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 (RNS) produced in cells in normal growth conditions and their enhanced production under stress conditions, are responsible for a variety of biochemical aberrations. 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 (DTT) 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 monodehydro-ascorbate 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- RNS, Reactive nitrogen species; GSNOR, S-nitrosoglutathione reductase; SNO, S-nitrosothiol; GSNO, S-nitrosoglutathione; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MDHAR, Monodehydroascorbate reductase; DTT, Dithioerythritol; NO, Nitric oxide; BST: Biotin switch technique

Introduction

Nitric oxide is a bioactive, gaseous free radical which plays critical roles as a diffusible intracellular signaling molecule in plant and animal cells. ubiquitous signaling molecule involved in various biochemical and physiological processes in plants (Neill et al. 2003, Mur et al. 2013). Nitric oxide is endogenously produced through established biochemical routes namely, action of arginine- dependent enzymatic pathway, nitrite reduction by nitrate reductase or in the apoplast through non-enzymatic mechanisms (del Rio et al. 2004). Thus, nitrite content in plant tissues indirectly correlates with the level of nitric oxide. 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). Nitric oxide-induced signaling is mediated through generation of a variety of molecules collectively termed as reactive nitrogen species (RNS). These include peroxynitrite ($ONOO^-$), NO_2, GSNO and N_2O_3 (Corpas and Barroso 2013). All these 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 is responsible for a variety of biochemical aberrations most of which are caused by post-translational 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. S-nitrosothiols (SNOs) are highly unstable and can thus be quickly diminished by low-molecular weight thiols, like glutathione (GSH). They are capable of mediating S-nitrosylation of specific target proteins and are involved in storage and transport of nitric oxide (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. Nitrosoglutathione 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 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. 2011). 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), dessication (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. 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), glutathione 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 evidences available from our previous publications on NO crosstalk with the above stated ROS scavenging enzymes, our current objective is to understand further these molecular interactions at the proteome level. In view of the crucial role known to be played by nitrite accumulation in the tissue, its correlation was also analyzed in seedling roots and cotyledons in response to salt stress. NaCl (120 m*M*) stress-induced modulation of nitrosothiol content and S-nitrosoglutathione reductase (GSNOR) was compared in seedling roots and cotyledons. In order to understand the role of nitric oxide 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 hr. Seed sterilization was done using 0.005% mercuric chloride ($HgCl_2$) and sterilized seeds were then imbibed in distilled water for 2 h which were thereafter sown on pre-soaked germination paper in plastic trays. Seedlings were grown in dark at 25°C and were irrigated with half-strength Hoagland nutrient solution. A treatment of sunflower seedlings with 120 m*M* 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 m*M*). Based on these earlier findings, 120 m*M* has been adopted for all further experiments since at this concentration the seedling survives though 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 provided by supplementing Hoagland solution with 120 m*M* 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^{o} C until further use. Present work is focused on the analysis of roots and cotyledons of 2-day old seedling only so as to monitor

various biochemical changes expected as rapid signaling response (within 48 hrs 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' Nitric Oxide Analyzer (NOA 280i, Munich, Germany). 500 mg root and cotyledon tissues were powdered in liquid nitrogen and mixed with 1 ml extraction buffer (50 m*M* Tris-HCl pH 7.5, 10 m*M* NEM, 2.5 m*M* EDTA) and followed by centrifugation twice at 10,000 *g* for 20 min each at 4^0C. 100 µl and 200 µl of the homogenate for nitrite and nitrosothiol content measurements, respectively, were injected into the reaction vessel which was filled with Trijodide solution containing iodine, potassium iodide and acetic acid. The homogenate was pre-treated 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 Biotin Switch Technique (BST)

S-nitrosylated proteins were isolated and purified using Biotin Switch Technique (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 m*M* Tris-HCl, 1 m*M* EDTA, 0.1 m*M* 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 µ*M* GSNO for 30 min at RT in dark. Samples treated with H_2O served as negative controls. All protein samples were treated with 25 m*M* MMTS (methylmethanethiosulphonate), 2.5% SDS and incubated at 50ºC for 20 min with frequent vortexing. Acetone precipitation of proteins was undertaken to eliminate residual MMTS. 1 m*M* sodium ascorbate (reducing agent) and 1 m*M* biotin-HPDP (to biotinylate the proteins) were then added and samples were mixed thoroughly and incubated at 37ºC for 1 hr. Acetone precipitation of proteins was done again to remove excess biotin and ascorbate. The biotin-labelled S-nitrosylated proteins were then subjected to Western blot analysis or were purified by affinity chromatography.

