# S-nitrosylation/denitrosylation as a regulatory mechanism of salt stress sensing in sunflower seedlings

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Nitric oxide (NO) and various reactive nitrogen species produced in cells in normal growth conditions, and their enhanced production under stress conditions are responsible for a variety of biochemical aberrations. The present findings demonstrate that sunflower seedling roots exhibit high sensitivity to salt stress in terms of nitrite accumulation. A significant reduction in S-nitrosoglutathione reductase (GSNOR) activity is evident in response to salt stress. Restoration of GSNOR activity with dithioerythritol shows that the enzyme is reversibly inhibited under conditions of 120 m$M$ NaCl. Salt stress-mediated S-nitrosylation of cytosolic proteins was analyzed in roots and cotyledons using biotin-switch assay. LC-MS/MS analysis revealed opposite patterns of S-nitrosylation in seedling cotyledons and roots. Salt stress enhances S-nitrosylation of proteins in cotyledons, whereas roots exhibit denitrosylation of proteins. Highest number of proteins having undergone S-nitrosylation belonged to the category of carbohydrate metabolism followed by other metabolic proteins. Of the total 61 proteins observed to be regulated by S-nitrosylation, 17 are unique to cotyledons, 4 are unique to roots whereas 40 are common to both. Eighteen S-nitrosylated proteins are being reported for the first time in plant systems, including pectinesterase, phospholipase D-alpha and calmodulin. Further physiological analysis of glyceraldehyde-3-phosphate dehydrogenase and monodehydroascorbate reductase showed that salt stress leads to a reversible inhibition of both these enzymes in cotyledons. However, seedling roots exhibit enhanced enzyme activity under salinity stress. These observations implicate the role of S-nitrosylation and denitrosylation in NO signaling thereby regulating various enzyme activities under salinity stress in sunflower seedlings.

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*Abbreviations* – ABC, ammonium bicarbonate; ACN, acetonitrile; APX, ascorbate peroxidase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; BSA, bovine serum albumin; BST, biotin-switch technique; DTT, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GSNO, S-nitrosoglutathione; GSNOR, GSNO reductase; GSSG, oxidized GSH; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hsp, heat shock protein; LC-MS/MS, liquid chromatrography- mass spectrometry; MDHAR, monodehydroascorbate reductase; MMTS, methylmethanethiosulfonate; NC, nitrocellulose; NR, nitrate reductase; PAGE, polyacrylamide gel electrophoresis; PD-10, protein desalting 10; PTM, posttranslational modification; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, room temperature; RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAM, S-adenosyl methionine; SDS, sodium dodecyl sulfate; SNO/RSNO, S-nitrosothiol; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; TSP, total soluble protein.

## Introduction

Nitric oxide (NO) is a bioactive, gaseous free radical, which plays a critical role as a diffusible intracellular signaling molecule in plant and animal cells. Ubiquitous signaling molecules are involved in various biochemical and physiological processes in plants (Neill et al. 2003, Mur et al. 2013). NO is endogenously produced through established biochemical routes namely, action of arginine-dependent enzymatic pathway, nitrite reduction by nitrate reductase (NR) or in the apoplast through non-enzymatic mechanisms (del Río et al. 2004). Thus, nitrite content in plant tissues indirectly correlates with the level of NO. Nitrite is considered as a biomarker of NO metabolism, thereby necessitating the quantification of nitrite levels in various plant organs and tissues (Salgado et al. 2013). NO has been shown to play critical role in seed germination (Beligni and Lamattina 2000), flowering (He et al. 2004), induction of lateral roots (Creus et al. 2005), abiotic and biotic stress tolerance (Jain and Bhatla 2017), adventitious root formation (Yadav et al. 2010) and various hormonal responses (Zhou et al. 2005). NO-induced signaling is mediated through generation of a variety of molecules collectively termed as reactive nitrogen species (RNS). These include peroxynitrite (ONOO$^-$), $NO_2$, S-nitrosoglutathione (GSNO) and $N_2O_3$ (Corpas and Barroso 2013). All RNS serve as nitrosative stress biomarkers in plants, and together with reactive oxygen species (ROS), their overproduction may be involved in cell damage/death. NO and various RNS produced in cells in normal conditions and their enhanced production under stress conditions are responsible for a variety of biochemical aberrations most of which are caused by posttranslational modifications (PTMs) of proteins.

S-nitrosylation is an established and important PTM in context of NO-mediated signaling in plants. It involves covalent attachment of NO to thiol groups of cysteine residues leading to the production of S-nitrosothiols (SNOs), which further play roles in signaling, transport and storage of NO. SNOs are highly unstable and can thus be quickly diminished by low-molecular weight thiols, such as glutathione (GSH). They are capable of mediating S-nitrosylation of specific target proteins and are involved in storage and transport of NO (Wang et al. 2006). Because of the labile nature of SNOs, the extent of S-nitrosylation of a protein is governed by the rate of nitrosylation and denitrosylation (Benhar 2015). Denitrosylation can occur both by enzymatic and non-enzymatic processes. GSNO reductase (GSNOR) is so far the most worked out and an established enzyme known to control nitrosylation in both plant and animal systems (Benhar et al. 2009, Xu et al. 2013). GSNOR indirectly affects the extent of protein nitrosylation by modulating the intracellular levels of GSNO by catalyzing the reduction of GSNO to oxidized GSH (GSSG) and ammonia in the presence of GSH (Kubienova et al. 2013). Impaired activity of GSNOR leads to increased levels of nitrosylated proteins in *Arabidopsis* thereby indicating the role of this enzyme in maintaining the levels of nitrosothiols in plant cells. Homeostasis of endogenous NO and RSNO appears to be strongly modulated by GSNOR (Feechan et al. 2005). A signaling role for GSNOR has also been proposed in plants and animals (Rusterucci et al. 2007). Plant stress responses, observed to be, modulated by GSNOR include disease resistance (Feechan et al. 2005), cell death (Chen et al. 2009) and protection against nitrosative stress (Lamotte et al. 2005). GSNOR activity has been observed to be induced by mechanical wounding, darkness and low and high temperatures (Corpas et al. 2008, Chaki et al. 2011a, 2011b). S-nitrosylation positively or negatively regulates enzyme activity by affecting protein function, cellular localization and stability (Astier et al. 2012).

A variety of abiotic stress conditions bring about noteworthy modulation of S-nitrosylation of proteins (Jain and Bhatla 2017). These include hypoxia (Perazzolli et al. 2004), low temperature (Abat and Deswal 2009), salt stress (Tanou et al. 2009, Camejo et al. 2013), desiccation (Bai et al. 2011) and high light (Lin et al. 2012). Among all these, S-nitrosylation has been most intensively examined under saline conditions. In vitro S-nitrosylation of proteins using NO donors such as, GSNO, has been studied in various plant species (Lindermayr et al. 2005). Although several studies have reported the impact of salt stress on total proteome in various plant species, reports on the modulation of S-nitroso proteome in the presence of NaCl are limited. A small proportion of S-nitrosylated proteome modified by NaCl stress has been shown in *Arabidopsis* (Wawer et al. 2010, Fares et al. 2011). In *Pisum sativum*, however, a detailed proteomic study of mitochondrial proteins revealed a reduction of S-nitrosylation in response to salt treatment (Camejo et al. 2013). Proteins of the pathways of photorespiration, respiration and antioxidant enzymes have been observed to exhibit changes in the pattern of S-nitrosylation (Fares et al. 2011, Camejo et al. 2013). Earlier reports suggest that S-nitrosylation generally leads to reduction of enzyme activity in plants (Abat and Deswal 2009). However, denitrosylation has been shown to activate enzyme activity (Mannick et al. 1999). S-nitrosylation and denitrosylation together regulate the activities of

various enzymes, especially under conditions of abiotic and biotic stress. Biotin-switch technique (BST), developed by Jaffrey and Snyder (2001), is the most widely used method for identification of S-nitrosylated proteins. Liquid chromatography- mass spectrometry (LC-MS/MS) analysis coupled with biotin-switch method allows identification of target proteins undergoing S-nitrosylation.

Author's laboratory has been engaged in extensive research over the past more than a decade in understanding physiology and biochemistry of oil body mobilization and its associated signaling mechanisms during seedling growth in sunflower (Sadegipour and Bhatla 2002, 2003), developing novel tools and methodologies for localization and biochemical detection of lipase (Gupta et al. 2003, Gupta and Bhatla 2005), phospholipase (Gupta and Bhatla 2007), lipoxygenase (Yadav and Bhatla 2011) and protease (Sadegipour and Bhatla 2003, Vandana and Bhatla 2006). In continuation of our deeper understanding of the signaling mechanisms associated with seed germination in this important oilseed crop, over the past decade, we have further undertaken critical analysis of various salt stress tolerance mechanisms in sunflower seedlings, both as a rapid response to salt and also in long-distance sensing of salt stress by the seedling cotyledons. In this context, we have very recently reported the crucial role of superoxide dismutase (Arora and Bhatla 2015), GSH reductase (Kaur and Bhatla 2016) and heme oxygenase (Singh and Bhatla 2016) in modulating salt stress tolerance by sunflower seedlings in coordination with endogenous NO. Thus, on the basis of these strong evidence available from our previous publications on NO crosstalk with the above-stated ROS-scavenging enzymes, our current objective is to further understand these molecular interactions at the proteome level. In view of the crucial role played by nitrite accumulation in the tissue, its correlation was also analyzed in seedling roots and cotyledons in response to salt stress. NaCl (120 mM) stress-induced modulation of nitrosothiol content and GSNOR was compared in seedling roots and cotyledons. To understand the role of NO in abiotic stress signaling, a comparative analysis of the status of S-nitrosylation in control and salt stress sunflower seedlings was undertaken. In terms of the extent of S-nitrosylation, many proteins of primary metabolism, cytoskeletal proteins, regulatory proteins, defense-related, transport, chaperones and other metabolic proteins exhibited significant modulation in the extent of their S-nitrosylation. These differential expression patterns in terms of S-nitrosylation have been discussed with reference to the impact of salt stress and its long-distance sensing.

## Materials and methods

### Seed germination and treatments

Sunflower seeds (*Helianthus annuus*, var. KBSH 54) were washed with a liquid detergent and kept under running tap water for 1 h. Seed sterilization was done using 0.005% mercuric chloride ($HgCl_2$) and sterilized seeds were then imbibed in distilled water for 2 h and were thereafter sown on presoaked germination paper in plastic trays. Seedlings were grown in dark at 25°C and were irrigated with half-strength Hoagland nutrient solution. The treatment of sunflower seedlings with 120 mM NaCl supplemented in Hoagland medium is based on our earlier investigations on the extent of salt stress tolerance by sunflower seedlings at varying concentrations of NaCl (20–180 mM). Based on these earlier findings, 120 mM has been adopted for all further experiments as at this concentration the seedling survives although it shows significant reduction in growth. Any further increase in NaCl concentration leads to seedling death. So we are working at a concentration at which the seedling is able to tolerate salt stress provided. Salt treatment was carried out by supplementing the Hoagland solution with 120 mM NaCl according to David et al. (2010). Cotyledon and root tissues from seedlings showing uniform growth pattern (hypocotyl length and root growth) were harvested and stored at −80°C until further use. The present work is focused on the analysis of roots and cotyledons of 2-day-old seedlings only so as to monitor various biochemical changes expected as rapid signaling response (within 48 h of salt application) both in the vicinity of the NaCl molecules in solution (i.e. roots) and also the effect of NaCl over long-distance signaling response (transcellular, from roots to cotyledons).

### Determination of nitrite and nitrosothiol contents

The amount of nitrite and nitrosothiol was determined using Sievers' NO Analyzer (NOA 280i, Munich, Germany). Five hundred mg of root and cotyledon tissues were powdered in liquid nitrogen and mixed with 1 ml extraction buffer [50 mM Tris–HCl pH 7.5, 10 mM N-ethylmaleimide (NEM) and 2.5 mM ethylenediaminetetraacetic acid (EDTA)] and followed by centrifugation twice at 10 000 g for 20 min each at 4°C. Hundred and 200 μl of the homogenate for nitrite and nitrosothiol content measurements, respectively, were injected into the reaction vessel, which was filled with Tri-iodide solution containing iodine, potassium iodide and acetic acid. The homogenate was pretreated with 5% sulfanilamide (prepared in 1 M HCl) to remove nitrite for measurement of SNO content. Sievers NO ANALYSIS SOFTWARE (v3.2) was used to quantify nitrite and SNO contents

by integrating peak areas and using standard curve of nitrite. Standard curve was generated using known concentrations of sodium nitrite.

## Analysis of S-nitrosylated proteins by BST

S-nitrosylated proteins were isolated and purified using BST, according to Lindermayr et al. (2005). Cotyledon and root tissues were ground to fine powder with liquid nitrogen and homogenized in grinding medium (50 mM Tris–HCl, 1 mM EDTA and 0.1 mM neocuproine pH 7.8). The homogenate was centrifuged at 10 000 $g$ for 20 min at 4°C to obtain total soluble protein (TSP). Protein was quantified according to Bradford (1976) and protein concentration was adjusted to 1 mg ml$^{-1}$. Samples were then treated with 250 μM GSNO for 30 min at room temperature (RT) in dark. Samples treated with $H_2O$ served as negative controls. All protein samples were treated with 25 mM methylmethanethiosulfonate (MMTS), 2.5% sodium dodecyl sulfate (SDS) and incubated at 50°C for 20 min with frequent vortexing. Acetone precipitation of proteins was undertaken to eliminate residual MMTS. Sodium ascorbate (1 mM; reducing agent) and 1 mM biotin-HPDP (pyridyldithiol-biotin; to biotinylate the proteins) were then added and samples were mixed thoroughly and incubated at 37°C for 1 h. Acetone precipitation of proteins was done again to remove excess biotin and ascorbate. The biotin-labeled S-nitrosylated proteins were then subjected to Western blot analysis or were purified by affinity chromatography.

