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Silencing *CaTPS1* Increases the Sensitivity to Low Temperature and Salt Stresses in Pepper

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Abstract: Trehalose, as a non-reductive disaccharide, plays a vital role in plant growth and development and resistance to abiotic stress. Trehalose-6-phosphate synthase (TPS) is a key enzyme in the synthesis mechanism of trehalose and *TPS1* genes play a crucial role in the response to abiotic stress in plants. However, it has rarely been reported that *CaTPS1* responds to cold and salt stresses in pepper. To verify the function of *CaTPS1* in response to cold and salt stresses, *CaTPS1* was silenced by virus-induced gene silencing (VIGS). Subsequently, the expressions of *CaTPS1*, plant morphology and some physiological indexes were analyzed after cold and salt stresses in pepper. The results showed that the expression of *CaTPS1* was significantly lower in *CaTPS1*-silenced (pTRV2-*CaTPS1*) plant than that in the non-VIGS (CK) and negative control (PTRV2-00) plants. The parameters of response to cold and salt stresses have changed accordingly. The chlorophyll content decreased, while the trehalose content, peroxidase (POD) activity, catalase (CAT) activity and ascorbate peroxidase (APX) activity increased in all treatments. However, these parameters of response to cold and salt stresses were significantly lower in pTRV2-*CaTPS1* plant than in CK and PTRV2-00 plants. This study suggested that *CaTPS1* was involved in the response to cold and salt stresses in pepper.

Keywords: pepper; trehalose-6-phosphate synthase gene; virus-induced gene silencing; low-temperature stress; salt stress

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1. Introduction

Chili peppers (*Capsicum annuum* L.) are widely grown around the world as vegetable and spice crops due to their high nutrient and capsaicin content [1,2]. However, many biotic and abiotic factors contributed to the decline in the production and quality of pepper [3]. Low temperature and salt are the primary abiotic stresses that seriously restrict the growth and development of plants, including pepper [4,5], which led to a significant drop in yield and quality in Europe [6] and Australia [7].

As an energy substance and a stress protectant, trehalose (α-D-glucopyranosyl-1; 1-α-D-glucopyranose-side) plays a very important role in the growth and development of plants [8,9]. Trehalose is considered to be a potential osmotic protective agent for plants, mainly in the following ways to enhance plant tolerance: First, trehalose absorbs nutrients by improving the integrity of the membrane and the water relationship of plants, reducing electrolyte leakage and lipid oxidation; second, trehalose improves the gas exchange characteristics, protects the photosynthetic mechanism from the oxidative damage caused by salinity, and causes the change in the ultrastructure in plants. Third, the antioxidant activity of plants and the expression of stress-responsive proteins and genes were improved. Fourthly, it participates in the signal connection with signal molecules and plant hormones [10]. In addition, as a carbon source and structural component in fungi, bacteria, invertebrates, and insects, trehalose also functions as a key protectant of proteins and membranes during stress conditions such as high salinity, dehydration, hypoxia, nutrient starvation, and cold [11]. The biosynthesis of trehalose is regulated by two main

enzymes, trehalose phosphate synthase (TPS) and trehalose-phosphatase (TPP). TPS first catalyzes glucose 6-phosphate (G6P) and uridine diphosphate glucose (UDP-Glc) to form trehalose 6-phosphate (T6P), then TPP catalyzes the dephosphorylation of T6P to form trehalose (Figure S1) [8,12,13]. TPS is a pivotal enzyme in the trehalose synthesis pathway and regulates the synthesis efficiency of trehalose.

TPS gene families have been identified in many species. For example, there is a gene family containing 53 *TPSs* members in cotton (*Gossypium hirsutum*) [14], 23 *TPSs* genes were found in soybean (*Glycine max*) [15], and eight *TPSs* genes were found in potato (*Solanum tuberosum*) [16]. Eleven *TPSs* genes have been found in pepper, Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) [9,17]. In addition, most *TPSs* genes have two typical characteristic structural domains, the N-terminal TPS (Pfam: Glyco_transf_20) and the C-terminal TPP (Pfam: Trehalose_PPase) [14]. A total of 11 pepper TPS genes were identified in the whole genome database of pepper, except *CaTPS3*, which contains one TPS and two TPP structural domains, while the rest contain one TPS and one TPP structural domain, respectively. The expression of *CaTPS1* in leaves is about five–six times higher than that in roots and stems, indicating that the ex-pression of *CaTPS1* has obvious tissue specificity [9]. Yeast functional complementation experiments showed that only *OsTPS1*, out of 11 *OsTPSs* genes, had TPSase activity but not TPPase activity in rice [18]. In Arabidopsis, only *AtTPS1* also has TPSase activity and no TPPase activity [17]. Currently, it was found that, among most *TPSs* gene families, only *TPS1* has TPS activity. Brewer's yeast (*Saccharomyces cerevisiae*) *ScTPS1* functions as a metabolic enzyme and stress responder and transcript levels of *TPS1* are often correlated with TPS activity and trehalose content [19].

Simultaneously, most *TPSs* genes in different organisms are also involved in stress-resistant processes in plants [20]. For example, the overexpression of *OsTPS1* improved the tolerance to high salinity, drought, and cold treatments in rice [21,22]. Transfer of the yeast *TPS* gene into potato revealed that, during drought treatment, the transgenic plants had delayed wilting and higher stomatal conductance, net photosynthetic rate, and CO_2 assimilation rate than wild-type plants, resulting in increased resistance to abiotic stresses. [23]. The overexpression of the *SlTPS* gene in tomatoes (*Solanum lycopersicum*) resulted in enhanced photosynthesis and increased tolerance to drought and salt stresses in transgenic plants [24]. Freezing stress induced the high expression of the *TaTPS* gene in winter wheat (*Triticum aestivum*), suggesting that the *TaTPS* gene could be involved in signaling pathways in response to freezing in winter wheat [25]. The overexpression of the *OsTPS1* gene in rice led to increased tolerance to low temperature, salt, and drought stresses of rice seedlings, as well as grown trehalose and proline content in rice plants, and up-regulated expression of some stress-related genes, including *OsWSI18*, *OsRAB16C*, *OsHSP70* and *OsELIP* genes [21]. The expression of *CsTPS1*, *CsTPS9* and *CsTPPA* were highest under at least one abiotic stress, and it was speculated that these three genes might play a key role in the response of tea plants (*Camellia sinensis* L.) to abiotic stresses [26]. Under cold stress, the expression level of *OsTPP1* would be improved, which would increase trehalose content and resistance to cold injury [27,28].

