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Overexpression of a Thioredoxin-Protein-Encoding Gene, *MsTRX*, from *Medicago sativa* Enhances Salt Tolerance to Transgenic Tobacco

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Abstract: Thioredoxin (TRX) is a small molecule protein that participates in the redox process and plays a decisive role in various functions of plants. However, the role of TRX in *Medicago sativa* (alfalfa), a widely cultivated perennial herb of legume, is still poorly understood. Here, we isolated *MsTRX* from alfalfa and determined the characteristics in improving salt tolerance by assaying the phenotype and physiological changes and the expression of stress-response genes in transgenic tobacco. The expression of *MsTRX* was similar in alfalfa roots, leaves, and inflorescences, and was downregulated in response to cold, drought, and salt treatment. The overexpression of *MsTRX* in tobacco promoted the accumulation of soluble sugar (SS) and proline; enhanced the activity of peroxidase (POD); and induced the upregulation of beta-amylase 1 (*BAM1*), lipid-transfer protein 1 (*LTP1*), candidate signal molecules/sensor relay proteins (*CBSX3*), superoxide dismutase [Cu-Zn] (*Cu/Zn-SOD*), superoxide dismutase [Mn] (*Mn-SOD*), protein gamma response 1 (*GR1*), dehydrin DHN1-like (*ERD10B*), and serine/threonine-protein kinase (*SnRK2*), as well as the downregulation of phyB activation-tagged suppressor1 (*BAS1*) and serine/threonine-protein kinase that phosphorylates LHCII protein 7 (*STN7*) under salt stress. These results indicated that *MsTRX* improves salt tolerance via maintaining osmotic homeostasis, scavenging reactive oxygen species (ROS), and regulating the transcription of stress-response genes in plants. In our study, we provided a new understanding of how *MsTRX* improves salt stress in plants and how *MsTRX* can be included in future breeding programs to improve salt tolerance in alfalfa.

Keywords: alfalfa; thioredoxin; salt stress; phenotype assay; physiological change; stress-response genes

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1. Introduction

Soil salinization is one of the main abiotic stresses that causes global crop yields and quality to decline. The salinization of the soil makes it difficult for plant roots to absorb water and even unable to grow [1]. The increase in Na^+ under salt stress can cause the ionic imbalance in plants, which hinders the transportation and absorption of K^+, Ca^{2+}, and Mg^{2+}, leading to the deficiency of HPO_4^{2-} [2]. Under salt stress, many metabolisms in plants are disordered, and free radical metabolisms are unbalanced, accompanied by the production of reactive oxygen species (ROS) [3]. The excessive accumulation of ROS in plant cells could induce the subcellular structure damage and membrane lipid oxidation and aggravate

the accumulation of toxic substances that intermediated nitrogen metabolism, including ammonia, isoleucine, ornithine, and arginine [4,5]. Therefore, we should pay much more attention to the improvement, rational development, and utilization of salinization land.

Thioredoxin (TRX) is a small molecule multifunctional protein with a catalytically active disulfide bond and an essential protein in regulating sulfhydryl redox [6]. In plants, six types of TRX (h, o, f, m, x, y) have been reported. TRXf, TRXm, TRXh, and TRXy are chloroplastic proteins, and TRXf and TRXm are involved in the regulation of photosynthesis [7]. They all play an essential role in hormone synthesis, enzymatic activation, translation, metabolism, and abiotic stress response [8]. The differential expression of *ThTrx* genes was observed in response to sodium chloride (NaCl) stress in *Tamarix hispida* [9]. *TRXh* has been reported to improve salt tolerance in *Nicotiana tabacum* (tobacco) [10]. The transcription of *OsTRXh1* in *Oryza sativa* (rice) was induced by salt, and the accumulation of ROS in the cytoplasmic exosomes of *OsTRXh1* overexpressed plants was similar to that of wild-type (WT) plants under salt treatment, while the apoplast of *OsTRXh1 RNAi* plants contains more ROS [11]. The expression level of *TRX* increased in both salt-tolerant and salt-sensitive soybean varieties under salt stress, but the upregulated level in salt-tolerant varieties was more significant [12]. The comparative analysis of *Medicago sativa* (alfalfa) leaf transcriptome under salt stress revealed that the expression of *TRX* involved in ROS scavenging and the expression level in salt-tolerant variety (*M. sativa* cv. Zhongmu No. 1) was much higher than in salt-sensitive variety (*M. sativa* cv. Xinjiang Daye) [13]. The NADP-thioredoxin reductases (NTR) with a distinctive extension of the TRX domain (NTRC) can interact with TRX to downregulate the expression of phyB-activation-tagged suppressor 1 (BAS1) to scavenge different peroxides produced by stress in *M. truncatula* [14].

Alfalfa is a perennial herb of the leguminous and is widely planted worldwide for its high crude protein content required for the development of animal husbandry [15]. However, the good quality and yield of alfalfa are affected by adverse environmental conditions, such as salt, drought, and cold stress. Salinity, irrigation, and low temperature are the main limiting factors in alfalfa growth and production in northern China. Although alfalfa is considered a moderately salt-tolerant plant, planting alfalfa can reduce the salt content and improve the soil's physical and chemical properties [16]. Therefore, a comprehensive study and understanding of the salt-tolerant genes of alfalfa are helpful for the molecular breeding of alfalfa with salt tolerance. In this paper, we aimed to investigate the positive role of the *MsTRX* gene in improving salt tolerance via assaying the phenotype and physiological changes and the expression of stress-response genes in transgenic tobacco.