Western blot analysis for detection of biotin-labelled S-nitrosylated proteins

The acetone precipitated biotin-labelled proteins were resuspended in HENS buffer (25 m*M* HEPES, 1 m*M* EDTA, 0.1 m*M* 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 75V for 0.5 h, on 100V for 0.5 h and 150V for 2 hrs. 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 NC 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% BSA, 0.2% Tween 20 in TBS] for 2 hrs 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 1:10000 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 BCIP/NBT (1 Sigma Fast tablet 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 m*M* HEPES pH 7.7, 0.5% Triton-X-100, 1 m*M* EDTA, 100 m*M* NaCl). 30 µl of equilibrated neutravidin agarose slurry/mg protein 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 minutes and supernatant was removed. The beads attached to biotinylated proteins were washed twice with 20 volumes of washing buffer (25 m*M* HEPES pH 7.7, 1 m*M* EDTA, 600 m*M* NaCl, 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 m*M* β-mercaptoethanol in neutralization buffer. The sample was centrifuged and the supernatant (containing S-nitrosylated proteins) was stored at -20ºC until further analysis.

Proteomic analysis of S-nitrosylated proteins

The preparation of samples and mass spectrometric analysis of S-nitrosylated proteins was done according to Merl et al. (2012) and von Toerne et al. (2013). The proteins were first purified using 1D Gel clean-up kit. Protein was quantified using BCA protein assay and 30 µg of each sample was loaded on 12% SDS-PAGE gel. 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% acetonitrile (ACN) for 10 min. 5 m*M* DTT was then added and gel pieces were incubated at 60ºC 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 IAA 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 37ºC for 15 min and 0.01 $\mu g.\mu l^{-1}$ trypsin solution (Sigma Aldrich, St. Louis, MO) 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 37ºC. The proteins were then eluted from the gel using 60% ACN/0.1% 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^{0}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 a 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-HPLC (Ultimate 3000, Dionex, Thermo Scientific, Waltham, MA) 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 nl 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 and 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.

S-nitrosoglutathione reductase (GSNOR) activity assay

GSNO reductase 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 total soluble protein (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. Reaction was started by adding 0.5 m*M* GSNO and absorbance was recorded for 5 minutes at 340 nm. Enzyme activity was calculated using molar extinction coefficient for NADH (6.22 mM^{-1} cm^{-1}).

Native PAGE for analysis of S-nitrosoglutathione reductase (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: 75V for 20 minutes, 150 V for 2 h) using Miniprotean Tetra Cell (Biorad, Hercules, CA). 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 analysed for the disappearance of the NADH fluorescence due to GSNOR activity (Chaki et al. 2011b).

Western blot analysis of S-nitrosoglutathione reductase (GSNOR)

Protein was extracted and quantified as described above was constituted in reducing Laemmli buffer and loaded on 12% precast gels (Biorad, Hercules, CA) (conditions: 75V for 20 minutes, 150 V for 2 h) using Miniprotean Tetra Cell (Biorad, Hercules, CA). 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 NC membrane. Subsequently the blot with transferred proteins was then incubated in blocking buffer (3% BSA, 0.2% Tween 20 in PBS, pH 7.4) for 2 hrs 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, for 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., St. Louis, MO) (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.

Glyceraldehyde-3- phosphate dehydrogenase (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 obtain total soluble proteins (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 was 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, 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 $mM^{-1}cm^{-1}$ 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.

