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S-nitrosylation of *SlAPX* Is Involved in Alleviating Oxidative Damage in Transgenic Tobacco under Nitrate Stress

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Abstract: Nitric oxide (NO) modulates plant response by post-translationally modifying proteins, mainly through S-nitrosylation. Ascorbate peroxidase (APX) in the ascorbate-glutathione (AsA-GSH) cycle participates in the removal of hydrogen peroxide (H_2O_2). However, the relationship between S-nitrosylation and the role of tomato APX (*SlAPX*) under nitrate stress is still unclear. In this study, the enzyme activity, mRNA expression, and S-nitrosylation level of *SlAPX* were significantly increased in tomato roots after nitrate treatment. *SlAPX* protein could be S-nitrosylated by S-nitrosoglutathione in vitro, and APX activity was significantly increased after S-nitrosylation. The *SlAPX* overexpressed tobacco plants grew better than the wild type (WT) plants under nitrate stress. Meanwhile, the transgenic plants showed lower reactive oxygen species and malondialdehyde content, higher APX, monodehydroascorbate reductase, glutathione reductase activities, ascorbic acid/dehydroascorbic acid, and reduced glutathione/oxidized glutathione ratio, proline, and soluble sugar contents than those in the WT plants under nitrate treatment. Moreover, overexpressed transgenic seeds showed higher tolerance to methyl viologen induced oxidative stress compared with the WT. The NO accumulation and S-nitrosylation APX level were higher in transgenic plants than in WT plants after nitrate stress treatment. Our results provide novel insights into the mechanism of *SlAPX* modulation excess nitrate stress tolerance involving the S-nitrosylation modification.

Keywords: ascorbate peroxidase; NO; AsA-GSH cycle; tomato

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1. Introduction

Salt stress is one of the major abiotic stresses affecting plant growth and global productivity [1,2]. Salt stress seriously affects the normal growth and development of plants, with low seed germination rate, leaf wilt, and yellowing, and it decreases photosynthesis metabolic capacity, eventually resulting in a decrease in yield [1,3–7]. When subjected to salt stress, plant cells produce excess reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical, singlet oxygen, and superoxide [8–11]. Accumulation of ROS can interfere with cellular redox and oxidation, leading to DNA damage, membrane protein polymerization, lipid peroxidation, and inactivation of cell enzymes, ultimately destroying membrane structure and leading to cell dysfunction [12–14].

As sessile organisms, plants have evolved non-enzymatic and enzymatic scavenging ROS mechanisms to adapt to saline environments [15–17]. The enzymes that remove ROS in the plants are superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), single monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and certain nonenzymatic antioxidants [18–21]. Among them, the highly efficient ascorbate-glutathione (AsA-GSH) cycle formed by APX, MDHAR, DHAR, and GR is a crucial antioxidant system involved in the removal of intracellular ROS under plant development and stress conditions [22–24]. The APX uses

AsA as an electron donor to reduce the H_2O_2 to water. Monodehydroascorbate (MDHA) and dehydroascorbate (DHA) were reduced by MDHAR and DHAR to AsA, respectively, with GSH, and, finally, glutathione disulfide (GSSG) was restored to GSH. Past studies have shown that APX played an important role in alleviating plant abiotic stresses by regulating the H_2O_2 contents [25].

Nitric oxide (NO) is a ubiquitous bioactive gas molecule. In plants, NO is involved in the regulation of various physiological processes, including seed germination, root development, stomatal closure, flowering, hormonal signaling, gene expression and regulation, stress response, and programmed cell death. In abiotic stresses, NO helps plant cells to maintain their homeostasis and alleviate various stresses. NO plays its role mainly through S-nitrosylation—that is, the NO group binds to the residues of the protein cysteine [26]. Various abiotic stress conditions significantly regulate the S-nitrosylation of proteins [27]. S-nitrosylation of Cys20 and Cys147 of potato DHAR1 reduced DHAR activity [28]. Previous research showed that APX enzyme activity can be mediated by S-nitrosylation [29]. The S-nitrosylation site of pea APX was Cys32, and the S-nitrosylation increased APX activity [30,31]. Cys32 of Arabidopsis cytosolic APX1 was a S-nitrosylation site, and S-nitrosylation enhanced its enzymatic activity [32]. However, the cytosolic APX decreased its activity after S-nitrosylation in tobacco [33]. Proteomic analysis also identified APX as a target protein of S-nitrosylation in Arabidopsis [34]. There is no report about S-nitrosylation modification on tomato APX.

Over-utilization of chemical fertilizer has caused secondary salinization in Chinese greenhouses. The excessively accumulated anion in the soil of the greenhouse is nitrate (NO_3^-) [35,36]. NO_3^- excess is common in greenhouse soils, imposing environmental risks and degrading vegetable quality. Shi et al. suggested that excess NO_3^- stress to plants might share the similar defense pathways with NaCl stress [37]. To gain more insight into the mechanism of the tomato *SlAPX* under nitrate stress, we investigated the response of *SlAPX* overexpressed tobacco plants under excess nitrate stress. Meanwhile, we also investigated the possibility of the regulation of APX activity by S-nitrosylation.

2. Materials and Methods

2.1. Plant Materials and Treatments

Tomato (*Solanum lycopersicum* L.) seeds were surface sterilized with 55 °C sterile water for 10 min, and they were germinated in vermiculite. Plants were then hydroponically grown for 4 weeks in plastic tanks containing 4 L of aerated nutrient solution, including $Ca(NO_3)_2{\cdot}4H_2O$ 590 mg·L^{-1}, KNO_3 404 mg·L^{-1}, $KH_2PO_4$136 mg·L^{-1}, $MgSO_4{\cdot}7H_2O$ 246 mg·L^{-1}, EDTA·Na_2-Fe 40 mg·L^{-1}, H_3BO_3 2.86 mg·L^{-1}, $MnSO_4{\cdot}4H_2O$ 2.13 mg·L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.22 mg·L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.08 mg·L^{-1}, and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.02 mg·L^{-1}. The nutrient solution pH was adjusted to 6.0–6.5 by the addition of 98% (*w/v*) H_2SO_4. The experiment was conducted in the greenhouse of Kunming University of Technology under natural conditions. The temperature was 23–28 °C during the day and 13–18 °C at night, with a photoperiod of 12–14 h. The six–week–old tomato seedlings were treated with normal nutrient solution (CK), normal nutrient solution adding 100 mM nitrate (NO_3^-), normal nutrient solution adding 100 μM NO donor sodium nitroprusside (SNP), and normal nutrient solution adding 100 mM nitrate + 100 μM SNP (NO_3^-+SNP) for 24 h.