## Western blot analysis for detection of biotin-labeled S-nitrosylated proteins

The acetone-precipitated biotin-labeled proteins were resuspended in HENS buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1 mM EDTA, 0.1 mM neocuproine and 1% SDS, pH 7.7). Protein was mixed with non-reducing Laemmli buffer containing 0.06 $M$ Tris, pH 6.8, 10% (v/v) glycerol, 0.008% (w/v) bromophenol blue and loaded on 10% gel for separation of proteins using Miniprotean Tetra Cell (Biorad, Hercules, CA). Electrophoresis was performed at 75 V for 0.5 h, at 100 V for 0.5 h and 150 V for 2 h. After electrophoresis, the gel was taken out of the cassette and washed in transfer buffer [0.025 $M$ Tris, 0.192 $M$ glycine, 0.1% (w/v) SDS and 20% methanol] at 4°C for at least 15 min. The gel was subjected to Western blot transfer of proteins onto nitrocellulose membrane at a current of 400 mA for 1 h at 4°C to achieve complete transfer of proteins (as judged by Ponceau staining). The blot with transferred proteins was then incubated in blocking buffer [3% bovine serum albumin (BSA) and 0.2% Tween 20 in Tris-buffered saline (TBS)] for 2 h at RT. The membrane was then incubated overnight with anti-mouse monoclonal antibody against biotin conjugated with alkaline phosphatase enzyme (anti-biotin antibody obtained from Sigma-Aldrich, St. Louis, MO) in a dilution of 1:10 000 in blocking buffer, at 4°C on an orbital shaker. Thereafter, the membrane was washed in wash buffer (0.2% Tween 20 in TBS) three times for 5 min each and developed using freshly prepared 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium (BCIP/NBT) (1 Sigma fast tablet (Sigma-Aldrich, St. Louis, MO) dissolved in 10 ml MilliQ water) for 10–15 min. Once the desirable color intensity was obtained, the membrane was placed in MilliQ water.

## Affinity chromatography for purification of biotinylated proteins

The biotinylated proteins were purified by affinity chromatography according to Sell et al. (2008). The proteins resuspended in HENS buffer were mixed with 2 volumes of neutralization buffer (25 mM HEPES pH 7.7, 0.5% Triton-X-100, 1 mM EDTA and 100 mM NaCl). Thirty μl of equilibrated neutravidin agarose slurry (mg protein)$^{-1}$ was then added to the protein mixture incubated at RT for 1–2 h with gentle shaking. The protein sample was centrifuged at 100 $g$ for 5 min and the supernatant was removed. The beads attached to biotinylated proteins were washed twice with 20 volumes of washing buffer (25 mM HEPES pH 7.7, 1 mM EDTA, 600 mM NaCl and 0.5% Triton-X-100). After washing out non-specific proteins, the proteins bound to the matrix were eluted by incubating for 20–30 min with 100 mM $\beta$-mercaptoethanol in neutralization buffer. The sample was centrifuged and the supernatant (containing S-nitrosylated proteins) was stored at −20°C until further analysis.

## Proteomic analysis of S-nitrosylated proteins

The preparation of samples and mass spectrometric analysis of S-nitrosylated proteins was carried out according to Merl et al. (2012) and von Toerne et al. (2013). The proteins were first purified using 1D Gel clean-up kit (GE Healthcare Life Sciences, Chicago, Illinois, USA). Protein was quantified using BCA protein assay and 30 μg of each sample was loaded on 12% gel and SDS-PAGE was performed. The gel with resolved proteins was then fixed and stained with Coomassie brilliant blue. Complete lanes were subjected to in-gel digestion. The gel was cut into cubes and gel pieces were then destained by washing for 10 min in 60% acetonitrile (ACN) and then with water for 10 min. The gel cubes were then dehydrated by further incubating with 100% ACN for 10 min.

Dithioerythritol (DTT; 5 m$M$) was then added and gel pieces were incubated at 60°C for 10 min to reduce the proteins. The reduced proteins were then incubated with 25 m$M$ iodoacetamide solution for 15 min at RT in dark. The excess DTT and iodoacetamide were removed by incubating the samples with 100% ACN for 10 min and samples were washed three times with 50 m$M$ ammonium bicarbonate (ABC), 100% ACN and 60% ACN. The washed samples were air-dried at 37°C for 15 min and 0.01 µg µl$^{-1}$ trypsin solution (Sigma Aldrich) in 50 m$M$ ABC was added and incubated for 10 min. Further, 25 m$M$ ABC was added and the solutions were left overnight at 37°C. The proteins were then eluted from the gel using 60% ACN/0.1% trifluoroacetic acid (TFA) and incubating for 15 min with gentle shaking. The supernatant so obtained was then transferred to a new tube and remaining gel pieces were incubated with 99.9%/0.1% TFA solution for 30 min. The supernatants containing the eluted peptides were then dried in speedvac and stored at −20°C until further analysis. The pre-fractionated dried samples were dissolved in 2% ACN/0.5% TFA and incubated at RT for 30 min under agitation. LC-MS/MS analysis was performed on an LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA). Samples were injected automatically and loaded onto the trap column and peptides were eluted after 5 min and separated on an analytical column by reversed phase chromatography operated on nano-high-performance liquid chromatography (Ultimate 3000, Dionex, Thermo Scientific) with a non-linear 170-min gradient using 5% ACN in 0.1% formic acid in water (A) and 0.1% formic acid in 98% ACN (B) at a flow rate of 300 µl min$^{-1}$. The gradient settings were: 5–140 min: 14.5–90% A, 140–145 min: 90% A − 95% B, 145–150 min: 95% B followed by equilibration for 15 min to starting conditions. From the MS prescan, the 10 most abundant peptide ions were selected for fragmentation in the linear ion trap if they exceeded an intensity of at least 200 counts and were at least doubly charged. During fragment analysis, a high-resolution (60 000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 200 to 1500 Da. For peptide identification, Swiss-Prot database was used and results were displayed using Scaffold software.

### GSNOR activity assay

GSNOR activity was measured spectrophotometrically according to Sakamoto et al. (2002) by monitoring the rate of NADH oxidation in the presence of GSNO at 340 nm. Briefly, protein was extracted from cotyledon and root tissues (1 g each) in 1 ml extraction buffer (0.1 $M$ Tris–HCl pH 7.5, 10% glycerol, 0.1 m$M$ EDTA,

0.2% Triton-X-100 and protease inhibitor cocktail). The homogenate obtained was then centrifuged at 10 000 $g$ for 20 min at 4°C to get TSP. Protein concentration was measured using Bradford reagent (Bradford 1976). Aliquot from each sample containing 50 µg protein was added to reaction mixture containing 20 m$M$ Tris–HCl buffer (pH 8.0), 0.2 m$M$ NADH and 0.5 m$M$ EDTA. The reaction was started by adding 0.5 m$M$ GSNO and the absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated using molar extinction coefficient for NADH (6.22 m$M^{-1}$ cm$^{-1}$).

### Native PAGE for analysis of GSNOR activity

Protein extracted and quantified as described above was loaded for single dimension separation on a 6% vertical native polyacrylamide gel at 4°C (conditions: 75 V for 20 min and 150 V for 2 h) using Miniprotean Tetra Cell (Biorad). The gel with resolved proteins was soaked in 0.1 $M$ sodium phosphate buffer, pH 7.4, containing 2 m$M$ NADH for 15 min in an ice bath. Excess buffer was drained and gels were covered with filter paper strips soaked in freshly prepared 3 m$M$ GSNO. After 10 min, the filter paper was removed and gels were exposed to ultraviolet radiation (340 nm) and analyzed for the disappearance of the NADH fluorescence due to GSNOR activity (Chaki et al. 2011b).

### Western blot analysis of GSNOR

Protein extracted and quantified as described above was constituted in reducing Laemmli buffer and loaded on 12% precast gels (Biorad) (conditions: 75 V for 20 min and 150 V for 2 h) using Miniprotean Tetra Cell (Biorad). The gel with resolved proteins was then washed in transfer buffer [0.025 $M$ Tris, 0.192 $M$ glycine, 0.1% (w/v) SDS and 20% methanol] at 4°C for at least 15 min. The gel was subjected to a current of 400 mA for 1 h at 4°C to achieve complete transfer of proteins onto nitrocellulose membrane. Subsequently, the blot with transferred proteins was then incubated in blocking buffer (3% BSA and 0.2% Tween 20 in phosphate-buffered saline, pH 7.4) for 2 h at RT. The membrane was then incubated with a rabbit polyclonal antibody against GSNOR (anti-GSNOR antibody obtained from Agrisera, Vännäs, Sweden) in a dilution of 1:2000 in blocking buffer overnight at 4°C on an orbital shaker. Thereafter, the membrane was washed in wash buffer (0.2% Tween 20 in TBS) three times for 5 min each and incubated in secondary antibody (antirabbit IgG conjugated to alkaline phosphatase antibody, Sigma-Aldrich Chemicals Pvt. Ltd.) (1:2500 in wash buffer) for 1 h at RT on an orbital shaker. Finally, the membrane was washed in wash buffer three times

for 5 min each and developed using freshly prepared BCIP/NBT (1 Sigma Fast tablet dissolved in 10 ml MilliQ water) for 10–30 min.

### GAPDH activity assay

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was measured according to Lindermayr et al. (2005) by estimating the increase in absorbance due to reduction of $NAD^+$ to NADH. Cotyledons (500 mg) and roots (1 g) were ground to fine powder with liquid nitrogen and homogenized in grinding medium (0.1 $M$ Tris–HCl, pH 7.5). The homogenates were then centrifuged at 10 000 $g$ for 20 min at 4°C to TSPs. The supernatant obtained was treated with 10 m$M$ DTT, 250 μ$M$ GSNO and 250 μ$M$ GSH for 20 min at RT. Residual DTT and GSNO were removed using PD-10 desalting columns. Protein was quantified according to Bradford (1976). Each aliquot containing 150 μg protein was added to a reaction mixture containing 0.1 $M$ Tris–HCl, 50 μ$M$ arsenate and 100 μg ml$^{-1}$ glyceraldehyde-3-phosphate, and the reaction volume was adjusted to 950 μl. The reaction was initiated by addition of 50 μl of 10 m$M$ of $NAD^+$ and the change in absorbance was monitored at 340 nm for 1 min. Enzyme activity was calculated using extinction coefficient of 6.22 m$M^{-1}$ cm$^{-1}$ for NADH. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 m$M$ $NAD^+$ min $^{-1}$ at 25°C.

### MDHAR activity assay

Spectrophotometric estimation of monodehydroascorbate reductase (MDHAR) activity was carried out according to Hossain et al. (1984), with some modifications. Cotyledons (500 mg) and roots (1 g) harvested from etiolated seedlings were homogenized in grinding medium (0.1 $M$ Tris–HCl, pH 7.0). The homogenates obtained after centrifugation at 10 000 $g$ for 20 min at 4°C were treated with 10 m$M$ DTT, 250 μ$M$ GSNO and 250 μ$M$ GSH for 20 min at RT. Residual DTT and GSNO were removed using PD-10 desalting columns. Protein content was estimated according to Bradford (1976). TSPs obtained from respective samples were analyzed for MDHAR activity by measuring decrease in absorbance due to oxidation of NADH at 340 nm. The reaction mixture (1 ml) contained 0.1 $M$ Tris–HCl (pH 7.0), 2.5 m$M$ ascorbate, 0.2 m$M$ NADH and sample. The reaction was initiated by addition of 1 U of ascorbate oxidase and, thereafter, monitoring the change in absorbance at 340 nm for 1 min. Enzyme activity was calculated using extinction coefficient of 6.22 m$M^{-1}$ cm$^{-1}$ for NADH. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 m$M$ NADH min$^{-1}$ at 25°C.

### Statistical analysis

The S-nitroso protein intensities in both control and salt-treated samples were normalized and log 2 fold changes (salt-treated/control) were calculated before comparison. SPSS 22.0 statistical program (SPSS Inc., Chicago, IL) was used for statistical analysis of the data. Effects of various treatments were analyzed using one-way ANOVA. The effects of treatments were accordingly categorized into various levels of significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All experiments were performed at least thrice.

## Results

### Seedling roots exhibit much higher sensitivity to salt stress than cotyledons in term of nitrite accumulation

Analysis of 2-day-old, dark-grown sunflower seedling cotyledons and roots raised in the absence or presence of 120 m$M$ NaCl showed striking differences in the content of nitrite in the respective control tissues (Fig. 1). In general, cotyledons were observed to be low accumulators of nitrite in contrast with roots. Roots had almost 23-fold higher nitrite content in the control seedlings. Additionally, salt stress sensitivity of roots was also much higher than that of cotyledons in terms of its impact on nitrite accumulation. Thus, as compared to the respective controls, salt-stressed roots exhibited 71.5% increase in nitrite content as compared to 27% in cotyledons. To sum up, salt stress-sensitized accumulation of nitrite content in seedling roots and cotyledons may be considered an index/marker of nitrosative stress.

### Roots and cotyledons exhibit similar sensitivity for accumulation of nitrosothiols in response to salt stress

In absolute terms, nitrosothiol content in the homogenates derived from seedling roots was found to be threefold higher than seedling cotyledons (control conditions). Roots derived from seedlings subjected to 120 m$M$ NaCl stress exhibited further increase in nitrosothiol content by 61% (Fig. 1). Seedling cotyledons also exhibited a substantial increase in nitrosothiol content in response to salt stress (72% of control). Thus, it is evident that both the tissue systems exhibit almost similar sensitivity for nitrosothiol accumulation in response to salt stress.

