



Tomato *SINTRB* improved excess nitrate stress tolerance by alleviating the oxidative damage in transgenic tobacco

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Abstract

NADPH-dependent thioredoxin reductase (NTR), which is a flavin protein family of disulfide oxidoreductases present in all living cells, reduces thioredoxin (Trx). The roles of NTR B under stress conditions have not been well characterized. In this study, a tomato NTR B (*SINTRB*) gene was cloned and functionally characterized. The mRNA transcript level of *SINTRB* was induced significantly after nitrate treatment. The seed germination rate of *SINTRB* overexpressed transgenic tobacco was higher than wild type (WT) under nitrate stress. The growth of *SINTRB* overexpressed plants was better than WT and the reactive oxygen species (ROS) accumulation were lower than WT under nitrate stress. The NTR activity, superoxide dismutase (SOD) activity, the GSH/GSSG ratio, and the expression of *Trx* were significantly higher than WT after nitrate stress treatment. The *SINTRB* overexpressed transgenic tobacco seeds showed higher tolerance to H₂O₂ and methyl viologen (MV) treatments than WT. The above results indicated that the *SINTRB* gene improved the plant nitrate stress tolerance by alleviating the oxidative damage.

Key message

Overexpression of tomato *SINTRB* gene in tobacco enhances the excess nitrate stress tolerance with less oxidative damage.

Keywords NADPH-dependent thioredoxin reductase · Nitrate stress · Transgenic tobacco · Oxidative damage

Introduction

Nitrogen (N), which is a component of biological macromolecules such as protein and DNA, is an important element for the normal growth and maintenance of plant development (Ortigosa et al. 2019). N participates in various important physiological or metabolic processes in plants, such as photosynthesis, photorespiration, respiration, amino acid synthesis, tricarboxylic acid (TCA) cycle, and chlorophyll synthesis (Makino 2011). In order to get better growth and higher returns, growers apply too much N fertilizer to the crops they grow. In fact, the absorption rate of N fertilizer

in fruits and vegetables is very low (Qu et al. 2020). Less than 50% of applied N fertilizer is used by plants and the rest is either leached out in soil or exhausted as agricultural runoffs (Qiao et al. 2017). Excessive N fertilizer inputs lead to a waste of fertilizer, which is reflected by a decline in nitrogen use efficiency and secondary salinization (Zhang et al. 2022). NO₃⁻ and Ca²⁺ are the main anion and cation of soil secondary salinization during greenhouse cultivation (Wang et al. 2016). Ca(NO₃)₂ alone significantly induced changes in the components of cell wall, anatomical structure, and expression profiles of several lignin biosynthetic genes (An et al. 2018). The growth of cucumber seedlings was significantly inhibited after the treatment of Ca(NO₃)₂ stress (Xing et al. 2015).

Plants produce excessive reactive oxygen species (ROS) under stress, resulting in the destruction of biological macromolecules such as proteins, nucleic acids and lipid membranes in the body, increasing the leakage of solute membranes (Cerqueira et al. 2023). Maintenance of ROS is an important event for whole lifespan of plants, however, in special cases, toxic ROS molecules are largely accumulated

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under excess stresses and diverse enzymes played as ROS scavengers (Cha et al. 2015). Superoxide dismutase (SOD), as the first line of defense in the system, can convert $O_2^{\cdot-}$ into hydrogen peroxide and oxygen, effectively eliminating the damage caused by superoxide anions. After that, peroxidase (POD) (Jacquot et al. 2009) and catalase (Abenavoli et al. 2016) continue to break down the products of SOD metabolism, and these antioxidant enzymes form a tight defense line to eliminate the harm caused by ROS (Wang et al. 2023). The thioredoxin (Trx) system has a vital function in cellular antioxidative defense via eliminating the excessive generation of ROS. It can be reduced by ferridoxin dependent Trx reductase (FTR) or niacinamide adenine dinucleotide phosphate (NADPH) dependent Trx reductase (NTR) (Jacquot et al. 2009). NTR is a flavin protein family of disulfide oxidoreductase present in all living cells (Dietz 2007). Each NTR subunit has two cofactor binding domains, one FAD-binding domain and one NADPH binding domain (Hagglund et al. 2016). NTR uses NADPH to specifically reduce Trx-S₂ to Trx-(SH)₂, the form of which is a powerful protein disulfide reductase (Serrato et al. 2004).

Plants contain three NADPH-thioredoxin reductases (NTR), NTRA located in the cytosol, NTRB located in the mitochondria and the NTRC in plastid, with important metabolic functions (Souza et al. 2023a). NTRC is a kind of NTR unique to photosynthetic autotrophs. It mainly exists in chlorophylls. NTRC is a single peptide bifunctional enzyme composed of N-terminal NTR domain and C-terminal Trx domain, and has high affinity to NADPH (Najera et al. 2017). The lack of NTRC affects the expression of proteins involved in stress and REDOX reactions (Ojeda et al. 2017). While NTRA is found in the cytoplasm, NTRB is mainly found in mitochondria and interferes with developmental processes by regulating auxin signaling (Cejudo et al. 2012). The combination of NTRA and NTRB with glutathione in Arabidopsis alters auxin transport and metabolism (Bashandy et al. 2010). In plants, NTRB is the main mitochondrial thioredoxin reductase, capable of reducing thioredoxin in the cytoplasm (Reichheld et al. 2005). The mitochondrial NTRB reduction system is an important metabolic factor in the TCA cycle, and the contents of various metabolic substances in the TCA cycle will be significantly changed after mutation of NTRB gene. At the same time, the NTR system lacking NTRB also has an impact on amino acid metabolism, inhibiting glycine/serine metabolism (Porto et al. 2022). In the previous experiment, transcriptome analysis was performed on tomatoes treated with nitrates. Compared with the control group, the expression of NTRB under nitrate treatment was higher than that of NTRC. In addition, no significant increase or decrease of NTRA expression was found (Supplemental Table S2).

Tomato (*Solanum lycopersicum*) is one of the most consumed fruit and vegetable crops in the world. In our research

of RNA-seq of tomato seedling under nitrate stress, we found *SINTRB* was significantly induced after nitrate stress treatment in tomato root. To further investigate the potential roles of *SINTRB*, we amplified the *SINTRB* gene from tomato and analyzed the function of *SINTRB* in transgenic tobacco plants under excess nitrate stress.

Materials and methods

Plant materials and stress treatment

Tomato seedlings were grown hydroponically in plastic tank containing aerated modified nutrient solution (Zhai et al. 2022). After 4 weeks, excess nitrate provided by KNO₃ and Ca(NO₃)₂ were added into the nutrient solution to reach a final NO₃⁻ ion concentration of 100 mM. The normal NO₃⁻ ion concentration of 9 mM was used as a control. After excess nitrate treatment for 24 h, the roots were harvest and put at - 80 °C for later analysis.

Gene cloning and phylogenetic analysis of *SINTRB*

Tomato *SINTRB* gene (GenBank No. XM_004232546.4) was cloned by PCR with the primer of ATGACCGGC AACTGTT and ATCACTCTTTCCCACTT. To further understand the relationship between *SINTRB* and other plant NTRB, we compared the derived *SINTRB* amino acid sequence with other protein sequences and build phylogenetic trees using MEGA X software.