2.2. Gene Expression Analysis

The RNA was extracted using the TRIzol Reagent (Takara, Dalian, China) and detected by 1% agarose gel. For qRT-PCR, reverse transcription of RNA was carried out according to the instruction of the SYBR® PrimeScript™ RT-PCR Kit II (Takara, Dalian, China). qRT-PCR was performed using the iCycler iQ Real-time PCR detection system (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed in three technical repetitions with complementary DNAs (cDNAs) synthesized from three biological replicates. The relative expression of specific genes was quantified using the $2^{-\Delta\Delta Ct}$ method. Tomato *Actin* was used as inner control for qRT-PCR analysis. These primer sequences are listed in Supplemental Table S1.

2.3. Plasmid Construction and Overexpressed Tobacco Characterization

The full length of the *SlAPX1* (accession no. NM_001247853) cDNA sequence amplified from tomato was 753 bp. The cDNA was amplified with the gene specific primers with restriction enzyme sites, *SlAPX*-F-*Bam*HI (5′-CGGGGGTACCGGATCCATGGGTAAGTGCTATCCTACTGT-3′), and *SlAPX*-R-*Bam*HI (5′-TCAGAATTCGGATCCTTAAG CTTCAGCAAATCCC-3′) using the Pfu DNA polymerase (Vazyme, Najing, China). The PCR fragment was ligated into the binary plant vector pRI101-GFP (Takara, Dalian, China) with the ClonExpress II one-step cloning kit (Vazyme, Najing, China). The pRI101-GFP plasmid was transformed into the *Agrobacterium tumefaciens* LBA4404 [38]. Transgenic tobacco was obtained with the leaf plate method [39].

To confirm the *SlAPX* gene integration into the tobacco genome, genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method [40]. The binary plant vector pRI101-*SlAPX*-GFP and wild-type (WT) genomic DNA were used as positive and negative controls, respectively. The transgenic plants were then identified by qRT-PCR and Western blot analysis.

2.4. Analysis of Transgenic Plants under Nitrate Stress

To test the nitrate tolerance of tobacco seedlings, transgenic and WT tobacco plants were grown in vermiculite and peat-filled pots (1:1). Six-week-old tobacco seedlings were watered with 50 mL of water as a control, or 50 mL of 150 mM nitrate solution (provided by same mol of KNO_3 and $Ca(NO_3)_2$) for 14 d for the nitrate treatment group. The experiment was also conducted in the greenhouse of Kunming University of Technology under natural conditions. The leaves were taken from 3 plants (n = 3), immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.5. Analysis of Transgenic Plants under Methyl Viologen (MV) Stress

Seeds from transgenic and WT tobacco plants were soaked in sterile water at 55 °C for 30 min, and then sterilised with 4% NaClO for 20 min before being washed three times with sterile water. After that, the seeds were sown on Murashige Skoog (MS) agar plates (9 cm) with 0 and 20 μM MV, and the survival rate was analyzed after 10 days.

To test the MV tolerance of tobacco seedlings, transgenic and WT tobacco plants were grown in vermiculite and peat-filled pots (1:1). Tobacco plants were treated with water (control) and 50 μM MV solution for 5 days. The phenotype was then photographed.

2.6. Endogenous ROS and NO Fluorescence Analysis

The tomato root tips were cut into 0.5~1 cm pieces, rinsed with pure water, and then soaked in EP tubes with either 2 μM H_2DCFDA solution for ROS accumulation analysis [41] or 5 μM DAF-FM DA for NO accumulation analysis [42]. The EP tubes were then exposed in darkness for 30 min and the root tips were stained. The root tips were washed 3 times for 15 min each with 20 mM HEPES-KOH (pH 7.8) buffer solution. Finally, the root tips of the washed tomato seedlings were placed under the epifluorescence microscope (model DMI6000B; Leica, Solms, Germany) to observe the accumulation of ROS (excitation 488 nm; emission 525 nm) or NO (excitation 485 nm; emission 515 nm).

2.7. Antioxidant Enzyme Activities Analysis

0.2 g leaves were grinded in an ice bath in precooled mortar. 1 mL of enzyme extract (50 mmol·L^{-1}, PH 7.8, phosphate buffer, 1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} AsA and 1% PVP) was added and then centrifuged at 12,000 rpm for 20 min at 4 °C. The resulting supernatant was finally collected for enzymatic activity analysis. The decrease in absorbance at 290 nm when the ascorbate was oxidized was used to determine APX activity, as described by Nakano and Asada [43]. The MDHAR activity was evaluated by monitoring the change in absorbance at 340 nm, as described by Miyake and Asada [44]. The rate of NADPH oxidation was used to calculate GR activity [45].

2.8. AsA, DHA, GSH, GSSG, Proline and Soluble Sugar Contents Analysis

The reduced AsA, oxidized DHA, GSH, and GSSH were analyzed according to Jiang and Zhang [46]. Free proline contents were analyzed using the ninhydrin assay as previously described [47]. The material was extracted for 15 min at 4 °C with 3% (*w/v*) sulfosalicylic acid. The free proline was determined using the supernatant after centrifugation. The soluble sugar content was determined using the method of Yemm and Willis [48].

2.9. Lipid Peroxidation Level and H_2O_2 Contents Analysis

The generation of malondialdehyde (MDA) was estimated using the thiobarbituric acid reaction method to measure lipid peroxidation [49]. H_2O_2 contents were analyzed using the method of Gay and Gebicki [50].

2.10. Measurement of Protein Expression

Protein expression level was analyzed with the Western blot, following the method of Bai et al. [51]. SDS-PAGE was used to separate the proteins, which were then transferred to PVDF membranes. APX peptides were used to immunize white mice to obtain an antibody for APX detection.