### 120 m$M$ NaCl reversibly inhibits GSNOR activity in sunflower seedling roots and cotyledons

GSNOR activity and its protein abundance were analyzed in 2-day-old, dark-grown sunflower seedling
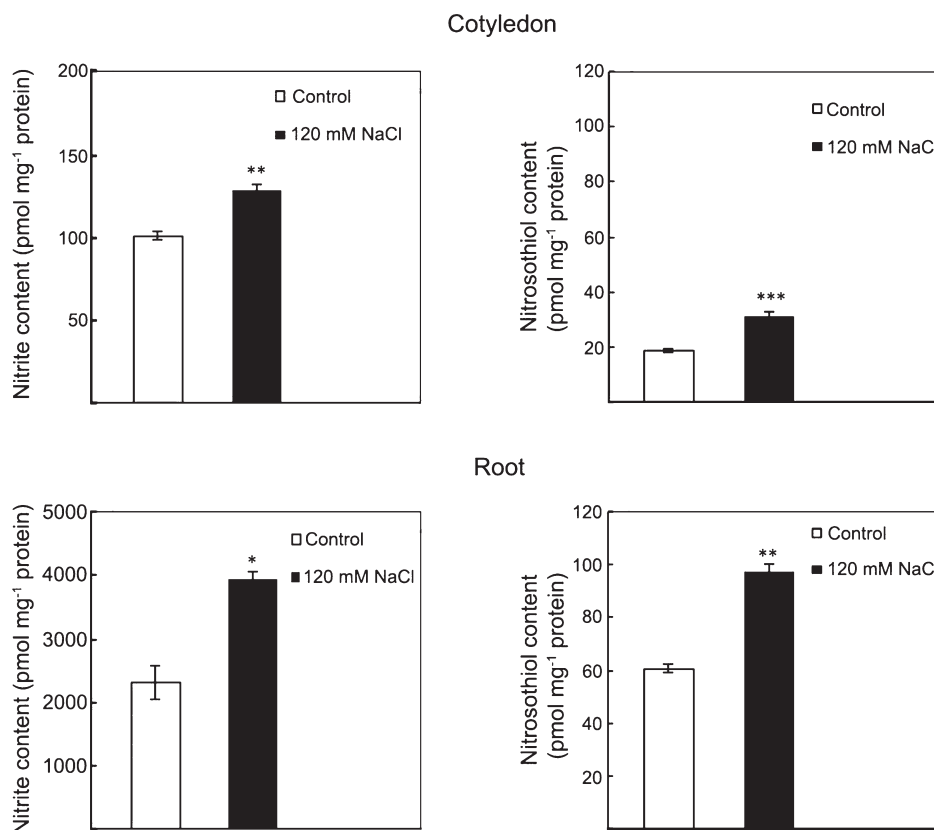
Cotyledon



Root



**Fig. 1.** Nitrite and nitrosothiol content in 10 000 g supernatant obtained from 2-day-old dark-grown sunflower seedling cotyledons and roots in the absence or presence of 120 mM NaCl stress. Data represent mean ± SE from three replicates. NaCl stress-induced changes were analyzed by one-way ANOVA using SPSS 22.0 and were found to be statistically significant ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$) in comparison to control [(−)NaCl].

cotyledons and roots grown in the absence or presence of 120 mM NaCl. GSNOR catalyzes the reduction of GSNO (a reservoir of NO) to oxidized GSH (GSSG) and ammonium ($NH_4^+$) using electrons from NADH. Spectrophotometric and zymographic (native PAGE) analyses of GSNOR activity revealed that GSNOR activity is higher in cotyledons than in roots. A significant reduction (approximately 50%) in enzyme activity was evident upon salt treatment both in sunflower seedling roots and cotyledons both from spectrophotometric and zymographic analyses (Fig. 2). However, treatment with 10 mM DTT (a reducing agent) reversed the inhibitory effect of salt treatment on enzyme activity, thereby suggesting that some kind of reversible oxidative modification, such as S-nitrosylation or sulfenation, is involved in impairing the catalytic activity of GSNOR under salt stress conditions. Western blot analysis interestingly showed no difference in the protein content under salt stress conditions as well as after DTT treatment (Fig. 2). This shows that salt stress modulates the activity of GSNOR. However, the amount of protein remained unaltered. Restoration of GSNOR activity (up to 80%)

with DTT both in seedling cotyledons and roots further reveals that enzyme is regulated by S-nitrosylation.

## S-nitrosylation of cytosolic proteins in sunflower seedling roots and cotyledons is modulated both by salt stress and GSNO

Raising sunflower seedlings in dark for 2 days in the absence or presence of 120 mM NaCl and also subjecting the 10 000 g supernatant from the homogenates of seedling roots and cotyledons to 250 μM GSNO revealed a general upregulation of the extent of S-nitrosylation of cytosolic proteins of varying molecular masses. Both salt stress and GSNO application brought about enhanced S-nitrosylation in sunflower seedling cotyledons as is evident from the indicated (arrowheads) 10 protein bands (Fig. 3). Compared to control (− NaCl), (+) NaCl condition (salt stress) led to enhanced S-nitrosylation in the homogenates which is further enhanced upon treatment with 250 μM of GSNO. In the case of seedling roots, the extent of S-nitrosylation of soluble proteins was less noteworthy than in cotyledons. Although
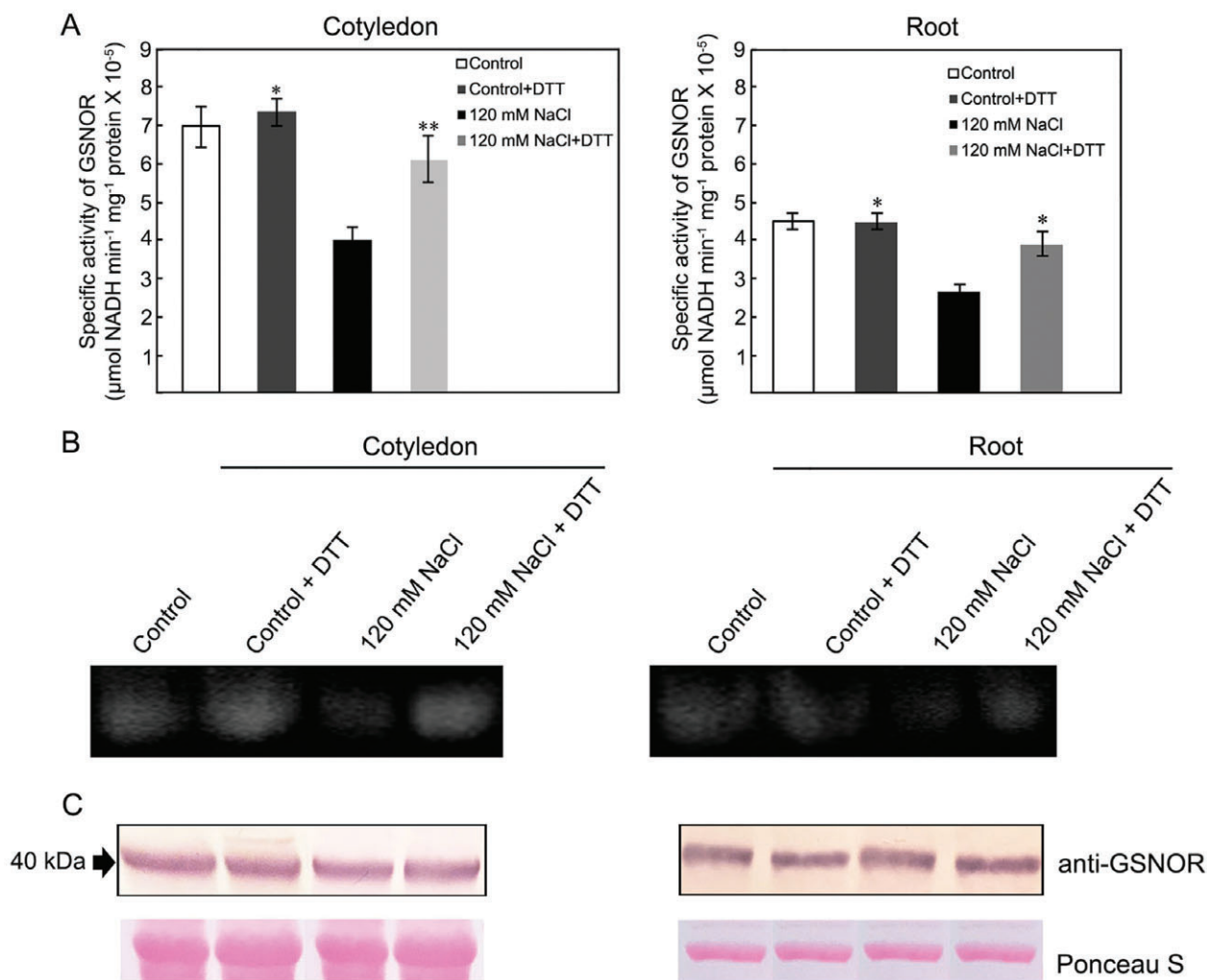
**Fig. 2.** Spectrophotometric (A), zymographic (B) and Western blot (C) analyses of salt stress-induced changes in the activity of GSNOR in 10 000 *g* supernatant obtained from cotyledons and roots of 2-day-old, dark-grown sunflower seedlings grown in the absence or presence of 120 m*M* NaCl stress. Treatment with 10 m*M* DTT leads to restoration of enzyme activity. Data represent mean ± SE from three replicates. NaCl stress-induced changes were analyzed by one-way ANOVA using SPSS 22.0 and were found to be statistically significant (*$P < 0.05$; **$P < 0.01$) in comparison to control [(−)NaCl].

enhanced S-nitrosylation was evident in most protein bands highlighted with arrowheads in response to salt stress and GSNO, proteins indicated in the highlighted zone clearly showed a downregulation of S-nitrosylation as a response to GSNO treatment in both (−) NaCl and (+) NaCl conditions (Fig. 3). It is evident that S-nitrosylation of cytosolic proteins is a stronger biomarker of stress in seedling cotyledons than in roots thereby highlighting its role in long-distance signaling. Samples treated with GSH and those devoid of ascorbate served as negative controls and these homogenates, therefore, did not exhibit any S-nitrosylation of proteins. These results show that biotin-switch method is a specific technique for the detection of S-nitrosylated proteins.

## LC-MS/MS analysis of S-nitrosylated proteins reveals opposite trends in seedling cotyledons and roots

Cotyledons and roots derived from 2-day-old sunflower seedlings (±120 m*M* NaCl) were homogenized and 10 000 *g* supernatant was subjected to biotinylation followed by affinity chromatography to purify S-nitrosylated proteins. The eluates so obtained were subjected to LC-MS/MS analysis to study the impact of salt stress on S-nitrosylation of cytosolic proteins. A total of 61 proteins both from seedling roots and cotyledons were found to be S-nitrosylated (Tables 1 and 2). These proteins were grouped into eight functional categories namely, those belonging to carbohydrate
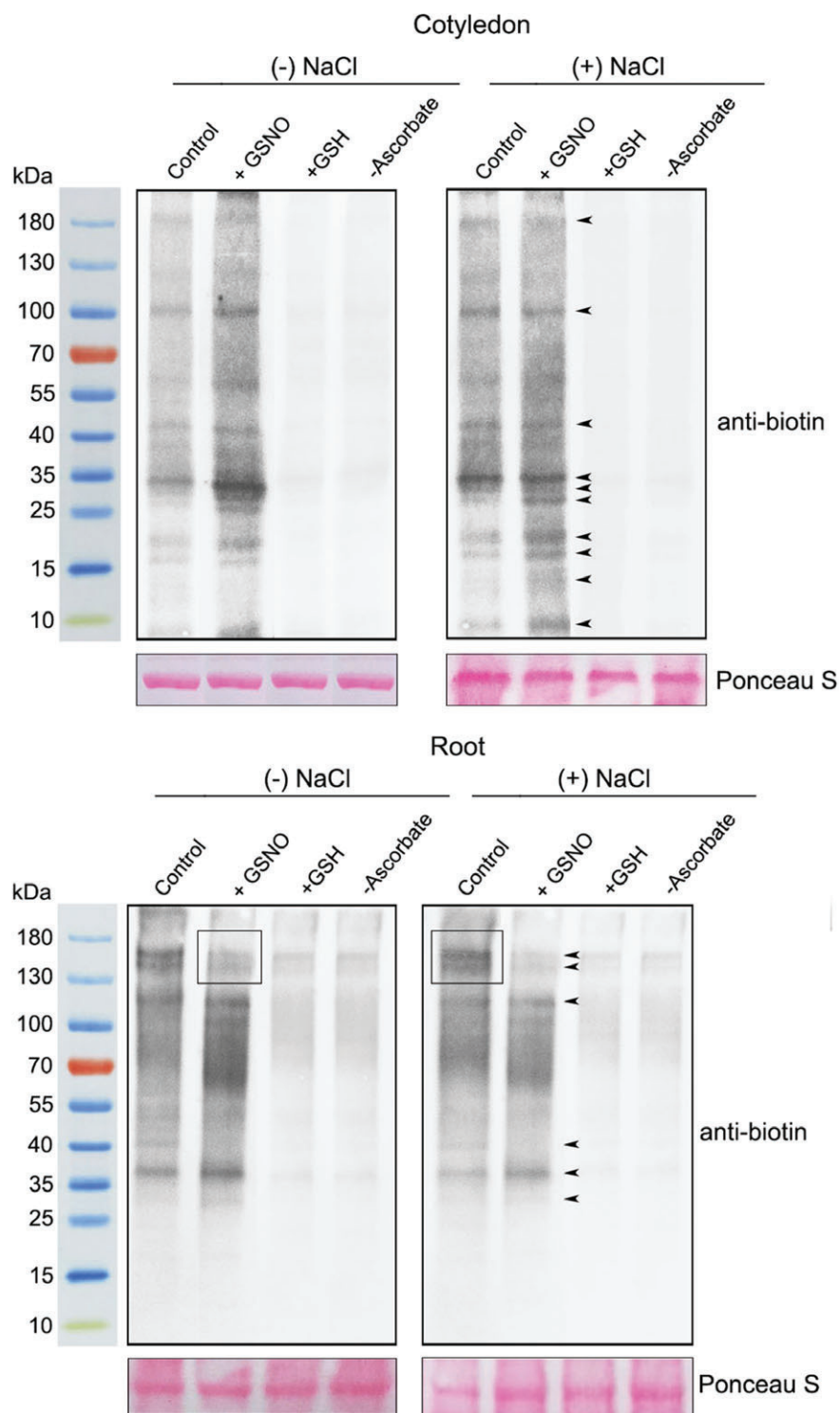
**Fig. 3.** Western blot analysis of S-nitrosylated proteins in cotyledons and roots of sunflower seedlings grown in the absence or presence of 120 m$M$ NaCl. S-nitrosylated proteins were extracted by biotin-switch method and analyzed using anti-biotin antibody. Homogenate samples treated with 250 µ$M$ GSNO served as positive controls while those treated with 250 µ$M$ GSH and untreated with ascorbate (reducing agent) served as negative controls.

metabolism, other metabolic proteins, chaperones, defense-related proteins, regulatory proteins, transport proteins, cytoskeletal proteins and miscellaneous proteins. In sunflower seedling cotyledons, highest number of proteins having undergone S-nitrosylation (37%) belonged to the category of carbohydrate metabolism followed by other metabolic proteins (18%), 9% each in the category of regulatory proteins, chaperones and defense-related proteins and 5 and 3% belonged to transport and cytoskeletal proteins, respectively (Fig. 4). The relative distribution of S-nitrosylated proteins in seedling roots remained by and large similar to that observed in seedling cotyledons (Fig. 4). Thus, both the tissue systems respond in a similar manner in terms of the proportion of S-nitrosylation of proteins of different categories. It was further noted that 18 S-nitrosylated proteins are being reported for the first time in plant systems of which 9 were common in both cotyledons and roots, 8 are specific to cotyledons only and 1 (calreticulin) was reported to be modulated by S-nitrosylation in roots (Table 3). Of the total proteins being regulated by S-nitrosylation, 17 are unique to cotyledons, 4 are unique to roots whereas 40 are common to both the tissue systems (Fig. 4).