2. Materials and Methods

2.1. Plant Growth Conditions and Treatments

Five hundred seeds of alfalfa (*M. sativa* L. cv. Longmu 801) were sterilized with 5% sodium hypochlorite (NaClO) solution for 5 min and 70% ethyl alcohol solution for 30 s. The sterilized seeds were rinsed four to five times with ultra-pure water. Then, the seeds were placed evenly in Petri dishes containing moist filter paper and transferred into a growth chamber at 25 °C without light. The germinated alfalfa seeds at five days old were transplanted into plastic pots filled with vermiculite in a greenhouse and watered with 1/2 strength Hoagland nutrient solution every two days [17]. Each pot contained one seedling, and 400 pots were used. The temperature of the cultivation environment was 25 °C, and the relative humidity was 55% during the day; the temperature at night was 20 °C and the relative humidity was 70%. Four weeks after transplanting, the healthy and uniform size seedlings were selected and divided into three groups and subjected to cold, drought, and salt stress treatment for 0 (control), 3, 6, 12, 24, and 48 h. In the cold treatment, alfalfa plants were moved into a growth chamber at 4 °C. In the treatment of drought and salt, the plants were transferred into a nutrient solution with 20% polyethylene glycol 6000 (PEG-6000) and 150 mM NaCl, respectively. All the roots and leaves were harvested and frozen in liquid nitrogen immediately and stored at −80 °C to evaluate the expression of *MsTRX* in response to abiotic stresses. Each treatment was sampled with three biological repeats, and

one biological repeat contained four alfalfa seedlings. For tissue-specific expression analysis of *MsTRX*, the roots, leaves, and inflorescence of 6-week-old seedlings were sampled. All the assays were repeated three times with three biological repeats.

2.2. Isolation and Cloning of the MsTRX Gene

The untreated alfalfa seedlings were used to extract plant total RNA with the Ultrapure RNA Kit (CWBIO, Beijing, China). The integrity and concentration of total RNA were assessed via NanoPhotometer (Implen, Munich, Germany). The complementary DNA (cDNA) was synthesized using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). The RNA (1 µg) mixed with RNA-free water (up to 12 µL) was incubated at 65 °C for 5 min and cooled on ice for 2 min. The genomic DNA was removed by appending 4 µL 4 × gDNA wiper mix and incubated at 42 °C for 2 min. Finally, the reaction solution was mixed with 2 µL 10 × RT mix 2 µL HiScript II Enzyme MiX, and the reverse transcription reaction was conducted at 25 °C for 5 min, 50 °C for 45 min, and 85 °C for 2 min. The coding DNA sequence (CDS) of *MsTRX* was isolated via homologous cloning. The degenerate primers (Table S1) were designed according to the sequence of *MtTRX* from *M. truncatula*, which is the model plant of legume, via Primer Express 5.0 software (Applied Biosystems, Carlsbad, CA, USA) using a 2×Unique^{TM} Taq Master Mix (with Dye) (Novogene, Beijing, China). The polymerase chain reaction (PCR) product was subsequently cloned and inserted into the PCE2 TA/Blunt-Zero-T vector (Vazyme, Nanjing, China) for sequencing.

2.3. Sequence Analysis of MsTRX

The National Center for Biotechnology Information (NCBI) open reading frame (ORF) was used to predict the ORFs (http://www.bioinformatics.org/sms2/orf_find accessed on 25 June 2020). SMART (http://smart.embl.de/smart accessed on 25 June 2020) was used to analyze the conserved motif, and ProtParam (http://web.expasy.org/Protparam accessed on 25 June 2020) was used to analyze the physicochemical properties. NCBI BLAST was used to identify TRX homologs by comparing TRX sequences from other plants. MEGA software (version 7.0.21) was used to construct the phylogenetic tree using the neighbor-joining method. SOPMA (http://pbil.ibcp.fr accessed on 25 June 2020) was used to predict the secondary structure of the MsTRX protein, and SWISS-MODEL provided the 3D structure (https://swissmodel.eu accessed on 25 June 2020).

2.4. Tissue-Specific and Stress-Response Expression of MsTRX

A quantitative real-time PCR (q-PCR) assay was conducted to study the tissue-specific expression of the *MsTRX* and its response to cold, drought, and salt stress. The RNA was extracted from each harvested alfalfa tissue and reverse transcribed into cDNA using the HiScript® II Q RT SuperMix for q-PCR (+gDNA wiper) Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. The q-PCR primers for *MsTRX* (Table S1) were designed using Premier 5.0 on the basis of the CDS of *MsTRX*. The alfalfa *β-actin* (*MsActin*) gene (Table S1) was used as a reference gene. The q-PCR assays were conducted using a Quantagene q225 Real-Time PCR apparatus (Novogene, Beijing, China) and ChamQTM Universal SYBR® q-PCR Master Mix (Vazyme, Nanjing, China). The reaction solution included 1 µL cDNA, 1 µL Primer F, 1 µL Primer R, 10 µL 2 × Cham Q Universal SYBR q-PCR Master Mix, and 7 µL dd H_2O. The reaction conditions were as follows: predenaturation at 95 °C for 30 s, followed by 40 cycles of cyclic reaction at 95 °C for 10 s; 58 °C for 30 s; and a dissociation curve at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative expression levels of *MsTRX* [18]. This experiment consisted of three biological replications and three replicate reactions for each sample.

2.5. Plant Transformation and Generation of Transgenic Plants

In order to construct the pCAMBIA1300-35S::MsTRX expression vector, the pCAMBIA 1300-35S-sGFP vector was digested with PstI and BamH1. The *Agrobacterium tumefaciens* strain EHA105 was inoculated with the recombinant vector and transformed into wild-type (WT) tobacco by agroinfiltration [19]. Twenty independent lines were selected on the basis of resistance to hygromycin (Hyg). Hygromycin was added to the germination medium at 20 mg/L for screening, and gene-specific primers for Hyg (Table S1) were used to identify the positive transgenic plants by PCR prior to sampling. Further confirmation of homozygous transformants (T2) was performed by q-PCR using primers for *MsTRX*, while the tobacco *Actin* gene (*NtActin*, Table S1) was used as an internal reference. Further stress tolerance tests and expression analysis were conducted using T_2-generation homozygous lines, including T-2, T-3, and T-5, along with WT tobacco plants.