2.11. The Biotin Switch Approach to Detect S-nitrosylation Protein

Detection of S-nitrosylation proteins was performed by the methods of Jaffrey and Snyder, with minor modifications [52]. 1 g plant was soaked in HEN buffer to extract soluble protein (containing1 mM EDTA, 0.1 mM neocuproine, 25 mM HEPES-NaOH, pH 7.7). The homogenate was centrifuged at 10,000× *g* for 20 min and the supernatant was measured and adjusted the protein concentration to 1 mg·mL^{-1}. All protein samples were incubated at 50 °C for 20 min with regular vortexing after being treated with 25 mM methylmethanethiosulfonate (MMTS) and 2.5% sodium dodecyl sulfate (SDS). The residual MMTS was eliminated by the acetone precipitation method. At 37 °C for 1.5 h, sodium ascorbate and biotin-HPDP were added, and the excess biotin and ascorbate were removed by acetone precipitation. SDS-PAGE was used to electrophorese the biotin–labeled S–nitrosylation protein, and the protein was then submitted to a western blot examination with the APX antibody.

2.12. Purification of Recombinant SlAPX Protein

The *SlAPX* fragment was ligated into the prokaryotic expression vector pDE1 vector (Beijing KoSo Biotechnology Co., Ltd., Beijing, China) with the ClonExpress II one-step cloning kit (Vazyme, Nanjing, China). The pDE1-*SlAPX1* was transformed into the Rosetta (DE3) plysS *E. coli* Strain (Ybscience, Beijing, China). The *SlAPX* gene recombinant expressing strain was inoculated into 25 mL LB medium at a ratio of 1:100 and incubated at 37 °C, 220 rpm·min^{-1} for about 4 to 6 h (OD 600 ≈ 0.5), with IPTG added at a final concentration of 1 mM and induced at 37 °C and 28 °C, respectively. A total of 2 mL bacterial fluid after induction at 0, 2, 4, and 6 h was centrifuged at 12,000 rpm·min^{-1} for 1 min, and bacteria were collected. The bacteria were resuspended in an appropriate amount of PBS, broken for 15 min by sonication, and then centrifuged at 12,000 rpm·min^{-1} for 1 min, and the supernatant was then collected and the precipitate was resuspended in an appropriate volume of PBS. A total of 10 μL of supernatant and 10 μL of precipitation resuspension were collected for SDS-PAGE analysis. Protein was purified using the MagneHis™ Protein Purification System (Promega, Madison, WI, USA) kit. Target proteins were eluted with 20 mM phosphate buffer containing 100 mM NaCl and a different concentration gradient of imidazole, and 10 μL samples were taken for 12% SDS-PAGE analysis.

2.13. Determination of S-nitrosylation and Activity of SlAPX Protein by GSNO Treatment In Vitro

In order to analyze the S-nitrosylation level of *SlAPX* protein in vitro, the purified *SlAPX* protein was treated with or without GSNO for 30 min, and analyzed by biotin conversion method with or without AsA. The *SlAPX* activity was analyzed after GSNO treatment.

2.14. Statistical Analysis

Each sample was statistically analyzed and the data was presented as mean ± standard errors (SE) of three independent experiments. One-way analysis of variance (ANOVA) was performed on the data. (p-values < 0.05 are summarized with one asterisk, and p-values < 0.01 are summarized with two asterisks).

3. Results

3.1. Effect of Nitrate Stress and NO Treatment on the Activity, Expression, and S-nitrosylation Level of SlAPX in Tomato

To determine the activity, expression, and S-nitrosylation level of *SlAPX* in tomato, 6-week-old tomato seedlings were treated with nitrate and NO donor SNP for 24 h. The activity of APX in roots increased significantly by 282.5% after 24 h nitrate treatment, compared with CK (Figure 1A). The APX enzyme activity in the roots increased by 14.85% after exogenous application of SNP compared to nitrate stress treatment alone, respectively. The *SlAPX* gene expression in tomato seedling roots was significantly (p < 0.05) increased by nitrate as well as by nitrate and SNP co-treatment compared with CK (Figure 1B). Compared with CK, *SlAPX* gene expression in tomato seedling roots increased 2.96-fold after nitrate treatment and 3.47-fold after nitrate and SNP treatment. Compared with CK, S-nitrosylation level of *SlAPX* protein was significantly increased in roots after nitrate treatment (Figure 1C). The result showed that nitrate stress enhanced S-nitrosylation of *SlAPX* in tomato roots.

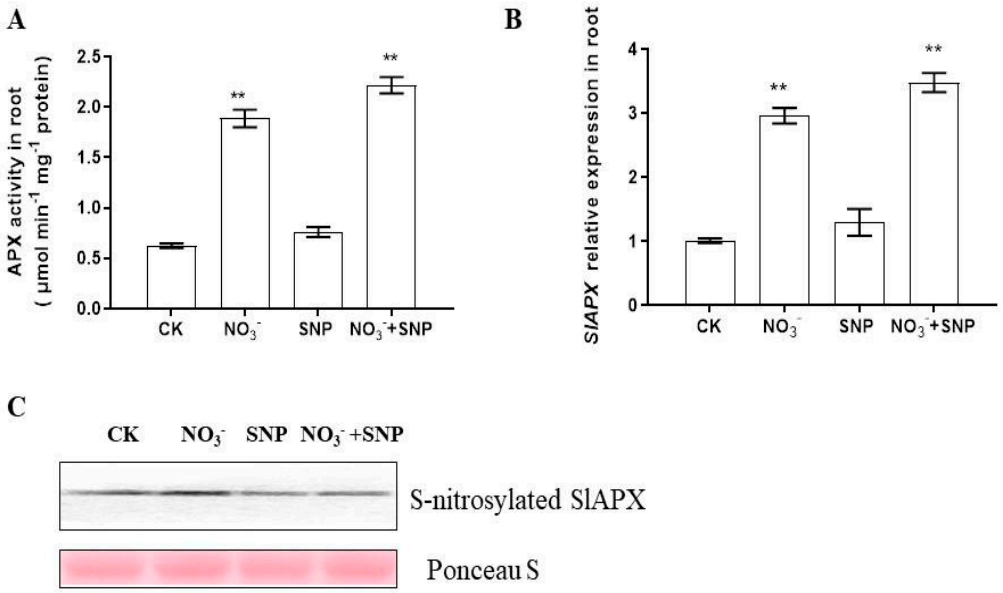

Figure 1. Effect of nitrate stress and SNP treatment on the enzyme activities, expression profile, and S-nitrosylation level of *SlAPX*. 6-week-old tomato seedlings were treated with control (CK), 100 mM nitrate, 100 μM SNP, or 100 mM nitrate + 100 μM SNP for 24 h. Subsequently, *SlAPX* enzyme activities (**A**) relative mRNA expression (**B**) and S-nitrosylation level (**C**) were analyzed. p-values < 0.01 are summarized with two asterisks.