### S-nitrosylation and denitrosylation together regulate GAPDH and MDHAR activities in sunflower seedling roots and cotyledons

Spectrophotometric analysis of GAPDH and MDHAR activities in sunflower seedling cotyledons revealed that the activities of both the enzymes decrease in the presence of 120 mM NaCl. It was also observed that the inhibition of enzyme activity under salt stress was reversible and could be restored upon treatment with 10 mM DTT (reducing agent). Treatment of homogenates with 250 μM GSNO led to reduction in enzyme activity thereby suggesting the role of S-nitrosylation in regulating enzyme activity in seedling cotyledons (Fig. 5). In case of sunflower seedling roots however, GAPDH and MDHAR activities increased in response to 120 mM NaCl stress. Treatment with 10 mM DTT led to restoration of enzyme activity in control homogenates (Fig. 5).

### Discussion

Nitrite is considered as a biomarker of NO metabolism, thereby necessitating the quantification of nitrite levels in various plant organs and tissues (Salgado et al. 2013). The non-enzymatic production of NO from nitrite in acidic conditions has been demonstrated in the apoplast of aleurone layer in barley (Bethke et al. 2004), in tobacco leaves and cell suspensions (Planchet et al. 2005), in the leaf homogenates of *Arabidopsis thaliana*

(Modolo et al. 2006) and in the chloroplasts of soybean leaves (Jasid et al. 2006). NO synthesis has also been reported by the action of nitrite-NO oxidoreductase activity in the root plasma membrane of *Nicotiana tabacum* (Stöhr et al. 2001, Stöhr and Stremlau 2006). The present observations have demonstrated preferential nitrite accumulation in seedling roots than in cotyledons. Furthermore, nitrite level in the tissue is enhanced both in response to NaCl stress and NO donor treatment. Thus, nitrite accumulation serves as a biomarker of salt and/or nitrosative stress. An increase in the levels of nitrite has been observed in citrus plants subjected to various abiotic stress factors, including salinity (Ziogas et al. 2013). It has been suggested that elevated levels of nitrite promote its reduction to NO either enzymatically by the action of NR or non-enzymatically at acidic pH (Yamasaki 2000, Rockel et al. 2002). Elevated levels of nitrite have also been reported in *Arabidopsis* leaves from NO-fumigated plants (Kuruthukulangarakoola et al. 2017). S-nitrosothiols (RSNOs), particularly GSNO, act as major intracellular reservoirs and carriers of NO in plants. Information on modulation of nitrosothiol content in response to a specific stress is limited as compared to that available on alterations in NO content in response to abiotic or biotic stress (Leterrier et al. 2012). Nitrosothiol content has been shown to regulate disease resistance in plants (Feechan et al. 2005). Salinity stress has been demonstrated to elevate total nitrosothiol content by twofold in olive plants (Valderrama et al. 2007). Similar observations have also been reported from the leaves of pea plants in response to mechanical wounding and low temperature (Corpas et al. 2008). The present observations also indicate an increase in total RSNO content upon 120 mM NaCl treatment in both seedling root and cotyledon tissues thereby suggesting role of RSNOs in plant response to salinity stress. *Arabidopsis* plants fumigated with NO also show 2.5 times higher RSNO levels than control plants suggesting that the endogenous level of NO rapidly increases after fumigation with NO (Kuruthukulangarakoola et al. 2017). SNOs are more stable as compared to NO in cell systems and participate in signal transduction pathway thus indicating the importance of this biomolecule in stress conditions.

GSNO is formed as a result of reaction between GSH and NO. The level of GSNO in plant cells is regulated by the activity of GSNOR (EC 1.1.1.284). It catalyzes the NADH-dependent reduction of GSNO to GSSG and $NH_4^+$. In the recent past, GSNOR-mediated reduction of GSNO has been demonstrated in tobacco (Diaz et al. 2003), *Arabidopsis* (Sakamoto et al. 2002) and pea (Barroso et al. 2006). GSNOR activity and its gene expression have been shown to be downregulated in pea plants in response to cadmium stress (Barroso et al. 2006).

**Table 1.** Proteomic analysis (LC-MS/MS) of S-nitrosylated proteins from the cotyledons (10 000 g supernatant) of sunflower seedlings (2-day-old, dark-grown) subjected to 120 m*M* NaCl stress. Fold change refers to protein abundance in salt-stressed cotyledons over control ones. The coloured regions in fold change in both figures indicate heat map where individual values have been represented with a gradation of colours. Lowest represented by red and highest by green.

| Protein | Accession number | kDa | Peptide sequence (ion score) | GO biological process | Fold change | Log 2 fold change |
|---|---|---|---|---|---|---|
| **Carbohydrate metabolism** | | | | | | |
| Aconitate hydratase | Q42560 | 98 | VLLQDFTGVPAVVDLACMR (57.3) SENAVQANMELEFQR (84.6) RGNDEVMAR (56.3) | Tricarboxylic acid cycle | 5.95 | 3 |
| Alcohol dehydrogenase 1 | P14673 | 41 | FGVTEFVNPK (77) | Oxidation−reduction process | 8.79 | 3 |
| 2,3-Bisphosphoglycerate-independent phosphoglycerate mutase | P35494 | 61 | GIDAQVASGGGR (94.1) LDQLQLLIK (64.7) GWDAQVLGEAPHK (43.8) | Glycolysis | 15.03 | 4 |
| Citrate synthase | Q9LXS6 | 57 | SSICYIDGDEGILR (88.6) LYDPGYLNTAPVR (58.7) | Tricarboxylic acid cycle | 14.92 | 4 |
| Enolase | P42896 | 48 | VNQIGSVTESIEAVR (96.3) SGETEDTFIADLSVGLATGQIK (85.5) AAVPSGASTGIYEALELR (91.5) | Glycolysis | 7.62 | 3 |
| GAPDH | P25861 | 37 | AGIALNDHFVK (69.9) VPTVDVSVVDLTVR (98) IGINGFGR (58.6) | Glycolysis | 10.11 | 3 |
| Isocitrate dehydrogenase | P50217 | 47 | EHYLNTEEFIDAVADELK (79.5) LIDDMVAYALK (70.2) | Glyoxylate cycle | 13.82 | 4 |
| Isocitrate lyase | P49297 | 64 | TVQGGITSTAAMGK (103.8) WSGANYYDR (60.2) | Tricarboxylic acid cycle | 7.95 | 3 |
| Malate dehydrogenase | O48905 | 36 | MELVDAAFPLLK (104.7) LNVQVSDVK (65.1) LDLTAEELTEEK (86.8) | Tricarboxylic acid cycle | 10.15 | 3 |
| Malate synthase | P08216 | 65 | ATVLIETLPAVFQMNEILYELR (100.1) YGVELDGDGLGVR (94.8) MIINALNSGAK (65.9) | Tricarboxylic acid cycle | 22.19 | 4 |
| Oxygen-evolving enhancer protein 1 | P85194 | 28 | LTYTLDEIEGPLEVSSDGTIK (90.2) GGSTGYDNAVALPAGGR (60.8) DGIDYAAVTVQLPGGER (60.2) | Photosynthesis | 6.31 | 3 |
| Phosphoenolpyruvate carboxykinase | P42066 | 74 | TTLSTDHNR (62.2) FGTVLENVVFDEHTR (81.6) EMVILGTQYAGEMK (109.9) | Gluconeogenesis | 18.44 | 4 |
| 6-Phosphogluconate dehydrogenase | Q9FFR3 | 53 | NPNLASLVVDPDFAK (63.9) LPANLVQAQR (79.5) | Pentose phosphate pathway | 7.77 | 3 |
| Phosphoribulokinase | P26302 | 45 | ANDFDLMYEQVK (60.8) | Reductive pentose phosphate cycle | 5.54 | 2 |
| Pyruvate phosphate dikinase | Q42736 | 105 | VMANADTPNDALTAR (96.4) DMMDIEFTVQENR (50.2) AALDLLLPYQR (52.8) | Photosynthesis | 16.51 | 4 |
| Ribulose bisphosphate carboxylase large chain | P45738 | 54 | FEFQAMDTLDTDK (95.7) DLATEGNEIIR (81.7) EITLGFVDLLRDDFIEK (76.9) | Carbon fixation, photosynthesis | 7.46 | 3 |
| Ribulose bisphosphate carboxylase small chain | P08705 | 20 | IIGFDNVR (52.8) SPGYYDGR (49.2) EYPQAWIR (47.6) KYETLSYLPPLTETQLAK (69.4) | Carbon fixation, photosynthesis | 9.76 | 3 |
| Serine hydroxymethyltransferase 1 | Q9SZJ5 | 57 | MGTPALTSR (39.8) LDESTGYIDYDQMEK (69.5) | Photorespiration | 3.58 | 2 |
| Sedoheptulose-1,7-bisphosphatase | P46285 | 42 | YTGGMVPDVNQIIVK (37.1) ATFDNPDYDK (44.4) | Reductive pentose phosphate cycle | 4.58 | 2 |
| Transketolase | O20250 | 80 | VTTTIGFGSPNK (94.6) TPGHPENFETPGIEVTTGPLC (100.7) | Reductive pentose phosphate cycle | 5.29 | 2 |
| Triosephosphate isomerase | P48493 | 21 | ALLNETNEFVGDK (83.9) VASPAQAQEVHAGLR (91.7) | Glycolysis | 1.82 | 1 |

**Table 1.** Continued.

| Protein | Accession number | kDa | Peptide sequence (ion score) | GO biological process | Fold change | Log 2 fold change |
|---|---|---|---|---|---|---|
| **Other metabolic proteins** | | | | | | |
| Adenosylhomocysteinase | P32112 | 53 | DSAAVFAWK (64.3) VAVVCGYGDVGK (62.7) ATDVMIAGK (71.8) | L-homocysteine biosynthesis | 13.49 | 4 |
| Adenylate kinase 4 | Q08480 | 27 | VLNFAIDDSILEER (90.7) GFILDGFPR (47.8) GELVSDDLVVGIIDEAMK (96.5) | Nucleotide metabolic process | 7.86 | 3 |
| Alanine aminotransferase 2 | Q9LDV4 | 60 | ALVVINPGNPTGQVLAEENQR (122.1) ATGAYSHSQGIK (65.2) | L-alanine catabolic process | 5.23 | 2 |
| Biotin carboxylase | O04983 | 58 | HIEFQVLADK (41.8) NAGVPTVPGSDGLLQSTEEAVR (52.5) | Fatty acid biosynthetic process | 12.98 | 4 |
| Ketol-acid reductoisomerase | O82043 | 63 | GSSSFNEAR (59.4) VNLAGHEEYIVR (40) | Amino acid biosynthesis | 20.78 | 4 |
| 5-Methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase | Q42662 | 85 | FALESFWDGK (68.9) YLFAGVVDGR (57.1) GNASVPAMEMTK (50.2) VVKLQEELDIDVLVHGEPER (57.6) | Amino acid biosynthesis | 5.68 | 3 |
| Peptidyl prolyl cis-trans isomerase | P21569 | 18 | VFFDMTVGGAPAGR (108.3) | Protein folding | 7.38 | 3 |
| Phospholipase D-alpha 1 | Q43007 | 92 | VLMLVWDDR (60.5) AYLPVQELLNGEEIDR (93.1) | Lipid catabolic process | 8.73 | 3 |
| S-adenosylmethionine synthase | P49613 | 41 | TNMVMVFGEITTK (89.6) VHTVLISTQHDETVTNDKIAA (66.5) | S-adenosylmethionine biosynthetic process | 14.66 | 4 |
| UDP-glucose 6-dehydrogenase 1 | Q9FZE1 | 53 | LPIYEPGLEDVVK (38.4) AADLTYWESAAR (46.2) | UDP-glucuronate biosynthetic process | 3.88 | 2 |
| **Chaperones** | | | | | | |
| Chaperone protein ClpB1 | P42730 | 101 | NNPVLIGEPGVGK (49.8) TAVVEGLAQR (55.7) VQLDSQPEEIDNLER (65.1) | Protein unfolding | 6.87 | 3 |
| Endoplasmin homolog | P35016 | 93 | ELISNASDALDK (81) LGIIEDAANR (70.2) SGTSAFVEK (65.1) | Protein folding | 8.45 | 3 |
| Hsp 90-1 | P27323 | 81 | ADLVNNLGTIAR (54.1) ELISNSSDALDK (62.2) KENEGEVEEVDEEK (72.9) | Protein folding | 7.74 | 3 |
| Luminal binding protein 2 | P24067 | 73 | DILLLDVAPLTLGIETVGGVMTK (87.4) DAGVIAGLNVAR (89.1) | Binding of protein assembly | 21.39 | 4 |
| T-complex protein 1 subunit alpha | P28769 | 59 | VLVELAELQDR (68.8) EVGDGTTSVVIVAAELLK (80.3) | Protein folding | 5.31 | 2 |
| **Defense-related proteins** | | | | | | |
| Annexin D1 | Q9SYT0 | 36 | TGTDEGALTR (94.1) ELSNDFER (50.8) | Abiotic stress response | 6.42 | 3 |
| L-ascorbate peroxidase | Q05431 | 28 | FDAEQAHGANSGIHIALR (49.7) | Abiotic and biotic stress response | 4.72 | 2 |
| Catalase | P45739 | 57 | APGVQTPVIVR (49.6) EGNFDIVGNNFPVFFIR (104.1) DEEIDYFPSR (56.9) | Hydrogen peroxide catabolism | 8.18 | 3 |
| 2-Cys peroxiredoxin | Q6ER94 | 28 | EGVIQHSTINNLAIGR (77.1) SGGLGDLNYPLISDVTK (54.9) | Hydrogen peroxide catabolism | 5.00 | 2 |
| MDHAR | Q42711 | 47 | EIDDADQLVEALK (74.1) EAVAPYERPALSK (48.5) | Oxidation–reduction process | 13.17 | 4 |