At present, as one of the most convenient methods for the indirect verification of gene function, virus-induced gene silencing (VIGS) has been widely used in some plants whose transgenic system is still immature [29]. Silencing *StSSH2*, *StWTF*, *StUGT*, *StBHP*, and *StFLTP* of potatoes by VIGS could accelerate plant senescence and decrease tuber number and size and dry matter contents [30]. The resistance to *M. grisea* of BMV (Brome mosaic virus): *OsMED16* and BMV: *OsMED25*-infiltrated seedlings was reduced by regulating H_2O_2 accumulation and the expression of defense-related genes. Additionally, the tolerance to cold of BMV: *OsMED16* infiltrated seedlings was reduced by increasing malondialdehyde (MDA) content and decreasing the expression of cold-responsive genes [31]. *CaPIF8*-silenced pepper plants obtained by VIGS showed a high sensitivity to cold and salt stresses significantly increased the relative electrolyte leakage (REL) and altered the expression of stress-related genes [32]. Silencing *GhGLK1* in cotton brought more damage to plants under drought and cold stresses compared to wild-type (WT) [33].

Given that *TPS1* genes play an important role in the response to abiotic stress in many plants, the response to abiotic stress of *CaTPS1* in pepper has seldom been reported. In this study, we explored the function of *CaTPS1* genes in cold and salt stresses in pepper by VIGS. It will be beneficial for understanding the biological function of *CaTPS1* and contribute to the study of the molecular mechanism of abiotic stress resistance in pepper, thus providing new genes for the molecular breeding of pepper low temperature and salt stress.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The cultivar 'Qiangfeng 101' (*Capsicum annuum* L.), provided by the College of Horticulture, Gansu agricultural university, China, was used in this study. Pick the pepper seeds with full grain and wrap them with gauze, soak them in hot water at 55 °C for 20 min, disinfect them with 20% trisodium phosphate for 20 min, germinate at 28 °C for 72 h, and sow them in the matrix of the nutrition bowl (vermiculite: Nutrient soil = 3:1), incubated in a light incubator. The pepper was planted in the nutrition bowl and grown in an artificial climate chamber with a 16 h light/8 h dark cycle at 28 °C and 23 °C, daytime light was 20,000 Lx.

2.2. Homologous Sequence Alignment of CaTPS1

In order to describe the sequence similarity between CaTPS1 and the TPS proteins that have been characterized in other species (Rice (OsTPS1), Arabidopsis (AtTPS1) and Zea mays maize (ZmTPS1)), we searched their respective protein sequences according to the literature [17,34], and then performed multiple sequence alignments on them using DNAMAN.6 software [35]. Set the parameter to the default value. Their domains were analyzed using the online web site SMART (https://smart.embl.de/smart) accessed on 16 January 2023 and visualized using Photosho.2020 software [36]

2.3. Construction of Silencing Vectors and Infection of Plants

The pTRV1 and pTRV2 vectors (tobacco rattle virus) were used to construct the VIGS system. *CaTPS1* was amplified by the gene-specific primers V2-CaTPS1 (Table 1). PCR product was purified and inserted into the pTRV2 vector with SoSoo homologous recombinase (Qingke, Beijing, China) at 50 °C for 15 min, and then transformed into *E.coli* DH5α. A single colony with an appropriate fragment size was cultured in LB liquid with 50 μg/mL kanamycin, and the bacterial liquid culture samples were sequenced to verify the results of the recombinant vector (Shenggong, Shanghai, China). Then, the recombinant plasmid of pTRV2-*CaTPS1* was obtained according to the sequencing results. The negative control empty pTRV2-00 recombinant plasmid was constructed in the same method as described above. Subsequently, the constructed vectors pTRV2-00 and pTRV2-*CaTPS1* were introduced into *Agrobacterium tumefaciens* GV3101.

Table 1. List of primers used in this study.

Usage	Primer Name	Primer Sequence(5′-3′)
pTRV2-*CaTPS1* construction	V2-CaTPS1	F: tgtgagtaaggttaccgaattcAACTTTTGTGAGTGAACTAAATGA R: tgagctcggtaccggatccTACTCACTGAAGTTATCATCTAAG
qRT-PCR	q-CaTPS1	F: TAAGTGGTGGAGGTCTCGTCAGC R: ATTCACACCTGCCCAACCAATCC
qRT-PCR	q-Actin	F: CCCGGAAGAGCACCCTGTC R: ATGCTGCTGGGAGCCAACG

Notes: Lowercase letters represent the sequence of the gene, and the uppercase letter represents the sequence of the vector.

The *A. tumefaciens* solutions carrying pTRV1, pTRV2-00 and pTRV2-*CaTPS1* were grown on LB medium with 50 μg/mL kanamycin, 50 μg/mL gentamicin and 50 μg/mL rifampicin. The reconstituted colony was transferred to LB liquid medium containing the

above antibiotics and incubated at 28 °C and 220 rpm for 24 h. *A. tumefaciens* was then concentrated by centrifugation and resuspended in an infiltration buffer IM. *A. tumefaciens* cells containing pTRV1 were mixed with cells containing the pTRV2-*CaTPS1* construct vector or the pTRV2 -00 empty vector in a 1:1 volume ratio. The mixed bacterial liquid was infiltrated into pepper seedlings at two true leaf periods using a 1 mL syringe and the infected plants were cultured in the light incubator at 16 h light/8 h dark cycle at 22 °C/18 °C, the light intensity was 20,000 Lx [37].

