

Proteomic Analysis of the Protective Effect of Sodium Nitroprusside on Leaves of Barley Stressed by Salinity

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ABSTRACT

Objective: The salinization of agricultural soils poses a serious challenge across the world. Although recent studies have shown that exogenous sodium nitroprusside (SNP) application can alleviate the harmful effects of salinity, the roles of SNP in the regulation of proteomic changes remain poorly understood.

Materials and Methods: To unravel the protective roles of exogenous SNP in alleviating salt-induced damage in barley (*Hordeum vulgare* L.), proteomic analysis was carried out on the leaves of seedlings exposed to 100 mM NaCl stress following 200 µM SNP pre-treatment.

Results: Our results indicated that SNP pre-treatment restored the seedling growth reduced by salinity stress. Comparing 2-DE gels from the treatments showed that 24 proteins were differentially accumulated under SNP and/or NaCl stress treatments. Among them, 15 proteins were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Gene ontology analysis demonstrated that several pathways were regulated by SNP and/or NaCl treatments, including photosynthesis, protein metabolism, stress defense, and energy metabolism. Exogenous SNP increased the expression levels of 20 kDa chaperonin, proteasome subunit beta type-2, 2-Cys peroxiredoxin BAS1, ferredoxin-NADP reductase, thiazole biosynthetic enzyme 1-1, S-adenosylmethionine synthetase 3, and elongation factor Tu proteins in the leaves of barley seedlings under NaCl stress.

Conclusion: Our results indicate that SNP pre-treatment may induce salinity tolerance through regulation of photosynthesis, activation of stress defence, degradation of damaged proteins, and the promoting of the synthesis of polyamines, proline, and GABA.

Keywords: Barley, nitric oxide, salinity stress, sodium nitroprusside, proteomics

INTRODUCTION

Plants are faced with a range of environmental constraints which can frequently cause significant reductions in growth and development. Of these, salinity is regarded as one of the most important environmental constraints that depress plant growth by inducing water deficiency and ion toxicity. It has been estimated that approximately 6% of the world's total land area is exposed to salinity (1,2). Since high soil salinity leads to a decline in crop productivity, improving salinity tolerance of agricultural plants has become an urgent priority. One of the ways to increase the adaptation of

plants to salt stress is exogenous applications of various chemicals such as phytohormones, antioxidants, signal molecules, and trace elements (3,4). Among those, nitric oxide (NO) has significant and diversified functions in mitigation of salt-induced adverse effects (5).

Nitric oxide is a gaseous free radical, and it regulates several physiological processes such as seed germination and seedling growth (6). Numerous studies have examined the ameliorative effects of exogenous sodium nitroprusside (SNP; NO donor) on salt-induced metal toxicity in plants (4,7). Different mechanisms have been suggested for NO-mediated salt tolerance. Yadu et al. (8)



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and Ahmad et al. (4) have demonstrated that NO improved the salinity tolerance of pea and tomato seedlings by enhancing the antioxidant capacity and synthesis of flavonoid, glycine betaine, and proline. Exogenous application of SNP has also been shown to improve photosynthetic capacity by protecting pigments, enhancing quantum-yield of photosystem II, and increasing stomatal conductance and RuBisCO activity (9,10).

Although the ameliorative effects of exogenous NO under salinity stress have been comprehensively studied, there have also been several attempts to unravel the molecular basis of NO-induced salinity tolerance (11,12). It has been suggested that exogenous NO helped maize seedlings to overcome salinity stress by enhancing the expression of G-protein-associated proteins and activating antioxidant system (11). Another previous study showed that exogenous NO up-regulated the abundance proteins functioning in energy metabolism, photosynthesis and stress response in mangrove plant *Avicennia marina* leaves under salinity stress (12). Since a few studies have examined the protective roles of NO at proteome levels, we used a 2-DE based proteomic analysis to identify protein alterations in response to salt stress and NO in barley leaves.