Plasmid construction and identification of overexpressed *SINTRB* tobacco

The cDNA sequence of the target gene *SINTRB* was cloned with the primer, F: TACGAACGATACTCGACCCCATGACCGGCAACTGTT and R: CTAGAGTCGA CGGATCCCCATCACTCTTTCCCACTT. The *SINTRB* gene was amplified using DNA polymerase (Vazyme, Nanjing, China). The plant expression vector of pRI101-*SINTRB*-GFP was constructed using ClonExpress II one-step cloning kit (Vazyme, Nanjing, China). The constructed plant expression vector was transformed into *Agrobacterium tumefaciens* (LB4404) and transgenic tobacco was obtained by leaf disk method (Horsch et al. 1985).

To verify that the *SINTRB* gene had been successfully integrated into tobacco, the genomic DNA of tobacco was extracted using the CTAB method. The PCR was done with the plasmid of pRI101-*SINTRB*-GFP vector used as positive control and wild-type (WT) genomic DNA as negative control. Then the transgenic plants were identified by qRT-PCR and western blot analysis.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from tobacco using TRIzol reagent (Dalian Takara, China) and detected by gel electrophoresis. SYBR PrimeScript™ RT-PCR Kit II (Takara, Dalian, China) was used for reverse transcription of RNA into cDNA. Real-time quantitative PCR was performed using Hieff™ qPCR SYBR® Green Master Mix kit (Shanghai Yishen Biotechnology Co., LTD.) and BioRad CFX 96™ instrument of real-time quantitative system. *NtActin* was used as an internal reference gene in tobacco. Tomato Actin was used as inner control for qRT-PCR analysis in tomato. The relative expression of specific genes was quantified using the $2^{-\Delta\Delta Ct}$ method. Three replicates were set up in each group to detect the expression of *SINTRB* gene and related gene. These primer sequences are listed in Supplemental Table S1.

Western blot assay

Western blot analysis was performed according to Leverone et al. (1991). The proteins were first transferred to PVDF membrane by SDS-PAGE gel electrophoresis and then transferred to PVDF membrane by micro-trans-blotting electrophoresis. After blocking with skim milk, the primary antibody is used with antibodies containing the target gene. The second antibody is horseradish peroxidase labeled anti-mouse IGG. After washing, ECL developer was used for color development (Beijing Conway Century Biotechnology Co., LTD.), and chemiluminescence imaging in dark room.

Seeds germination and seedlings growth analysis under excess nitrate stress

Transgenic tobacco seeds were placed in a 1.5 mL centrifuge tube and soaked in sterile water for 3 h, sterilized with 60% sodium hypochlorite solution for 3 min, and washed with sterile water for 30 min. Spot seeds evenly on MS medium containing 0 mM or 150 mM nitrate. The seeds were cultured in a tissue culture room with a constant temperature of 25 °C and photocycle of 16 h/8 h. The germination data were recorded daily.

In order to determine the tolerance of tobacco seedlings to nitrate stress, transgenic tobacco and WT tobacco of four weeks in peat and perlite (1:1) matrix were used for stress treatment. The experimental group was treated with 150 mM nitrate (50 mL) every day, while the control group was treated with water. The experiment was treated for three weeks, and then the samples were taken.

Seeds germination analysis of *SINTRB* overexpressed transgenic tobacco under oxidative treatment

In order to observe the tolerance of *SINTRB* overexpressed tobacco to oxidative stress, the overexpressed tobacco seeds were treated with H_2O_2 and methyl viologen (MV). After the tobacco seeds were disinfected as described above, they were uniformly located in normal MS and MS medium containing 60 μ M MV and 20 mM H_2O_2 . The germination data were recorded every day.

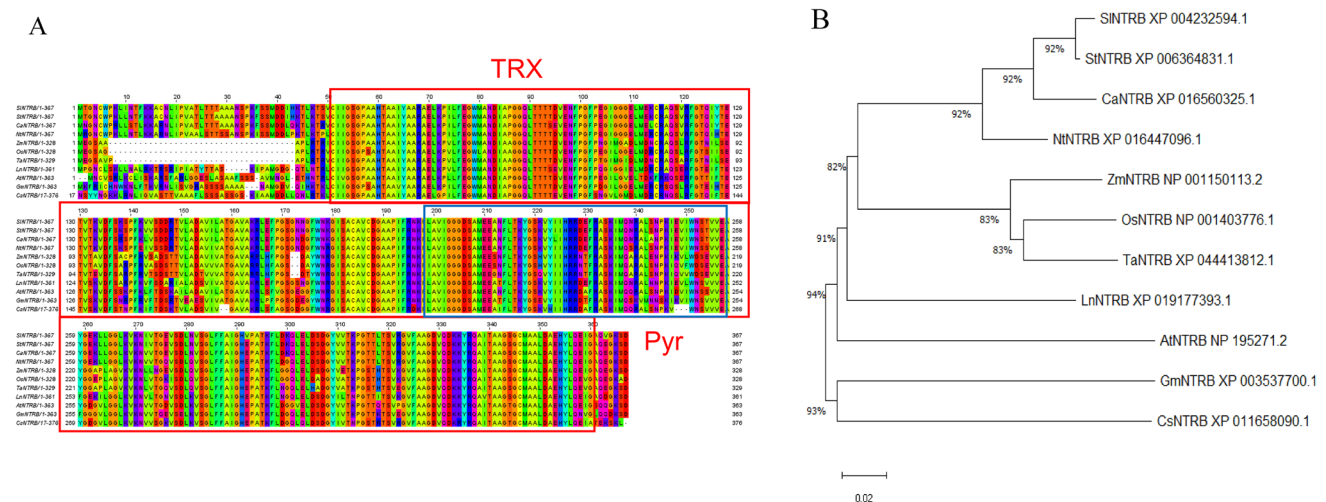


Fig. 1 Main structural analysis of *SINTRB*. **A** Analysis of the active domains in the *SINTRB* amino acid sequence identified by NCBI Blast. **B** Phylogenetic tree analysis of *SINTRB* with other NTRB pro-

teins. TRX (thioredoxin-disulfide reductase); Pyr (Pyridine nucleotide-disulphide oxidoreductase)

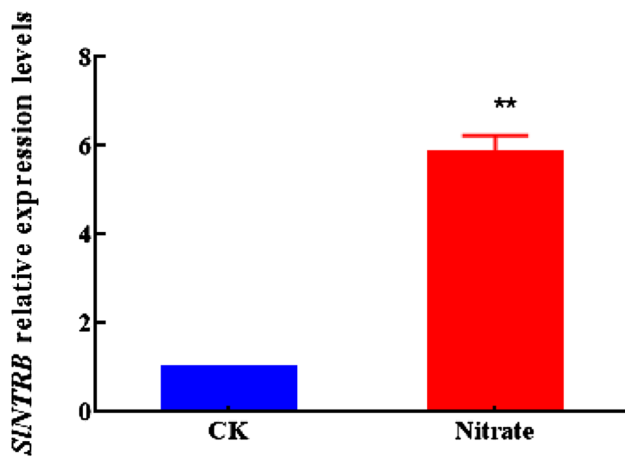


Fig. 2 Effects of excess nitrate stress on the expression of *SINTRB* in tomato seedlings. The expression of *SINTRB* in tomato roots was analyzed by qRT-PCR. Tomato plants were treated with 100 mM nitrate (KNO_3 , 50 mM and $\text{Ca}(\text{NO}_3)_2$, 25 mM) for 24 h. The normal NO_3^- ion concentration of 9 mM was used as a control (CK). The results represented mean \pm standard deviation (SD) of three biological replicates. Data was analyzed with Student's t-test compared with CK under similar conditions and indicated by * $P < 0.1$; ** $P < 0.05$

Fluorescence analysis of endogenous ROS and NO

ROS in root tips was observed using 2, 7-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$). The washed root tips were placed in a dye containing 2 μM $\text{H}_2\text{DCF-DA}$, dyed for 30 min, and then washed three times with 20 mM HEPES–KOH (pH 7.8) buffer for 45 min, and photographed with a Inverted fluorescence microscope of Leica (DMI 3000B model) (excited at 488 nm; Emission 525 nm) (Mazel et al. 2004).