2.4. The Plant Growth of Silenced Plants

Silent Plants (the non-VIGS (CK), Negative control/empty vector (pTRV2-00), and CaTPS1-silenced (pTRV2-*CaTPS1*) plants) Plant height and stem diameter were measured in silenced plants (CK, pTRV2-00 and pTRV2-*CaTPS1* plants) 15, 30 and 45 days after infiltration.

2.5. RNA Isolation and qRT-PCR Analysis

Total RNA was extracted using the RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) and following the kit's instructions. cDNA was synthesized using Prime Script^{TM} RT kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. To identify the silencing efficiency of VIGS and the response of *CaTPS1* to cold and salt stresses, the relative expression of the *CaTPS1* levels of the CK, pTRV2-00 and pTRV2-*CaTPS1* were measured by qRT-PCR under cold and salt stresses. qRT-PCR was performed on StepOnePlus (U.S. Applied Biosystems China Company Products, Beijing, China) and the conditions of qRT-PCR were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 5 s, annealing at 60 °C for 30 s. Actin (GenBank Accession: GQ339766.1) was used as an internal control to normalize PCR efficiency. The relative expression level was calculated using the $2^{-\triangle\triangle CT}$ method [38].

2.6. Salt and Cold Stresses Assays

The infected seedlings with seven or eight true leaves were used for abiotic stress. For cold treatment, normal plants and silent Plants (the CK, TRV2-00, and TRV2-*CaTPS1* plants) were cultured at 15 °C day/10 °C night for 3 days [39]. For salt treatment, initially, 100 mM NaCl was used, then the NaCl concentration in the Hoagland nutrient solution was gradually increased to 150, 200 and 300 mM at 12-h intervals and poured into the matrix of the plants to be treated to prevent salt shock from the high NaCl. The pepper leaves of normal plants and silent Plants (the CK, TRV2-00, and TRV2-CaTPS1 plants) were disposed of 300 mM NaCl treatment for 3 days [40]. Seedlings under normal growth conditions were used as control. Normal plants were collected after 0, 1, 3, 6, 12, 24, 36, 48, and 72 h of treatments and silent plants were collected after 0, 12, 36, and 48 h of treatments, immediately frozen in liquid nitrogen, and stored at −80 °C.

2.7. Determination of Chlorophyll and Trehalose Content, and Antioxidant Enzyme Activities

All physiological indicators of CK, pTRV2-00 and pTRV2-*CaTPS1* plants were measured after 36 h of cold and salt treatment and normal control.

The chlorophyll contents and trehalose contents of CK, pTRV2-00, and pTRV2-*CaTPS1* plants were measured. A SPAD-502 chlorophyll (Konica Minolta Co., Tokyo, Japan) was used to determine the chlorophyll content of pepper leaves [41]. The determination of trehalose content was carried out according to the instructions of the trehalose content detection kit (Sinobestbio, Shanghai, China).

The metabolic enzyme activities of CK, pTRV2-00, and pTRV2-*CaTPS1* plants were measured. Peroxidase (POD) activity was measured by the nitroblue tetrazolium (NBT) illumination method, catalase (CAT) activity was determined by the guaiacol method, and ascorbate peroxidase (APX) activity was measured by estimating the decreasing rate of ascorbate oxidation at 290 nm [39,42].

2.8. Statistical Analysis

The results were subjected to analysis of variance (ANOVA) using the SPSS statistical package (version 22.0, SPSS, Chicago, IL, USA). The statistical divergence among treatments was analyzed through Duncan's multiple range test ($p < 0.05$).

3. Results

3.1. Homologous Sequence Alignment of CaTPS1

From Figure 1, the length of the TPS structural domain domain of TPS1 in these four species is much larger than that of TPP. The TPS and TPP structural domains of CaTPS1 and AtTPS1, OsTPS1, ZmTPS1 are all relatively conserved, and the sequence similarity is basically greater than or equal to 75%. It can be concluded that CaTPS1 may have the protein function of catalyzing G6p like AtTPS1, OsTPS1 and ZmTPS1.

