E3 ligases can have tangible consequences for both stress adaptation and agronomic performance. In rice, *OsSPT38* was identified as a previously uncharacterized SUMO E3 ligase, and a rare gain-of-function allele enhances abiotic stress resilience while increasing grain yield, highlighting breeding potential by tuning SUMO E3 ligase activity. This progress in cereals underscores the importance of systematically characterizing *SIZ*-type ligases in tomato and evaluating their potential roles in both stress tolerance and productivity [52,53].

Taken together, phylogenetic relationships, chromosomal distribution patterns, domain structures, motif compositions, and physicochemical properties collectively indicate that the *SISIZ* gene family is structurally well conserved. At the same time, divergence in auxiliary domains and motif patterns suggests that functional specialization has occurred within the family, providing a genomic and structural basis for the diverse regulatory roles of *SISIZ* genes in tomato.

4.2. Expression Patterns and Regulatory Responses of *SISIZ* Genes Under Abiotic Stress

Recent evidence positions SUMOylation as a highly dynamic post-translational regulatory mechanism that integrates multiple environmental stresses with hormonal and transcriptional networks across plant species. Accordingly, tissue-specific expression profiles showed substantial variation among the four *SISIZ* genes, indicating functional divergence among family members (Figure 9) [53].

Tissue-specific expression profiles showed substantial variation among the four *SISIZ* genes, indicating functional divergence among family members (Figure 9). *SISIZ4* displayed consistently high expression across the most examined tissues, suggesting that it functions

as a broadly expressed member of the family. *SISIZ1* was expressed in key tissues, such as roots, leaves, flowers, and fruits, whereas *SISIZ2* showed low expression in these tissues. In addition, *SISIZ3* accumulated predominantly in roots and immature fruits. Specifically, these expression features of SIZ ligases were also reported in other plants. In *A. thaliana*, *AtSIZ1* transcripts were detected in almost all organs, and *atsiz1* mutants showed defects in growth, cell proliferation, and drought responses [4,50]. In rice (*O. sativa*), *OsSIZ1* was constitutively expressed during vegetative and reproductive development with strong promoter activity in vascular bundles, and *ossiz1* mutants exhibited altered root architecture and reduced fertility [8,51,54]. Thus, the above examples and our results indicate that *SIZ* genes are widely expressed and distributed in plant tissues, providing evidence that *SISIZs* may be important regulators for tomato plant growth. In pepper (*C. annuum*), *CaSIZ1* is induced by ABA and NaCl, localizes to the nucleus, interacts with *CaABI5*, and *CaSIZ1* knockdown reduces ABA-induced SUMO conjugate accumulation and attenuates salt tolerance, supporting a conserved ABA–SIZ–SUMOylation module within *Solanaceae* crops [54]. However, how tomato *SISIZ* proteins mediate plant growth through judging SUMOylated modification requires further study.

Subsequently, the promoter *cis*-acting regulatory element analysis revealed that the *SISIZ* genes contain ABRE-, DRE-, and MYB-binding sites associated with stress and hormone signaling (Figure 8) [11]. ABRE- and DRE-type *cis*-elements are also present in the promoters of *SIZ1* homologs in *A. thaliana* and *O. sativa*, consistent with their roles in ABA- and stress-related signaling [55–57]. Generally, ABRE and DRE elements represent two major classes of *cis*-acting elements, mediating ABA-dependent and ABA-independent transcriptional responses to drought and salinity, as shown for *AtDREB2A* and other stress-inducible gene promoters in *A. thaliana* [55–57]. Likewise, *OsSIZ1* in *O. sativa* was strongly induced by salt and drought stress [8]. When *OsSIZ1* was overexpressed in crop species, plants showed improved tolerance to drought, heat, and salinity [8,14]. Similarly, our study showed that the expression of *SISIZ1* and *SISIZ4* was strongly and persistently induced under salt and ABA treatments. This interpretation is further strengthened by the functional evidence from pepper, indicating that SIZ-type ligases can mediate ABA-associated SUMOylation and thereby influence stress tolerance in *Solanaceae* species [54]. In addition, light-responsive elements were found in all *SISIZ* promoters (Figure 8). Previous studies showed that the promoters of other SUMO E3 ligase genes, such as *AtSIZ1* and *OsSIZ1*, were enriched in light-responsive elements. In addition, their transcriptional levels were changed by photoperiod, circadian, or light-induced oxidative stress [58]. These findings imply possible interactions between light signaling and the SIZ proteins during circadian regulation or photooxidative stress responses, which remains to be elucidated.