The root tips were rinsed with clean water and placed in an EP tube containing 5 μM DAF-FMDA dye and left in darkness for 30 min. The root tips were removed and placed in 20 mM HEPES–KOH (pH 7.7) buffer. The root tips were repeatedly washed 3 times for 15 min each time. The cleaned samples were placed under an inverted fluorescence microscope to observe the content of NO in the root tips and photographed (excitation 495 nm; Emission 515 nm) (Zhao et al. 2009).

The fluorescence images taken were analyzed by Image j software (<https://imagej.en.softonic.com/>).

Determination of NTR enzyme activities

The NTR activity was determined according to Ye et al. (1997). The 0.5 g sample was ground into powder in a mortar with liquid nitrogen, and 3 mL 25 mM PBS buffer (1 M DTNB, 0.2 mM EDTA, 1% PVP) was added. After grinding, it was thoroughly mixed in a centrifuge tube and centrifuged at 4 $^\circ\text{C}$ and 12,000 rpm for 15 min. After centrifugation, 1 mL of supernatant was taken into a water bath at 55 $^\circ\text{C}$ for 10 min, then left on ice for 5 min and centrifuged at 4 $^\circ\text{C}$ and 12,000 rpm for 10 min. Take 1 mL of reaction solution with 25 mM PBS buffer (0.2 mM EDTA, 1 M DTNB), 50 μL reduced Coenzyme II (NADPH), 100 μL bovine Serum protein (BSA), add 200 μL sample, and determine the absorption value at OD412 with BSA as the control.

Analysis of H_2O_2 content

Xylenol orange method was used to determine the H_2O_2 content, and its light absorption value at OD560 was determined according to Gay and Gebicki (2003).

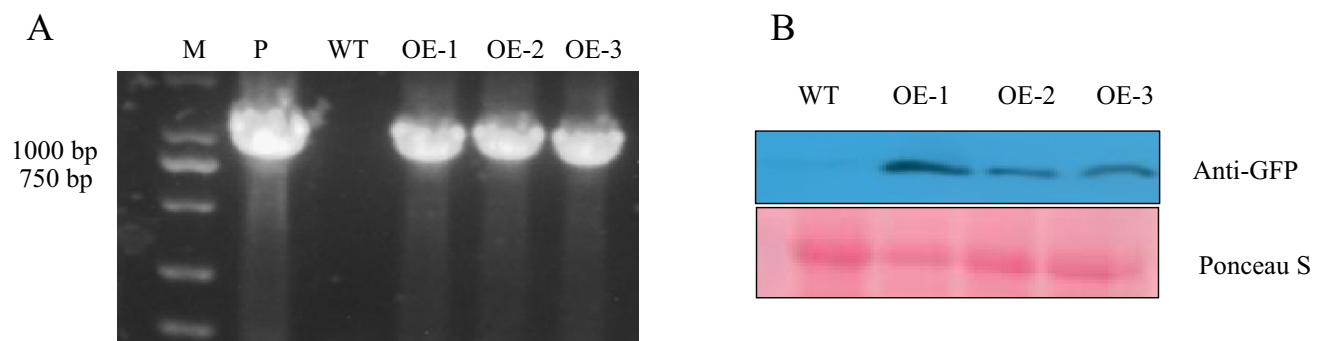


Fig. 3 Identification and molecular characteristics of *SINTRB* overexpressed transgenic tobacco. **A** Genomic PCR analysis of *SINTRB* in transgenic plants with specific primers. M: marker; P: positive control of the plasmid; WT: wild type; OE-1, 2, 3: different transgenic lines. **B** Western blot analysis of the *SINTRB* transgenic plants. All

the results represented mean \pm standard deviation (SD) of three biological replicates. Data was analysed with Student's t-test compared with control (CK) under similar conditions and indicated by * $P < 0.1$; ** $P < 0.05$