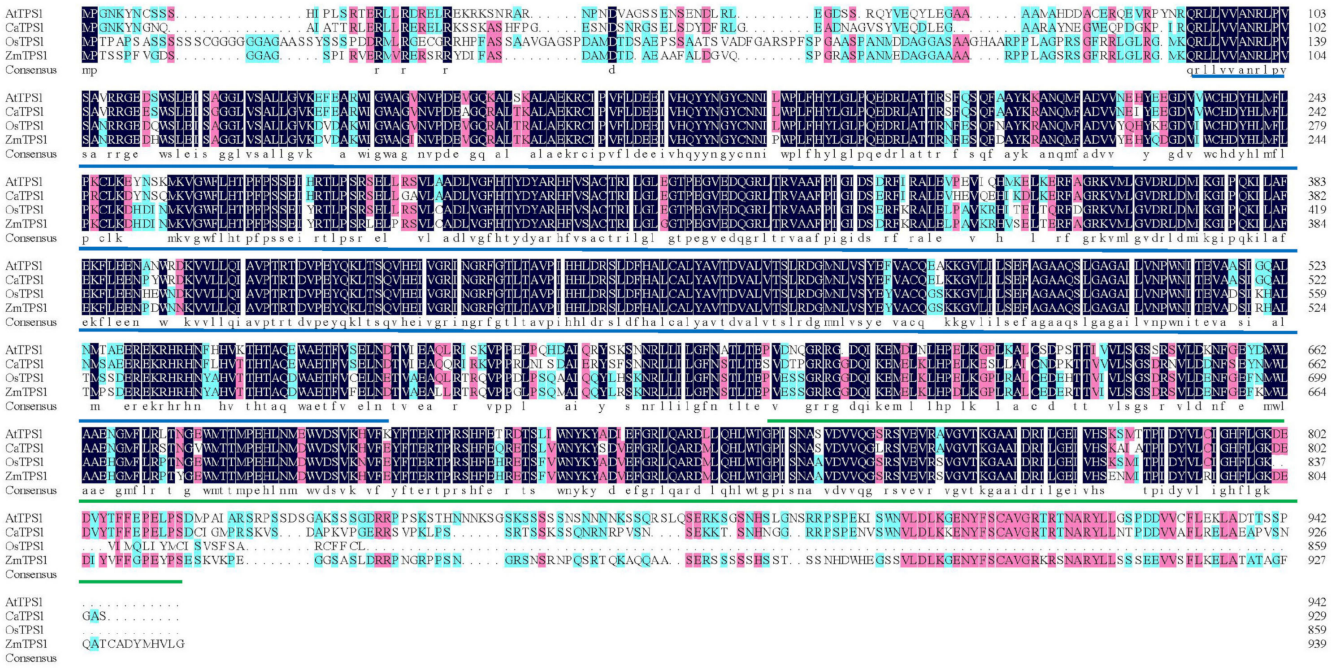

Figure 1. Alignment of pepper (CaTPS1), Rice (OsTPS1), *Arabidopsis* (AtTPS1) and maize (ZmTPS1) protein sequences. The TPS domain is underlined in blue and TPP domain in green. Amino acid residues with 100% similarity in all sequences are shaded in dark blue, while letters with greater than or equal to 75% similarity are shaded in pink and greater than or equal to 50% similarity in blue.

3.2. The Expression of CaTPS1 among Different Stress Treatments

TPS1 plays a crucial role in all aspects of plant development and stress response, and its function has been less studied in pepper. To explore the expression pattern of the *CaTPS1* gene under abiotic stress, we analyzed the expression levels of this gene in the pepper material "Qiangfeng 101" after treatment with low temperature and NaCl (Figure 2). Under low-temperature treatment, we found that the relative expression of *CaTPS1* peaked at 36 h, with *CaTPS1* expression rising by 100% relative to 0 h. In NaCl treatment, *CaTPS1* expression rose and then fell and then rose and then fell again, with its highest expression exhibited after 36 h of salt treatment.

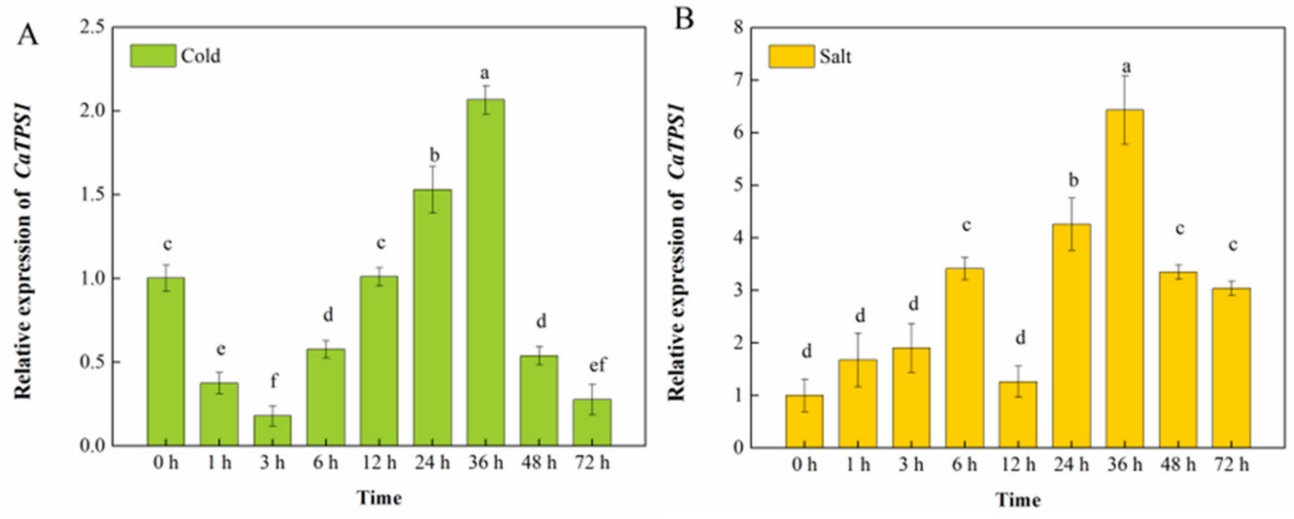

Figure 2. Relative expression of *CaTPS1*. Relative expression of *CaTPS1* in leaves from pepper under cold (**A**) and salt (**B**) treatments for 0 h, 1 h, 3 h, 6 h,12 h, 24 h, 36 h, 48 h and 72 h. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

3.3. The Plant Growth of Silenced Plants

To observe the effect of infection on plant growth, plant height (Figure 3A), and stem diameter (Figure 3B) were measured at different periods after infection. Plant height and stem diameter of CK, pTRV2-00 and pTRV2-*CaTPS1* plants increased significantly with increasing infestation time. The plant height of pTRV2-00 and pTRV2-*CaTPS1* plants was significantly reduced after 15, 30 and 45 days of infiltration, indicating that the TRV2 virus affects plant height. At the same time, the significant reduction of pTRV2-*CaTPS1* plants compared to CK, and pTRV2-00 plants indicates that *CaTPS1* affects the plant height of pepper plants. The stem diameter of pTRV2-00 and pTRV2-*CaTPS1* plants was reduced after 15, 30 and 45 days of infiltration, indicating that TRV2 and *CaTPS1* also affect the stem diameter of the plants. There was no significant difference in stem thickness between pTRV2-00 and pTRV2-*CaTPS1* plants after 15 and 30 days of infiltration. However, after 45 days of infiltration, the stem thickness of pTRV2-*CaTPS1* plants was significantly lower than that of CK and pTRV2-00 plants. These results indicate that the increase in plant height and stem diameter of pTRV2-*CaTPS1* is significantly reduced, further affecting normal plant growth and development, suggesting that *CaTPS1* may be associated with plant growth.