Monodehydroascorbate reductase (MDHAR) activity assay

Spectrophotometric estimation of monodehydroascorbate reductase (MDHAR) activity was done 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 4ºC 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 was removed using PD-10 desalting columns. Protein content was estimated according to Bradford (1976). Total soluble proteins (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* NAPH 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 mM^{-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 (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). 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 3-fold 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 seedlings cotyledons and roots grown in the absence or presence of 120 m*M* NaCl. GSNOR catalyzes the reduction of GSNO (a reservoir of NO) to oxidized glutathione (GSSG) and ammonium (NH_4^+) using electrons from NADH. Spectrophotometric and zymographic (native-PAGE) analysis of GSNOR activity revealed that GSNOR activity is higher in cotyledons than in roots. A significant reduction (~50%) in enzyme activity was evident upon salt treatment both in sunflower seedling roots and cotyledons both from spectrophotometric and zymographic analysis (Fig. 2). However, treatment with 10

m*M* 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 m*M* NaCl and also subjecting the 10 000 *g* supernatant from the homogenates of seedling roots and cotyledons to 250 µ*M* 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 µ*M* of GSNO. In the case of seedling roots, the extent of S-nitrosylation of soluble proteins was less noteworthy than in cotyledons. Although enhanced S-nitrosylation was evident in most protein bands highlighted with arrow heads 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 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 (Table 1, 2). These proteins were grouped into 8 functional categories namely, those belonging to carbohydrate 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 activity in sunflower seedling roots and cotyledons

Spectrophotometric analysis of GAPDH and MDHAR activity in sunflower seedling cotyledons revealed that the activities of both the enzymes decrease in the presence of 120 m*M* NaCl. It was also observed that the inhibition of enzyme activity under salt stress was reversible and could be restored upon treatment with 10 m*M* DTT (reducing agent). Treatment of homogenates with 250 µ*M* 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 activity increased in response to 120 m*M* NaCl stress. Treatment with 10 m*M* 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 (Ni-NOR) activity in the root plasma membrane of *Nicotiana tabaccum* (Stöhr et al. 2001, Stöhr and Stremlau 2006). 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 nitric oxide either enzymatically by the action of nitrate reductase (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. 2016). S-nitrosothiols (RSNOs), particularly GSNO, act as major intracellular reservoirs and carriers of nitric oxide 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. 2012a). 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 2-fold 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). Present observations also indicate an increase in total RSNO content upon 120 m*M* 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. 2016). 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 (S-nitrosoglutathione) is formed as a result of reaction between glutathione (GSH) and NO. The level of GSNO in plant cells is regulated by the activity of GSNOR (S-nitrosoglutathione reductase; 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). 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 present observations, it has also been suggested earlier that a downregulation of GSNOR activity, leading to accumulation of RSNOs, may mediate nitrosative stress in plants (Airaki et al. 2011, Lee et al. 2008, Barroso et al. 2006). Signaling network controlled by salicylic acid has also been observed to be positively regulated by the modulation of cellular levels of S-nitrosothiols and GSNOR activity in *Arabidopsis thaliana* (Feechan et al. 2005). In this context transgenic plants of *Arabidopsis* 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 *Pisum sativum*. These investigations highlighted diverse changes in GSNOR activity as well as the parallel changes in GSNOR protein levels. In contrast, present observations highlight the impact of salt stress on modulation of GSNOR activity without any noteworthy changes in GSNOR protein levels.

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 atleast 9 proteins and denitrosylation of 8 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 treatment (Camejo et al. 2013). Similar observations were also recorded in citrus plants in response to varied abiotic stress conditions (Ziogas et al. 2013). 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 GSNO reductase and thioredoxin/thioredoxin reductase have recently been classified as denitrosylases and these enzymes might themselves 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. Since 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 pertains to *Arabidopsis*, 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 and Deswal 2009, Abat et al. 2008, 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 nitric oxide can modulate the concentration of various metabolites and energy balance in salt stressed seedlings. S-nitrosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase and phosphoglycerate kinase has earlier been reported in *Arabidopsis 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 (PRK), 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, 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 TCA 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 cotyledons and roots (present work). Both malate dehydrogenase and aconitase have been earlier shown to be inhibited by S-nitrosylation (Ortega-Galisteo et al. 2012). Present results along with supporting data from previous reports on S-nitrosylation of proteins in various plant proteins suggests 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**", 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 as 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, though in opposite manner. The common proteins getting modulated in this category in both the tissue systems include 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 monodehydroascorbate reductase 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) v/s 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 includes 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 post-transcriptional and post-translational regulation of Mn-SOD, AOX and Prx II. In view of present observations on the modulation of S-nitrosylation status of APX and MDAR in sunflower seedling cotyledons and roots, it may be possible that S-nitrosylation plays a critical role in the ascorbate-glutathione 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 suggests the possible role of nitric oxide in protein synthesis. Lindermayr et al. (2005) identified different elongation and initiation factors as targets for S-nitrosylation in *Arabidopsis* in line with 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). 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, chaperone protein ClpB1.