MATERIALS AND METHODS

Plant Material and Treatments

Seeds of barley (*Hordeum vulgare* L. cv. Tarm-92) were obtained from Ankara Field Crops Central Research Institute. After surface sterilization of the seeds in 1% NaOCl solution, the seeds were washed 5 times with sterile distilled water. The sterilized seeds were germinated in a controlled climate cabin (23°C, dark, 60% humidity) for 48 h. The uniformly germinated seedlings were placed into perforated tubes with 3 seedlings per tube. Five tubes were placed in a pot containing 0.5 L of modified Hoagland nutrient solution (pH 6.0). The seedlings were grown for 3 d under relative humidity of 60%, 14 h photoperiod (250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and constant temperature of 25 °C in the growth chamber. For SNP pre-treatment, 200 μM NO donor SNP was applied along with the nutrient solution for 48 h. After pre-treatment, salinity stress was induced using a nutrient solution supplemented with 100 μM NaCl. The treatments of SNP and NaCl were as follows: (1) control; 0 mM NaCl, (2) SNP; 200 μM SNP, (3) NaCl; 100 μM NaCl, and (4) SNP+NaCl; 200 μM SNP + 100 μM NaCl. Experiments were arranged in a randomized complete block design. After seven days of NaCl treatment, the seedlings were separated into roots and shoots, and the fresh weight (FW) was weighed. Dry weights (DW) of root and shoot tissues were determined after drying at 80 °C for 2 d.

Protein Extraction and Two-Dimensional Gel Electrophoresis

The total proteins from fresh barley leaves were extracted using the phenol extraction protocol described by Ahsan et al. (13). One gram of mixed root tissues was ground with liquid nitrogen, and fine powders were transferred into the extraction solution (0.5 M Tris-HCl (pH 8.3), 2% β -mercaptoethanol, 2% NP-40, 0.7 M sucrose, 20 μM MgCl₂, and 1 μM phenylmethyl sulfonyl fluoride), and fractionated with Tris-buffered (pH 8.0) phenol. After centrifugation, phenol phase was mixed with methanol containing ammonium acetate to precipitate the proteins. The Bradford assay was used to

quantify protein concentration by dissolving air-dried pellets in a lysis buffer containing 7 M urea, 2 M thiourea, 40 μM DL-dithiothreitol (DTT), 0.2% biolytes (pH 3–10), and 4% CHAPS (14).

For separation of proteins via 2-DE, IPG strips (pH 4–7, Bio-Rad) were used. Dry IPG strips were passively rehydrated with 300 μL of rehydration buffer containing 80 μg proteins. Isoelectric focusing of the rehydrated strips was performed on the Proteans i12™ IEF System for 80 kVh. For the reduction and alkylation of the sulfhydryl groups, the strips were equilibrated with buffer A (0.05 M Tris-HCl pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 1% DTT), and then with buffer B (2.5% iodoacetamide instead of DTT). Second dimension electrophoresis was carried out on 12.5% SDS-PAGE gels using a Protean II XL Cell System (Bio-Rad). The preparative gels containing 500 μg proteins were stained with a modified method of colloidal Coomassie brilliant blue (15), and analytical gels were silver-stained (16).

Image Analysis and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF MS)

Images of the triplicate silver-stained gels were obtained using the ChemiDoc™ MP System and they were evaluated with PDQuest 8.01 (Bio-Rad). Before spot matching, spot detection and background subtraction were performed. The volume of each protein spot was normalized on the basis of total density of gels. The student's t-test with a significance level of 95% was performed to evaluate the significant changes in spots among treatments ($p < 0.05$). A protein showing a more than 1.5-fold increase in abundance in at least one treatment was considered as a differentially expressed protein.

Trypsin digestion was carried out using a commercial kit (ThermoFisher Scientific). A solution of digested peptides was purified using ZipTip C₁₈ pipette tips (Millipore), mixed with the matrix solution (α -cyano-hydroxy cinnamic acid), and then spotted onto a MALDI plate. Peptide mass spectra were obtained using an AB Sciex TOF/TOF 5800 mass spectrometer.

The obtained MS/MS spectra were used to search the Swiss-Prot database with the MASCOT search engine (<http://www.matrix-science.com>). The search criteria were as follows: Taxonomy, Viridiplantae (green plants); database, Swiss-Prot; Enzyme, Trypsin; Mass accuracy, 50 ppm; Peptide MS tolerance, ± 0.4 Da; Variable modifications, Oxidation of methionine; Fixed modification, Carbamidomethylation of cysteine; and allowance of one missed cleavage. The differentially expressed proteins were classified on the basis of functions according to the database at <http://www.uniprot.org/uniprot>. To explore the relationships in the protein-protein interaction (PPI) network, proteins identified in the current study were analysed by STRING 11.0 (17). BiNGO was used to estimate biological processes and molecular functions (18).