3.4. The Expression of CaTPS1 among Silenced Plants under Different Treatments

In this study, we would attempt to silence *CaTPS1* by VIGS assay in pepper. The results showed that there was little expressional difference in *CaTPS1* between pTRV2-00 and CK plants, but the expression of *CaTPS1* was significantly lower in pTRV2-*CaTPS1* than that in pTRV2-00 and CK plants respectively (Figure 4A). Compared to CK, the expression of *CaTPS1* in the pTRV2-*CaTPS1* plant was reduced by 64.3%, which indicated that the expression of *CaTPS1* was efficiently inhibited in pTRV2-*CaTPS1* plants by VIGS.

After low-temperature treatment, the expression of *CaTPS1* increased first and then decreased with the extension of time and peaked at 36 h (Figure 4B). Generally, the expression of *CaTPS1* was lower at different stages in the pTRV2-*CaTPS1* plant than in pTRV2-00 and CK plants, and they were significantly different among pTRV2-*CaTPS1* and pTRV2-00 plants at 0, 36 and 48 h, except for 12 h.

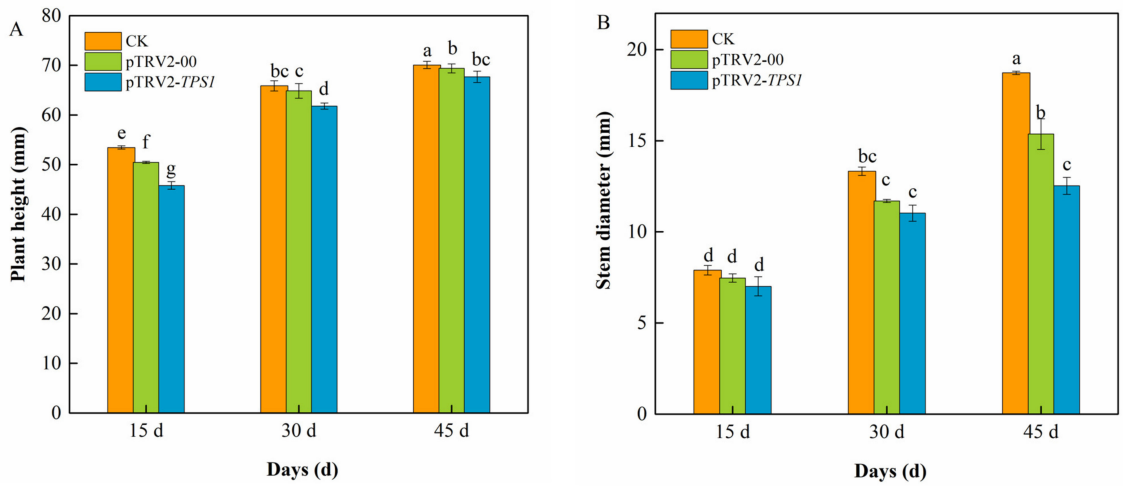

Figure 3. Changes in plant growth after infection. Plant height (**A**), stem diameter (**B**) of CK, pTRV2-00, and pTRV2-*CaTPS1* at 15 d, 30 d, and 45 d after inoculation. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