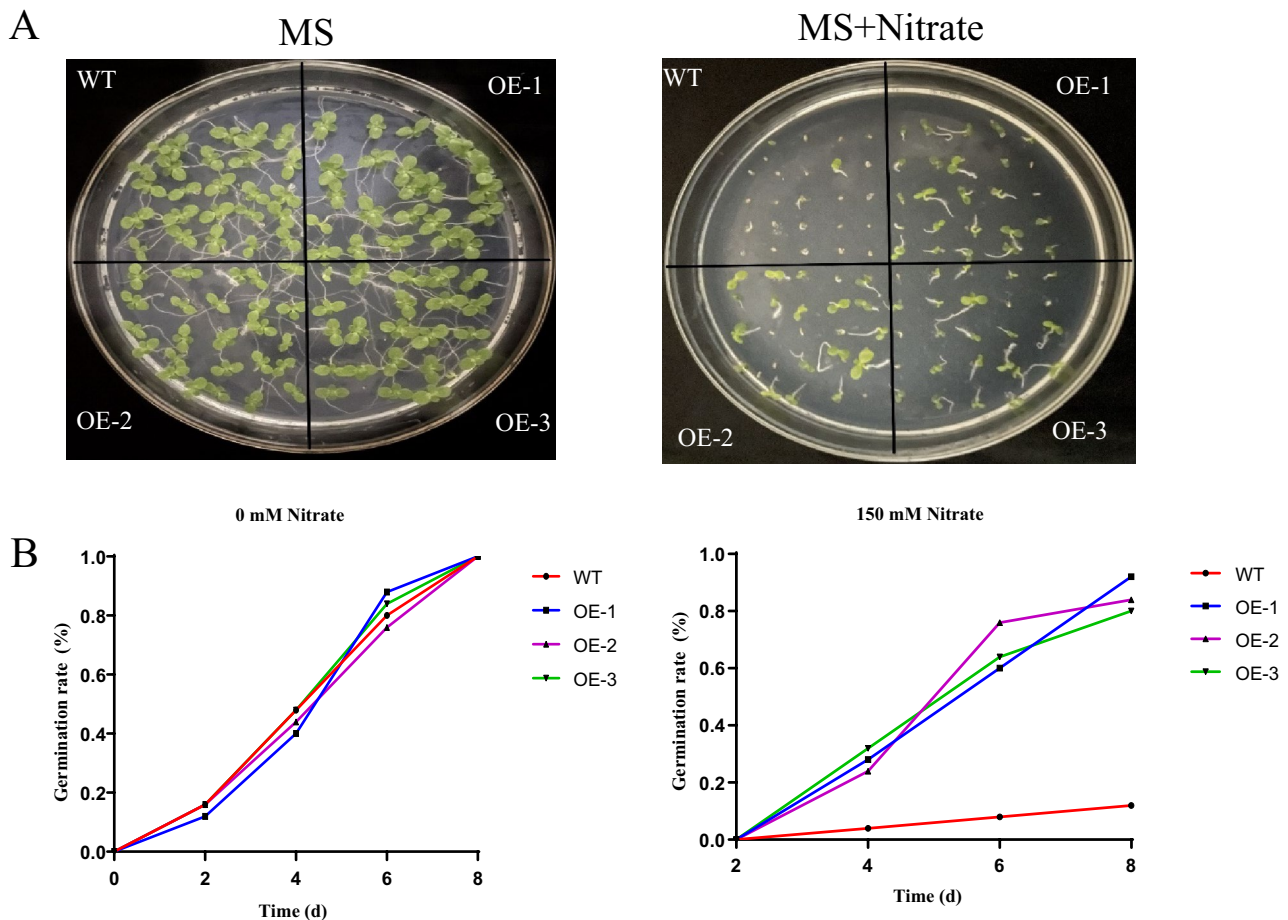


Fig. 4 Effect of excess nitrate stress on the germination of *SINTRB* overexpressed transgenic seeds. **A** Phenotype of WT and *SINTRB* transgenic plants grown on MS and MS added excess nitrate. **B** Ger-

mination rate of WT and *SINTRB* transgenic plants grown on MS and MS added excess nitrate

Determination of antioxidant activities and antioxidant substance contents

The activity of superoxide dismutase (SOD) was determined by Wang et al. (2021). After adding the reaction solution, the determination was performed at 560 nm. The determination of GSH and GSSG was referred to the method of Jiang and Zhang (2001).

Phylogenetic analysis of NTRB

The NTRB protein sequences of different species were downloaded from the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis of *SINTRB* with nsNTRB from other plants. The deduced amino acid sequences were aligned multiple times to

analyze their same conserved structures using Jalview software. In addition, the phylogenetic trees of NTRBs in different plants were constructed using the NJ method in MEGA 11 software. The accession number of the database is shown in parentheses after the plant name.

Data processing and analysis

Graphpad Prism 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for data statistics and processing. Student's t-test was used to determine the significance of the difference (* denotes $P < 0.05$; ** indicates $P < 0.01$; **** indicates $P < 0.0001$). The experimental data were composed of the mean \pm standard deviation (SD) values of three repeated experiments.

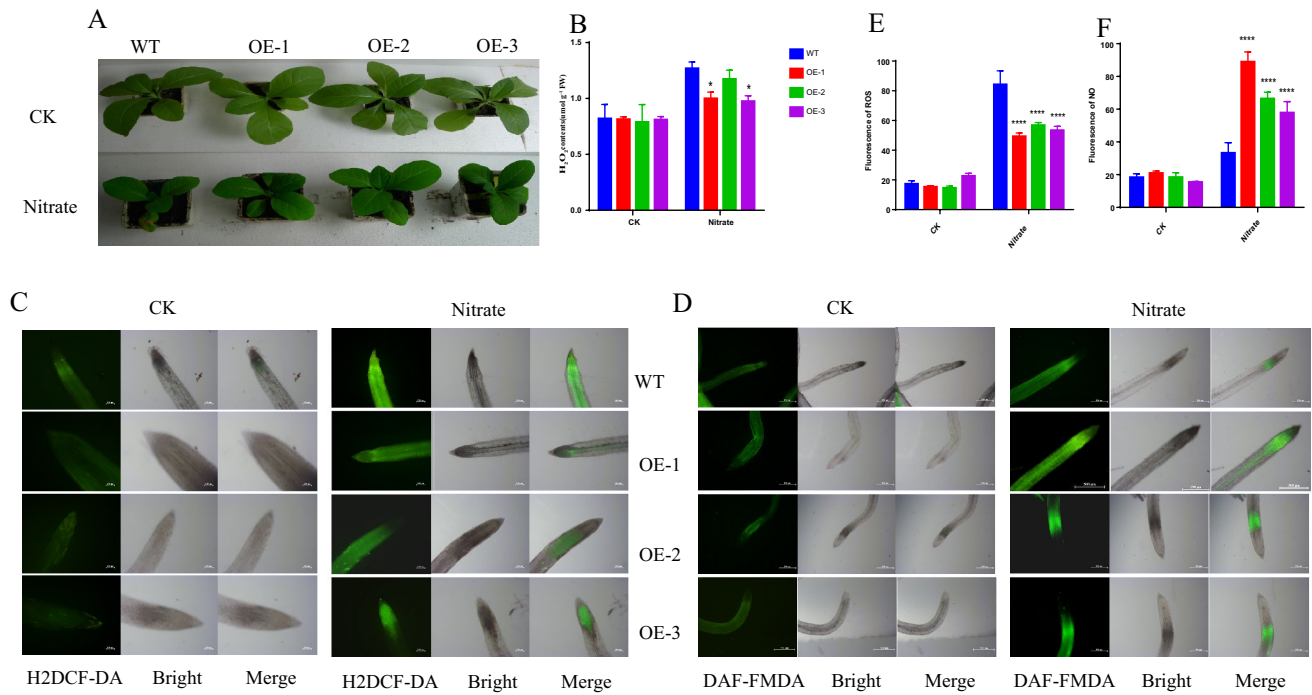


Fig. 5 Effects of excess nitrate stress on the phenotype (A), H₂O₂ contents (B), ROS accumulation (C, E) and NO accumulation (D;F) of *SINTRB* overexpressed tobacco and WT seedlings. *SINTRB* transgenic tobacco and WT plants were treated with 0 or 150 mM nitrate

solution for 3 weeks. All the results represented mean \pm standard deviation (SD) of three biological replicates. Data was analysed with Student's t-test compared with control (CK) under similar conditions and indicated by *P < 0.1; **P < 0.05; **** indicates P < 0.0001

Results

Bioinformatics analysis of *SINTRB*

The identified full-length cDNA of *SINTRB* was 1104 bp, which encodes a 368-amino-acid protein with a molecular mass of 40 kDa. The *SINTRB* conservative sequence belongs to Trx reductive superfamily (Fig. 1A). Conservative domains are included TRX (thioredoxin-disulfide reductase) and Pyr (Pyridine nucleotide-disulphide oxidoreductase).

A phylogenetic tree was constructed using *SINTRB* proteins with other proteins of tobacco, *Arabidopsis*, potato and pepper, *Zea mays*, cotton, rice (Fig. 1B). The result showed that *SINTRB* was closely related to NTRB of potato.

The expression of *SINTRB* in tomato seedlings under nitrate stress

The tomato seedlings were treated with 100 mM nitrate for 24 h, and the gene expression of *SINTRB* was then measured by qRT-PCR. Under nitrate stress, the expression of *SINTRB* was up-regulated by 4.9-fold of the control

(Fig. 2). These results indicated that *SINTRB* gene was involved in the regulation of nitrate stress in tomato.

Identification of *SINTRB* overexpressed tobacco

To further investigate the function of *SINTRB* under nitrate stress, overexpression transgenic plants were obtained. Three *SINTRB* overexpressed tobacco lines were selected for the identification by PCR and Western blot analysis. The genomic PCR showed three specific bands, all of which were consistent with the size of the plasmid PCR bands (Fig. 3A). Western blot assay showed that NTRB protein expression was significantly increased in OE-1, OE-2, and OE-3 in the transgenic plants compared with WT (Fig. 3B). The above results proved that the transgenic tobacco of these three lines were *SINTRB* overexpressed tobacco.