4.3. Functional Validation of *SISIZ1* and *SISIZ4* Under Salt Stress

Salt stress inhibits normal plant growth and development through multiple mechanisms, including osmotic stress, ion toxicity, and oxidative damage [1,2,17,18]. In tomato seedlings, typical symptoms of salt injury include leaf chlorosis and curling, reduced photosynthetic efficiency, and, ultimately, decreased yield and fruit quality [1,2]. Given the tissue-specific expression patterns of *SISIZ1* and *SISIZ4* and their potential association with salt-induced physiological damage, we employed virus-induced gene-silencing (VIGS) methods to clarify their roles in salt tolerance. Only the plants that exhibited 60–80% VIGS efficiency were selected for the subsequent salt treatments, which was a strategy that was commonly adopted in tomato VIGS studies to improve reproducibility (Figure 11) [37]. Overall, our experimental analyses indicate that *SISIZ1* and *SISIZ4* contribute to maintaining growth and photosynthetic capacity in tomato seedlings under salt stress. In addition, the loss of either gene weakened the ability of tomato seedlings to withstand salt-induced

damage (Figure 11). This salt-sensitive phenotype is consistent with reports on homologous genes in *A. thaliana* and rice. In *A. thaliana*, *AtSIZ1* modulated the activity and stability of multiple SUMOylated target proteins, thereby supporting the function of photosynthetic tissues under salt and drought stress [4,50]. In rice, *OsSIZ1* was essential for vegetative and reproductive development, and mutants of *OsSIZ1* exhibited dwarfism, reduced tillering, and decreased pollen fertility, together with increased sensitivity to abiotic stresses, including salinity [8,51]. These examples indicate that SIZ1-like SUMO E3 ligases may play conserved roles in coordinating growth and stress tolerance across plant species.

Salt stress leads to excessive accumulation of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which are highly toxic to cells and can cause membrane lipid peroxidation, protein oxidation, and DNA damage [17,18,59]. Under salt stress, changes in the levels of proline (Pro), malondialdehyde (MDA), and electrolyte leakage (EL) serve as key physiological and biochemical indicators of stress damage and tolerance [2,39,41,43,60]. Pro is generally regarded as a positive protective factor, and an increase in Pro usually reflects enhanced osmotic adjustment [60]. In contrast, elevated MDA and EL indicate aggravated membrane lipid peroxidation and membrane damage and are considered negative indicators of stress tolerance [2,41,44]. Consistent with this, salt-tolerant cultivars of wheat usually maintained lower MDA and less EL but accumulated more Pro when compared with sensitive genotypes under saline conditions [61]. These physiological parameters are also closely associated with ROS overaccumulation and membrane lipid peroxidation under abiotic stresses [17,18,41]. Therefore, to mitigate salt-induced damage, plants activate a series of defense mechanisms, including the antioxidant system. Moreover, plants alleviate oxidative damage by increasing the activities of antioxidant enzymes to scavenge excess ROS [17,18]. In *A. thaliana*, *siz1* mutants similarly show salt-induced ROS overaccumulation together with reduced activities of antioxidant enzymes, such as SOD, POD, CAT, and APX [4,50]. Mechanistically, recent work in *A. thaliana* directly links SIZ1-dependent SUMOylation with ROS homeostasis and chloroplast-associated redox regulation. Specifically, *SIZ1* contributes to ROS-triggered increases in SUMO conjugates, whereas *siz1* mutants display elevated sensitivity to oxidative stress accompanied by altered hydrogen peroxide and superoxide accumulation across development. These findings support the notion that SIZ1-type ligases participate in redox control, which is consistent with the enhanced ROS accumulation and compromised antioxidant enzyme activities observed in *SISIZ1*- and *SISIZ4*-silenced tomato seedlings under salinity. In this study, *SISIZ1*- and *SISIZ4*-silenced plants accumulated much higher levels of H_2O_2 and O_2^- , consistent with the increase of MDA and EL under salt treatment when compared with WT and TRV:00 control plants (Figures 12 and 13). These changes indicate more severe membrane lipid peroxidation and structural damage. By contrast, Pro content was markedly reduced in the silenced lines, implying impaired osmotic adjustment capacity. Enzyme activity assays further showed that the activities of SOD, POD, CAT, and APX were all clearly reduced in the silenced plants, similar to the patterns observed in *A. thaliana* *siz1* mutants (Figure 14E–H). Taken together, these results suggest that the silencing of *SIZ* genes may disrupt ROS homeostasis and membrane stability, thereby weakening the ability of plants to cope with salt stress. Mechanistically, SIZ-type SUMO E3 ligases may regulate ROS-scavenging capacity mainly through SUMOylation of transcriptional regulators rather than by directly modifying antioxidant enzymes. In *A. thaliana*, SIZ1-mediated SUMOylation of the R2R3-MYB transcription factor MYB30 is salt responsive and promotes salt tolerance by reinforcing the MYB30–AOX1a module, thereby improving cellular redox homeostasis via alternative respiration. This provides a concrete example that *SIZ*-dependent SUMOylation of transcription factors can reshape downstream redox networks. Accordingly, the elevated ROS levels together with reduced SOD, POD, CAT,