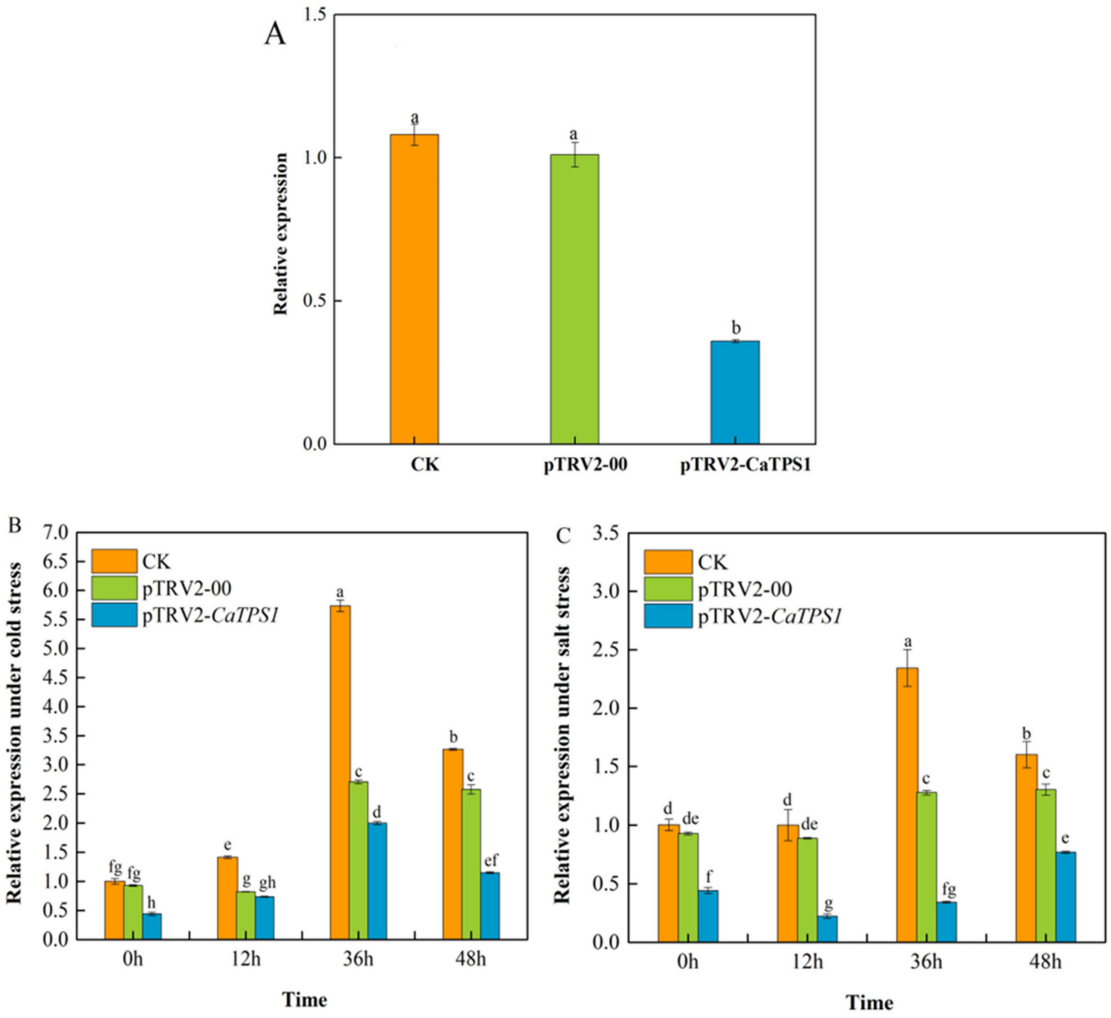

Figure 4. Relative expression of *CaTPS1*. (**A**) Relative expression of *CaTPS1* in leaves from *CaTPS1*-silenced and control pepper plants. Relative expression of *CaTPS1* in leaves from *CaTPS1*-silenced

and control pepper plants after cold (**B**) and salt (**C**) treatments for 0 h, 12 h, 36 h, and 48 h. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

After salt treatment, the expression of *CaTPS1* increased first and then decreased with the extension of time and peaked at 36 h in the CK plant. However, it showed a decrease first and then an increase in the pTRV2-*CaTPS1* plant (Figure 4C). In general, the expression of CaTPS1 was significantly lower at every stage in the pTRV2-*CaTPS1* plant than in the pTRV2-00 and CK plants, respectively.

3.5. Chlorophyll Content of Silencing CaTPS1 under Cold and Salt Stresses

The chlorophyll contents of leaves in CK, pTRV2-00 and pTRV2-*CaTPS1* plants were observed under cold and salt stresses. Whether under untreated or under low temperature and salt stresses, the total chlorophyll content of the pTRV2-*CaTPS1* plant was significantly lower than that of CK and pTRV2-00 plants (Figure 5). Under low temperatures and salt stresses, the total chlorophyll contents were obviously decreased in all samples, especially in the pTRV2-*CaTPS1* plant. It was also shown that the decrements of chlorophyll contents under salt stress were more than that under cold stress in the pTRV2-*CaTPS1* plant (Figure 5).

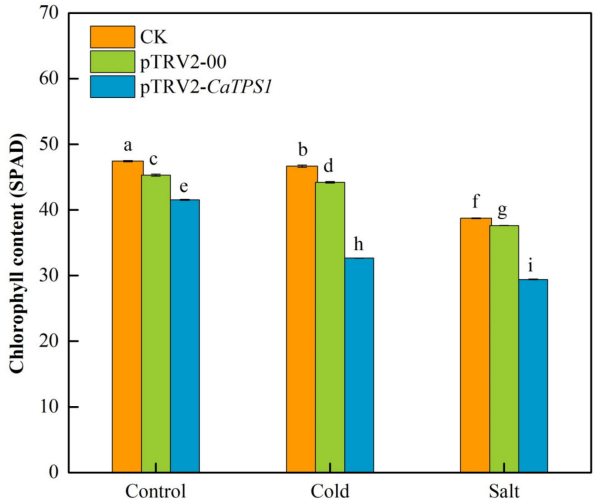

Figure 5. The chlorophyll content under cold and salt stresses. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison tests for each gene was presented. Different letters stand for significant differences between CK, pTRV2-00, and pTRV2-*CaTPS1* plants. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

3.6. Trehalose Content of Silencing CaTPS1 under Cold and Salt Stresses

Compared to the control, the trehalose contents improved significantly in CK, pTRV2-00 and pTRV2-*CaTPS1* plants under cold stress and salt stresses (Figure 6). In addition, the trehalose contents were significantly higher under salt stress than under cold stress. Further, the trehalose content is obviously lower in the pTRV2-*CaTPS1* plant than in CK and pTRV2-00 plants under control, cold and salt stresses.

3.7. Antioxidant Enzymes of Silencing CaTPS1 under Cold and Salt Stresses

The activities of peroxidase (POD) (Figure 7A), catalase (CAT) (Figure 7B), and ascorbate peroxidase (APX) (Figure 7C) were measured in the CK, pTRV2-00 and pTRV2-*CaTPS1* plants under normal, cold and salt stress conditions.