2.6. Stress Tolerance Tests

A total of 400 seeds of the transgenic lines and WT plants were grown on 1/2-strength MS medium plates for a two-day incubation period at 4 °C. Following germination, the seedlings were transplanted into vermiculite-filled plastic pots inside a 25 °C growth chamber. Each pot contained one seedling, and 200 pots were used. Twenty-one days after transplanting, the healthy and uniform size seedlings were selected and used for stress tolerance assays. In the cold tolerance test, the plants were grown in a growth chamber at 4 °C for 7 days. For drought and salt tolerance tests, the plants were watered for 7 days with a solution containing 20% PEG-6000 or 150 mM NaCl, respectively. Leaf samples were harvested immediately, frozen in liquid nitrogen, and stored at 80 °C to examine physiological and expression changes. Three biological repeats were taken from each transgenic tobacco line and WT plant, and each biological repeat contained three tobacco seedlings.

2.7. Measurement of Physiological Changes

The absorbance of WT and transgenic tobacco lines was observed using a spectrophotometer to assess the physiological changes. The accumulation of malondialdehyde (MDA) was measured by a modified thiobarbituric acid (TBA) method [20]. The Bradford method was used to determine the soluble protein (SP) content [21]. The Dreywood method was used to determine the soluble sugar (SS) content [22]. The free proline content was determined according to the ninhydrin method [23]. The $O_2^{\cdot -}$ content was determined on the basis of the changes in absorption values at 530 nm [24]. The content of reduced glutathione (GSH), a non-enzyme antioxidant, was fluorometrically estimated [25]. The activity of superoxide dismutase (SOD) and peroxidase (POD) were determined using the nitroblue tetrazolium (NBT) and guaiacol methods, respectively [26]. All the assays were repeated three times with three biological repeats, and one biological repeat contained three tobacco seedlings.

2.8. Expression Analysis of Stress-Response Genes

The expression levels of 10 stress-response genes were analyzed further to illustrate the role of *MsTRX* in stress tolerance. These 10 genes, namely, beta-amylase 1 (*BAM1*), phyB activation-tagged suppressor1 (*BAS1*), candidate signal molecules/sensor relay proteins (*CBSX3*), superoxide dismutase [Cu-Zn] (*Cu/Zn-SOD*), superoxide dismutase [Mn] (*Mn-SOD*), dehydrin DHN1-like (*ERD10B*), protein gamma response 1 (*GR1*), lipid-transfer protein 1 (*LTP1*), serine/threonine-protein kinase (*SnRK2*), and serine/threonine-protein kinase that phosphorylates LHCII protein 7 (*STN7*), were associated with the physiological changes and played important roles in stress tolerance. *NtActin* was used as the internal reference gene in this experiment, and the primers were summarized and listed in Table S1.

2.9. Statistical Analysis

In this study, all the experiments were evaluated three times with three biological replicates. The statistical analysis was conducted with SPSS 22.0 software, which used Student's *t*-test and one-way analysis of variance (ANOVA) to find significant differences at $\alpha = 0.05$.

3. Results

3.1. Isolation and Characteristics of MsTRX

The complete CDS of *MsTRX* was 363 bp (Genbank accession: MG197809), which encoded a protein of 120 amino acids (Figure S1). The relative molecular weight was 13.35 kD, and the theoretical isoelectric point was 4.99. The average hydrophilicity coefficient was −0.160, with a predicted stability coefficient of 23.06, which indicated that *MsTRX* is a stable and hydrophilic protein. The comparison of *MsTRX* amino acid sequences with seven other homologs indicated that the TRX functional domain was highly conserved in *MsTRX* (Figure S1). *MsTRX* was found to be most homologous with *MtTRX* (98.35%) in the phylogenetic tree constructed by neighbor-joining and was situated on an adjacent branch to MtTRX (Figure S2). As predicted by the three-dimensional structure of the *MsTRX* protein, the *MsTRX* protein contains 48.33% helices, 10.83% turns, 20.01% extended strands, and 20.83% random coils (Figure S2), which was consistent with the protein secondary structure prediction by SWISS-MODEL.

3.2. Expression Patterns of MsTRX

The expression patterns of *MsTRX* in various alfalfa tissues and their responses to cold, drought, and salt stress were investigated using q-PCR analysis. There was no significant difference in the expression of *MsTRX* in alfalfa roots, leaves, and inflorescences (Figure 1A). The expression level of *MsTRX* in alfalfa gradually decreased under cold stress and reached the minimum level at 12 h (Figure 1B). Under drought stress, the expression of *MsTRX* decreased rapidly in alfalfa leaves and roots (Figure 1C). The lowest expression level of *MsTRX* in alfalfa leaves and roots was at 3 h, which changed 0.26-fold and 0.70-fold, respectively. The expression level of *MsTRX* declined with fluctuation in both alfalfa leaves and roots under salt stress (Figure 1D). Herein, the expression of *MsTRX* in alfalfa was not tissue-specific, and its transcription level was downregulated under cold, drought, and salt stresses, which indicated that *MsTRX* might play a role in the climate adaptation of alfalfa.

3.3. Stress Tolerance Tests of Transgenic Tobacco

The recombinant vector pCAMBIA1300-35S::MsTRX was used to overexpress *MsTRX* to investigate the abiotic stress tolerance of MsTRX in transgenic tobacco (Figure 2A). The T_2 generation of transgenic tobacco plants was generated and confirmed by q-PCR analysis. The expression of *MsTRX* in transgenic tobacco T02, T03, and T05 lines was visibly higher than that in the WT plants (Figure 2B). Finally, the transgenic tobacco grew better than the WT plant under control condition and was more tolerant of cold, drought, and salt treatments (Figure 2C). In particular, only a few leaves were yellow in transgenic tobacco under salt treatment.