Effects of nitrate stress on germination of *SINTRB* overexpressed tobacco seeds

In order to verify the nitrate resistance of *SINTRB* overexpressed tobacco seeds, tobacco germination test was conducted. As shown in the Fig. 4, there is no significant difference of the germination rate of WT and overexpression of *SINTRB* transgenic tobacco seeds in MS medium (Fig. 4A).

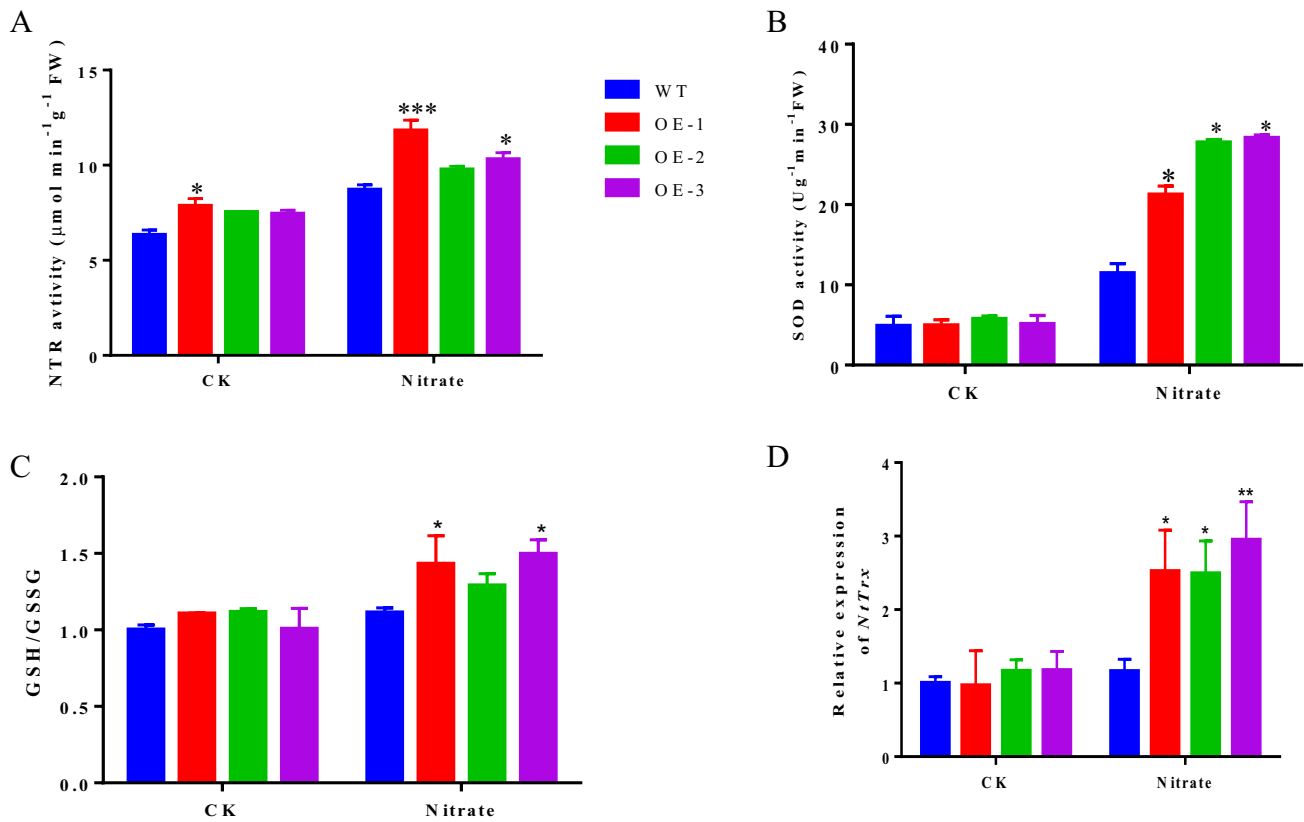


Fig. 6 Effects of excess nitrate stress on the enzyme activities of NTR and SOD, ratio of GSH/GSSG and expression of *NtTrx* of *SINTRB* overexpressed tobacco and WT seedlings. *SINTRB* transgenic tobacco and WT were treated with 0 or 150 mM nitrate solution for 3 weeks,

the data was analyzed. All the results represented mean \pm standard deviation (SD) of three biological replicates. Data was analysed with Student's t-test compared with control (CK) under similar conditions and indicated by * $P < 0.1$; ** $P < 0.05$

The germination rate of WT seeds in nitrate medium was 12.0% on the 8th day. The germination rates of *SINTRB* overexpressed tobacco seeds of OE-1, OE-2, OE-3 were 92.0%, 84.0% and 80.0%, respectively on the 8th day after nitrate treatment (Fig. 4B).

Effects of nitrate stress on the growth, oxidative damage and NO accumulation in *SINTRB* overexpressed tobacco seedlings

In the control, there is no difference of the growth size of overexpression tobacco and WT seedlings. However, after nitrate treatment, it was observed that the growth of *SINTRB* overexpressed tobacco was better than that of WT plants (Fig. 5A). The H_2O_2 contents were then analyzed. As shown in Fig. 5B, there is no difference of the H_2O_2 contents between WT and overexpression plants. After nitrate treatment, the H_2O_2 contents were significantly lower than WT. The fluorescence intensity of ROS in lateral roots of WT plants was more than that of *SINTRB* overexpressed tobacco, indicated that there is less oxidative damage to overexpression transgenic plants than WT plants (Fig. 5C, E). In the

NO detection experiment, we found that after nitrate stress treatment, the fluorescence intensity of NO in the lateral roots of the three overexpressed tobacco was higher than that of WT tobacco (Fig. 5D, F). These results suggested that overexpression of *SINTRB* increased the nitrate stress tolerance of tobacco by alleviating the oxidative damage.

Effect of nitrate stress on NTR and SOD activities, GSH/GSSG ratio and *NtTrx* expression in *SINTRB* overexpressed tobacco

The NTR activity in *SINTRB* overexpressed tobacco was significantly higher than that in WT tobacco under normal treatment. After nitrate stress, NTR activity in WT and overexpression transgenic plants were all increased, and NTR activities in OE-1, OE-2, and OE-3 were 137.2%, 111.5% and 118.1% of WT plants (Fig. 6A). In the control group, there was no significant difference in SOD activity between WT and overexpressed tobacco. After nitrate stress, SOD activity of overexpressed tobacco increased significantly (Fig. 6B). There was no significant difference of the GSH/GSSG ratio under normal treatment. After nitrate stress