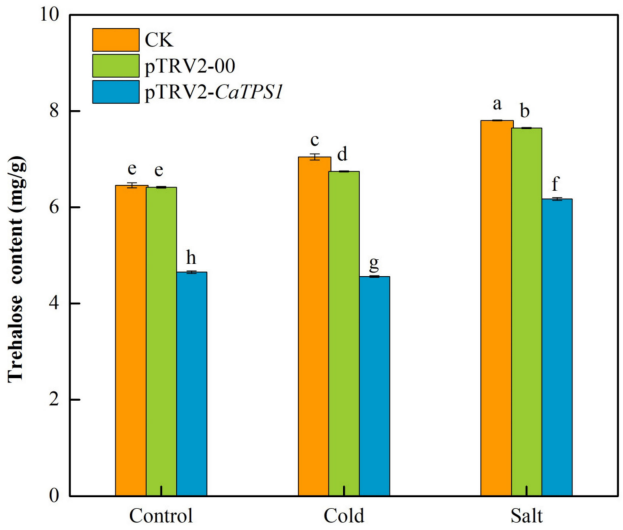

Figure 6. The trehalose content under cold and salt stresses. Each value is shown as the mean ± standard error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

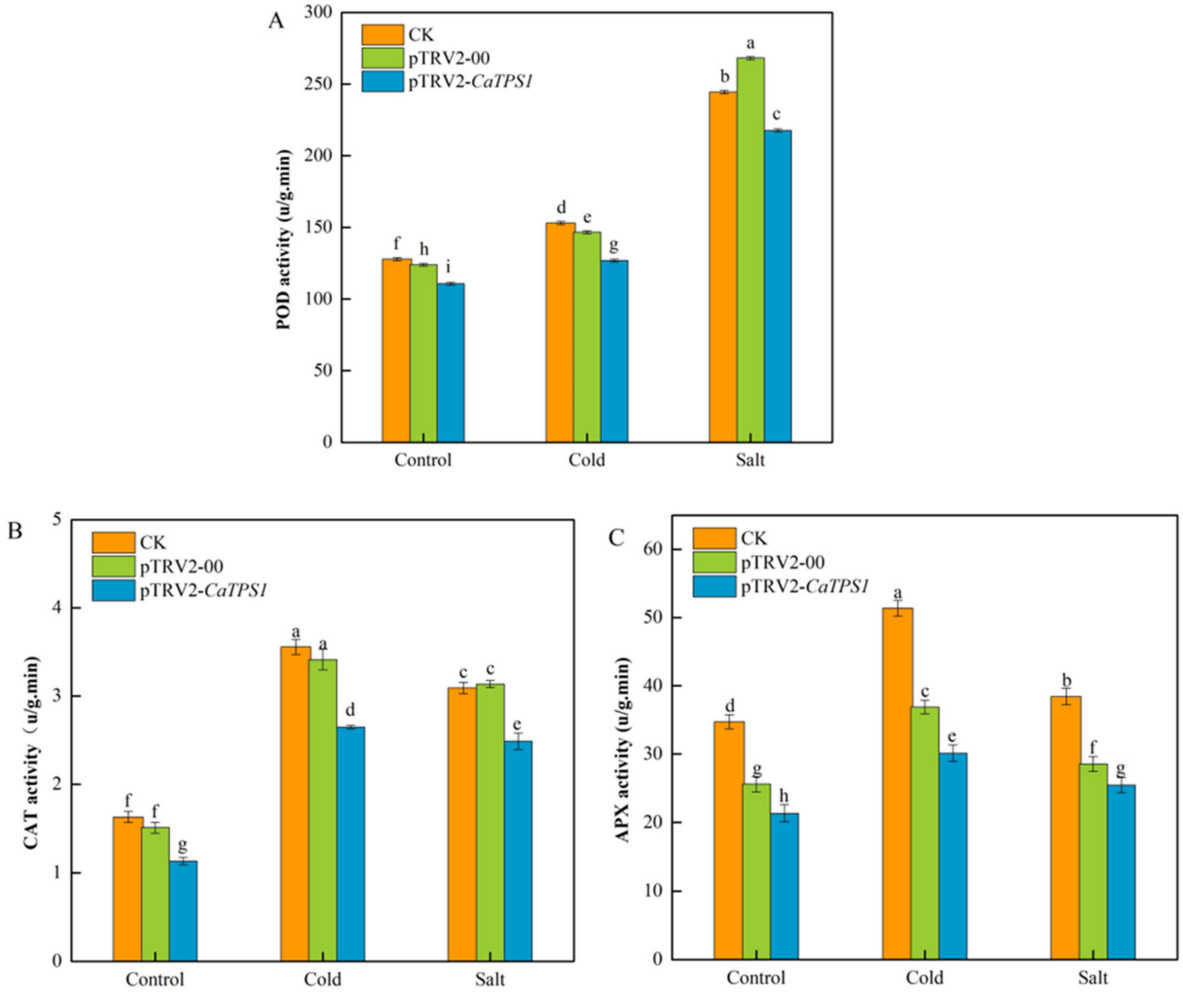

Figure 7. The antioxidant enzymes under cold and salt stresses. POD activity (**A**), CAT activity (**B**), and APX activity (**C**) of CK, pTRV2-00, and pTRV2-*CaTPS1*. Each value is shown as the mean ± standard

error of three biological replicates, and the multiple comparison test for each gene was presented. Different letters stand for significant differences between CK, TRV2-00, and TRV2-*CaTPS1* plants (Duncan's test, $p < 0.05$).

It was indicated that the activities of POD, CAT and APX increased in CK, pTRV2-00 and pTRV2-*CaTPS1* plants under cold and salt stresses. However, the activities of POD, CAT and APX were obviously lower in the pTRV2-*CaTPS1* plant than in CK and pTRV2-00 plants under all treatments. Additionally, it was also suggested that the activities of POD were higher under salt stress than under cold stress. However, the activities of CAT and APX were higher under cold stress than under salt stress.