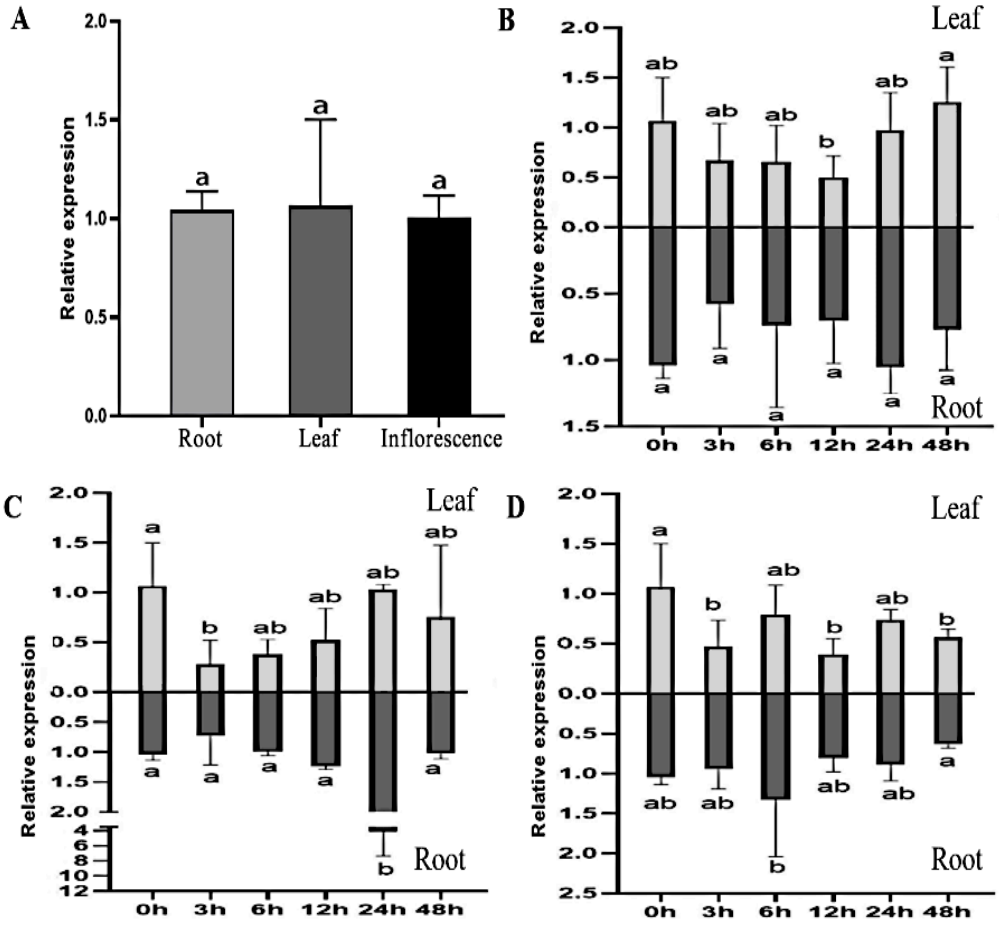

Figure 1. Expression patterns of *MsTRX*. (**A**) Expression of *MsTRX* in alfalfa roots, leaves, and inflorescences. (**B**) Expression of *MsTRX* in alfalfa root and leaf under cold stress. (**C**) Expression pattern of *MsTRX* in alfalfa root and leaf under drought stress. (**D**) Expression of *MsTRX* in alfalfa root and leaf under salt stress. Alfalfa seedlings at 6 weeks old were sampled for tissue-specific expression. *MsActin* was used as the internal reference. The error bars represented the means ± SDs of three independent biological replicates. The different lowercase represented significant differences ($\alpha = 0.05$).

3.4. Physiological Varieties in Overexpressed MsTRX Tobacco

The physiological varieties in transgenic tobacco and WT plants under cold, drought, and salt treatments were investigated further to analyze the role of *MsTRX* in salt stress resistance. The content of MDA changed differently in transgenic lines under cold, drought, and salt stress (Figure 3A). The content of SP changed slightly under abiotic stresses, and the differences we observed among WT and transgenic plants were associated with the expression level of *MsTRX* in transgenic lines (Figure 3B). The content of SS and proline increased sharply in transgenic tobacco under salt stress (Figure 3C,D). There was no significant change in the content of $O_2^{\cdot -}$ in overexpressed *MsTRX* tobacco under salt stresses (Figure 3E). Interestingly, the GSH content and the SOD activity in line T02 were significantly lower than that in WT plants under salt stress (Figure 3F,G). Salt stress resulted in higher POD activity in overexpressed *MsTRX* tobacco than in WT plants (Figure 3H).