Statistical Analysis

All experiments with triplicate were performed twice. Statistical analyses were performed by analysis of variance (ANOVA) using SPSS 22.0 software. Means from physiological analyses were statistically analysed with Duncan's multiple range test (DMRT).

RESULTS

Effect of SNP on Barley Growth under NaCl Stress

The effect of 200 μ M SNP pre-treatment and 100 μ M NaCl stress on growth of barley seedlings in this study is given in Table 1. NaCl stress resulted in significant reductions in both shoot and root fresh and dry weights compared to the control ($p < 0.05$). However, SNP pre-treatment completely restored the growth parameters except for shoot fresh weight. Additionally, SNP pre-treatment resulted in an increase in all growth parameters compared to the control (Table 1).

Table 1. The effects of SNP pre-treatment and NaCl stress on fresh (FW) and dry weights (DW) of barley seedlings

Treatments	Shoot	Shoot	Root	Root
	FW	DW	FW	DW
mg.seedling ⁻¹				
Control	647 \pm 26 ^b	63.7 \pm 1.2 ^a	223 \pm 8.6 ^b	14.0 \pm 0.4 ^b
SNP	737 \pm 25 ^a	68.2 \pm 2.4 ^a	249 \pm 13.9 ^a	16.3 \pm 0.2 ^a
NaCl	409 \pm 36 ^d	49.4 \pm 2.6 ^b	160 \pm 15.3 ^c	12.4 \pm 0.8 ^c
SNP + NaCl	542 \pm 21 ^c	64.0 \pm 4.9 ^a	233 \pm 6.4 ^{ab}	16.4 \pm 1.0 ^a

* Different letters (a-d) within the same column indicate significant differences between treatments at $P < 0.05$, according to DMRT. Each value is the average of six replicates, in which 10 seedlings are sampled per replicates. Standard error (\pm SE).

Effect of SNP on NaCl-Induced Proteome Alterations

In this study, proteome profiles of proteins extracted from leaf tissues of barley seedlings from control, SNP, NaCl and SNP+NaCl treatments were obtained using the 2-DE (IEF/SDS-PAGE) analysis (Figure 1). In the 2-DE gel profiles, changes in the expression of proteins were analysed using the PDQuest software. According to image analysis, 24 protein spots which showed at least a 1.5-fold difference in expression level were determined in the leaf tissue. Among them, 15 proteins were successfully identified by MALDI-TOF/TOF analysis and Swiss-Prot database searching (Table 2).

The identified proteins were grouped into various categories according to the biological functions such as photosynthesis, stress defence, energy metabolism, protein metabolism, and primary metabolism (Table 2). In the present study, 6 proteins (spots 1, 2, 3, 4, 10 and 12) associated with photosynthesis were differently accumulated in SNP and/or NaCl treatments compared to the control (Figure 1, Table 2). The expression level of the RuBisCO small subunit (RBCS, spot 1) increased in NaCl treatment, while it decreased to control level in SNP+NaCl application. The expression levels of another RuBisCO small subunit (spot 2) and cytochrome b6-f complex iron-sulphur subunit (spot 3) were down-regulated in SNP+NaCl treatment. Additionally, the expression levels of RuBisCO large subunit (RBCL, spot 4) and RuBisCO activase A (RCA, spot 12) proteins were down-regulated by NaCl treatment, while SNP pre-treatment removed this inhibition. Additionally, the expression level of the ferredoxin-NADP reductase (FNR, spot 10) protein was up-regulated by SNP and SNP+NaCl treatments (Table 2).