S-nitrosylation/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, Romero-Puertas et al. 2007, Belenghi et al. 2007, Palmieri et al. 2010). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and monodehydroascorbate reductase (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 activity 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, present observations have demonstrated that nitrite accumulation serves as a biomarker of salt and/or nitrosative stress. S-nitrosothiols (RSNOs), particularly GSNO, act as major intracellular reservoirs and carriers of nitric oxide in plants. 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 (Fig. 2,3). 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 GSNO reductase and thioredoxin/thioredoxin reductase have recently been classified as denitrosylases and these enzymes might themselves also be regulated by NO. Furthermore, non-enzymatic denitrosylation of proteins has also 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.

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Disclosures

The authors have no conflicts of interest to declare.

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Table I: Proteomic analysis (LC-MS/MS) of S-nitrosylated proteins from the cotyledons (10,000g supernatant) of sunflower seedlings (2-day old, dark grown) subjected to 120 mM NaCl stress. Fold change refers to protein abundance in salt-stressed cotyledons over control ones.

Protein	Accession no.	kDa	Peptide sequence (Ion score)	GO Biological process	Fold change	Log 2 fold change
Carbohydrate metabolism						
Aconitate hydratase	Q42560	98	VLLQDFTGVPAV VDLACMR (57.3) SENAVQANMELE FQR (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) GWDAQVLGEAP HK (43.8)	Glycolysis	**15,03**	**4**
Citrate synthase	Q9LXS6	57	SSICYIDGDEGILR (88.6) LYDPGYLNTAPV R (58.7)	Tricarboxylic acid cycle	**14,92**	**4**
Enolase	P42896	48	VNQIGSVTESIEA VR (96.3) SGETEDTFIADLS VGLATGQIK (85.5) AAVPSGASTGIYE ALELR (91.5)	Glycolysis	**7,62**	**3**
Glyceraldehyde-3-phosphate dehydrogenase	P25861	37	AGIALNDHFVK (69.9) VPTVDVSVVDLT VR (98)	Glycolysis	**10,11**	**3**

			IGINGFGR (58.6)			
Isocitrate dehydrogenase	P50217	47	EHYLNTEEFIDAV ADELK (79.5) LIDDMVAYALK (70.2)	Glyoxylate cycle	**13,82**	**4**
Isocitrate lyase	P49297	64	TVQGGITSTAAM GK (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	ATVLIETLPAVFQ MNEILYELR (100.1) YGVELDGDGLGV R (94.8) MIINALNSGAK (65.9)	Tricarboxylic acid cycle	**22,19**	**4**
Oxygen-evolving enhancer protein 1	P85194	28	LTYTLDEIEGPLE VSSDGTIK (90.2) GGSTGYDNAVAL PAGGR (60.8) DGIDYAAVTVQL PGGER (60.2)	Photosynthesis	**6,31**	**3**
Phosphoenolpyruvate carboxykinase	P42066	74	TTLSTDHNR (62.2) FGTVLENVVFDE HTR (81.6) EMVILGTQYAGE MK (109.9)	Gluconeogenesis	**18,44**	**4**
6-	Q9FFR3	53	NPNLASLVVDPD	Pentose	**7,77**	**3**

phosphogluconate dehydrogenase			FAK (63.9) LPANLVQAQR (79.5)	phosphate pathway		
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	P48493	21	ALLNETNEFVGD	Glycolysis	**1,82**	**1**