3.2. S-nitrosylation of SlAPX In Vitro and the Effect of S-nitrosylation on APX Activity

Strains with a recombinant expression of the *SlAPX* gene were grown in LB medium, and expression was induced using IPTG. Cultures at 0, 2, 4, and 6 h after 28 °C and 37 °C induction were analyzed by SDS-PAGE. The results showed that the induced *SlAPX* recombinant protein size was approximately 28 kD, as expected (Figure S1A). SDS-PAGE analysis indicated the expression of homogeneous target proteins in the supernatant and precipitate, but mainly concentrated in the supernatant (Figure S1B). The supernatant was purified using a Ni^{2+} NTA affinity column, and 100 and 150 mM of imidazole eluates were

eluted, with a clear single band of about 28 kD, indicating a high purity of *SlAPX* protein (Figure S1C).

The purified *SlAPX* protein was treated with or without 1 mM S-nitrosylation agent GSNO for 30 min, and then the S-nitrosylation of *SlAPX* protein in the presence or absence of AsA was analyzed. The results showed that S-nitrosylation of *SlAPX* protein occurred only when GSNO and AsA were added at the same time (Figure 2A).

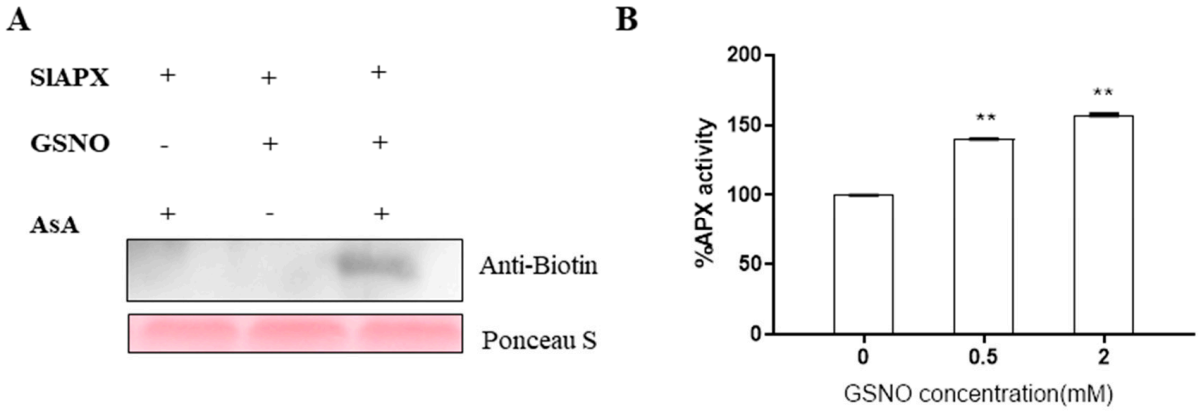

Figure 2. Effect of GSNO treatment on the S-nitrosylation level and enzyme activity of *SlAPX* protein. (**A**): The *SlAPX* protein was treated with or without 1 mM GSNO for 30 min, and then the S-nitrosylation level of *SlAPX* protein in vitro was measured with or without AsA. (**B**): Effect of 0, 0.5, and 2 mM GSNO treatment on the activity of *SlAPX* protein. p values < 0.01 are summarized with two asterisks.

To investigate the activity of *SlAPX* after S-nitrosylation, the recombinant *SlAPX* protein were purified and treated with GSNO, and *SlAPX* activity was measured. *SlAPX* activity increased significantly with increasing GSNO concentration (Figure 2B). The *SlAPX* activity increased by 40.14% and 57.45% after incubation with 0.5 and 2 mM of GSNO at room temperature for 30 min, respectively. These results suggested that tomato *SlAPX* activity might be increased after S-nitrosylation.

3.3. The Characterization of Overexpressed SlAPX Transgenic Tobacco

We identified 12 positive overexpressed *SlAPX* transgenic lines by kanamycin screening. Genomic PCR showed the target band in the transgenic plants with specific primers for NPTII (Figure 3A). qRT-PCR, Western blot, and enzyme activities were then conducted. The qRT-PCR results showed that the *SlAPX* gene overexpressed transgenic lines of OE2, OE3, and OE4increased 3.64, 3.75, and 3.47 times, respectively, compared with WT (Figure 3B). The results of Western blot showed that APX protein expression was significantly increased in OE2, OE3, and OE4 in the transgenic plants compared with WT (Figure 3C). The APX activity was significantly increased in OE2, OE3, and OE4 compared with WT (Figure 3D). The above results indicated that *SlAPX* successfully overexpressed in tobacco.

3.4. Overexpression of SlAPX Reduced Oxidative Damage in Tobacco under Nitrate Stress

The phenotype of WT leaves was smaller and yellower than that of overexpressed transgenic plants after nitrate treatment (Figure 4A). After nitrate treatment, ROS fluorescence accumulation was dramatically lower in the *SlAPX* overexpressed transgenic lines than that in the WT (Figure 4B). Under normal growth, the H_2O_2 content of the transgenic plants was lower than that of the WT; after nitrate treatment, the OE2, OE3, and OE4 transgenic plants have a significantly lower H_2O_2 content than WT (Figure 4C). Similar to the H_2O_2 content, MDA contents increased in both WT and transgenic tobacco after nitrate treatment, and the contents in transgenic tobacco was significantly lower than in WT plants (Figure 4D).