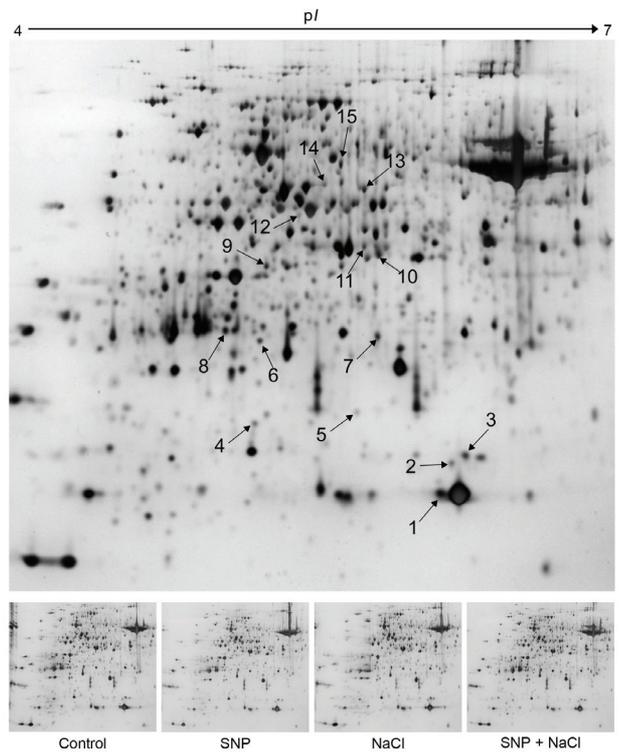


Figure 1. Two-dimensional (2-D) electrophoretic profiles of proteins extracted from leaf tissues of barley seedlings exposed to control, SNP, NaCl, and SNP + NaCl treatments. Total proteins (80 μ g) were loaded on 17 cm IPG strips (pH 4-7), and SDS-PAGE was performed on 12% gel. Proteins were visualised by silver staining. Arrow indicates the differentially accumulated proteins (Table 2).

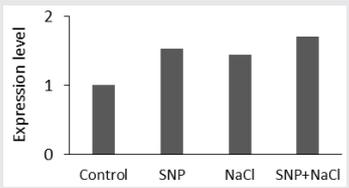
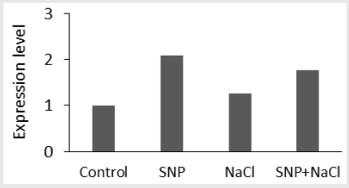
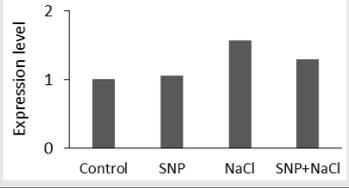
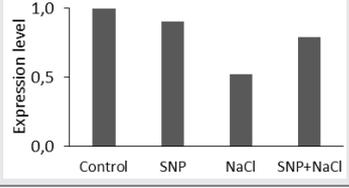
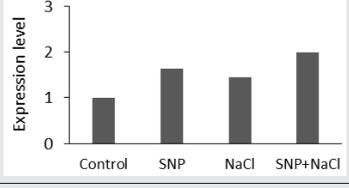
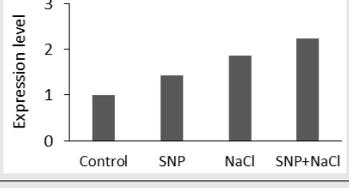
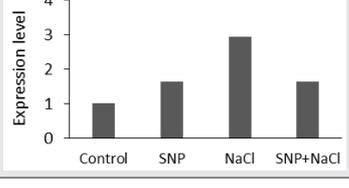
Four proteins (spots 5, 6, 7, and 14) related to protein synthesis and metabolisms were differentially expressed in SNP and/or NaCl treatments (Figure 1, Table 2). The expression level of the eukaryotic translation initiation factor 5A-3 (ELF5A-3, spot 5) protein was down-regulated by SNP and NaCl treatments while it decreased to the control level in SNP+NaCl treatment. Proteasome subunit beta type-2 (spot 7) protein was up-regulated by SNP+NaCl treatment. Additionally, 20 kDa chaperonin (CPN20, spot 6) and elongation factor Tu (EFTU, spot 14) proteins increased gradually (Table 2).

The expression level of the 2-Cys peroxylodoxin BAS1 (BAS, spot 8) protein was up-regulated by SNP+NaCl treatment. However, the abundance of thiazole biosynthetic enzyme 1-1 (THI1, spot 9) was up-regulated by SNP and SNP+NaCl treatments (Table 2). A glycolysis related protein fructose-bisphosphate aldolase (FBA, spot 11) was increased by NaCl treatment, while SNP pre-treatment removed this increase. S-adenosylmethionine synthetase 3 (SAMS, spot 13) protein was increased by SNP and SNP+NaCl treatments. Finally, the abundance of glutamate decarboxylase (GAD, spot 15) protein, which plays a role in the synthesis of gamma-aminobutyric acid (GABA), increased in all applications compared to control (Table 2).

Table 2. Proteins identified by MALDI-TOF/TOF mass spectrometry in leaf tissues of barley seedlings exposed to SNP pre-treatment and/or NaCl stress.