isomerase			K (83.9) VASPAQAQEVHA GLR (91.7)			
Other metabolic proteins						
Adenosylhomocysteinase	P32112	53	DSAAVFAWK (64.3) VAVVCGYGDVG K (62.7) ATDVMIAGK (71.8)	L-homocysteine biosynthesis	**13,49**	**4**
Adenylate kinase 4	Q08480	27	VLNFAIDDSILEE R (90.7) GFILDGFPR (47.8) GELVSDDLVVGII DEAMK (96.5)	Nucleotide metabolic process	**7,86**	**3**
Alanine aminotransferase 2	Q9LDV4	60	ALVVINPGNPTG QVLAEENQR (122.1) ATGAYSHSQGIK (65.2)	L-alanine catabolic process	**5,23**	**2**
Biotin carboxylase	O04983	58	HIEFQVLADK (41.8) NAGVPTVPGSDG LLQSTEEAVR (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-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	Q42662	85	FALESFWDGK (68.9) YLFAGVVDGR (57.1) GNASVPAMEMT K (50.2) VVKLQEELDIDV LVHGEPER (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**
Heat shock protein 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	Binding of protein	**21,39**	**4**

			(87.4) DAGVIAGLNVAR (89.1) VLVELAELQDR (68.8) EVGDGTTSVVIV AAELLK (80.3)	assembly		
T-complex protein 1subunit alpha	P28769	59		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	FDAEQAHGANSG IHIALR (49.7)	Abiotic and biotic stress response	**4,72**	**2**
Catalase	P45739	57	APGVQTPVIVR (49.6) EGNFDIVGNNFP VFFIR (104.1) DEEIDYFPSR (56.9)	Hydrogen peroxide catabolism	**8,18**	**3**
2-Cys peroxiredoxin	Q6ER94	28	EGVIQHSTINNLA IGR (77.1) SGGLGDLNYPLIS DVTK (54.9)	Hydrogen peroxide catabolism	**5,00**	**2**
Monodehydroas corbate reductase	Q42711	47	EIDDADQLVEAL K (74.1) EAVAPYERPALS K (48.5)	Oxidation-reduction process	**13,17**	**4**
Regulatory proteins						
40S ribosomal protein S3a	P49198	30	VFEVSLADLQND EDHSYR (62.2) LDRPAEEAAPEA TEVIGA (46.6)	Translation	**5,18**	**2**
14-3-3-like protein	O65352	29	VVAAADGGEELT IEER (120) QAFDEAIAELDTL	Signaling	**10,08**	**3**

			GEDSYK (101.3) DSTLIMQLLR (57.3)			
CBS domain-containing protein CBSX3	Q9LEV3	23	VGDIMTEENK (64.1)	Cell redox homeostasis	**10,94**	**3**
Elongation factor 2	Q9ASR1	94	STLTDSLVAAAGI IAQEVAGDVR (110.4) NMSVIAHVDHGK (65) EGALAEENMR (64.5) DSVVAGFQWAS K (68.4)	Translation	**4,59**	**2**
Eukaryotic initiation factor 4A-3	Q9CAI7	47	MFVLDEADEMLS R (95.4) GLDVIQQAQSGT GK (96.7)	Translation	**16,07**	**4**
Transport proteins						
ATP synthase subunit alpha	P18260	55	AAELTTLLESR (62.3) ISNFYTNFQVDEI GR (88) TAIAIDTILNQK (87)	ATP hydrolysis/ synthesis	**5,63**	**2**
ATP synthase subunit beta	Q1KXV2	59	DVNEQDVLLFVD NIFR (64.3) ELQDIIAILGLDEL SEEDR (80.5) GMDVIDTGAPLS VPVGGATLGR (67.8)	ATP synthesis, ion transport	**10,77**	**3**
Aquaporin PIP1-3	Q08733	31	QPIGTSAQTDK (44.8) SLGAAIIYNK	Water transport	**6,88**	**3**

			(61.8)			
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) EADVDGDGQINY EEFVK (86.5)	Calcium ion binding	**4,32**	**2**
Cell division cycle protein 48 homolog	P54774	90	NAPSIIFIDEIDSIA PK (78.2) ETVVEVPNVSWE DIGGLENVK (67)	Cell cycle	**4,93**	**2**
11S globulin seed storage protein G3	P19084	56	VQIVDNQGNSVF DNELR (115.9) TNDNAMIANLAG R (109) EGQVVVIPQNFA VIK (92.6)	Storage protein	**18,95**	**4**
Oleosin	P29529	19	GTLQDAGEYAGQ K (72.6) QTAGSVPESLDY VK (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)	Chlorophyll biosynthesis	**5,26**	**2**

Table II: Proteomic analysis (LC-MS/MS) of S-nitrosylated proteins from the roots (10,000g supernatant) of sunflower seedling (2-day old, dark-grown) subjected to 120 mM NaCl stress. Fold change refers to protein abundance in salt-stressed roots over control ones.