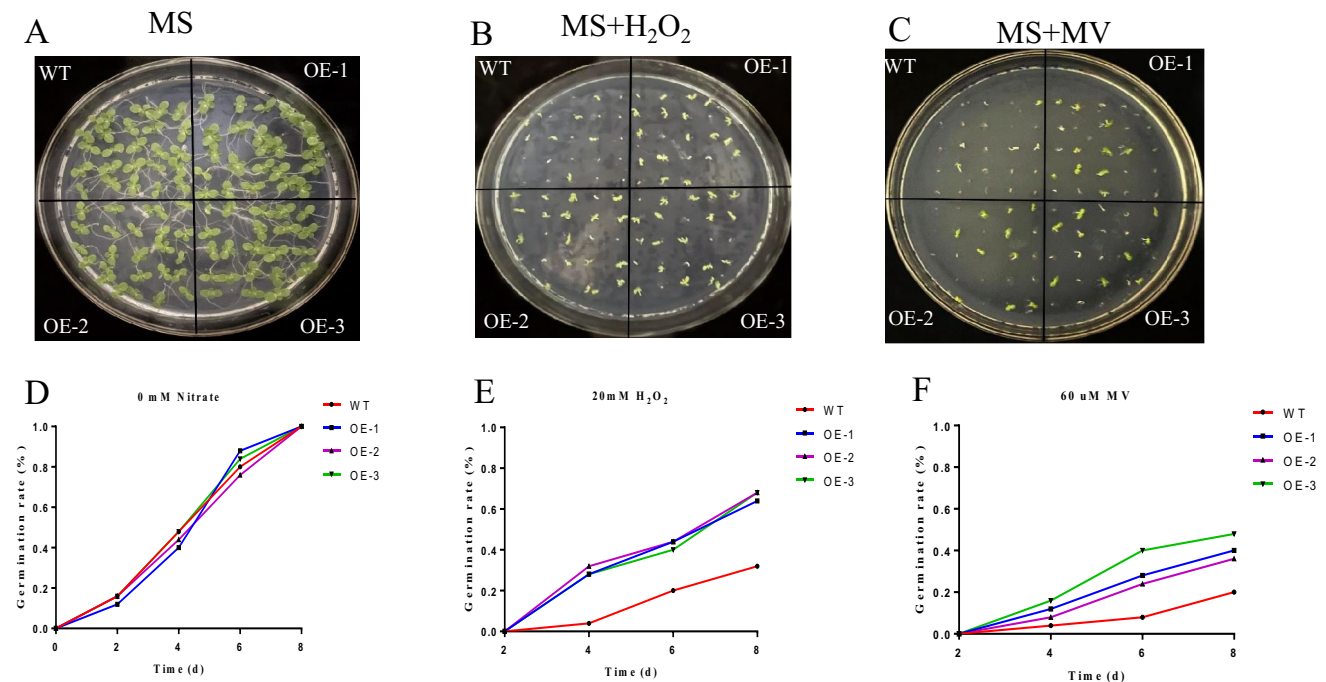


Fig. 7 Germination rate of *SINTRB* overexpressed tobacco under H_2O_2 and MV stress conditions. A, B, C: Phenotypes of tobacco seeds grown on MS medium containing 0 (A), 20 mM H_2O_2 (B) or

60 μM of MV (C) for 8 days. D, E, F: Germination rate of tobacco seeds grown on MS medium containing 0 (D), 20 mM H_2O_2 (E) or 60 μM of MV (F) for 8 days

treatment, the ratio in overexpression tobacco was significantly increased, compared with WT (Fig. 6C). As shown in Fig. 6D, under normal treatment, the expression level of *NiTrx* genes in overexpressed tobacco had little difference with those in WT plants. However, under nitrate treatment, the expression of *NiTrx* genes in OE-1, OE-2 and OE-3 was 2.3-, 2.2- and 2.8-fold of WT.

Analysis of oxidative stress tolerance of *SINTRB* overexpressed tobacco

In order to test the tolerance of *SINTRB* overexpressed tobacco to oxidative stress, the overexpressed tobacco seeds were treated with H_2O_2 and MV. As shown in Fig. 7A, D, the germination rate of overexpression and WT tobacco in MS was basically similar. In the germination test treated with H_2O_2 , the germination rate of WT tobacco was 32.2%, while the germination rate of *SINTRB* overexpressed tobacco was 64.3%, 68.2%, and 68.2%, respectively (Fig. 7B, E). After MV treatment, the germination rate of WT was only 20.4%, while the germination rate of overexpressed tobacco was 40.4%, 36.3%, and 48.6%, respectively (Fig. 7C, F). These results showed that *SINTRB* overexpressed tobacco enhanced the tolerance to oxidative damage.

Discussion

Nitrate content is an essential indicator of the quality of vegetables but can cause stress at high levels (Abd-Elrahman et al. 2022). Redox regulation in heterotrophic organisms relies on NADPH, thioredoxins (Trxs), and an NADPH-dependent Trx reductase (NTR) (Gonzalez et al. 2019). Trx reductases control the redox state of Trx ubiquitous proteins that regulate a spectrum of enzymes by dithiol-disulfide exchange reactions (Buey et al. 2021). Arabidopsis and rice contain 3 NTRs which transfer reducing power to Thioredoxin/Peroxiredoxin system for scavenging ROS (Cha et al. 2015). In this study, a tomato NTR was isolated which belong to Trx reductive superfamily. Overexpression of Trx CDSP32 gene can alleviate oxidative damage in tobacco leaves under Cd exposure by regulating antioxidant defense system (Zhang et al. 2023). Ca and EGTA stimulated chickpea thioredoxin and NTR activities when compared to Cd-stressed (Sakouhi et al. 2021). In our study, tomato *SINTRB* was significantly induced after nitrate treatment.

Plants contain three NTR located in the cytosol/mitochondria (NTRA/B) and the plastid (NTRC) with important metabolic functions (Souza et al. 2023b). Of the NTRA expressed by adjusting the quantity of ROS oxidation and drought tolerance in arabidopsis thaliana (Cha et al. 2014).

Due to this functional redundancy between NTRA and NTRB, their physiological functions under environmental stresses are not clearly classified and remain elusive to date. Both *ntrc* and *ntrbc* mutants showed reduced growth and substantial metabolic alterations as compared to the wild type (Souza et al. 2023b). The *ntra ntrb* plants were characterized as producing wrinkled seeds, exhibiting slow rates of plant growth and accumulating high levels of anthocyanins (Reichheld et al. 2007; Bashandy et al. 2009), which could be expected to increase tolerance to abiotic stresses such as drought (Kovinich et al. 2014). It has been reported that *ntra ntrb* double mutant plant exhibits UV-C tolerance due to high accumulation of anthocyanin (Bashandy et al. 2009). In our study, the germination rate and seedling growth of *SINTRB* transgenic tobacco were better than WT, suggesting that overexpression of *SINTRB* enhanced the nitrate stress tolerance of tobacco.

Under oxidative stress, toxic ROS are largely accumulated in plant cells lead to cell damage and cause damage of DNA, RNA, protein and lipid (Rasheed et al. 2022). Thioredoxins (Trx) implicated redox reactions, conferred stress tolerance by enhancing the ROS scavenging ability of plants (Considine and Foyer 2021). To date, function of three NTRs in Arabidopsis was association with plant protection against oxidative stress (Lepisto et al. 2013; Cejudo et al. 2021). Under nitrate stress, the ROS fluorescence accumulation increased, while the contents in the overexpression *SINTRB* plants were lower than WT plants. Besides, in our study, the *SINTRB* overexpression plants showed high survival rates than WT under MV and H₂O₂ stress, which are well known to induce large accumulations of ROS and cause oxidative damage in plant cells. These results suggested that that *SINTRB* overexpression improved the tolerance to the oxidative stress. The activity of NTR and expression of *Trx* were increased under nitrate stress in *SINTRB* overexpression transgenic plants, suggesting that NTRB regulated cellular ROS levels via activated Trx systems in plant cells and protects the plants against stress.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11240-023-02661-w>.