**Table 1.** Continued.

| Protein | Accession number | kDa | Peptide sequence (ion score) | GO biological process | Fold change | Log 2 fold change |
|---|---|---|---|---|---|---|
| **Regulatory proteins** | | | | | | |
| 40S ribosomal protein S3a | P49198 | 30 | VFEVSLADLQNDEDHSYR (62.2) LDRPAEEAAPEATEVIGA (46.6) | Translation | 5.18 | 2 |
| 14-3-3-like protein | O65352 | 29 | VVAAADGGEELTIEER (120) QAFDEAIAELDTLGEDSYK (101.3) DSTLIMQLLR (57.3) | Signaling | 10.08 | 3 |
| CBS domain-containing protein CBSX3 | Q9LEV3 | 23 | VGDIMTEENK (64.1) | Cell redox homeostasis | 10.94 | 3 |
| Elongation factor 2 | Q9ASR1 | 94 | STLTDSLVAAAGIIAQEVAGDVR (110.4) NMSVIAHVDHGK (65) EGALAEENMR (64.5) DSVVAGFQWASK (68.4) | Translation | 4.59 | 2 |
| Eukaryotic initiation factor 4A-3 | Q9CAI7 | 47 | MFVLDEADEMLSR (95.4) GLDVIQQAQSGTGK (96.7) | Translation | 16.07 | 4 |
| **Transport proteins** | | | | | | |
| ATP synthase subunit alpha | P18260 | 55 | AAELTTLLESR (62.3) ISNFYTNFQVDEIGR (88) TAIAIDTILNQK (87) | ATP hydrolysis/synthesis | 5.63 | 2 |
| ATP synthase subunit beta | Q1KXV2 | 59 | DVNEQDVLLFVDNIFR (64.3) ELQDIIAILGLDELSEEDR (80.5) GMDVIDTGAPLSVPVGGATLGR (67.8) | ATP synthesis, ion transport | 10.77 | 3 |
| Aquaporin Plasma membrane intrinsic proteins (PIP)1−3 | Q08733 | 31 | QPIGTSAQTDK (44.8) SLGAAIIYNK (61.8) | Water transport | 6.88 | 3 |
| **Cytoskeletal proteins** | | | | | | |
| Actin-2 | Q96292 | 42 | AGFAGDDAPR (71.1) DAYVGDEAQSK (53.3) EITALAPSSMK (44.2) | Cytoplasmic streaming, cell growth | 14.63 | 4 |
| Tubulin alpha-2 chain | Q6VAG0 | 50 | EDAANNFAR (48.2) EDLAALEK (67) | Microtubule-based process | 5.92 | 3 |
| **Miscellaneous proteins** | | | | | | |
| Calmodulin | P04464 | 17 | DQDGFISAAELR (61.5) EADVDGDGQINYEEFVK (86.5) | Calcium ion binding | 4.32 | 2 |
| Cell division cycle protein 48 homolog | P54774 | 90 | NAPSIIFIDEIDSIAPK (78.2) ETVVEVPNVSWEDIGGLENVK (67) | Cell cycle | 4.93 | 2 |
| 11S globulin seed storage protein G3 | P19084 | 56 | VQIVDNQGNSVFDNELR (115.9) TNDNAMIANLAGR (109) EGQVVVIPQNFAVIK (92.6) | Storage protein | 18.95 | 4 |
| Oleosin | P29529 | 19 | GTLQDAGEYAGQK (72.6) QTAGSVPESLDYVK (63.1) | Storage protein | 11.91 | 4 |
| Pectinesterase | P83218 | 34 | VGSDLSAFYR (60) TDPNQNTGIVIQK (73.2) | Cell wall modification | 8.35 | 3 |
| Porphobilinogen deaminase | Q43316 | 41 | ILSQPLADIGGK (49.2) GSPLALAQAYETR (96.6) | Chlorophyll biosynthesis | 5.26 | 2 |

The above-stated earlier reports together with present observations on sunflower seedling roots and cotyledons indicate that in general plant tissues maintain a relatively higher level of SNOs. This can be attributed to both enhanced NO/nitrite level and downregulation of GSNOR activity in response to stress. This is evident through the present observations both in roots (which are in direct contact with the stress inducer, NaCl) as well as in cotyledons as a long-distance salt stress response. As evident from the present observations, it has also been suggested earlier that the downregulation of GSNOR activity, leading to accumulation of RSNOs, may mediate nitrosative stress in plants (Barroso et al. 2006, Lee et al. 2008, Airaki et al. 2011). Signaling networks controlled by salicylic acid have also been observed to be positively regulated by the modulation of cellular levels of SNOs and GSNOR activity in *A. thaliana* (Feechan et al. 2005). In this context, transgenic plants of *Arabidopsis*

**Table 2.** Proteomic analysis (LC-MS/MS) of S-nitrosylated proteins from the roots (10 000 $g$ supernatant) of sunflower seedling (2-day-old, dark-grown) subjected to 120 m$M$ NaCl stress. Fold change refers to protein abundance in salt-stressed roots over control ones. The coloured regions in fold change in both figures indicate heat map where individual values have been represented with a gradation of colours. Lowest represented by red and highest by green.

| Protein | Accession number | kDa | Peptide sequence (ion score) | Gene ontology (GO) biological process | Fold change | Log 2 fold change |
|---|---|---|---|---|---|---|
| **Carbohydrate metabolism** | | | | | | |
| Aconitate hydratase | Q42560 | 98 | VLLQDFTGVPAVVDLACMR (57.3) SENAVQANMELEFQR (84.6) RGNDEVMAR (56.3) | Tricarboxylic acid cycle | 0.34 | −2 |
| Alcohol dehydrogenase 1 | P14673 | 41 | FGVTEFVNPK (77) | Oxidation−reduction process | 0.32 | −2 |
| GAPDH | P25861 | 37 | AGIALNDHFVK (69.9) VPTVDVSVVDLTVR (98) IGINGFGR (58.6) | Glycolysis | 0.09 | −3 |
| Isocitrate dehydrogenase | P50217 | 47 | EHYLNTEEFIDAVADELK (79.5) LIDDMVAYALK (70.2) | Glyoxylate cycle | 0.15 | −3 |
| Isocitrate lyase | P49297 | 64 | TVQGGITSTAAMGK (103.8) WSGANYYDR (60.2) | Tricarboxylic acid cycle | 0.19 | −2 |
| Malate dehydrogenase | O48905 | 36 | MELVDAAFPLLK (104.7) LNVQVSDVK (65.1) LDLTAEELTEEK (86.8) | Tricarboxylic acid cycle | 0.06 | −4 |
| Malate synthase | P08216 | 65 | ATVLIETLPAVFQMNEILYELR (100.1) YGVELDGDGLGVR (94.8) MIINALNSGAK (65.9) | Tricarboxylic acid cycle | 0.14 | −3 |
| Oxygen-evolving enhancer protein 1 | P85194 | 28 | LTYTLDEIEGPLEVSSDGTIK (90.2) GGSTGYDNAVALPAGGR (60.8) DGIDYAAVTVQLPGGER (60.2) | Photosynthesis | 0.20 | −2 |
| 6-Phosphogluconate dehydrogenase | Q9FFR3 | 53 | NPNLASLVVDPDFAK (63.9) LPANLVQAQR (79.5) | Pentose phosphate pathway | 0.13 | −3 |
| Phosphoglycerate kinase | Q42961 | 50 | GVSLLLPSDVVIADK (57.7) ELDYLVGAVSNPK (78.9) | Glycolysis | 0.04 | −5 |
| Phosphoribulokinase | P26302 | 45 | ANDFDLMYEQVK (60.8) | Reductive pentose phosphate cycle | 0.26 | −2 |
| Ribulose bisphosphate carboxylase large chain | P45738 | 54 | FEFQAMDTLDTDK (95.7) DLATEGNEIIR (81.7) EITLGFVDLLRDDFIEK (76.9) | Carbon fixation, photosynthesis | 0.23 | −2 |
| Serine hydroxymethyltransferase 1 | Q9SZJ5 | 57 | MGTPALTSR (39.8) LDESTGYIDYDQMEK (69.5) | Photorespiration | 0.33 | −2 |
| Transketolase | O20250 | 80 | VTTTIGFGSPNK (94.6) TPGHPENFETPGIEVTTGPLC (100.7) | Reductive pentose phosphate cycle | 0.18 | −2 |
| Triosephosphate isomerase | P48493 | 21 | ALLNETNEFVGDK (83.9) VASPAQAQEVHAGLR (91.7) | Glycolysis | 0.14 | −3 |
| **Other metabolic proteins** | | | | | | |
| Adenosylhomocysteinase | P32112 | 53 | DSAAVFAWK (64.3) VAVVCGYGDVGK (62.7) ATDVMIAGK (71.8) | L-homocysteine biosynthesis | 0.07 | −4 |
| Alanine aminotransferase 2 | Q9LDV4 | 60 | ALVVINPGNPTGQVLAEENQR (122.1) ATGAYSHSQGIK (65.2) | L-alanine catabolic process | 0.38 | −1 |
| Biotin carboxylase | O04983 | 58 | HIEFQVLADK (41.8) NAGVPTVPGSDGLLQSTEEAVR (52.5) | Fatty acid biosynthetic process | 0.59 | −1 |
| Ketol-acid reductoisomerase | O82043 | 63 | GSSSFNEAR (59.4) VNLAGHEEYIVR (40) | Amino acid biosynthesis | 0.18 | −2 |
| 5-Methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase | Q42662 | 85 | FALESFWDGK (68.9) YLFAGVVDGR (57.1) GNASVPAMEMTK (50.2) VVKLQEELDIDVLVHGEPER (57.6) | Amino acid biosynthesis | 0.26 | −2 |
| Peptidyl prolyl *cis-trans* isomerase | P21569 | 18 | VFFDMTVGGAPAGR (108.3) | Protein folding | 0.20 | −2 |
| Phospholipase D-alpha 1 | Q43007 | 92 | VLMLVWDDR (60.5) AYLPVQELLNGEEIDR (93.1) | Lipid catabolic process | 0.07 | −4 |

**Table 2.** Continued.

| Protein | Accession number | kDa | Peptide sequence (ion score) | GO biological process | Fold change | Log 2 fold change |
|---|---|---|---|---|---|---|
| S-adenosylmethionine synthase | P49613 | 41 | TNMVMVFGEITTK (89.6) VHTVLISTQHDETVTNDKIAA (66.5) | S-adenosylmethionine biosynthetic process | 0.08 | −4 |
| **Chaperones** | | | | | | |
| Calreticulin | Q9ZPP1 | 48 | GIQTSEDYR (52.5) LLSGDVDQK (55.6) KPEGYDDIPK (47.7) | Protein folding | 0.07 | −4 |
| Endoplasmin homolog | P35016 | 93 | ELISNASDALDK (81) LGIIEDAANR (70.2) SGTSAFVEK (65.1) | Protein folding | 0.07 | −4 |
| Hsp 90-1 | P27323 | 81 | ADLVNNLGTIAR (54.1) ELISNSSDALDK (62.2) KENEGEVEEVDEEK (72.9) | Protein folding | 0.11 | −3 |
| Luminal binding protein 2 | P24067 | 73 | DILLLDVAPLTLGIETVGGVMTK (87.4) DAGVIAGLNVAR (89.1) | Binding of protein assembly | 0.06 | −4 |
| **Defense-related proteins** | | | | | | |
| Annexin D1 | Q9SYT0 | 36 | TGTDEGALTR (94.1) ELSNDFER (50.8) | Cellular oxidant detoxification | 0.07 | −4 |
| MDHAR | Q42711 | 47 | EIDDADQLVEALK (74.1) EAVAPYERPALSK (48.5) | Oxidation−reduction process | 0.04 | −5 |
| Phenylalanine ammonia-lyase | O04058 | 72 | EINSVNDNPLINVSR (87.2) TAEAVDILK (38.6) | Cinnamic acid biosynthetic process | 0.10 | −3 |
| Superoxide dismutase (Mn) | Q9LYK8 | 27 | LVVETTANQDPLVTK (64.2) | Superoxide metabolic process | 0.07 | −4 |
| **Regulatory proteins** | | | | | | |
| 14-3-3-like protein | O65352 | 29 | QAFDEAIAELDTLGEDSYK (101.3) DSTLIMQLLR (57.3) | Signaling | 0.10 | −3 |
| 40S ribosomal protein S3a | P49198 | 30 | VFEVSLADLQNDEDHSYR (62.2) LDRPAEEAAPEATEVIGA (46.6) | Translation | 0.12 | −3 |
| Elongation factor 2 | Q9ASR1 | 94 | STLTDSLVAAAGIIAQEVAGDVR (110.4) NMSVIAHVDHGK (65) EGALAEENMR (64.5) DSVVAGFQWASK (68.4) | Translation | 0.12 | −3 |
| **Transport proteins** | | | | | | |
| ATP synthase subunit alpha | P18260 | 55 | AAELTTLLESR (62.3) ISNFYTNFQVDEIGR (88) TAIAIDTILNQK (87) | ATP hydrolysis/synthesis | 0.30 | −2 |
| ATP synthase subunit beta | Q1KXV2 | 59 | DVNEQDVLLFVDNIFR (64.3) ELQDIIAILGLDELSEEDR (80.5) GMDVIDTGAPLSVPVGGATLGR (67.8) | ATP synthesis, ion transport | 0.07 | −4 |
| Aquaporin PIP1−3 | Q08733 | 31 | QPIGTSAQTDK (44.8) SLGAAIIYNK (61.8) | Water transport | 0.19 | −2 |
| **Cytoskeletal proteins** | | | | | | |
| Actin-2 | Q96292 | 42 | AGFAGDDAPR (71.1) DAYVGDEAQSK (53.3) EITALAPSSMK (44.2) | Cytoplasmic streaming, cell growth | 0.09 | −3 |
| Tubulin alpha-2 chain | Q6VAG0 | 50 | EDAANNFAR (48.2) EDLAALEK (67) | Microtubule-based process | 0.17 | −3 |
| **Miscellaneous proteins** | | | | | | |
| Calmodulin | P04464 | 17 | DQDGFISAAELR (61.5) EADVDGDGQINYEEFVK (86.5) | Calcium ion binding | 0.15 | −3 |
| Cell division cycle protein 48 homolog | P54774 | 90 | NAPSIIFIDEIDSIAPK (78.2) ETVVEVPNVSWEDIGGLENVK (67) | Cell cycle | 0.47 | −1 |
| 11S globulin seed storage protein G3 | P19084 | 56 | VQIVDNQGNSVFDNELR (115.9) TNDNAMIANLAGR (109) EGQVVVIPQNFAVIK (92.6) | Storage protein | 0.24 | −2 |
| Pectinesterase | P83218 | 34 | VGSDLSAFYR (60) TDPNQNTGIVIQK (73.2) | Cell wall modification | 0.31 | −2 |
| Porphobilinogen deaminase | Q43316 | 41 | ILSQPLADIGGK (49.2) GSPLALAQAYETR (96.6) | Chlorophyll biosynthesis | 0.11 | −3 |

exhibiting low GSNOR activity show enhanced resistance against *Peronospora parasitica* (Rusterucci et al. 2007). Two cultivars of sunflower with different sensitivity to the pathogen *Plasmopara halstedii* also show difference in GSNOR activity (Chaki et al. 2009b). Detailed investigations undertaken by Kubienova et al. (2014) on the impact of a variety of stress factors (heat, cold, injury and light) have demonstrated the significance of GSNOR activity modulation both during normal plant development and also in response to a variety of abiotic stress conditions in *Cucumis* and *P. sativum*. These investigations highlighted diverse changes in GSNOR activity as well as the parallel changes in GSNOR protein levels. In contrast, the present observations highlight the impact of salt stress on modulation of GSNOR activity without any noteworthy changes in GSNOR protein levels.