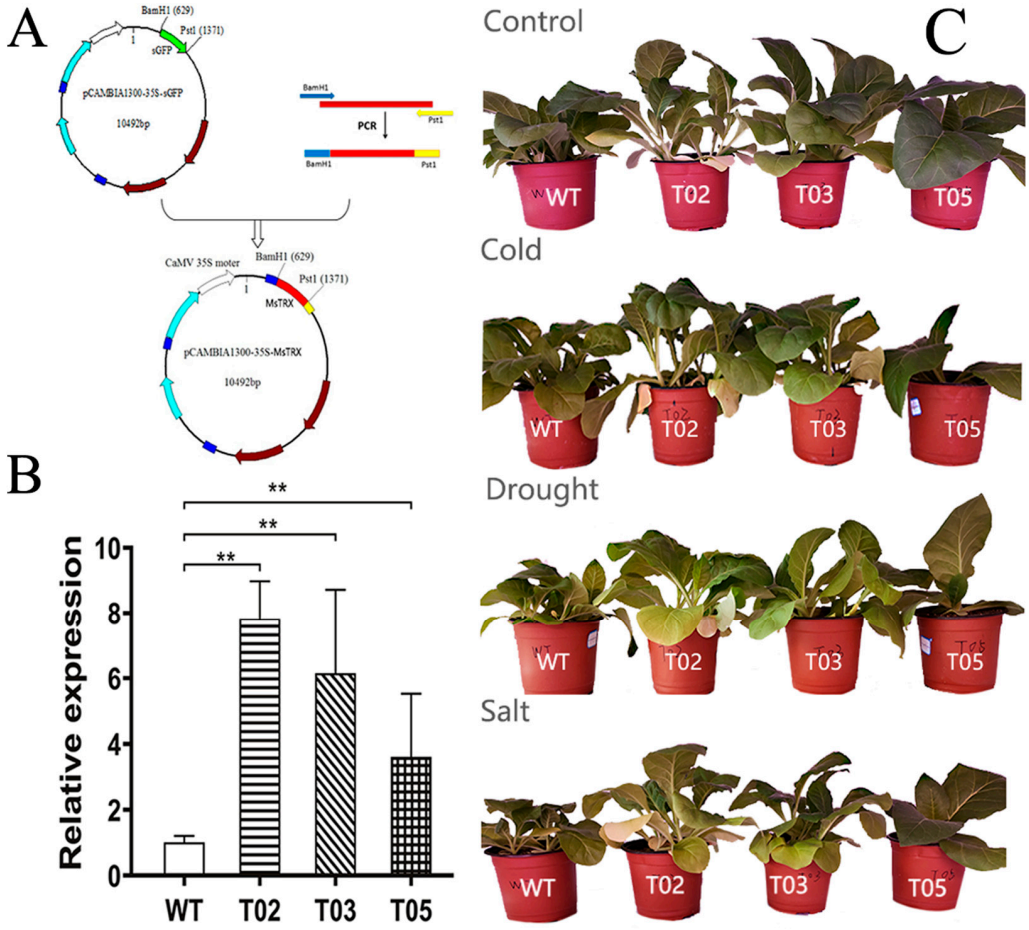

Figure 2. Stress tolerance tests of transgenic tobacco overexpressing *MsTRX*. (**A**) The illustration of pCAMBIA1300-35S::*MsTRX* recombinant vector. (**B**) Expression of *MsTRX* in the T2 generation of transgenic tobacco and WT plants. (**C**) The phenotypes of overexpressed *MsTRX* tobacco and WT plants under control and cold, drought, and salt treatments. The transgenic tobacco and WT plants at 4 weeks old were treated with 4 °C, 20% PEG-6000, and 150 mM NaCl, individually. WT: wild type. T02: line 2. T03: line 3. T05: line 5. **, $p < 0.01$.

3.5. Expression Analysis of Stress-Response Genes in Overexpressed MsTRX Tobacco under Salt Stress

To further illustrate the possible regulatory mechanism of *MsTRX* in salt tolerance, we investigated the expression level of 10 stress-response genes, namely, *BAM1*, *BAS1*, *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *ERD10B*, *GR1*, *LTP1*, *SnRK2*, and *STN7*, in transgenic tobacco and WT plants treated with 150 mM NaCl. As compared to WT plants, transgenic tobacco showed significantly lower transcription levels for *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *ERD10B*, *GR1*, *LTP1*, and *SnRK2*. The expression levels of *BAS1* and *STN7* in line T05 were markedly higher than those in WT plants (Figure 4). Despite the upregulation of BAM1 and LTP1 in transgenic tobacco plants under salt stress, no significant differences were found between transgenic lines and WT plants. Salt stress upregulated the expression of *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *GR1*, *ERD10B*, and *SnRK2*, but the expression levels of *Cu/Zn-SOD*, *Mn-SOD*, and *GR1* were much lower in transgenic tobacco plants. Meanwhile, the expression levels of *ERD10B* and *SnRK2* in line T03 were higher than that in WT plants. The expression of both *BAS1* and *STN7* was downregulated in transgenic tobacco under salt stress, but the expression level of *BAS1* was significantly lower than that in WT plants. Conversely, the expression level of *STN7* was higher than that in WT plants.