and APX activities in *SISIZ1*- and *SISIZ4*-silenced tomato seedlings may reflect impaired SUMOylation-dependent regulation of stress/redox-related transcriptional programs under salinity. ABA signaling is tightly coupled to ROS production and detoxification during salt stress, and SUMOylation adds an additional regulatory layer that modulates the magnitude and duration of ABA outputs. Notably, *SIZ1*-dependent SUMOylation of the bZIP transcription factor ABI5 attenuates ABA signaling in *A. thaliana*, demonstrating that SIZ proteins can tune ABA-responsive gene expression through post-translational modification of a central ABA regulator. In *Solanaceae*, pepper *CaSIZ1* interacts with *CaABI5*, and ABA promotes SUMO-conjugate accumulation, whereas *CaSIZ1* knockdown reduces ABA-induced SUMOylation and compromises salt tolerance. Together with ABRE enrichment in *SISIZ* promoters and the ABA inducibility of *SISIZ1/SISIZ4* in our study, these findings support a working model in which *SISIZ1/SISIZ4* coordinate ABA-responsive transcriptional networks and antioxidant defenses via SUMOylation of ABA/stress-related transcription factors, ultimately constraining ROS overaccumulation and membrane damage under salinity. At the same time, SIZ1-like SUMO E3 ligases may help to alleviate ROS damage in coordinating salt stress tolerance across plant species. Given that *SISIZ1/SISIZ4* are predicted to be embedded in a SUMOylation-centered interaction module (STRING; Figure 6), it is plausible that some key substrates under salinity are nuclear regulatory proteins rather than ROS-scavenging enzymes per se. In *A. thaliana*, the SIZ1-type SUMO E3 ligase regulates diverse stress and developmental processes through SUMOylation of downstream transcription factors [4,9,50], and *AtSIZ1* has been shown to SUMOylate MYB30 to modulate ABA responses [10]. Moreover, *AtSIZ1*-mediated SUMOylation of ABI5 fine-tunes ABA signaling and stress-responsive gene expression [62]. In *Solanaceae* crops, pepper *CaSIZ1/CaDSIZ1* also participates in ABA-related stress signaling and drought tolerance by affecting SUMOylation and transcription factor stability [11,12]. By analogy, *SISIZ1/SISIZ4* may target tomato ortholog(s) of ABI5-like bZIPs and MYB30-like R2R3-MYBs, thereby indirectly controlling antioxidant gene expression and ROS homeostasis during salt stress. Future identification of *SISIZ1/SISIZ4*-interacting proteins and SUMOylated substrates under salinity will be essential to validate these candidate targets. Another report found that *AtSIZ1* mediated the SUMOylated modification of ABI5 to fine-tune ABA-dependent expression of stress-responsive genes [62]. In our study, the tomato *SISIZ* promoters consisted of ABA-responsive *cis*-elements (Figure 8). In addition, the transcriptional abundances of *SISIZ1* and *SISIZ4* could be mediated by ABA (Figure 10C). Thus, there may exist a relationship between *SISIZs* and ABA in plant responses to salt stress, which needs to be detected in the future.