The present investigations on deciphering the qualitative and quantitative variations in the S-nitrosylation of cytosolic proteins from sunflower seedling roots and cotyledons in response to salt stress highlight noteworthy differences in the pattern and extent of their S-nitrosylation. Immunoblot analysis of S-nitrosylated proteins in seedling cotyledons consistently shows accumulation in at least 10 protein zones in response to salt stress as well as GSNO treatment resolved by electrophoresis. The number of zones of proteins from root homogenates showing upregulation of S-nitrosylation in response to salt stress and GSNO treatment was, however, less than that in seedling cotyledons. Interestingly, proteins in the indicated zone showed downregulation in response to GSNO treatment irrespective of salt stress. Vanzo et al. (2014) analyzed the abundance of S-nitrosylated proteins in callus and leaf extracts from poplar and observed enrichment of S-nitrosylation of several proteins in response to ozone stress. In the recent past, differential modulation of S-nitrosoproteome of *Brassica juncea* by low temperature revealed both enhanced S-nitrosylation of at least nine proteins and denitrosylation of eight proteins (Abat and Deswal 2009). Similar pattern of S-nitrosylation was also observed in citrus plants in response to salinity (Tanou et al. 2009). NaCl stress has been reported to bring about minor changes in the extent of S-nitrosylation of proteins in the homogenates from *Arabidopsis* cell suspensions (Fares et al. 2011). Furthermore, analysis of pea mitochondrial proteins showed a decrease in the extent of S-nitrosylation in response to short- and long-term salt treatments (Camejo et al. 2013). Similar observations were also recorded in citrus plants in response to varied abiotic stress conditions (Ziogas et al. 2013). The present observations and earlier reports thus indicate that stress can lead to both enhanced S-nitrosylation and also denitrosylation of different proteins. In this context, it may be

noted that GSNOR and thioredoxin/thioredoxin reductase have recently been classified as denitrosylases and these enzymes might also be regulated by NO (Vanzo et al. 2014). Furthermore, non-enzymatic denitrosylation of proteins has also been reported in response to changes in the cellular redox environment (Singh et al. 1995). LC-MS/MS analysis of S-nitrosylated proteins in sunflower seedlings shows that many enzymes belonging to primary carbohydrate metabolism undergo S-nitrosylation (upregulation in seedling cotyledons and downregulation in seedling roots). These enzymes belong to glycolysis, photorespiration, photosynthesis, tricarboxylic acid cycle, glyoxylate cycle and gluconeogenesis. As these investigations have been undertaken on dark-grown seedling cotyledons and roots, the observed enhanced or decreased S-nitrosylation of various enzymes from the above-stated pathways is on expected lines. It is, however, not clear which of these enzymes upon getting S-nitrosylated or denitrosylated will show enhanced or reduced enzyme activity. S-nitrosylation of proteins is often observed to inhibit the enzyme activity while denitrosylation may promote the enzyme activity (Lindermayr et al. 2006, Romero-Puertas et al. 2007, Palmieri et al. 2010, Yun et al. 2011). Thus, denitrosylation may be associated with fast upregulation of various metabolic processes required to combat stress in seedling roots. This view, however, needs to be proved through further detailed biochemical analysis of the respective enzymes. It may further be noted that sunflower is an unconventional research material for proteomic analysis under abiotic stress on two accounts: (1) most of the available data on proteomic analysis pertain to *Arabidopsis* and (2) most of the cell volume in cotyledon tissue is filled with oil bodies encased in a phospholipid monolayer impregnated with a variety of intrinsic and extrinsic structural and signaling proteins. Keeping these facts in view, the noteworthy large number of enzymes exhibiting upregulation of S-nitrosylation highlights the metabolic tendency of the seedling cotyledons to modulate various carbohydrate biosynthetic and catabolic routes to enable the seedling to survive in the harsh conditions of salt stress. It may further be noted that most of these observations on S-nitrosylation of proteins are in agreement with earlier reports (Marcus et al. 2003, Lindermayr et al. 2005, Abat et al. 2008, Abat and Deswal 2009, Tanou et al. 2012, Vanzo et al. 2014).

Substantial S-nitrosylation of a variety of enzymes associated with carbohydrate metabolism in response to salt stress observed in the present work suggests that NO can modulate the concentration of various metabolites and energy balance in salt-stressed seedlings. S-nitrosylation of GAPDH, triose phosphate
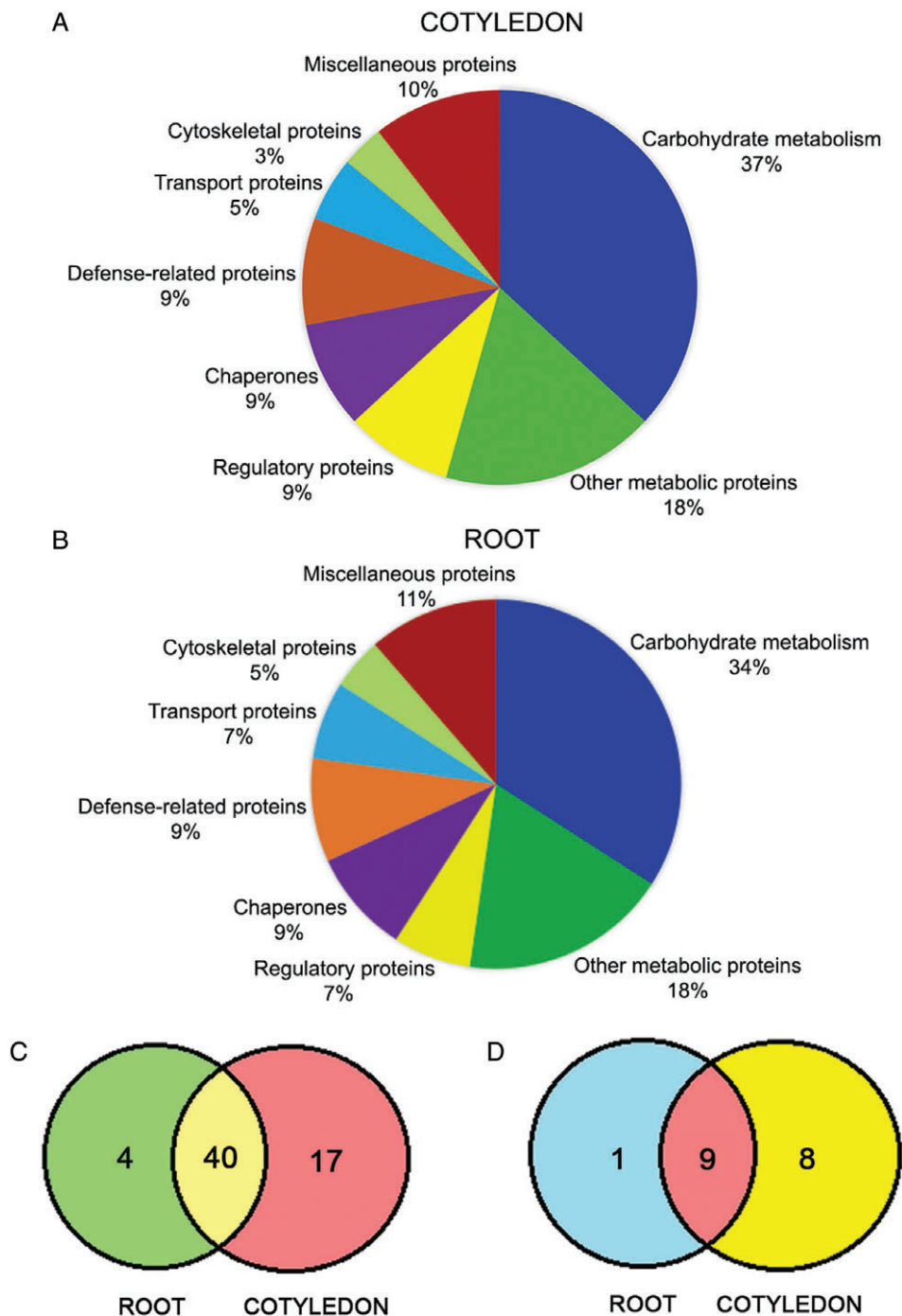
**Fig. 4.** Pie chart showing categories of proteins regulated by S-nitrosylation in the 10 000 *g* -supernatant from 2-day-old sunflower seedling cotyledons (A) and roots (B) in response to salt stress (120 m*M* NaCl). Venn diagram depicting the distribution of total (C) and novel (D) S-nitrosylated proteins in 2-day-old sunflower seedling roots and cotyledons in response to salt stress. Novel proteins refer to those which are being reported for the first time under S-nitrosylation category.

**Table 3.** Novel S-nitrosylated proteins in sunflower seedling cotyledons and roots (new reports) in response to salt stress. Bold "C" represents cysteine residue which is the site of S-nitrosylation.

| Protein | Subcellular localization | Prediction of S-nitrosylation sites (using GPS-SNO 1.0) | |
| --- | --- | --- | --- |
| | | Position | Sequence |
| Both cotyledons and roots | | | |
| Malate synthase | Glyoxysome | 533 | KGMYKEA**C**KMFTRQC |
| Phospholipase D-alpha 1 | Cell membrane | 214 741 | KNYEPHR**C**WEDIFDA QRPESLE**C**VQKVNRI |
| Biotin carboxylase | Chloroplast | 147 | SAAISRG**C**TMLHPGY |
| Ketol-acid reductoisomerase | Chloroplast | 8 386 579 | MAAVTSS**C**STAISAS CMEILYE**C**YEDVASG VRPELRQ**C**SN |
| Endoplasmin homolog | Endoplasmic reticulum (lumen) | 21 675 | CPSLSSS**C**QGRKIHA NRLANTP**C**VVVTSKY |
| Aquaporin PIP1−3 | Cell membrane | 140 | VFYIVMQ**C**LGAICGA |
| Pectinesterase | Cell wall | 170 | AAVVLQD**C**DIHARRP |
| Calmodulin | Cytoplasm, cell membrane | – | – |
| 11S globulin seed storage protein G3 | Protein body | 32 312 | RQQQQNQ**C**QLQNIEA NGVEETI**C**SMKFKVN |
| Only cotyledons | | | |
| Citrate synthase | Peroxisome | 110 293 312 | APVRSSI**C**YIDGDEG HAEHEMN**C**STAAARH GVDVYTA**C**AGAVGAL |
| Pyruvate phosphate dikinase | Chloroplast | 324 380 | GTAVNIQ**C**MVFGNMG DLATMET**C**MPEAYRE |
| UDP-glucose 6-dehydrogenase | Cytoplasm, nucleus | 5 | ***MVKI**C**CIGAGYV |
| Adenylate kinase | Cytoplasm | – | – |
| T-complex protein 1 subunit alpha | Cytoplasm | 154 400 | GKVPLIN**C**AKTSMSS RALHDAL**C**IVKRTLE |
| Chaperone protein ClpB1 | Cytoplasm, nucleus, chloroplast | 311 742 | LARGQLR**C**IGATTLE TMEVARD**C**VMREVRK |
| CBS domain-containing protein CBSX3 | Mitochondria | 36 | AIQPSVF**C**SRSESTQ |
| Oleosin | Oil body membrane | – | – |
| Only roots | | | |
| Calreticulin | Endoplasmic reticulum (lumen) | – | – |

isomerase and phosphoglycerate kinase has earlier been reported in *A. thaliana* (Lindermayr et al. 2005). GAPDH exhibits S-nitrosylation-dependent reversible modulation of enzyme activity, indicating how such a modification can affect protein function by the inactivation of cysteine residue in the active center of the protein (Padgett and Whorton 1995, Lindermayr et al. 2005). This enzyme is involved in various nuclear events such as RNA transport, DNA replication and transcription (Sirover 1999). S-nitrosylation of cytosolic GAPDH has been shown to inhibit its catalytic activity (Holtgrefe et al. 2008, Zaffagnini et al. 2013). Phosphoribulokinase, an enzyme of the reductive pentose phosphate pathway, primarily functions to generate reducing equivalents (NADPH) and provides ribose 5-phosphate for nucleotide biosynthesis. Thus, S-nitrosylation of such an enzyme (as observed in the present work) is likely to affect its above-stated functions. As also

observed in the present work, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), the key enzyme of Calvin−Benson cycle, has earlier been reported to exhibit S-nitrosylation of its large subunit in *Arabidopsis* in response to NO donor treatment (Lindermayr et al. 2005). The Cys residue in this subunit of RuBisCO residing adjacent to the active site is believed to play a role in the modulation of enzyme activity and degradation of the protein thereby altering its turnover (Marcus et al. 2003). RuBisCO and triose phosphate isomerase have been characterized and shown to be inhibited by S-nitrosylation (Abat et al. 2008, Abat and Deswal 2009). Enzymes of tricarboxylic acid cycle, such as malate dehydrogenase and aconitase, were also found to be regulated by S-nitrosylation upon salt stress. Malate dehydrogenase is involved in ATP generation through NADH and it shows modulation of its S-nitrosylation pattern accompanying salt stress in sunflower seedling
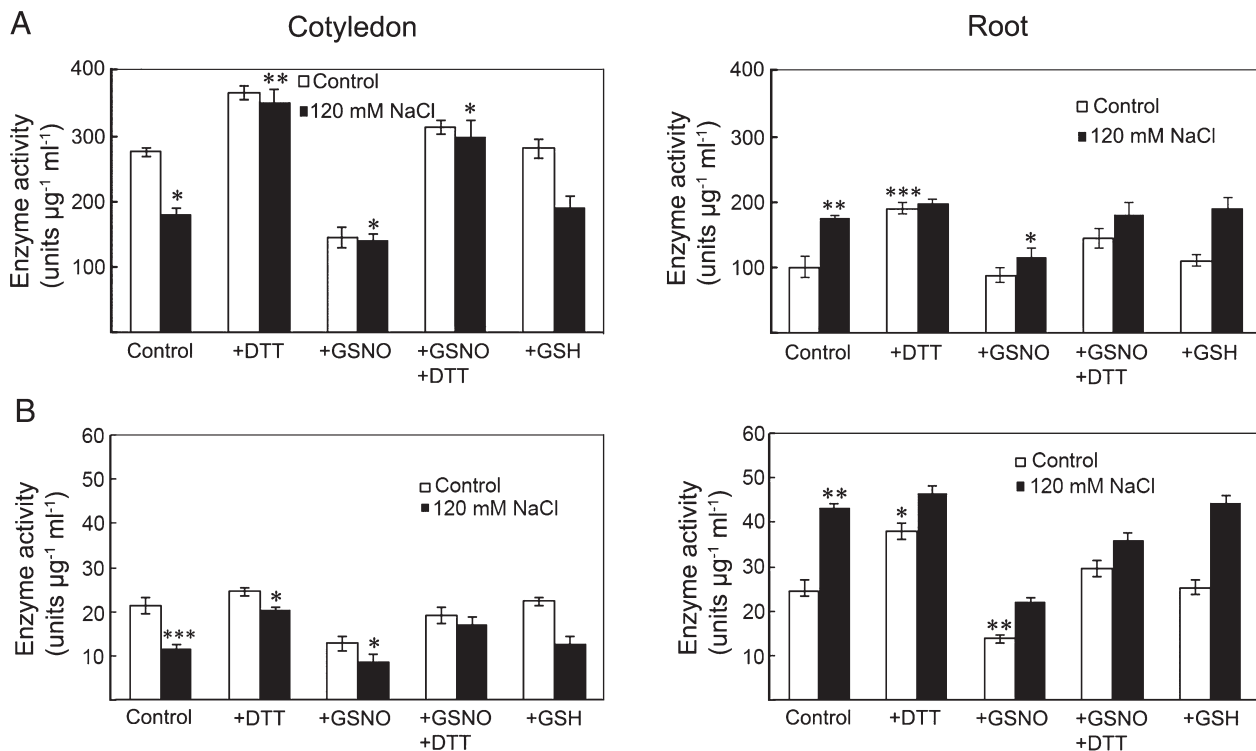
**Fig. 5.** Effect of GSNO and DTT (reducing agent) treatments on GAPDH (A) and MDHAR (B) activities in 10 000 *g* supernatant obtained from cotyledons and roots of 2-day-old, dark-grown sunflower seedlings grown in the absence or presence of 120 m*M* NaCl. Data represent mean ± SE from three replicates. NaCl stress- and treatment-induced changes were analyzed by one-way ANOVA using SPSS 22.0 and were found to be statistically significant ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

cotyledons and roots (present work). Both malate dehydrogenase and aconitase have earlier been shown to be inhibited by S-nitrosylation (Ortega-Galisteo et al. 2012). The present results along with supporting data from previous reports on S-nitrosylation of proteins in various plant proteins suggest that different enzymes involved in various plant metabolic pathways are regulated differentially in response to salt stress or NO donor treatment or any other kind of stress stimuli.