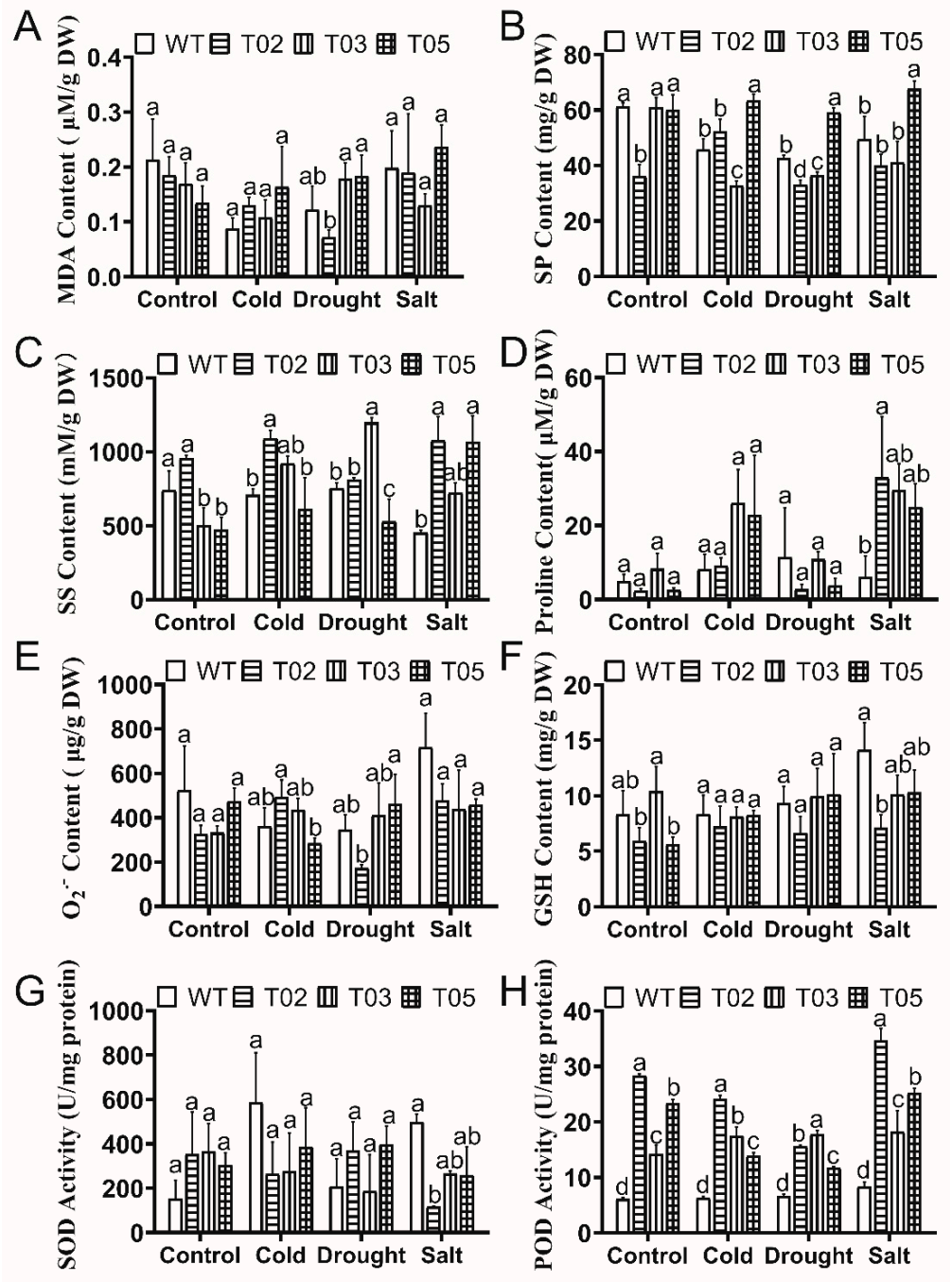

Figure 3. Physiological varieties in overexpressed *MsTRX* tobacco overexpressing under cold, drought, and salt treatments. (**A**) Content of malondialdehyde (MDA). (**B**) Content of soluble protein (SP). (**C**) Content of soluble sugar (SS). (**D**) Content of proline. (**E**) Content of superoxide anion ($O_2^{\cdot-}$). (**F**) Content of reduced glutathione (GSH). (**G**) Activity of superoxide dismutase (SOD). (**H**) Activity of peroxidase (POD). WT: wild type. T02: line 2. T03: line 3. T05: line 5. The error bars represented the means ± SDs of three independent biological replicates. The different lowercase represented significant differences ($\alpha = 0.05$).

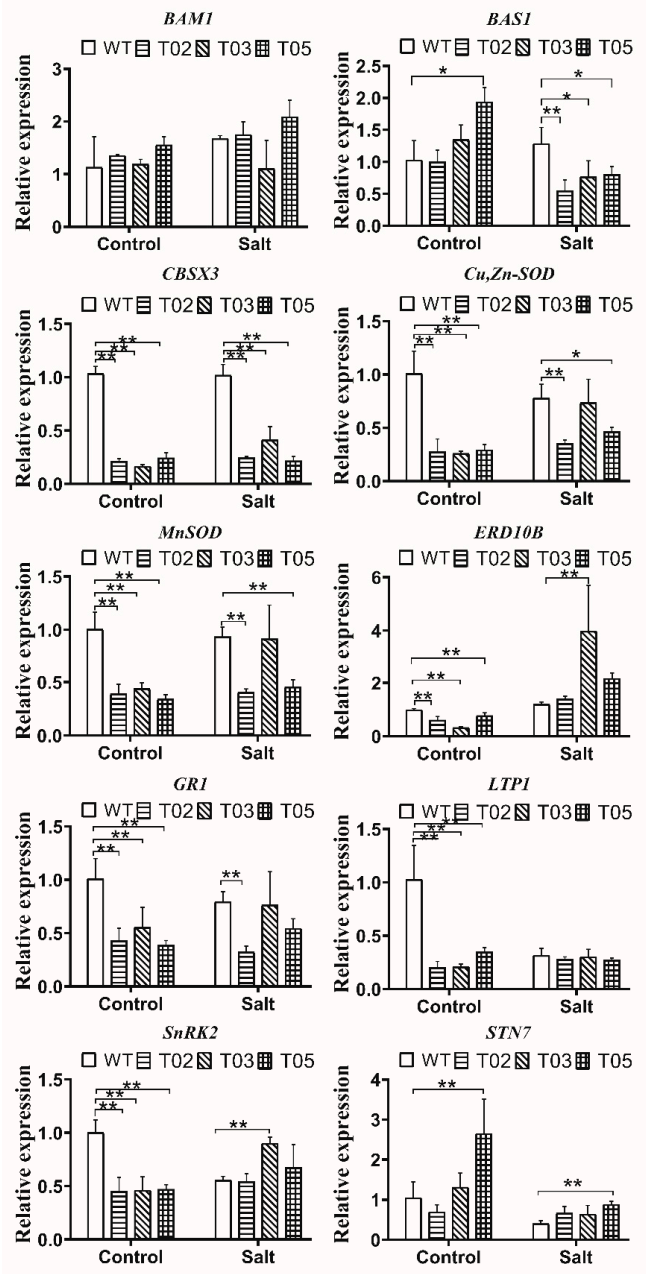

Figure 4. Expression pattern of stress-response genes in overexpressed *MsTRX* tobacco under salt stress. *BAM1*: beta-amylase 1. *BAS1*: phyB activation-tagged suppressor 1. *CBSX3*: candidate signal molecules/sensor relay proteins. *Cu/Zn-SOD*: superoxide dismutase [Cu-Zn]. *Mn-SOD*: superoxide dismutase [Mn]. *ERD10B*: dehydrin DHN1-like. *GR1*: protein gamma response 1. *LTP1*: lipid-transfer protein 1. *SnRK2*: serine/threonine-protein kinase. *STN7*: serine/threonine-protein kinase that phosphorylates LHCII protein 7. WT: wild type. T02: line 2. T03: line 3. T05: line 5. The error bars represent the means ± SDs of three independent biological replicates. *, $p < 0.05$. **, $p < 0.01$.