Spot	Accession number	Protein	Score	MW/pI	Seq. cover.	MP	Expression level
1	RBS_HORVU	Ribulose biphosphate carboxylase small chain	293	19.4/8.98	55%	22	
2	RBS_HORVU	Ribulose biphosphate carboxylase small chain	52	19.4/8.98	27%	8	
3	UCRIA_WHEAT	Cytochrome b6-f complex iron-sulfur subunit	264	23.7/8.47	57%	15	
4	RBL_HORVU	Ribulose biphosphate carboxylase large chain	316	53.0/6.22	13%	14	
5	IF5A3_ARATH	Eukaryotic translation initiation factor 5A-3	60	17.2/5.56	32%	7	
6	CH10C_ARATH	20 kDa chaperonin	47	26.8/8.86	11%	4	
7	PSB2_ORYSJ	Proteasome subunit beta type-2	63	23.5/5.42	16%	5	
8	BAS1_HORVU	2-Cys peroxiredoxin BAS1	83	23.3/5.48	23%	13	

Table 2. Continued.

Spot	Accession number	Protein	Score	MW/pI	Seq. cover.	MP	Expression level
9	THI41_MAIZE	Thiazole biosynthetic enzyme 1-1	260	37.1/5.22	24%	17	
10	FENR1_PEA	Ferredoxin--NADP reductase	203	40.2/8.56	31%	19	
11	ALFC_ORYSJ	Fructose-bisphosphate aldolase, chloroplastic	146	41.9/6.38	16%	11	
12	RCAA_HORVU	Ribulose bisphosphate carboxylase/oxygenase activase A	246	51.0/8.04	29%	26	
13	METK3_HORVU	S-adenosylmethionine synthetase 3	160	42.7/5.51	33%	17	
14	EFTU_PEA	Elongation factor Tu	209	53.0/6.62	12%	8	
15	DCE_SOLLC	Glutamate decarboxylase	92	56.7/5.97	9%	8	

Based on STRING analysis, protein-protein interactions of identified proteins are shown in Figure 2a. RCA, FNR, FBA and BAS proteins were found to be important interaction points, suggesting that photosynthesis, energy metabolism and stress defence are very important for response to NaCl stress and exogenous SNP. Differently expressed proteins

were analysed using BiNGO to obtain statistically under- and over-represented categories of molecular functions and biological pathways related to NaCl stress and exogenous SNP (Figure 2b). NaCl stress and exogenous SNP promoted predominantly stress-responsive proteins and less photosynthesis-related proteins.