In the category of 'other metabolic proteins', the present observations on the modulation of the extent of S-nitrosylation/denitrosylation in sunflower seedling cotyledons and roots in response to salt stress are in conformity with S-nitrosylation of adenosylhomocysteinase, methionine synthase and S-adenosylmethionine synthetase in *Arabidopsis* (Lindermayr et al. 2005). These enzymes are part of methylMet cycle, which provides activated methyl groups such as S-adenosyl methionine (SAM) for methylation of various cell components such as flavonoids, lignin and DNA. SAM is also the precursor for ethylene biosynthesis in plants and S-nitrosylation of SAM synthetase or the associated enzymes of methylMet cycle are likely to mediate a crosstalk between NO and ethylene. A significant

modulation of S-nitrosylation of chaperones in response to salt stress is evident in sunflower seedling cotyledons and roots, although in opposite manner. The common proteins getting modulated in this category in both the tissue systems include heat shock protein (Hsp) 90-1, endoplasmin homolog and luminal binding protein. Camejo et al. (2013) reported S-nitrosylation of Hsp 90 from the mitochondrial proteins. Similar reports on S-nitrosylation of Hsp 70 and Hsp 90 are also available (Lindermayr et al. 2005, Molassiotis et al. 2010, Fares et al. 2011). Calreticulin is a new report from the present work under the category of chaperones exhibiting denitrosylation in seedling roots in response to salt stress. In the category of defense-related proteins, well-known ROS-scavenging enzymes have been found to be modulated by S-nitrosylation in both the tissue systems, although MDHAR and annexin D1 are the two which are common to both the tissue systems and exhibit enhanced S-nitrosylation in cotyledons and denitrosylation in seedling roots. This observation highlights the significant role these two proteins might be playing in tolerating salt stress in direct contact with salt (roots) vs long-distance mechanisms to combat NaCl stress in seedling cotyledons through

changes in ROS-scavenging mechanisms. The other defense-related proteins exhibiting modulation of the extent of S-nitrosylation/denitrosylation in sunflower include phenylalanine ammonia lyase and Mn-SOD in seedling roots (denitrosylation) and L-ascorbate peroxidase, 2-Cys peroxiredoxin and catalase in seedling cotyledons (upregulation). In this context, Camejo et al. (2013) reported S-nitrosylation of Mn-SOD and peroxiredoxin in the mitochondrial proteins of pea 14 days after salinity stress. In citrus plants, salt stress leads to modulation of S-nitrosylation of Fe-SOD and Mn-SOD (Tanou et al. 2009). Furthermore, Marti et al. (2011) reported that pea plants subjected to salinity stress exhibited posttranscriptional and posttranslational regulation of Mn-SOD, alternative oxidase AOX and peroxiredoxin II. In view of the present observations on the modulation of S-nitrosylation status of ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) in sunflower seedling cotyledons and roots, it may be possible that S-nitrosylation plays a critical role in the ascorbate–GSH cycle. Both these enzymes are essential components of this pathway and they have been reported to be induced at the level of both transcription and translation in response to salt stress (Begara-Morales et al. 2014, 2015). Furthermore, APX has been found to be S-nitrosylated by salt stress leading to enhanced activity of the enzyme (Begara-Morales et al. 2014). These investigators reported that S-nitrosylation of APX at Cys-32 enhanced its activity thereby promoting $H_2O_2$ turnover, causing enhanced resistance to oxidative stress. In contrast, blocking of S-nitrosylation results in reduction of APX activity (Begara-Morales et al. 2014). S-nitrosylation of MDAR under salinity stress has earlier been found to inhibit enzyme activity. Differential status of S-nitrosylation of various ROS-scavenging enzymes suggests the role of these enzymes in regulating cellular redox homoestasis. Changes in the S-nitrosylation status of various regulatory proteins observed in the present work as a response to salt stress suggest the possible role of NO in protein synthesis. Lindermayr et al. (2005) identified different elongation and initiation factors as targets for S-nitrosylation in *Arabidopsis* in line with the present observation on sunflower seedlings. A variety of cytoskeletal proteins such as actin, actin polymerizing factor, tubulin alpha and tubulin beta have earlier been reported to undergo variations in S-nitrosylation in *Arabidopsis* (Lindermayr et al. 2005). The present work highlights both up- and downregulation of actin-2 and tubulin alpha 2 in sunflower seedling cotyledons and roots, respectively, in response to salt stress. The opposite trends of S-nitrosylation patterns of these cytoskeletal proteins in sunflower seedling roots and cotyledons signify the role of cytoskeleton

filaments in transfer of rapid long-distance signals in response to stress factors. Any conformational changes in the components of cytoskeleton due to S-nitrosylation might affect loading of vesicles with various metabolites and their long-distance transport. Quite a few novel proteins have also been identified as targets of S-nitrosylation in sunflower seedlings. These include pyruvate phosphate dikinase, oleosins, calmodulin, calreticulin, pectinesterases, aquaporin, phospholipase D, malate synthase, 11S globulin seed storage protein, citrate synthase and chaperones such as endoplasmin homolog and chaperone protein ClpB1.

S-nitrosylation and denitrosylation together regulate the activities of various enzymes. It has been demonstrated that S-nitrosylation often leads to inhibition of enzyme activity while denitrosylation promotes enzyme activity (Lindermayr et al. 2006, Belenghi et al. 2007, Romero-Puertas et al. 2007, Palmieri et al. 2010). GAPDH and MDHAR are two important regulatory enzymes, which were found to be S-nitrosylated in sunflower seedling roots and cotyledons (present work). Salt stress-induced inhibition of GAPDH and MDHAR activities in sunflower seedling cotyledons correlates with LC-MS/MS data. However, denitrosylation of proteins in seedling roots in the presence of 120 m$M$ NaCl promotes activities of both the enzymes. Denitrosylation of proteins in sunflower seedling roots leading to increase in enzyme activity might act as an early response to salt stress. These results together highlight the role of both S-nitrosylation and denitrosylation by regulating enzyme activities during long-distance sensing of salt stress in sunflower seedlings.

To sum up, the present observations have demonstrated that nitrite accumulation serves as a biomarker of salt and/or nitrosative stress. RSNOs, particularly GSNO, act as major intracellular reservoirs and carriers of NO in plants. The present observations also suggest the role of RSNOs in plant response to salinity stress. SNOs are more stable as compared to NO in cell systems and participate in signal transduction pathway thus indicating the importance of this biomolecule in stress conditions. In general, plant tissues maintain a relatively higher level of SNOs. This can be attributed to both enhanced NO/nitrite level and downregulation of GSNOR activity in response to stress (Figs 2 and 3). Downregulation of GSNOR activity, leading to accumulation of RSNOs, may mediate nitrosative stress in plants. Stress can lead to both enhanced S-nitrosylation and also denitrosylation of different proteins. In this context, it may be noted that GSNOR and thioredoxin/thioredoxin reductase have recently been classified as denitrosylases and these enzymes might also be regulated by NO. Furthermore, non-enzymatic denitrosylation of proteins has also
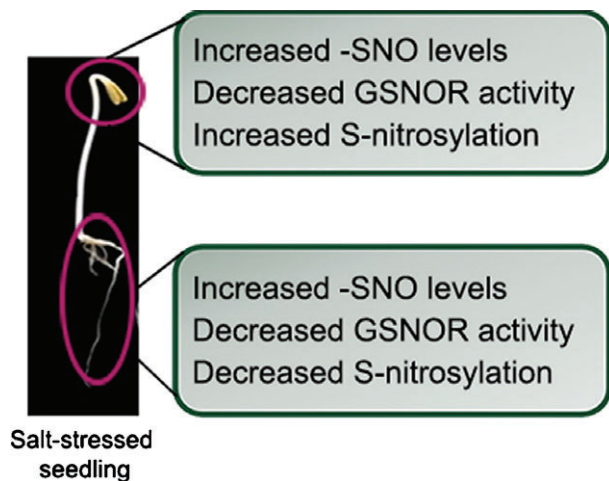
**Fig. 6.** S-nitrosylation status in sunflower seedling roots and cotyledons.

been reported in response to changes in the cellular redox environment.

LC-MS/MS analysis of S-nitrosylated proteins in sunflower seedlings has shown that many enzymes belonging to primary carbohydrate metabolism undergo S-nitrosylation (upregulation in seedling cotyledons and downregulation in seedling roots; Fig. 6). Further work is required to demonstrate which of these enzymes upon getting S-nitrosylated or denitrosylated will show enhanced or reduced enzyme activity. S-nitrosylation of proteins is often observed to inhibit the enzyme activity while denitrosylation may promote the enzyme activity. Thus, denitrosylation may be associated with fast upregulation of various metabolic processes required to combat stress in seedling roots. The noteworthy large number of enzymes exhibiting upregulation of S-nitrosylation highlights the metabolic tendency of the seedling cotyledons to modulate various carbohydrate biosynthetic and catabolic routes to enable the seedling to survive in the harsh conditions of salt stress.

## References

Abat JK, Deswal R (2009) Differential modulation of S-nitrosoproteome of *Brassica juncea* by low temperature: change in S-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity. Proteomics 9: 4368–4380

Abat JK, Mattoo AK, Deswal R (2008) S-nitrosylated proteins of a medicinal CAM plant *Kalanchoe pinnata*–ribulose-1,5-bisphosphate carboxylase/

oxygenase activity targeted for inhibition. FEBS J 275: 2862–2872

Airaki M, Sánchez-Moreno L, Leterrier M, Barroso JB, Palma JM, Corpas FJ (2011) Detection and quantification of S-nitrosoglutathione (GSNO) in pepper (*Capsicum annuum* L.) plant organs by LC-ES/MS. Plant Cell Physiol 52: 2006–2015

Arora D, Bhatla SC (2015) Nitric oxide triggers a concentration-dependent differential modulation of superoxide dismutase (FeSOD and Cu/ZnSOD) activity in sunflower seedling roots and cotyledons as an early and long distance signaling response to NaCl stress. Plant Signal Behav 10: e1071753. https://doi.org/10.1080/15592324.2015.1071753

Astier J, Kulik A, Koen E, Besson-Bard A, Bourque S, Jeandroz S, Lamotte O, Wendehenne D (2012) Protein S-nitrosylation: what's going on in plants? Free Radic Biol Med 53: 1101–1110

Bai X, Yang L, Tian M, Chen J, Shi J, Yang Y, Hu X (2011) Nitric oxide enhances desiccation tolerance of recalcitrant *Antiaris toxicaria* seeds via protein S-nitrosylation and carbonylation. PLoS One 6: e20714

Barroso JB, Corpas FJ, Carreras A, Rodríguez-Serrano M, Esteban FJ, Fernández-Ocaña A, Chaki M, Romero-Puertas MC, Valderrama R, Sandalio LM, del Rio LA (2006) Localization of S-nitrosoglutathione and expression of S-nitrosoglutathione reductase in pea plants under cadmium stress. J Exp Bot 57: 1785–1793

Begara-Morales JC, Sanchez-Calvo B, Chaki M, Valderrama R, Mata-Perez C, Lopez-Jaramillo J, et al. (2014) Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. J Exp Bot 65: 527–538

Begara-Morales JC, Sanchez-Calvo B, Chaki M, Mata-Perez C, Valderrama R, Padilla MN, Carreras A, Corpas FJ, Barroso JB (2015) Differential molecular response of monodehydroascorbate reductase and glutathione reductase by nitration and S-nitrosylation. J Exp Bot 66: 5983–5996

Belenghi B, Romero-Puertas MC, Vercammen D, Brackenier A, Inzé D, Delledonne M, van Breusegem F (2007) Metacaspase activity of *Arabidopsis thaliana* is regulated by S-nitrosylation of a critical cysteine residue. J Biol Chem 282: 1352–1358

Beligni MV, Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta 210: 215–221

Benhar M (2015) Nitric oxide and the thioredoxin system: a complex interplay in redox regulation. Biochim Biophys Acta 1850: 2476–2484

Benhar M, Forrester MT, Stamler JS (2009) Protein denitrosylation: enzymatic mechanisms and cellular functions. Nat Rev Mol Cell Biol 10: 721–732

Bethke PC, Badger MR, Jones RL (2004) Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell 16: 332–341

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Camejo D, Romero-Puertas MC, Rodríguez-Serrano M, Sandalio LM, Lázaro JJ, Jiménez A, Sevilla F (2013) Salinity-induced changes in S-nitrosylation of pea mitochondrial proteins. J Proteomics 79: 87–99

Chaki M, Fernández-Ocaña AM, Valderrama R, Carreras A, Esteban FJ, Luque F, Gomez-Rodriguez MV, Begara-Morales JC, Corpas FJ, Barroso JB (2009) Involvement of reactive nitrogen and oxygen species (RNS and ROS) in sunflower–mildew interaction. Plant Cell Physiol 50: 265–279