Beyond salinity, recent studies in other species emphasize that SUMO E3 ligases can protect yield-related processes under environmental stress. In *A. thaliana*, *SIZ1*-mediated SUMOylation stabilizes WRINKLED1 (WRI1) to safeguard seed filling and fatty acid biosynthesis under prolonged high-temperature stress, illustrating how *SIZ1* can buffer stress-induced protein instability to sustain productivity. In rice, a newly characterized SUMO E3 ligase *OsSPT38* carries a rare gain-of-function mutation that enhances abiotic stress resilience while increasing grain yield, highlighting breeding potential by tuning SUMO E3 ligase activity. Together with our tomato data, these cross-species advances support the broader relevance of *SISIZ1/SISIZ4* as candidate regulatory nodes for improving stress tolerance and potentially productivity, depending on downstream targets and trade-offs [52–64] (WRI1–heat/seed filling) (*OsSPT38*–stress + yield).

Taken together, these findings indicate that *SISIZ1* and *SISIZ4* play important roles in tomato responses to salt stress. However, this study still has several limitations. Firstly, it remains unclear whether *SISIZ1* and *SISIZ4* are functionally redundant or mainly regulate distinct sets of targets. In *A. thaliana*, analysis of *AtSIZ1* and *MMS21/HPY2* double mutants

has revealed overlapping yet distinct functional networks in development and stress responses [4]. Therefore, generating *SISIZ1*- and *SISIZ4*-related double-silenced lines in tomato will be essential for dissecting their functional relationship. Accordingly, the conclusions drawn here primarily reflect seedling-stage salt responses, and direct validation during flowering and fruit set will be required to determine yield-relevant roles. Secondly, this study primarily examined salt responses at the seedling stage, and the roles of *SISIZ1* and *SISIZ4* in salt tolerance during reproductive development, such as flowering and fruit set, remain unclear. Previous studies have shown that loss of *OsSIZ1* function in rice leads to reduced pollen fertility and abnormal panicle architecture, suggesting that *SISIZ1* and *SISIZ4* may also influence reproductive development in tomato [51,54]. Thus, a detailed investigation of how *SISIZ1* and *SISIZ4* affect fruit development and quality formation under salt stress may be of considerable agronomic importance. Finally, the identification of the direct SUMOylation substrates of *SISIZ1* and *SISIZ4* is crucial for fully elucidating their molecular mechanisms, which may also provide potential molecular targets and a theoretical basis for improving stress resistance in tomato crops [3,4]. A schematic summary of the proposed *SISIZ*-mediated salt tolerance pathway is presented in Figure 16.