Protein	Accession no.	kDa	Peptide sequence (Ion score)	GO Biological process	Fold change	Log 2 fold change
Carbohydrate metabolism						
Aconitate hydratase	Q42560	98	VLLQDFTGVPAVV DLACMR (57.3) SENAVQANMELE FQR (84.6) RGNDEVMAR (56.3)	Tricarboxylic acid cycle	**0,34**	**-2**
Alcohol dehydrogenase 1	P14673	41	FGVTEFVNPK (77)	Oxidation-reduction process	**0,32**	**-2**
Glyceraldehyde-3-phosphate dehydrogenase	P25861	37	AGIALNDHFVK (69.9) VPTVDVSVVDLTV R (98) IGINGFGR (58.6)	Glycolysis	**0,09**	**-3**
Isocitrate dehydrogenase	P50217	47	EHYLNTEEFIDAV ADELK (79.5) LIDDMVAYALK (70.2)	Glyoxylate cycle	**0,15**	**-3**
Isocitrate lyase	P49297	64	TVQGGITSTAAMG K (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	ATVLIETLPAVFQ	Tricarboxylic	**0,14**	**-3**

			MNEILYELR (100.1) YGVELDGDGLGVR (94.8) MIINALNSGAK (65.9)	acid cycle		
Oxygen-evolving enhancer protein 1	P85194	28	LTYTLDEIEGPLEVSSDGTIK (90.2) GGSTGYDNAVLPAGGR (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) VASPAQAQEVHA GLR (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	ALVVINPGNPTGQ VLAEENQR (122.1) ATGAYSHSQGIK (65.2)	L-alanine catabolic process	**0,38**	**-1**
Biotin carboxylase	O04983	58	HIEFQVLADK (41.8) NAGVPTVPGSDGL LQSTEEAVR (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-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	Q42662	85	FALESFWDGK (68.9) YLFAGVVDGR (57.1) GNASVPAMEMTK (50.2) VVKLQEELDIDVL VHGEPER (57.6)	Amino acid biosynthesis	**0,26**	**-2**
Peptidyl prolyl cis-trans isomerase	P21569	18	VFFDMTVGGAPA GR (108.3)	Potein folding	**0,20**	**-2**
Phospholipase D alpha 1	Q43007	92	VLMLVWDDR (60.5) AYLPVQELLNGEE	Lipid catabolic process	**0,07**	**-4**

			IDR (93.1)			
S-adenosylmethionine synthase	P49613	41	TNMVMVFGEITTK (89.6) VHTVLISTQHDET VTNDKIAA (66.5)	S-adenosylmethionine biosynthetic process	**0,08**	**-4**
Chaperones						
Calreticulin	Q9ZPP1	48	GIQTSEDYR (52.5) LLSGDVDQK (55.6)	Protein folding	**0,07**	**-4**
Endoplasmin homolog	P35016	93	KPEGYDDIPK (47.7 ELISNASDALDK (81) LGIIEDAANR (70.2) SGTSAFVEK (65.1)	Protein folding	**0,07**	**-4**
Heat shock protein 90-1	P27323	81	ADLVNNLGTIAR (54.1) ELISNSSDALDK (62.2) KENEGEVEEVDEE K (72.9)	Protein folding	**0,11**	**-3**
Luminal binding protein 2	P24067	73	DILLLDVAPLTLGI ETVGGVMTK (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**
Monodehydroascorbate reductase	Q42711	47	EIDDADQLVEALK (74.1) EAVAPYERPALSK (48.5)	Oxidation-reduction process	**0,04**	**-5**
Phenylalanine ammonia-lyase	O04058	72	EINSVNDNPLINVS R (87.2)	Cinnamic acid biosynthetic	**0,10**	**-3**