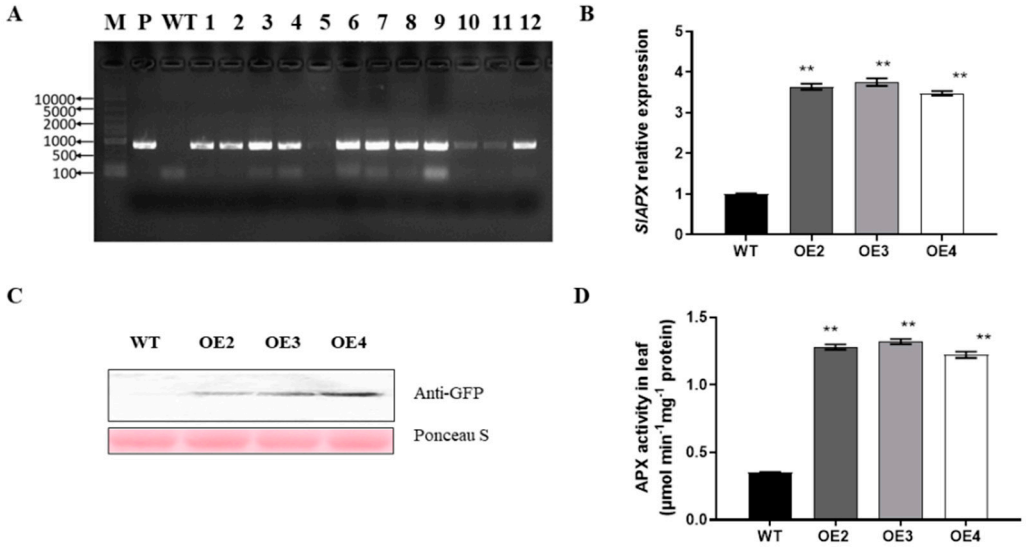

Figure 3. The genomic PCR (**A**), relative expression (**B**), protein expression (**C**), and APX activity (**D**) analysis in *SlAPX* transgenic tobacco plants. OE2, OE3, and OE4 were three different *SlAPX* overexpressed transgenic lines. *p* values < 0.01 are summarized with two asterisks.

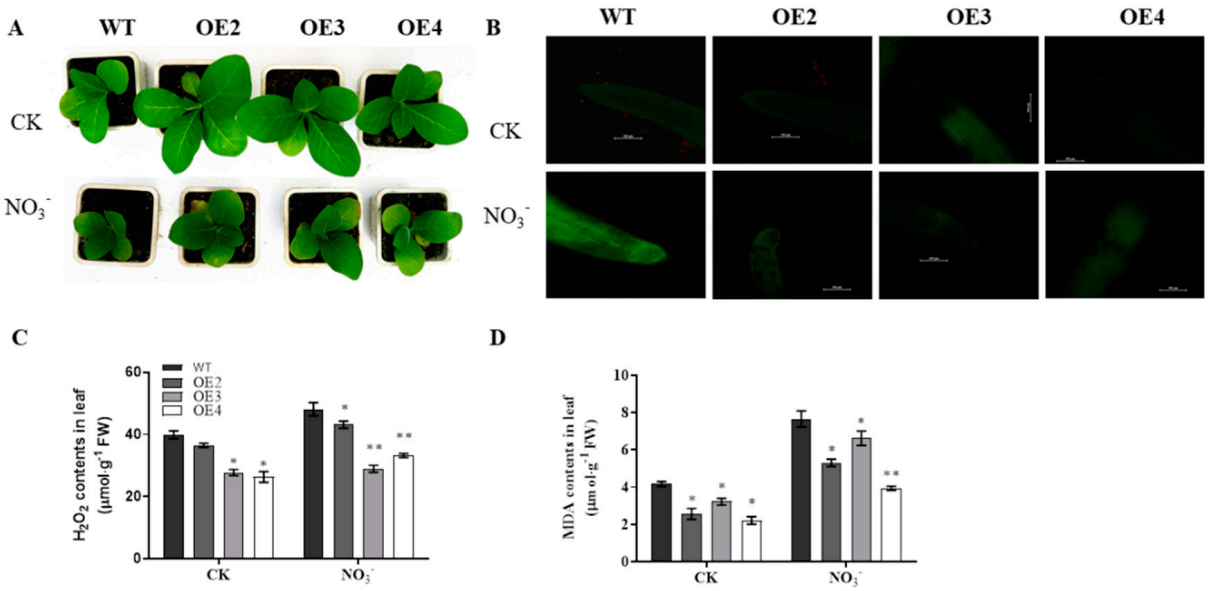

Figure 4. Effects of nitrate stress on the phenotype (**A**), ROS fluoresence (**B**), H_2O_2 contents (**C**), and MDA contents (**D**) in *SlAPX* overexpressing and WT plants. Transgenic and WT tobacco plants were watered with 50 mL of water as a control, or 50 mL of 150 mM nitrate solution once every 2 days for 2 weeks. Scale bar = 100 μM. *p* values < 0.05 are summarized with one asterisk, and *p* values < 0.01 are summarized with two asterisks.

3.5. SlAPX Overexpressed Tobacco Plants Had Increased Oxidative Stress Tolerance

To further investigate the tolerance of *SlAPX* overexpressing tobacco to oxidative stress, seeds of transgenic lines were transferred to MS medium containing MV induced oxidative stress, and MS medium without MV was used as a control (Figure 5A). There was no significant difference in seed germination between the WT and transgenic plants on the MS medium without MV. On MS medium containing 20 μM MV, the growth of WT and overexpressed tobacco was significantly inhibited and the leaves showed photobleaching, but the survival rate of transgenic tobacco was significantly higher than that of WT (Figure 5B).