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Author contributions HNX designed the project. YTN and STQ conducted the experiments and analyzed the data and wrote the manuscript. KZL helped in the writing of the article. All authors have read and agreed to the published version of the manuscript.

Data availability The data presented in this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

- Abd-Elrahman SH, Saady HS, Abd El-Fattah DA, Hashem FAE (2022) Effect of irrigation water and organic fertilizer on reducing nitrate accumulation and boosting lettuce productivity. *J Soil Sci Plant Nutr* 22(2):2144–2155. <https://doi.org/10.1007/s42729-022-00799-8>
- Abenavoli MR, Longo C, Lupini A, Miller AJ, Araniti F, Mercati F, Princi MP, Sunseri F (2016) Phenotyping two tomato genotypes with different nitrogen use efficiency. *Plant Physiol Biochem* 107:21–32. <https://doi.org/10.1016/j.plaphy.2016.04.021>
- An YH, Zhou H, Yuan YH, Li L, Sun J, Shu S, Guo SR (2018) 24-Epi-brassinolide-induced alterations in the root cell walls of *Cucumis sativus* L. under Ca(NO₃)₂ stress. *Protoplasma* 255(3):841–850. <https://doi.org/10.1007/s00709-017-1187-8>
- Bashandy T, Taconnat L, Renou JP, Meyer Y, Reichheld JP (2009) Accumulation of flavonoids in an *ntra ntrb* mutant leads to tolerance to UV-C. *Mol Plant* 2(2):249–258. <https://doi.org/10.1093/mp/ssn065>
- Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld JP (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. *Plant Cell* 22(2):376–391. <https://doi.org/10.1105/tpc.109.071225>
- Buey RM, Fernandez-Justel D, Gonzalez-Holgado G, Martinez-Julvez M, Gonzalez-Lopez A, Velazquez-Campoy A, Medina M, Buchanan BB, Balsera M (2021) Unexpected diversity of ferredoxin-dependent thioredoxin reductases in cyanobacteria. *Plant Physiol* 186(1):285–296. <https://doi.org/10.1093/plphys/kiab072>
- Cejudo FJ, Ferrandez J, Cano B, Puerto-Galan L, Guinea M (2012) The function of the NADPH thioredoxin reductase C-2-Cys peroxiredoxin system in plastid redox regulation and signalling. *FEBS Lett* 586(18):2974–2980. <https://doi.org/10.1016/j.febslet.2012.07.003>
- Cejudo FJ, González MC, Pérez-Ruiz JM (2021) Redox regulation of chloroplast metabolism. *Plant Physiol* 186(1):9–21. <https://doi.org/10.1093/plphys/kiab062>
- Cerqueira JVA, de Andrade MT, Rafael DD, Zhu F, Martins SVC, Nunes-Nesi A, Benedito V, Fernie AR, Zsögön A (2023) Anthocyanins and reactive oxygen species: a team of rivals regulating plant development? *Plant Mol Biol* 112(4–5):213–223. <https://doi.org/10.1007/s11103-023-01362-4>
- Cha JY, Kim JY, Jung IJ, Kim MR, Melencion A, Alam SS, Yun DJ, Lee SY, Kim MG, Kim WY (2014) NADPH-dependent thioredoxin reductase A (NTRA) confers elevated tolerance to oxidative stress and drought. *Plant Physiol Biochem* 80:184–191. <https://doi.org/10.1016/j.plaphy.2014.04.008>
- Cha JY, Barman DN, Kim MG, Kim WY (2015) Stress defense mechanisms of NADPH-dependent thioredoxin reductases (NTRs) in plants. *Plant Signal Behav* 10(5):e1017698. <https://doi.org/10.1080/15592324.2015.1017698>
- Considine MJ, Foyer CH (2021) Oxygen and reactive oxygen species-dependent regulation of plant growth and development. *Plant Physiol* 186(1):79–92. <https://doi.org/10.1093/plphys/kiab077>
- Dietz KJ (2007) The dual function of plant peroxiredoxins in antioxidant defence and redox signaling. *Subcell Biochem* 44:267–294. https://doi.org/10.1007/978-1-4020-6051-9_13
- Gay CA, Gebicki JM (2003) Measurement of protein and lipid hydroperoxides in biological systems by the ferric-xylenol orange