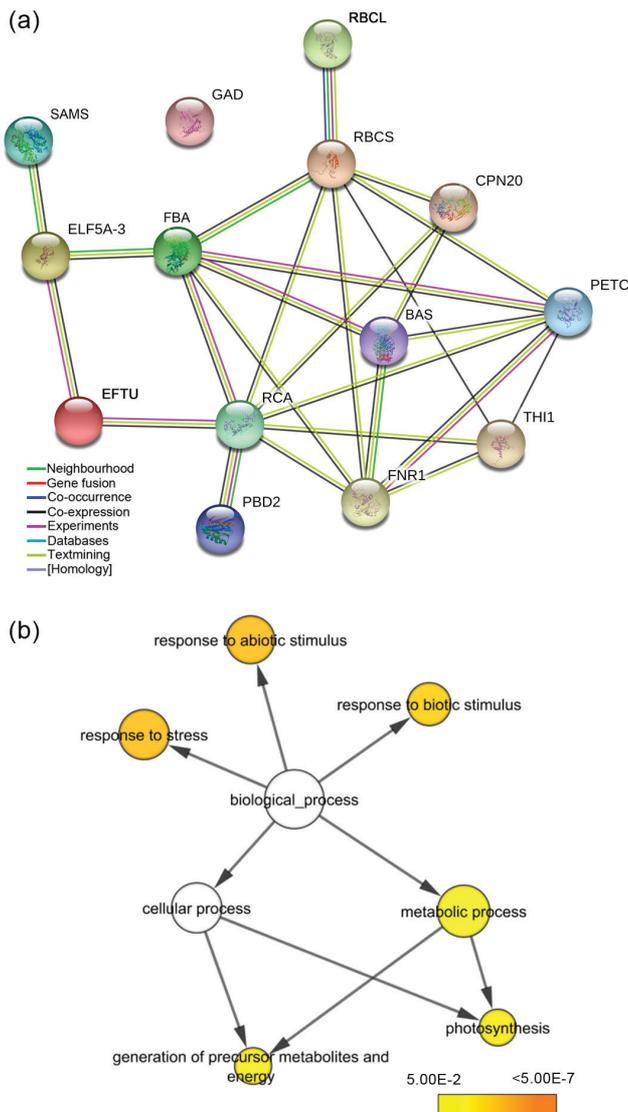


Figure 2. a) Protein-protein interactions were created using the STRING system (<http://string.embl.de>). Lines of different colours indicate different evidence types for the association of the proteins. b) The molecular function networks generated by BiNGO. The size of the node is proportional to the number of molecules within this group, and the colour of the node represents the significance of enrichment.

DISCUSSION

Nitric oxide has been reported to be a signalling molecule that alleviates the reduction in growth of plants under diversified biotic and abiotic stresses (4,19,20). In our study, SNP pre-treatment alleviated the detrimental effects of NaCl stress on seedling growth. These results have also been shown in many plant species exposed to NaCl stress (4,7,8). It has been reported that NO can loosen the cell wall, affect the phospholipid layer, increase membrane fluidity, and promote plant growth by increasing cell expansion (21). Dong et al. (22) re-

ported that NO increases stem and root elongation by improving cytoplasmic viscosity and increasing the osmotic pressure under high salinity. However, it has been demonstrated that NO-mediated induction of salt tolerance is associated with increased activities of antioxidant enzymes and the accumulation of osmoprotectants (4,8). Additionally, NO has been shown to interact with salicylic acid and hydrogen peroxide to mediate the elimination of oxidative damage in many stress conditions (23). Overall, SNP-induced salt tolerance in barley seedlings may be associated with a high accumulation of important osmotic protective compounds or promoting the antioxidant defence system.

High soil salinity limits the productivity of agricultural crops in arid and semi-arid regions. Thus, understanding the molecular basis of salt stress tolerance in plants is useful for the development of plant species that can tolerate salt stress. In the present study, the effects of SNP and/or NaCl stress on leaf tissues of barley seedlings were evaluated using a proteomic approach. The differential regulation of salt-responsive proteins by SNP pre-treatment stress is discussed below.

It is well-known that salinity stress can severely inhibit the photosynthetic pathway (24). The decrease in photosynthetic activity under salt stress conditions is directly related to the expression levels of proteins associated with photosynthesis. It has been reported that the decrease in the photosynthetic activity reduces the ability of plants to overcome stress (3). In our study, proteomic analysis showed that 6 photosynthetic proteins involved in CO₂ fixation and light reactions were expressed differently in NaCl stress and/or SNP treatments (Table 2). The abundance of RBCL and RCA proteins decreased in salt stress conditions, but this decrease was restored by SNP pre-treatment. Additionally, salt stress caused an approximately 2-fold increase in the expression level of the RBCS protein compared to the control. RCA has been shown to play a significant role in maintaining assimilation at low CO₂ levels caused by salt stress (25). FNR plays a role in photosynthetic electron flow from reduced ferredoxin to NADP⁺ and is required for CO₂ fixation in plants (26). Additionally, ferredoxin has been reported to play a role in different biological pathways including phenolic biosynthesis, nitrogen fixation, biogenesis of iron-sulphur clusters, and detoxification of xenobiotics (27). However, overexpression of the chloroplastic FNR enzyme in agricultural plants has been reported to increase oxidative stress tolerance (28). In our study, the expression level of FNR protein was increased by SNP and SNP+NaCl treatments. As a result, a higher activity of photosynthetic enzymes after SNP treatments could provide a better photosynthetic activity and improve salt tolerance of barley seedlings.

Salt stress can seriously affect protein synthesis and cause ER stress by disrupting protein folding (29). In our study, the abundance of chloroplastic EFTU protein increased in NaCl, and its expression further increased in SNP+NaCl treatment. Previous research has shown that EFTU plays a significant role in the high temperature tolerance of *Brassica campestris* plants (30). SNP-induced up-regulation of EFTU protein under salt stress

conditions may repair the damaged photosynthetic proteins in chloroplasts by increasing protein biosynthesis under salt stress. However, the expression level of molecular chaperone CPN20 protein was up-regulated by SNP pre-treatment in NaCl-stressed seedlings. Chaperonins help protein folding and assembling and function in protecting and repairing proteins under stress conditions (31). Additionally, the abundance of the proteasome subunit beta type-2 protein was up-regulated by SNP+NaCl treatment. This protein has been reported to play a role in ubiquitin-mediated protein degradation (32). This result suggests that NO can alleviate salt stress-induced damage by increasing the degradation of misfolded or damaged proteins. As a result, an SNP-induced increase in these proteins can lead to a general improvement in cellular processes during stress conditions.