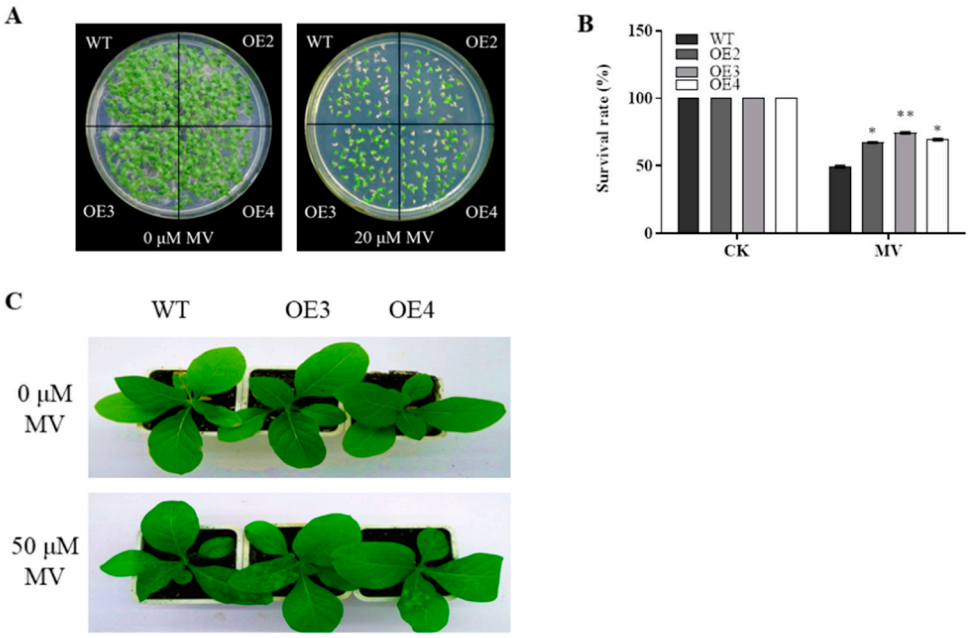

Figure 5. Effects of MV stress on the seed germination and seedling growth in *SlAPX* overexpressing and WT plants. (**A**): The phenotype of *SlAPX* overexpressing and WT seeds sown on MS agar plates with 0 or 20 μM MV. (**B**): The survival rate was assayed after 10 days of MV treatment. (**C**): Tobacco plants were sprayed with water or 50 μM MV solution for 5 days. The phenotype was then observed. p values < 0.05 are summarized with one asterisk, and p values < 0.01 are summarized with two asterisks.

To investigate the oxidative stress tolerance of transgenic plants seedlings, leaves were sprayed with solutions containing 0 μM (control) or 50 μM MV for 5 days. There was no significant difference in growth between WT and transgenic plants in the control. After treatment with 50 μM MV solution, the leaves of WT plants showed severe damage, while the leaves of transgenic plants showed less damage symptoms (Figure 5C).

3.6. SlAPX Overexpressed Plants Had Higher Antioxidant Enzyme Activities, and the AsA/DHA, GSH/GSSG Ratio under Nitrate Stress

Under nitrate treatment, the mRNA expression level and activity of APX were significantly higher in transgenic plants than in WT plants (Figure 6A,B). Activities of antioxidant enzymes in the AsA-GSH cycle were then analyzed. As shown in Figure 6C,D, the activities of MDHAR, and GR were significantly higher in transgenic plants than in WT plants under normal conditions. After nitrate treatment, MDHAR and GR activities in overexpressed plants were significantly higher than those in WT. The AsA/DHA and GSH/GSSG ratio of *SlAPX* overexpressed plants was significantly higher than that of WT plants under control and nitrate stress conditions (Figure 6E,F).

3.7. SlAPX Overexpressed Plants Had Enhanced Accumulation of Osmotic Substance under Nitrate Stress

As shown in Figure 7A, proline contents were significantly higher in transgenic lines than in WT plants under normal conditions and nitrate stress. There is no significant difference in soluble sugar contents between transgenic and WT plants under control. After nitrate treatment, the soluble sugar contents in transgenic plants were significantly higher than WT plants (Figure 7B). We then analyzed the mRNA expression of several osmotic stress marker genes by qRT-PCR, including *Ntosmotin*, *NtP5CS*, *NtDREB*, and *NtLEA5*. As shown in Figure 7C–F, there was no significant difference between the WT and transgenic lines under normal conditions. The mRNA transcript levels of *NtP5CS*, *NtDREB2*, *Ntosmotin*, and *NtLEA5* genes were dramatically increased after nitrate stress treatment.

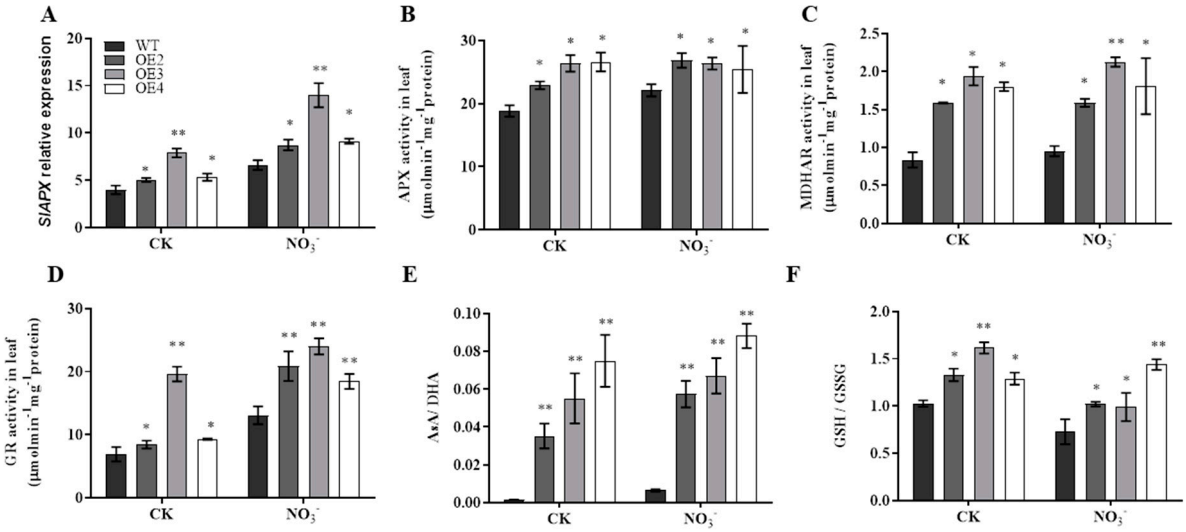

Figure 6. Effects of nitrate treatment on the key antioxidant enzymes activities and AsA/DHA, GSH/GSSG ratio in *SlAPX* transgenic and WT plants. Transgenic and WT tobacco plants were watered with 50 mL of water as a control, or 50 mL of 150 mM nitrate solution once every 2 days for 2 weeks. (**A**): Relative expression of *SlAPX*. (**B**–**D**): Activities of APX, MDHAR, GR. (**E**,**F**):AsA/DHA and GSH/GSSG ratio. p values < 0.05 are summarized with one asterisk, and p values < 0.01 are summarized with two asterisks.