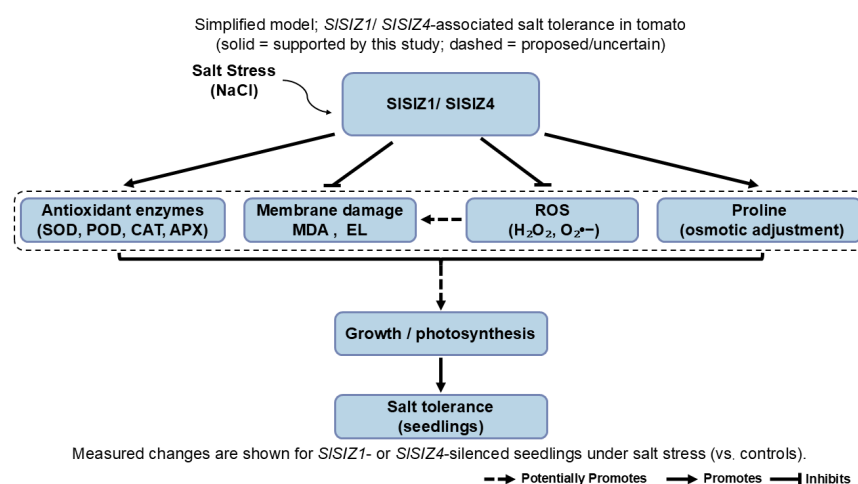


Figure 16. A simplified model of *SISIZ1*- and *SISIZ4*-mediated salt tolerance in tomato seedlings. Salt stress (NaCl) induces the expression of *SISIZ1* and *SISIZ4*, which act as positive regulators of salt tolerance. These SUMO E3 ligases enhance the activities of antioxidant enzymes (SOD, POD, CAT, and APX) and promote proline accumulation for osmotic adjustment. Simultaneously, they inhibit the overaccumulation of reactive oxygen species (ROS, such as H_2O_2 and $O_2^{\bullet-}$) and mitigate membrane damage, as indicated by reduced malondialdehyde (MDA) content and electrolyte leakage (EL). These coordinated physiological responses maintain growth and photosynthetic capacity under salinity, ultimately enhancing seedling salt tolerance. Solid lines indicate regulatory pathways supported by experimental data in this study, while dashed lines represent proposed or uncertain mechanisms. Arrows and T-bars represent promotion and inhibition, respectively.

5. Conclusions

In summary, we identified four SIZ-type SUMO E3 ligase genes in tomato and showed that they are structurally conserved but functionally differentiated in development and stress responses. Promoter and phylogenetic analyses indicated that the tomato *SISIZ* genes form evolutionarily conserved evolutionary relationships with SIZ homologs from representative monocot and dicot species (e.g., *A. thaliana* and *O. sativa*; Table S1). In addition, *SIZ* members may be tightly connected to ABA-, drought-, light-, and defense-related signaling networks. Expression analysis showed that *SISIZ* genes exhibited distinct expression patterns across different tissues and stress treatments: *SISIZ1* and *SISIZ4* were highly expressed in several tissues and were markedly upregulated by salt and ABA,

whereas *SISIZ2* and *SISIZ3* showed relatively low expression levels. Functional assays using virus-induced gene silencing (VIGS) further demonstrated that *SISIZ1* and *SISIZ4* act as positive regulators of salt stress tolerance, since their silencing under salt stress resulted in inhibited plant growth, reduced chlorophyll accumulation, disruption of redox and osmotic homeostasis, and downregulation of antioxidant- and ABA-related genes. This work provides a concise framework for the *SISIZ* gene family and offers candidate genes for improving salt stress tolerance in tomato through molecular breeding or genetic engineering. Importantly, the family-wide *SISIZ* reference generated here goes beyond prior single-gene studies and facilitates future mechanistic and stage-specific investigations of SUMOylation-mediated regulation in tomato.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16040422/s1>, Table S1: Gene/locus IDs and protein sequence identifiers of SIZ/PIAS-type SUMO E3 ligases from tomato and representative plant species used for phylogenetic analysis.

Author Contributions: All authors contributed to the conception and design of the study. Conceptualization, C.W.; funding acquisition, Q.W. and C.W.; original draft—writing, C.W. and Q.W.; review and editing, Q.W., Z.L. and C.W.; visualization, Q.W. and C.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Supporting Funds for Youth Mentor of Gansu Agricultural University (GAU-QDFC-2024-15), the National Natural Science Foundation of China (No. 32460753 and No. 32102370), the Fuxi Young Talents Fund of Gansu Agricultural University (Gaufx-03Y07), and the Natural Science Foundation of Gansu Province, China (23JRRA1406).

Institutional Review Board Statement: The experiments involving tomato plants were conducted in accordance with institutional and national guidelines for the use of plants in scientific research. The cultivated tomato cultivar ‘Micro-Tom’ used in this study did not involve any endangered or protected species and, therefore, did not require specific permits.

Data Availability Statement: The data presented in this study are available in this article. For further information, please contact the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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