4. Discussion

Plants are inevitably subjected to various abiotic stresses in the natural environment, such as drought, extreme temperatures, salinity, heavy metals, and ultraviolet radiation [27]. Salinity, drought, and low temperature are the main environmental factors in northern China. Salt stress can cause the disruption of osmotic homeostasis, accumulation of ROS and toxic ions, and cellulator abnormalities, as well as severely limiting the yields and quality of crops [28]. Although *TRX* genes have been implicated in abiotic stress regulation in plants such as *Glycine max* (soybean), *Gossypium spp* (cotton), *Zea mays* (corn), and

others, there have been few studies on *TRX* genes in alfalfa [12,29–32]. In this study, we cloned the CDS sequence of *TRX* from alfalfa and named it *MsTRX*. The protein homology analysis indicated that *MsTRX* belonged to the *TRX* family and contained a highly conserved WC(G/P)PC motif, which was the same as previous studies [33]. The expression level of *MsTRX* in alfalfa leaf was the highest, and the expression level in root and inflorescence also maintained a high level (Figure 1). The results were consistent with the expression of *TRX* in *Arabidopsis* and demonstrated the critical role of TRX in alfalfa growth and development [34]. Stresses such as cold, drought, and salt significantly altered the expression level of *MsTRX*. It was the same as the expression of *AtTRX* and *GmTRXh1* under abiotic stress and indicated that *MsTRX* was involved in the response of alfalfa to abiotic stress [35].

To further characterize the function of *MsTRX* under abiotic stress, we transferred *MsTRX* into tobacco. The observed phenotype changes showed that transgenic tobacco overexpressing *MsTRX* grew better than WT plants and was more tolerant of cold, drought, and salt treatments (Figure 2). Physiological changes were also measured to reflect the capacity of plants to cope with stressful environments. The content of MDA is an important indicator of membrane lipid peroxidation and membrane damage [36]. Free proline, SS, and SP play a crucial role in regulating the osmotic homeostasis and enhancing plant tolerance to abiotic stress [37]. In this study, the content of MDA changed slightly under cold, drought, and salt stress (Figure 3). The significant aggregation in the content of SP and SS varied from plant to plant in transgenic tobacco under abiotic stress. The content of proline only increased significantly in T02 lines under salt stress. These results indicated that the overexpression of *MsTRX* can participate in the osmotic regulation of tobacco plants in response to cold, drought, and stress. Excessive ROS can cause oxidative destruction of cells and tissues and even plant death [38]. Plants have developed antioxidant defense systems that include antioxidant enzymes such as SOD and POD and the non-enzymatic system such as GSH that assists in coping with oxidative damage under abiotic stress [39–41]. Here, all the transgenic lines under salt stress had lower $O_2^{\cdot -}$ content than WT plants, which may be related to the sharply enhanced POD activity in these lines. Therefore, the overexpression of *MsTRX* can intensify the acculturation of SS and proline, and the activity of POD to maintain a balance in osmotic and oxidation to improve abiotic stress tolerance of transgenic tobacco.

Due to the obvious changes in phenotype and physiological indicators in salt-treated transgenic tobacco, the expression of stress-response genes was investigated in overexpressed *MsTRX* tobacco under salt stress to succeed in providing evidence of the role of *MsTRX* in salt tolerance of plants. *BAM1* plays a role in starch degradation in guard cells to keep the stomata open during the day [42]. *LTP1* encodes a lipid transfer protein that can improve stress resistance by regulating the structure of cell membranes [43]. We found that transgenic tobacco was upregulated in *BAM1* and *LTP1*, but the levels of expression of the two genes were not different. These results showed there was a slight role of *MsTRX* in preventing membrane damage in transgenic tobacco under salt stress, as indicated by the change in MDA content. *CBSX3* activates the o-type thioredoxin (*TRX-o2*) in the mitochondria and regulates plant active oxygen homeostasis depending on the ADP/ATP ratio [44]. *Cu/Zn-SOD* and *Mn-SOD* are two subtypes of SOD, an antioxidant enzyme in scavenging oxygen free radicals [45]. NADPH-dependent glutathione reductase (*GR*), a member of the FAD-binding disulfide bond reductase superfamily, acts as a scavenger of active oxygen and a stabilizer of proteins and membranes to relieve oxidative stress and supplements the NTR function in plants' abiotic stress tolerance [46]. Plants regulate the production of dehydrin via *ERD10B*, which protects various nuclear and cytoplasmic macromolecules from coagulation during dehydration [47]. ABA signaling pathway member *SnRK2* was upregulated by salt stress in plants, and salt stress can upregulate ABA signaling pathway member *SnRK2* [48]. *Arabidopsis* plants that express *TaSnRK2.3* from *Triticum aestivum* (common wheat) can be more resistant to salt stress [49]. In this study, the transcription level of *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *GR1*, *ERD10B*, and *SnRK2* were

all upregulated in transgenic tobacco under salt stress. Despite that, the expression level of *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, and *GR1* were lower in transgenic tobacco than those of the WT plants, and the expression levels of *ERD10B* and *SnRK2* in line T03 were higher than those of the WT plants. In addition to the changes in the content of $O_2^{\cdot -}$ and GSH, as well as SOD and POD activities, we found that the overexpression of MsTRX leads to the upregulation of genes involved in the process of NTRC and ROS scavenging to maintain oxidative balance and improve the salt tolerance of plants. As a key component of antioxidant defense in photosynthetic metabolism, *BAS1* plays a key role in the pathway of *TRX* [50]. The ferredoxin–thioredoxin system regulates protein kinase *STN7* redox state and the plastoquinone pool [51]. The antioxidant protein kinase *STN7* was inhibited in tobacco chloroplasts by overexpressing *TRXm* [52]. In this study, the expression of *BAS1* and *STN7* in transgenic tobacco under salt stress was downregulated, indicating the specific role of *MsTRX* in ROS scavenging, such as the sharp aggravation of POD activity.