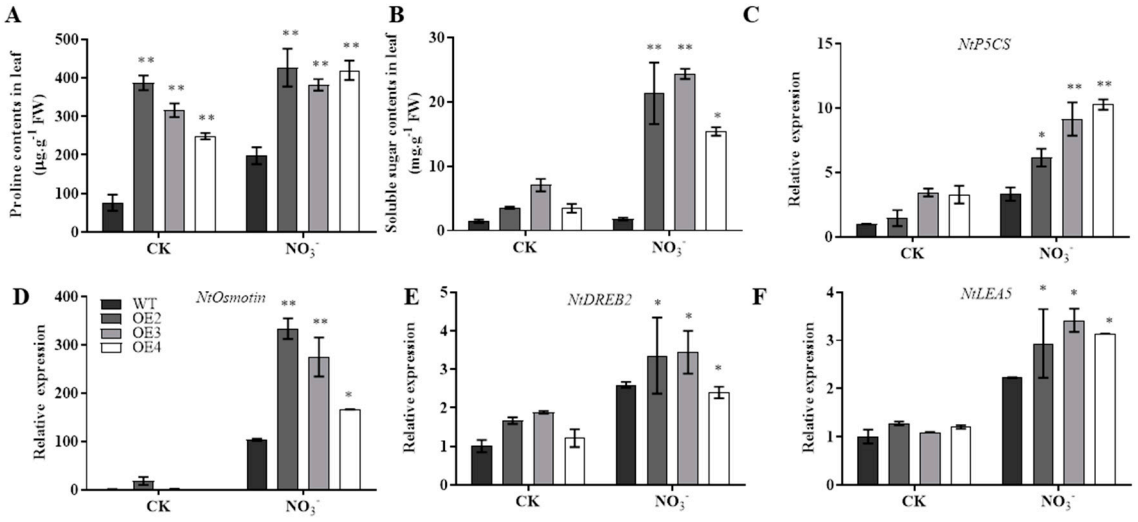

Figure 7. Effects of nitrate stress on proline (**A**) and soluble sugar (**B**) contents and expression of *NtP5CS* (**C**), *NtOsmotin* (**D**), *NtDREB* (**E**), *NtLEA5* (**F**) in *SlAPX* overexpressing and WT plants. Transgenic and WT tobacco plants were watered with 50 mL of water as a control, or 50 mL of 150 mM nitrate solution once every 2 days for 2 weeks. p values < 0.05 are summarized with one asterisk, and p values < 0.01 are summarized with two asterisks.

3.8. SlAPX Overexpressed Transgenic Plants Had Higher S-nitrosylation Level of APX under Nitrate Stress

To investigate whether *SlAPX* was S-nitrosylated under nitrate stress, the NO accumulation was first assayed. There was no dramatical difference in NO accumulation between WT and *SlAPX* overexpressed plants in the control (Figure 8A). When tobacco seedlings were exposed to nitrate stress, NO content increased significantly in WT and transgenic plants, especially in the latter. As shown in Figure 8B, the S-nitrosylation APX levels of overexpressed transgenic plants were significantly higher than those of WT plants under normal growth and nitrate treatment.

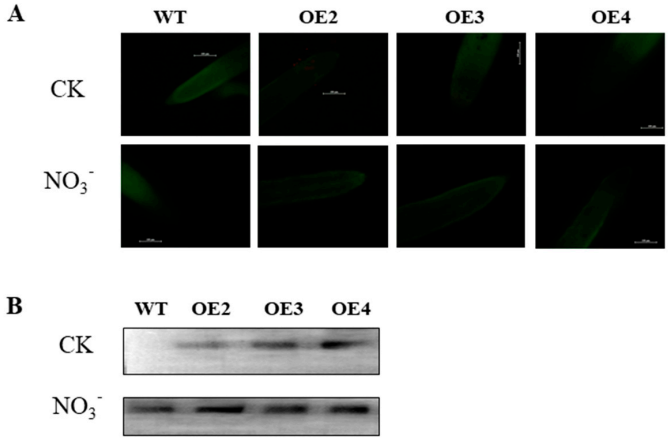

Figure 8. Effect of nitrate stress on the NO fluorescence (**A**) and S-nitrosylation APX level (**B**) in *SlAPX* overexpressed and WT tobacco. Transgenic and WT tobacco plants were watered with 50 mL of water as a control, or 50 mL of 150 mM nitrate solution once every 2 days for 2 weeks. The NO accumulation and S-nitrosylation level of APX were then analysed.

4. Discussion

APX, one of the important enzymes in the AsA-GSH cycle, is involved in regulating H_2O_2 levels under plant growth and stress conditions [53–56]. Salinity caused an increase in the activity of APX in sugarcane [57]. Compared with salt stress, the enzyme activity of APX was significantly increased in wheat with SNP and salt stress [58]. In this study, the activity and transcription levels of *SlAPX* were significantly increased after nitrate treatment (Figure 1A,B).

Overexpression of APX has been reported to enhance plant tolerance under environmental stress. The transgenic plants overexpressing the Populus *PpAPX* gene increased APX activity under salt stress, and they also significantly increased salt tolerance during the vegetative period [59]. Overexpressing the *LmAPX* gene of *Lycium chinense* Mill. in tobacco showed high APX activity under salt stress, which improved the salt tolerance of tobacco [60]. The transgenic *Arabidopsis* plants overexpressing *Oncidium OgCytAPX1* showed highly efficient ROS scavenging activity and salt tolerance [61]. In this study, under nitrate stress, the growth of *SlAPX* overexpressed transgenic tobacco was better than WT, indicating that *SlAPX* overexpression enhanced nitrate stress tolerance of tobacco (Figure 4A).