The over-production of reactive oxygen species is a well-documented indirect effect of salinity stress (33). In our study, the abundance of BAS and THI1 protein was increased by SNP+NaCl treatment. It has been reported that a thiol specific antioxidant BAS is localized to chloroplasts and it has antioxidant and chaperone activity during photosynthesis and plant development (34). THI1 is an important enzyme functioning in the thiamine biosynthesis pathway and participates in stress tolerance mechanisms in yeast, bacteria, and *Arabidopsis thaliana* by repairing DNA damage (35,36). Additionally, thiamine has been shown to alleviate the effects of oxidative stress in plants during environmental stresses (37). SNP-induced up-regulation of BAS and THI1 proteins may alleviate NaCl-induced oxidative stress in barley leaves by reducing oxidative stress and maintaining DNA stability.

Regulation of energy metabolism is one of the important strategies for overcoming salinity stress (38). In our study, the level of expression of the glycolysis-related FBA protein was up-regulated by salt stress. FBA catalyses the reversible separation of fructose-1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Yin et al. (39) found that FBA is important in maintaining the physiological functions of *B. napus* leaves under biotic stress conditions. However, overexpression of the *Sesuvium portulacastrum* FBA gene (*SpFBA*) in *E. coli* bacteria enhanced salinity tolerance (40). It suggests that increased glycolysis activity may be a possible plant strategy to overcome salt stress.

S-adenosylmethionine synthase (SAMS) catalyse the biosynthesis of SAM from methionine and ATP, and SAM functions as the precursor of phytohormones such as polyamines and ethylene (41). Transgenic experiments have proven that SAM contributes to stress tolerance by increasing accumulation of polyamines (41,42). However, it has been stated that the abundance of SAMS protein was higher in salt-tolerant barley than in salt-sensitive barley (43). In our study, SNP pre-treatment resulted in an increase in the abundance of SAMS protein in both control and salt stress conditions. It can be suggested that an increased abundance of SAMS may increase salt tolerance by promoting polyamine biosynthesis.

Glutamate decarboxylase (GAD) is a Ca²⁺-dependent calmodulin-binding protein that catalyses the synthesis of amino-aminobutyric acid (GABA). GABA plays a role in various biological pathways including cytosolic pH regulation, nitrogen metabolism, and carbon flow to the TCA cycle (44). Renault et al. (45) reported that GABA accumulation was associated with GAD activity in Arabidopsis plants under salinity stress. Additionally, exogenous GABA application has been reported to increase salt tolerance by increasing antioxidant capacity and photosynthetic activity, ensuring osmotic adjustment and regulating water use efficiency (46). The increased GAD activity has been shown to be associated with the rapid accumulation of proline which increases salt tolerance in sesame (47). In our study, the expression level of GAD protein increased approximately 3-fold in NaCl application, and 1.6-fold in SNP+NaCl applications. This result suggested that the accumulation of GAD protein may contribute to salt tolerance of barley seedlings.

CONCLUSION

SNP application significantly increased biomass and helped maintain the growth of barley seedlings under salt stress. In our study, proteins that have a role in important biological functions and are differentially expressed in response to SNP application and/or NaCl stress in barley seedlings were identified by MALDI-TOF/TOF mass spectrometry. It can be argued that these proteins, which are mostly up-regulated, affect plant metabolism in order to overcome the negative effects of salinity stress. Exogenous SNP increased the expression levels of CPN20, BAS, FNR, THI1, SAMS, EFTU, and proteasome subunit beta type-2, proteins in the leaves of barley seedlings under NaCl stress. SNP has contributed greatly to the increase of salt tolerance by regulating photosynthesis, protein metabolism and defense systems. These findings will allow for a better understanding of SNP-induced salt tolerance mechanisms in barley plants.

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Conflict of Interest: The authors declare that they have no conflicts of interest to disclose.

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