Chaki M, Valderrama R, Fernandez-Ocana AM, Carreras A, Gomez-Rodriguez MV, Lopez-Jaramillo J, Begara-Morales JC, Sanchez-Calvo B, Luque F, Leterrier M, Corpas FJ, Barroso JB (2011a) High temperature triggers the metabolism of S-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin-NADP reductase by tyrosine nitration. Plant Cell Environ 34: 1803–1818

Chaki M, Valderrama R, Fernández-Ocaña AM, Carreras A, Gómez-Rodríguez MV, Pedrajas JR, Begara-Morales JC, Sanchez-Calvo B, Luque F, Leterrier M, Corpas FJ, Barroso JB (2011b) Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in S-nitrosothiols in sunflower (*Helianthus annuus*) seedlings. J Exp Bot 62: 1803–1813

Chen R, Sun S, Wang C, Li Y, Liang Y, An F (2009) The *Arabidopsis* paraquat resistant 2 gene encodes an S-nitrosoglutathione reductase that is a key regulator of cell death. Cell Res 19: 1377–1387

Corpas FJ, Barroso JB (2013) Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. New Phytol 199: 633–635

Corpas FJ, Chaki M, Fernandez-Ocana A, Valderrama R, Palma JM, Carreras A, Begara-Morales JC, Airaki M, del Rio LA, Barroso JB (2008) Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. Plant Cell Physiol 49: 1711–1722

Creus CM, Graziano M, Casanovas EM, Pereyra MA, Simontacchi M, Puntarulo S, Barassi CA, Lamattina L (2005) Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. Planta 221: 297–303

David A, Yadav S, Bhatla SC (2010) Sodium chloride stress induces nitric oxide accumulation in root tips and oil body surface accompanying slower oleosin degradation in sunflower seedlings. Physiol Plant 140: 342–354

del Río LA, Corpas FJ, Barroso JB (2004) Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry 65: 783–792

Diaz M, Achkor H, Titarenko E, Martinez MC (2003) The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. FEBS Lett 543: 136–139

Fares A, Rossignol M, Peltier JB (2011) Proteomics investigation of endogenous S-nitrosylation in Arabidopsis. Biochem Biophys Res Commun 416: 331–336

Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ (2005) A central role for S-nitrosothiols in plant disease resistance. Proc Natl Acad Sci USA 102: 8054–8059

Gupta A, Bhatla SC (2005) Spatial and temporal changes in lipase activity sites during oil body mobilization in protoplasts from sunflower seedling cotyledons. Plant Growth Regul 46: 11–17

Gupta A, Bhatla SC (2007) Preferential phospholipase A2 activity on the oil bodies in cotyledons during seed germination in *Helianthus annuus* L. cv. Morden. Plant Sci 172: 535–543

Gupta A, Sadegipour HR, Bhatla SC (2003) Subcellular detection of lipase activity in plant protoplasts using fluorescence microscopy. Plant Growth Regul 41: 259–264

He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM, Pei ZM (2004) Nitric oxide represses the Arabidopsis floral transition. Science 305: 1968–1971

Holtgrefe S, Gohlke J, Starmann J, Druce S, Klocke S, Altmann B, Wojtera J, Lindermayr C, Scheibe R (2008) Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. Physiol Plant 133: 211–228

Hossain MA, Nakano Y, Asada K (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol 25: 385–395

Jaffrey SR, Snyder SH (2001) The biotin switch method for the detection of S-nitrosylated proteins. Sci STKE 86: pl1

Jain P, Bhatla SC (2017) Molecular mechanisms accompanying nitric oxide signalling through tyrosine nitration and S-nitrosylation of proteins in plants. Funct Plant Biol https://doi.org/10.1071/FP16279

Jasid S, Simontacchi M, Bartoli CG, Puntarulo S (2006) Chloroplasts as a nitric oxide cellular source: effect of reactive nitrogen species on chloroplastic lipids and proteins. Plant Physiol 142: 1246–1255

Kaur H, Bhatla SC (2016) Melatonin and nitric oxide modulate glutathione content and glutathione reductase activity in sunflower seedling cotyledons accompanying salt stress. Nitric Oxide 59: 42–53

Kubienova L, Kopecny D, Tylichova M, Briozzo P, Skopalova J, Sebela M, Navratil M, Tache R, Luhova L, Barroso JB, Petrivalsky M (2013) Structural and functional characterization of a plant S-nitrosoglutathione reductase from *Solanum lycopersicum*. Biochimie 95: 889–902

Kubienova L, Ticha T, Jahnova J, Luhova L, Mieslerova B, Petřivalsky M (2014) Effect of abiotic stress stimuli on S-nitrosoglutathione reductase in plants. Planta 239: 139–146

Kuruthukulangarakoola GT, Zhang J, Albert A, Winkler B, Lang H, Buegger F, Gaupels F, Heller W, Michalke B, Sarioglu H, Schnitzler JP, Hebelstrup KH, Durner J, Lindermayr C (2017) Nitric oxide-fixation by non-symbiotic hemoglobin proteins in *Arabidopsis thaliana* under N-limited conditions. Plant Cell Environ 40(1): 36–50

Lamotte O, Curtois C, Barnavon L, Pugin A, Wendehenne D (2005) Nitric oxide in plants: the biosynthesis and cell signaling properties of a fascinating molecule. Planta 221: 1–4

Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E (2008) Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in Arabidopsis. Plant Cell 20: 786–802

Leterrier M, Airaki M, Palma JM, Chaki M, Barroso JB, Corpas FJ (2012) Arsenic triggers the nitric oxide (NO) and S-nitrosoglutathione (GSNO) metabolism in Arabidopsis. Environ Pollut 166: 136–143

Lin A, Wang Y, Tang J, Xue P, Li C, Liu L, Hu B, Yang F, Loake GJ, Chu C (2012) Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice. Plant Physiol 158: 451–464

Lindermayr C, Saalbach G, Durner J (2005) Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiol 137: 921–930

Lindermayr C, Saalbach G, Bahnweg G, Durner J (2006) Differential inhibition of Arabidopsis methionine adenosyltransferases by protein S-nitrosylation. J Biol Chem 281: 4285–4291

Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS (1999) Fas-induced caspase denitrosylation. Science 284: 651–654

Marcus Y, Altman-Gueta H, Finkler A, Gurevitz M (2003) Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation. J Bacteriol 185: 1509–1517

Marti MC, Florez-Sarasa I, Camejo D, Ribas-Carbo M, Lazaro JJ, Sevilla F, Jimenez A (2011) Response of mitochondrial thioredoxin PsTrxo1, antioxidant enzymes, and respiration to salinity in pea (*Pisum sativum* L.) leaves. J Exp Bot 62: 3863–3874

Merl J, Ueffing M, Hauck SM, von Toerne C (2012) Direct comparison of MS-based label-free and SILAC quantitative proteome profiling strategies in primary retinal Müller cells. Proteomics 12: 1902–1911

Modolo LV, Augusto O, Almeida IMG, Pinto-Maglio CAF, Oliveira HC, Seligman K, Salgado I (2006) Decreased arginine and nitrite levels in nitrate reductase-deficient *Arabidopsis thaliana* plants impair nitric oxide synthesis and the hypersensitive response to *Pseudomonas syringae*. Plant Sci 171: 34–40

Molassiotis A, Tanou G, Diamantidis G (2010) NO says more than 'YES' to salt tolerance: salt priming and systemic nitric oxide signaling in plants. Plant Signal Behav 5: 209–212

Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJ, Hebelstrup KH, Gupta KJ (2013) Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants 5: pls052

Neill SJ, Desikan R, Hancock JT (2003) Nitric oxide signalling in plants. New Phytol 159: 11–35

Ortega-Galisteo AP, Rodriguez-Serrano M, Pazmin DM, Gupta DK, Sandalio LM, Romero-Puertas MC (2012) S-nitrosylated proteins in pea (*Pisum sativum* L.) leaf peroxisomes: changes under abiotic stress. J Exp Bot 63: 2089–2103

Padgett CM, Whorton AR (1995) S-nitrosoglutathione reversibly inhibits GAPDH by S-nitrosylation. Am J Physiol 269: C739–C749

Palmieri MC, Lindermayr C, Bauwe H, Steinhauser DJ (2010) Regulation of plant glycine decarboxylase by S-nitrosylation and glutathionylation. Plant Physiol 152: 1514–1528

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C, Delledonne M (2004) Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. Plant Cell 16: 2785–2794

Planchet E, Kapuganti JG, Sonoda M, Kaiser WM (2005) Nitric oxide emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J 41: 732–743

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J Exp Bot 53: 103–110

Romero-Puertas MC, Laxa M, Matte A, Zaninotto F, Finkemeier I, Jones AM, Perazzolli M, Vandelle E, Dietz KJ, Delledonne M (2007) S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. Plant Cell 19: 4120–4130

Rusterucci C, Espunya MC, Díaz M, Chabannes M, Martínez MC (2007) S-nitrosoglutathione reductase affords protection against pathogens in Arabidopsis, both locally and systemically. Plant Physiol 143: 1282–1292

Sadegipour HR, Bhatla SC (2002) Differential sensitivity of oleosins to proteolysis during oil body mobilization in sunflower seedlings. Plant Cell Physiol 43: 1117–1126

Sadegipour HR, Bhatla SC (2003) Light enhanced oil-body mobilization in sunflower seedlings accompanies faster protease action in oleosins. Plant Physiol Biochem 41: 259–264

Sakamoto A, Ueda M, Morikawa H (2002) Arabidopsis glutathione dependent formaldehyde dehydrogenase is an S-nitrosoglutathione reductase. FEBS Lett 515: 20–24

Salgado I, Martínez MC, Oliveira HC, Frungillo L (2013) Nitric oxide signaling and homeostasis in plants: a focus on nitrate reductase and S-nitrosoglutathione reductase in stress-related responses. Braz J Bot 32: 89–98

Sell S, Lindermayr C, Durner J (2008) Identification of S-nitrosylated proteins in plants. Methods Enzymol 440: 283–293

Singh N, Bhatla SC (2016) Nitric oxide and iron modulate heme oxygenase activity as a long distance signaling response to salt stress in sunflower seedling cotyledons. Nitric Oxide 53: 54–64

Singh RJ, Hogg N, Joseph J, Kalyanaraman B (1995) Photosensitized decomposition of S-nitrosothiols and 2-methyl-2-nitrosopropane. Possible use for site-directed nitric oxide production. FEBS Lett 360: 47–51

Sirover MA (1999) New insight into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1432: 159–184

Stöhr C, Stremlau S (2006) Formation and possible roles of nitric oxide in plant roots. J Exp Bot 57: 463–470

Stöhr C, Strube F, Marx G, Ullrich WR, Rockel P (2001) A plasma-membrane-bound enzyme of tobacco roots catalyzes the formation of nitric oxide from nitrite. Planta 212: 835–841

Tanou G, Job C, Rajjou L, Arc E, Belghazi M, Diamantidis G, Molassiotis A, Job D (2009) Proteomics reveals the overlapping roles of hydrogen peroxide and nitric oxide in the acclimation of citrus plants to salinity. Plant J 60: 795–804

Tanou G, Filippou P, Belghazi M, Job D, Diamantidis G, Fotopoulos V, Molassiotis A (2012) Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. Plant J 72: 585–599

Valderrama R, Corpas FJ, Carreras A, Fernandez-Ocana A, Chaki M, Luque F, Gomez-Rodriguez MV, Colmenero-Varea P, del Rio LA, Barroso JB (2007) Nitrosative stress in plants. FEBS Lett 581: 453–461

Vandana S, Bhatla SC (2006) Evidence for the probable oil body association of a thiol protease, leading to oleosin degradation in sunflower. Plant Physiol Biochem 44: 714–723

Vanzo E, Ghirardo A, Merl-Pham J, Lindermayr C, Heller W, Hauck SM, Durner J, Schnitzler JP (2014) S-nitroso-proteome in poplar leaves in response to acute ozone stress. PLoS One 9: e106886

von Toerne C, Kahle M, Schäfer A, Ispiryan R, Blindert M, Hrabe de Angelis M, Neshcen S, Ueffing M, Hauck SM (2013) Apoe, Mbl2, and Psp plasma protein levels correlate with diabetic phenotype in NZO Mice: an optimized rapid workflow for SRM-based quantification. J Proteome Res 12: 1331–1343

Wang Y, Yun BW, Kwon E, Hong JK, Yoon J, Loake GJ (2006) S-nitrosylation: an emerging redox-based post-translational modification in plants. J Exp Bot 57: 1777–1784

Wawer I, Bucholc M, Astier J, Anielska-Mazur A, Dahan J, Kulik A, Wyslouch-Cieszynska A, Zareba-Koziol M, Krzywinska E, Dadlez M, Dobrowolska G, Wendehenne D (2010) Regulation of *Nicotiana tabacum* osmotic stress-activated protein kinase and its cellular partner GAPDH by nitric oxide in response to salinity. Biochem J 429: 73–83

Xu S, Guerra D, Lee U, Vierling E (2013) S-nitrosoglutathione reductases are low-copy number, cysteine-rich proteins in plants that control multiple developmental and defense responses in Arabidopsis. Front Plant Sci 4: 430

Yadav MK, Bhatla SC (2011) Localization of lipoxygenase activity on oil bodies and in protoplasts using a novel fluorescence imaging method. Plant Physiol Biochem 49: 230–234

Yadav S, David A, Bhatla SC (2010) Nitric oxide modulates specific steps of auxin-induced adventitious rooting in sunflower. Plant Signal Behav 5: 1163–1166. https://doi.org/10.4161/psb.5.10.12159

Yamasaki H (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo. Philos Trans R Soc Lond B Biol Sci 355: 1477–1488

Yun BW, Feechan A, Yin M, Saidi NB, Le Bihan T, Yu M, Moore JW, Kang JG, Kwon E, Spoel SH, Pallas JA, Loake GJ (2011) S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature 478: 264–268

Zaffagnini M, Morisse S, Bedhomme M, Marchand CH, Festa M, Rouhier N, Lemaire SD, Trost P (2013) Mechanisms of nitrosylation and denitrosylation of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. J Biol Chem 288: 22777–22789

Zhou B, Guo Z, Xing J, Huang B (2005) Nitric oxide is involved in abscisic acid-induced antioxidant activities in *Stylosanthes guianensis*. J Exp Bot 56: 3223–3228

Ziogas V, Tanou G, Filippou P, Diamantidis G, Vasilakakis M, Fotopoulos V, Molassiotis A (2013) Nitrosative responses in citrus plants exposed to six abiotic stress conditions. Plant Physiol Biochem 68: 118–126

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