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Article

SlTrxh functions downstream of SlMYB86 and positively regulates nitrate stress tolerance via S-nitrosation in tomato seedling

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Abstract

Nitric oxide (NO) is a redox-dependent signaling molecule that plays a crucial role in regulating a wide range of biological processes in plants. It functions by post-translationally modifying proteins, primarily through S-nitrosation. Thioredoxin (Trx), a small and ubiquitous protein with multifunctional properties, plays a pivotal role in the antioxidant defense system. However, the regulatory mechanism governing the response of tomato Trxh (SlTrxh) to excessive nitrate stress remains unknown. In this study, overexpression or silencing of *SlTrxh* in tomato led to increased or decreased nitrate stress tolerance, respectively. The overexpression of *SlTrxh* resulted in a reduction in levels of reactive oxygen species (ROS) and an increase in S-nitrosothiol (SNO) contents; conversely, silencing *SlTrxh* exhibited the opposite trend. The level of S-nitrosated SlTrxh was increased and decreased in *SlTrxh* overexpression and RNAi plants after nitrate treatment, respectively. SlTrxh was found to be susceptible to S-nitrosation both *in vivo* and *in vitro*, with Cysteine 54 potentially being the key site for S-nitrosation. Protein interaction assays revealed that SlTrxh physically interacts with SlGrx9, and this interaction is strengthened by S-nitrosation. Moreover, a combination of yeast one-hybrid (Y1H), electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR), and transient expression assays confirmed the direct binding of SlMYB86 to the *SlTrxh* promoter, thereby enhancing its expression. SlMYB86 is located in the nucleus and *SlMYB86* overexpressed and knockout tomato lines showed enhanced and decreased nitrate stress tolerance, respectively. Our findings indicate that SlTrxh functions downstream of SlMYB86 and highlight the potential significance of S-nitrosation of SlTrxh in modulating its function under nitrate stress.

Introduction

Nitrogen (N) is an essential macronutrient for plant growth. The rates of nitrogen fertilizer supplies have experienced a significant surge, particularly in vegetable production under protected agricultural systems. Nevertheless, plants uptake less than 50% of the N fertilizer provided, while the remaining amount is either lost through leaching in the soil or discharged as agricultural runoffs [1]. Excess nitrate (NO_3^-) may be accumulated in soils and their quality deteriorates faster in greenhouse vegetable production than in conventional crop rotations [2]. Previous studies have reported substantial differences in ion accumulation between protected agricultural land and coastal areas. The soluble salt components, ranked in order of percentage, were as follows: $NO_3^- > SO_4^{2-} > Ca^{2+} > Cl^- > Na^+$, based on mass [3]. Greenhouse soils often accumulate nitrates in higher levels, which can pose environmental hazards and result in a decline in the quality of vegetables [4]. Regarding plant physiological issues, NO_3^- excess caused oxidative stress in spinach [5], limited cucumber plant development [6], modifications in the composition of the cell as well as in its structure, and patterns of genes expression linked to lignin synthesis in *Brassica napus* [7]. Furthermore, nitrate excess altered the accumulation of proteins linked to photosynthetic processes, stress and oxidative damage responses [8].

Nitric oxide (NO) is a gaseous molecule and a crucial biological messenger that plays a pivotal role in various plant physiological processes, including morphogenesis, development, and tolerance to biotic and abiotic stress conditions [9–12]. NO primarily functions through S-nitrosation [13, 14]. This process involves the covalent attachment of a NO molecule to a cysteine thiol group on a protein or peptide, leading to the formation of S-nitrosothiols (SNOs). The level of S-nitrosation of cellular protein is linked to the equilibrium between S-nitrosation and denitrosation. The main protein denitrosylases in human cells are glutathione (GSH), S-nitrosoglutathione reductase (GSNOR), and thioredoxin (Trx). The role of GSH as a denitrosylating agent was well established [15]. The natural NO donor, S-nitrosoglutathione (GSNO), synthesized by the reaction of NO with GSH, acts as a reservoir of NO bioactivity [16]. GSNOR influences the bioavailability of NO via

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Luciferase complementation assay (LCA)

The pCAMBIA1300-35S-cLUC and pCAMBIA1300-35S-nLUC vectors were utilized to insert the coding sequence of SlTrxh and SlGrx9, resulting in the cLUC-SlTrxh and SlGrx9-nLUC constructs for the LCA assay. *Agrobacterium* GV3101 strains carrying the mentioned constructs were infiltrated into the leaves of 4-week-old *N. benthamiana* tobacco plants and incubated for 48–72 hours. To investigate the interaction of S-nitrosation of SlTrxh with SlGrx9, the 54th cysteine was mutated to Tryptophan or Serine with DpnI enzyme (Takara, China). Nitrate and GSNO were added in the LCA experiment. Subsequently, the leaves were sprayed with a solution of 1 mM D-luciferin Firefly (Gold Biotechnology, St Louis, MO, USA) for 5 minutes, and the luciferase signals in the infiltrated region were detected using the Tanon 5200 Multi Chemiluminescent Imaging System (Tanon, China). The relative fluorescence intensity was carried out using ImageJ software. The list of primers used can be found in Table S9 (see online supplementary material).

Co-immunoprecipitation assays (Co-IP)

To perform co-immunoprecipitation assays, SlTrxh and SlGrx9 were tagged with GFP and FLAG tags, respectively, using the pCAMBIA1300-GFP and pCAMBIA1300-FLAG vectors. The properly constructed vectors were co-injected into the leaves of 4-week-old *N. benthamiana* plants and incubated for 48–72 hours. Subsequently, a Co-IP assay was conducted using the Beyotime Anti-Flag Affinity Gel Kit (Beyotime, China). All primers used can be found in Table S10 (see online supplementary material).

Subcellular localization analysis

The coding sequence of *SlMYB86* was inserted into the pRI101-GFP vector to create the pRI101-GFP-SlMYB86 plasmid, which included a 35S promoter, and then transferred into the *A. tumefaciens* EHA105 strain. Subsequently, the *Agrobacterium* carrying the pRI101-GFP-SlMYB86 and pRI101-GFP constructs were infiltrated into the epidermal cells of 4-week-old *N. benthamiana* leaves. Fluorescence images were captured using a laser confocal microscope model Olympus FV1000 (Olympus, Tokyo, Japan). The list of primers used can be found in Table S11 (see online supplementary material).

Statistical analysis

The experimental design involved testing and analysing the results for three biological replicates where each replicate was evaluated using three technical repetitions. The differences between treatments were assessed using a one-way ANOVA and Duncan's multiple range test, in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) and SPSS software (IBM, USA). Distinct letters represent statistically significant variances at a significance level of $P < 0.05$.

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Author contributions

H.X. designed the experiments and wrote the manuscript. S.Z., X.S., J.Z., and X.L. performed the experiments and analysed the data. X.S., P.G.-C., H.N., and K.L. helped in revising the manuscript.

Data availability

The whole of the data used in this study are available in the publication and its supplementary information files.

Conflict of interest statement

We declare the absence of any conflicts of interest.

Supplementary data

Supplementary data is available at *Horticulture Research* online.

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