4. Discussion

Abiotic stress is the key factor plaguing crop production, and studies on tolerance to low temperatures and salt have been a focus of attention [43]. The most sensitive part of the plant to stress is the cell membrane system, which is also the key structure for sensing cold damage and resisting it [44]. At low temperatures and salt stresses, plants are generally damaged, and leaves are prone to premature [45]. Numerous transgenic studies have also demonstrated the involvement of trehalose in the induction of stress-response genes [46,47]. Plant endogenous synthesized trehalose can be involved in signal transduction and induced the expression of stress-related genes [26]. In addition, as a key enzyme in trehalose synthesis, most *TPS* genes are also involved in stress tolerance in plants. Rice resistance to high salinity, drought and cold treatments was increased after overexpression of *OsTPS1* [22]. *CsTPS1*, *CsTPS9* and *CsTPPA* were the most highly expressed in response to at least one abiotic stress and hypothesized that these three genes may be involved in the abiotic stress pathway in tea tree [26]. The high expression of the *TaTPS* gene in winter wheat was induced by freezing stress, indicating that the *TaTPS* gene may participate in the signal pathway of winter wheat coping with freezing damage [25].

In this study, the *CaTPS1* gene was efficiently silenced by VIGS and the expression of *CaTPS1* was reduced by 64.3% in pTRV2-*CaTPS1* compared to the control. While *CaTPS1* was up-regulated first and then down-regulated under cold and salt stresses, the expression of *CaTPS1* in the pTRV2-*CaTPS1* plant was significantly lower than that in CK and pTRV2-00 plants. This result indicates that low temperature and salt stresses can induce the expression of *CaTPS1* to respond to cold and salt stresses in the short term in pepper. There was a higher expression of *CsTPS1*, *CsTPS9* and *CsTPPA* in abiotic stress, which is consistent with the above results [26].

The difference between CK (blank control) and pTRV2-00 (negative control) without injection of TRV2 vectors was mainly to rule out detection errors caused by the viral vector itself. While some indicators in pTRV2-00 were significantly lower than those in CK, pTRV2-*CaTPS1* was also significantly lower than those in pTRV2-00, indicating that TRV2 virus was responsible for these indicators. (Plant height, stem diameter, chlorophyll content, POD, APX enzyme activity) The impact is greater, but the change of each indicator after *CaTPS1* is silenced is not affected.

The chlorophyll content is a key indicator for monitoring plant physiological status, assessing plant health and estimating photosynthetic potential [44]. In this study, chlorophyll content was significantly lower in all samples under low temperature and salt stresses, and chlorophyll content of the pTRV2-*CaTPS1* plant was significantly lower than that of the CK and pTRV2-00 plants, illustrating that *CaTPS1* is a positive response to the effects of abiotic stress on chlorophyll content. Numerous experimental results have shown that chlorophyll content decreases under abiotic stress [48].

Interestingly, trehalose might be a ubiquitous sugar and a protective agency in response to stress in many organisms. Trehalose content also was on the rise under cold and salt stresses, but the trehalose content of the pTRV2-*CaTPS1* plant was significantly lower than the CK and pTRV2-00 plants. On the one hand, abiotic stress could increase trehalose content, on the other hand, trehalose synthesis decreased after *CaTPS1* silencing, resulting

in a decrease in trehalose content, which was related to the changes in the sugar metabolism and were both convergent and divergent in response to different stresses [17,26].

Furthermore, when plants undergo different environmental stresses, the balance between productivity and clearance of ROS in plants is disrupted, which will lead to remaining toxic ROS, the toxicity negatively impacts the growth and development of plants [31]. Endogenous protective enzymes play an important role in protecting against and reversing ROS-induced damage and improving the ability of plants to respond to stresses by altering their activity [31,32]. Antioxidant enzymes are important substances to scavenge ROS, and the change in their content can indirectly measure the degree of damage caused by ROS to plants. CAT, POD and APX can reduce the production rate of superoxide anion radicals and the content of hydrogen peroxide [10,39]. CAT, POD and APX are important antioxidant enzymes and play a crucial role in protecting plant tissues under abiotic stress. In this study, the POD activities, APX activities, and CAT activities of the CK, pTRV2-00, and pTRV2-*CaTPS1* plants increased after suffering from cold and salt stresses, but the values of the pTRV2-*CaTPS1* plant were lower than the CK and pTRV2-00 plants. The culture environment and other conditions of CK, pTRV2-00 and pTRV2-*CaTPS1* plants were consistent. Since the seedlings after VIGS treatment were cultured in a climate box at 22 °C during the day and 18 °C at night, the control group and salt stress may have some low-temperature stress. The slightly lower temperature of the culture environment resulted in mild stress, resulting in significantly higher antioxidant enzyme activity in the CK group than in the pTRV2-*CaTPS1* group under normal conditions (similar to that in the low-temperature stress group). This suggests that low temperature and salt stresses increase antioxidant enzyme activity and that *CaTPS1* is positively regulated to abiotic stress-induced damage. Silencing of *CaDHN4* in salt and low-temperature stresses treatment resulted in a significant increase in the enzymatic activities of antioxidant enzymes such as SOD, POD, CAT and APX activities, which was similar to the results of this experiment [48]. Besides, POD, SOD and CAT activities were raised with the cold and NaCl treatments in wheat, showing that the content of antioxidant enzymes will increase basically under abiotic stress [45].

In conclusion, the expression of *CaTPS1* in CK, pTRV2-00 and pTRV2-*CaTPS1* plants increased first and then decreased with the accumulation of time under low temperature and salt stresses, reaching a maximum at 36 h of stress treatment. Meanwhile, the expression of *CaTPS1* in the pTRV2-*CaTPS1* plant was significantly lower than that in CK and pTRV2-00 plants. In low temperature and salt stresses, chlorophyll content of the pTRV2-*CaTPS1* plant decreased; the trehalose content, POD activity, CAT activity and APX activity of pTRV2-*CaTPS1* plant increased but were significantly lower than those of CK and PTRV2-00 plants. Based on the experimental results obtained above, it can be concluded that silencing of the *CaTPS1* gene made the plants more sensitive to low temperature and salt stresses, indicating that the *CaTPS1* gene plays an important role in plant growth and development and response to abiotic stresses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020319/s1, Figure S1: The main pathway of trehalose metabolism [8,12,13,49,50].

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