- method. *Anal Biochem* 315(1):29–35. [https://doi.org/10.1016/s0003-2697\(02\)00606-1](https://doi.org/10.1016/s0003-2697(02)00606-1)
- Gonzalez M, Delgado-Requerey V, Ferrandez J, Serna A, Cejudo FJ (2019) Insights into the function of NADPH thioredoxin reductase C (NTRC) based on identification of NTRC-interacting proteins in vivo. *J Exp Bot* 70(20):5787–5798. <https://doi.org/10.1093/jxb/erz326>
- Hagglund P, Finnie C, Yano H, Shahpiri A, Buchanan BB, Henriksen A, Svensson B (2016) Seed thioredoxin h. *Biochim Biophys Acta* 1864(8):974–982. <https://doi.org/10.1016/j.bbapap.2016.02.014>
- Jacquot JP, Eklund H, Rouhier N, Schurmann P (2009) Structural and evolutionary aspects of thioredoxin reductases in photosynthetic organisms. *Trends Plant Sci* 14(6):336–343. <https://doi.org/10.1016/j.tplants.2009.03.005>
- Jiang M, Zhang J (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant Cell Physiol* 42(11):1265–1273. <https://doi.org/10.1093/pcp/pce162>
- Kovinich N, Kayanja G, Chanoca A, Riedl K, Otegui MS, Grotewold E (2014) Not all anthocyanins are born equal: distinct patterns induced by stress in Arabidopsis. *Planta* 240(5):931–940. <https://doi.org/10.1007/s00425-014-2079-1>
- Lepisto A, Pakula E, Toivola J, Krieger-Liszakay A, Vignols F, Rintamaki E (2013) Deletion of chloroplast NADPH-dependent thioredoxin reductase results in inability to regulate starch synthesis and causes stunted growth under short-day photoperiods. *J Exp Bot* 64(12):3843–3854. <https://doi.org/10.1093/jxb/ert216>
- Leverone LA, Stroup TL, Caruso JL (1991) Western blot analysis of cereal grain prolamins using an antibody to carboxyl-linked indoleacetic acid. *Plant Physiol* 96(4):1076–1078. <https://doi.org/10.1104/pp.96.4.1076>
- Makino A (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. *Plant Physiol* 155(1):125–129. <https://doi.org/10.1104/pp.110.165076>
- Mazel A, Leshem Y, Tiwari BS, Levine A (2004) Induction of salt and osmotic stress tolerance by overexpression of an intracellular vesicle trafficking protein AtRab7 (AtRabG3e). *Plant Physiol* 134(1):118–128. <https://doi.org/10.1104/pp.103.025379>
- Najera VA, Gonzalez MC, Perez-Ruiz JM, Cejudo FJ (2017) An event of alternative splicing affects the expression of the NTRC gene, encoding NADPH-thioredoxin reductase C, in seed plants. *Plant Sci* 258:21–28. <https://doi.org/10.1016/j.plantsci.2017.02.001>
- Ojeda V, Pérez-Ruiz JM, González M, Nájera VA, Sahrawy M, Serrato AJ, Geigenberger P, Cejudo FJ (2017) NADPH thioredoxin reductase C and thioredoxins act concertedly in seedling development. *Plant Physiol* 174(3):1436–1448. <https://doi.org/10.1104/pp.17.00481>
- Ortigosa F, Valderrama-Martin JM, Avila C, Canovas FM, Canas RA (2019) Understanding plant nitrogen nutrition through a laboratory experiment. *Biochem Mol Biol Educ* 47(4):450–458. <https://doi.org/10.1002/bmb.21239>
- Porto NP, Bret RSC, Souza PVL, Candido-Sobrinho SA, Medeiros DB, Fernie AR, Daloso DM (2022) Thioredoxins regulate the metabolic fluxes throughout the tricarboxylic acid cycle and associated pathways in a light-independent manner. *Plant Physiol Biochem* 193:36–49. <https://doi.org/10.1016/j.plaphy.2022.10.022>
- Qiao P, Zhao F, Liu M, Gao D, Zhang H, Yan Y (2017) Hydrogen sulfide inhibits mitochondrial fission in neuroblastoma N2a cells through the Drp1/ERK1/2 signaling pathway. *Mol Med Rep* 16(1):971–977. <https://doi.org/10.3892/mmr.2017.6627>
- Qu ZM, Qi XC, Shi RG, Zhao YJ, Hu ZP, Chen Q, Li CL (2020) Reduced N fertilizer application with optimal blend of controlled-release urea and urea improves tomato yield and quality in greenhouse production system. *J Soil Sci Plant Nut* 20(4):1741–1750. <https://doi.org/10.1007/s42729-020-00244-8>
- Rasheed R, Ashraf MA, Ahmad SJN, Parveen N, Hussain I, Bashir R (2022) Taurine regulates ROS metabolism, osmotic adjustment, and nutrient uptake to lessen the effects of alkaline stress on *L. plants*. *S Afr J Bot* 148:482–498. <https://doi.org/10.1016/j.sajb.2022.05.023>
- Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227(4691):1229–1231. <https://doi.org/10.1126/science.227.4691.1229>
- Reichheld JP, Meyer E, Khafif M, Bonnard G, Meyer Y (2005) AtNTRB is the major mitochondrial thioredoxin reductase in Arabidopsis thaliana. *FEBS Lett* 579(2):337–342. <https://doi.org/10.1016/j.febslet.2004.11.094>
- Reichheld JP, Khafif M, Riondet C, Droux M, Bonnard G, Meyer Y (2007) Inactivation of thioredoxin reductases reveals a complex interplay between thioredoxin and glutathione pathways in Arabidopsis development. *Plant Cell* 19(6):1851–1865. <https://doi.org/10.1105/tpc.107.050849>
- Sakouhi L, Kharbech O, Ben Massoud M, Gharsallah C, Ben Hassine S, Munemasa S, Murata Y, Chaoui A (2021) Calcium and ethylene glycol tetraacetic acid mitigate toxicity and alteration of gene expression associated with cadmium stress in chickpea (*Cicer arietinum* L.) shoots. *Protoplasma* 258(4):849–861. <https://doi.org/10.1007/s00709-020-01605-x>
- Serrato AJ, Perez-Ruiz JM, Spinola MC, Cejudo FJ (2004) A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana. *J Biol Chem* 279(42):43821–43827. <https://doi.org/10.1074/jbc.M404696200>
- Souza PVL, Hou LY, Sun H, Poeker L, Lehman M, Bahadar H, Domingues AP, Dard A, Bariat L, Reichheld JP, Silveira JAG, Fernie AR, Timm S, Geigenberger P, Daloso DM (2023a) Plant NADPH-dependent thioredoxin reductases are crucial for the metabolism of sink leaves and plant acclimation to elevated CO₂. *Plant Cell Environ* 46(8):2337–2357. <https://doi.org/10.1111/pce.14631>
- Souza PVL, Hou LY, Sun H, Poeker L, Lehman M, Bahadar H, Domingues AP, Dard A, Bariat L, Reichheld JP, Silveira JAG, Fernie AR, Timm S, Geigenberger P, Daloso DM (2023b) Plant NADPH-dependent thioredoxin reductases are crucial for the metabolism of sink leaves and plant acclimation to elevated CO₂. *Plant Cell Environ* 46(8):2337–2357. <https://doi.org/10.1111/pce.14631>
- Wang Y, Xu L, Tang M, Jiang H, Chen W, Zhang W, Wang R, Liu L (2016) Functional and integrative analysis of the proteomic profile of radish root under Pb exposure. *Front Plant Sci* 7:1871. <https://doi.org/10.3389/fpls.2016.01871>
- Wang XY, Jiang JX, Wu ZH, Li XM, Dong SK (2021) Relationship between soil moisture content and superoxide dismutase activity in soybean leaves in northeast China. *J Biobased Mater Bioenergy* 15(6):799–804. <https://doi.org/10.1166/jbmb.2021.2148>
- Wang G, Zhang L, Zhang S, Li B, Li J, Wang X, Zhang J, Guan C, Ji J (2023) The combined use of a plant growth promoting *Bacillus* sp. strain and GABA promotes the growth of rice under salt stress by regulating antioxidant enzyme system, enhancing photosynthesis and improving soil enzyme activities. *Microbiol Res* 266:127225. <https://doi.org/10.1016/j.micres.2022.127225>
- Xing WW, Li L, Gao P, Li H, Shao QS, Shu S, Sun J, Guo SR (2015) Effects of grafting with pumpkin rootstock on carbohydrate metabolism in cucumber seedlings under Ca(NO₃)₂ stress. *Plant Physiol Biochem* 87:124–132. <https://doi.org/10.1016/j.plaphy.2014.12.011>
- Ye B, Gitler C, Gressel J (1997) A high-sensitivity, single-gel, polyacrylamide gel electrophoresis method for the quantitative determination of glutathione reductases. *Anal Biochem* 246(2):159–165. <https://doi.org/10.1006/abio.1996.9985>
- Zhai J, Qi Q, Wang M, Yan J, Li K, Xu H (2022) Overexpression of tomato thioredoxin h (SlTrxh) enhances excess nitrate stress tolerance in transgenic tobacco interacting with SlPrx protein. *Plant Sci* 315:111137. <https://doi.org/10.1016/j.plantsci.2021.111137>

- Zhang YJ, Yu SG, Li ZJ, Chang TT, Xu QC, Xu HL, Zhang J (2022) Effects of excessive nitrogen fertilizer and soil moisture deficiency on antioxidant enzyme system and osmotic adjustment in tomato seedlings. *Int J Agr Biol Eng* 15(2):127–134. <https://doi.org/10.25165/j.ijabe.20221502.5555>
- Zhang HB, Yao TT, Wang Y, Wang JC, Song JQ, Cui CC, Ji GX, Cao JN, Muhammad S, Ao H, Zhang HH (2023) CDSP32-overexpressing tobacco plants improves cadmium tolerance by modulating antioxidant mechanism. *Plant Physiol Biochem* 194:524–532. <https://doi.org/10.1016/j.plaphy.2022.11.036>
- Zhao MG, Chen L, Zhang LL, Zhang WH (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation

and freezing tolerance in Arabidopsis. *Plant Physiol* 151(2):755–767. <https://doi.org/10.1104/pp.109.140996>

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