Therefore, we hypothesized that *MsTRX* might be involved in a complex regulatory network that affects physiological changes and gene expression in plants (Figure 5). The overexpression of MsTRX produced an increase in SS and proline accumulation to maintain osmotic homeostasis, as well as an increase in POD activity to scavenge ROS, and induced the upregulation of *BAM1*, *LTP1*, *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *GR1*, *ERD10B* and *SnRK2*, as well as the downregulation of *BAS1* and *STN7* under salt stress.

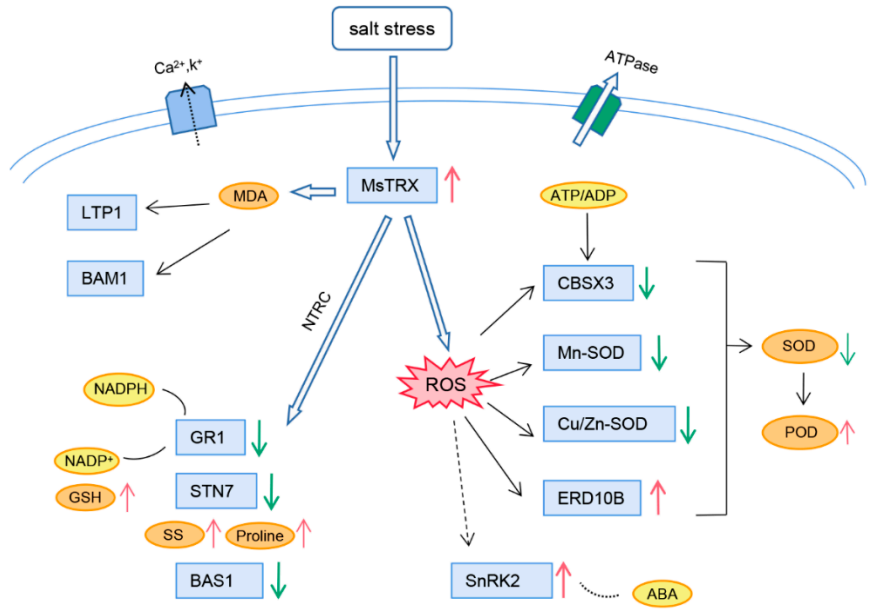

Figure 5. Possible molecular mechanisms of *MsTRX* in response to salt stress. ABA: abscisic acid. ADP: adenosine diphosphate. ATP: adenosine triphosphate. BAM1: beta-amylase 1. BAS1: phyB activation-tagged suppressor 1. CBSX3: candidate signal molecules/sensor relay proteins. Cu/Zn-SOD: superoxide dismutase [Cu-Zn]. ERD10B: dehydrin DHN1-like. GR1: protein gamma response 1. GSH: reduced glutathione. LTP1: lipid-transfer protein 1. MDA: malondialdehyde. Mn-SOD: superoxide dismutase [Mn]. NADP: nicotinamide adenine dinucleotide phosphate. NADPH: nicotinamide adenine dinucleotide phosphate. NTRC: NADP-thioredoxin reductases (NTR) with a distinctive extension of the TRX domain. POD: peroxidase. ROS: reactive oxygen species. SnRK2: serine/threonine-protein kinase. SOD: superoxide dismutase. SS: soluble sugar. STN7: serine/threonine-protein kinase that phosphorylates LHCII protein 7. The red arrow indicates the upregulation of stress-response genes or the increase in physiological indicators. The green arrow indicates the downregulation of stress-response genes or the decrease in physiological indicators. Dotted lines indicate the possible adjustment mechanisms.

5. Conclusions

Taken together, we isolated *MsTRX*, a thioredoxin protein-encoding gene, from alfalfa and determined the characteristics in improving salt tolerance by assaying the phenotype and physiological changes and the expression of stress-response genes in transgenic tobacco. The expression of *MsTRX* was similar in alfalfa roots, leaves, and inflorescences, and was downregulated in response to cold, drought, and salt treatment. The overexpression of *MsTRX* in tobacco promoted the accumulation of SS and proline; enhanced POD activity; and induced the upregulation of *BAM1*, *LTP1*, *CBSX3*, *Cu/Zn-SOD*, *Mn-SOD*, *GR1*, *ERD10B*, and *SnRK2*, as well as the downregulation of *BAS1* and *STN7* under salt stress. These results indicated that *MsTRX* improves salt tolerance via maintaining osmotic homeostasis, scavenging ROS, and regulating the transcription of stress-response genes in plants. Future research should pay more attention to the gene interaction or post-translational modification of *MsTRX* in salt tolerance, and the role of *MsTRX* in the regulation of cold and drought tolerance also needs to be illustrated. In our study, we provided a new understanding of how *MsTRX* improves salt stress in plants and how *MsTRX* can be included in future breeding programs to improve salt tolerance in alfalfa.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy12061467/s1, Figure S1. Alignment of *MsTRX* amino acid sequence. Figure S2. Phylogenetic tree of *TRX*. Figure S3. Three-dimensional model of the *MsTRX* protein. Table S1. List of primers in this study.

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