Salt stress leads to excessive production of ROS in plants, leading to oxidative stress, thus affecting the growth and development of the plant. MDA content, as a product of lipid peroxidation, was used to evaluate the extent of oxidative damage [62]. APX plays a key role in stress regulation responses by enhancing ROS clearance and maintaining ROS homeostasis, regulating H_2O_2 content, and reducing MDA content. *Populus euphratica PeAPX2* can promote the accumulation of cAPX under salt stress, scavenging ROS and reducing the content of H_2O_2 and MDA, thus enhancing the salt tolerance of poplar [63]. In our study, the ROS, H_2O_2, and MDA contents in *SlAPX* overexpressed transgenic tobacco plants were lower than WT plants, suggesting that the oxidative damage caused by excessive nitrate was lower in overexpressed plants than WT (Figure 4). Visual assessment of transgenic and control lines exposed to MV confirmed that overexpression of Populus *PpAPX* minimized leaf damage, indicating that *APX* transgenic plants improved oxidative damage in response to abiotic stress [59]. The transgenic tobacco plants overexpressing *APX* showed increased tolerance to oxidative stress caused by application of MV [64]. In our study, the survival rate of *SlAPX* overexpressed plants was higher than WT plants under MV treatment (Figure 5), indicating that *SlAPX* when overexpressed showed higher tolerance to oxidative stress than WT.

Our study showed that the *SlAPX* transgenic plants have higher antioxidant enzyme activity of APX, MDHAR, and GR to enhance nitrate stress tolerance (Figure 6). The

alteration in AsA and GSH production may contribute to increased resistance to abiotic and biotic stresses [65]. Under drought stress, the AsA and DHA contents of *CytAPX* overexpressed tobacco increased, the AsA/DHA ratio decreased, and the GSH/GSSG ratio increased [66]. In a sensitive genotype of pea under NaCl stress, the total AsA content in the soluble fraction decreased, with a slightly lower AsA/DHA ratio, and a 60% decrease in the glutathione pool compared with the control group [19]. Our study found that AsA/DHA and GSH/GSSG ratios were significantly higher in *SlAPX* overexpressing plants under control and nitrate stress conditions (Figure 6F,G), indicating that the transgenic plants had more reducing agents to clear ROS.

Our study found that under normal conditions and after nitrate stress treatment, the contents of proline in transgenic tobacco were significantly higher than those in WT plants (Figure 7). Osmotin proteins have been shown to be induced in plants in response to abiotic and biotic stresses, involved in protecting plants from these stresses [67]. Tobacco *osmotin* transgenic plants showed significantly increased accumulation of free proline compared to WT plants, resulting in better stress tolerance [68]. The transgenic tomato plants carrying the tobacco *osmotin* gene exhibited enhanced salt stress tolerance compared with WT plants, indicating that overexpression of the *osmotin* gene enhanced salt tolerance in transgenic tomato plants [69]. A bifunctional enzyme, delta(1)-pyrrolin-5-carboxylate synthetase (P5CS), controls the glutamate pathway in proline biosynthesis in plants and positively regulates plant response to salt stress [70,71]. Overexpression of *P5CS* in soybean transgenic lines increased the content of proline and showed high salt tolerance [72]. Previous studies have confirmed that dehydration responsive element binding protein (DREB) is a transcription factor that responds to salt stress by enhancing transcription expression and activating salt tolerance related genes in plants [73]. Under salt stress, transgenic tobacco plants with the soybean *GmDREB6* gene increased the transcription levels of *GmDREB6* and *NtP5CS* genes, thus improving salt tolerance [74]. Late embryogenetic abundant (LEA) proteins play an important role in plant responses to abiotic stresses as osmotic regulatory materials and protective materials for cell membrane structure [75]. In our study, the expression levels of *Ntosmotin*, *NtP5CS*, *NtDREB2*, and *NtLEA5* genes in *SlAPX* transgenic tobacco were significantly higher than WT plants after nitrate stress treatment, suggesting that *SlAPX* may regulate osmotic potential to enhance nitrate stress tolerance (Figure 7).

NO, as a signaling molecule, regulates plant growth and development mainly through S-nitrosylation [26]. Proteomic studies have shown that APX acts as an S-nitrosylation target protein [34]. Studies have shown that S-nitrosylation increased APX activity [30]. After treatment of *Antiaris toxicaria* with NO gas, the S-nitrosylation of APX increased its enzyme activity and contributed to seed drying [76]. Proteomic analysis of *Arabidopsis* roots showed that cytoplasmic APX (APX1) could undergo S-nitrosylation, and the activity of recombinant APX1 was increased after S-nitrosylation [77]. In the present study, the APX S-nitrosylation level was increased in tomato after nitrate treatment (Figure 1C). *SlAPX* was S-nitrosylated by GSNO treatment and its activity was increased (Figure 2). In addition, *SlAPX* overexpressed transgenic tobacco plants had higher S-nitrosylation level of APX and activity when compared to WT (Figure 8B). Our results suggest that APX are regulated by S-nitrosylation, thus highlighting the close involvement of interactions between NO metabolism and antioxidant enzymes related to ROS metabolism in stress tolerance. The key cysteine cite of S-nitrosylation will be studied further in the future.

5. Conclusions

SlAPX protein was S-nitrosylated in tomato under nitrate stress and in vitro. The growth of *SlAPX* overexpressed plants was significantly better than that of WT under nitrate stress, with lower ROS accumulation, higher AsA/DHA ratio and antioxidant enzyme activities, and higher proline and soluble sugar contents. These results suggest that *SlAPX* is involved in mitigating oxidative damage under nitrate stress related to the S-nitrosylation of APX.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13051322/s1, Figure S1: Induction and purification of tomato *SlAPX* recombinant protein prokaryotically; Table S1: Specific primers for qRT-PCR amplification.

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