			TAEAVDILK (38.6)	process		
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) EADVDGDGQINY EEFVK (86.5)	Calcium ion binding	**0,15**	**-3**
Cell division cycle protein 48 homolog	P54774	90	NAPSIIFIDEIDSIAP K (78.2) ETVVEVPNVSWE DIGGLENVK (67)	Cell cycle	**0,47**	**-1**
11S globulin seed storage protein G3	P19084	56	VQIVDNQGNSVFD NELR (115.9) TNDNAMIANLAG R (109) EGQVVVIPQNFAV IK (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**

Table III: Novel S-nitrosylated proteins in sunflower seedling cotyledons and roots (new reports) in response to salt stress

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

protein CBSX3			
Oleosin	Oil body membrane	-	-
Only roots			
Calreticulin	Endoplasmic reticulum (lumen)	-	-

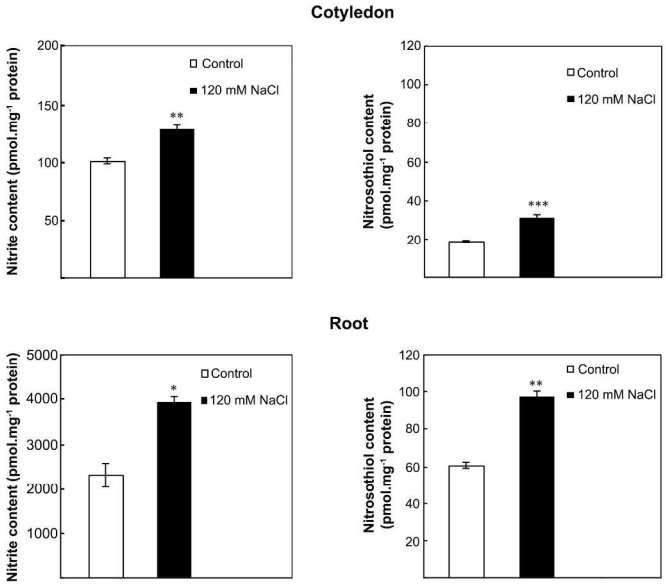

Fig. 1: Nitrite and nitrosothiol content in 10,000g supernatant obtained from 2d old dark-grown sunflower seedling cotyledons and roots in the absence or presence of 120 mM NaCl stress. Data represent mean ± SE from three replicates. NaCl-stress induced changes were analyzed by one-way ANOVA using SPSS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

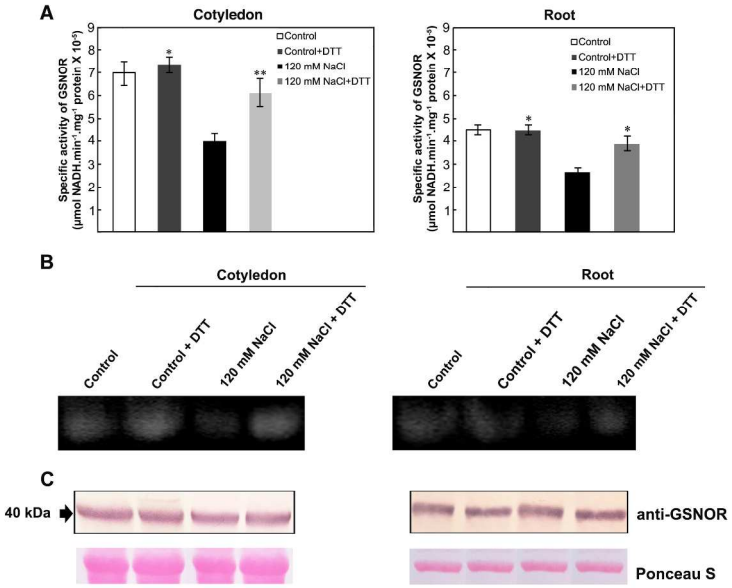

Fig. 2: Spectrophotometric (A), zymographic (B) and Western Blot (C) analyses of salt stress-induced changes in the activity of S-nitrosoglutathione reductase (GSNOR) in 10,000g supernatant obtained from cotyledons and roots of 2d old, dark-grown sunflower seedlings grown in the absence or presence of 120 mM NaCl stress. Treatment with 10 mM dithiothreitol (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 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) in comparison to control [(-)NaCl].

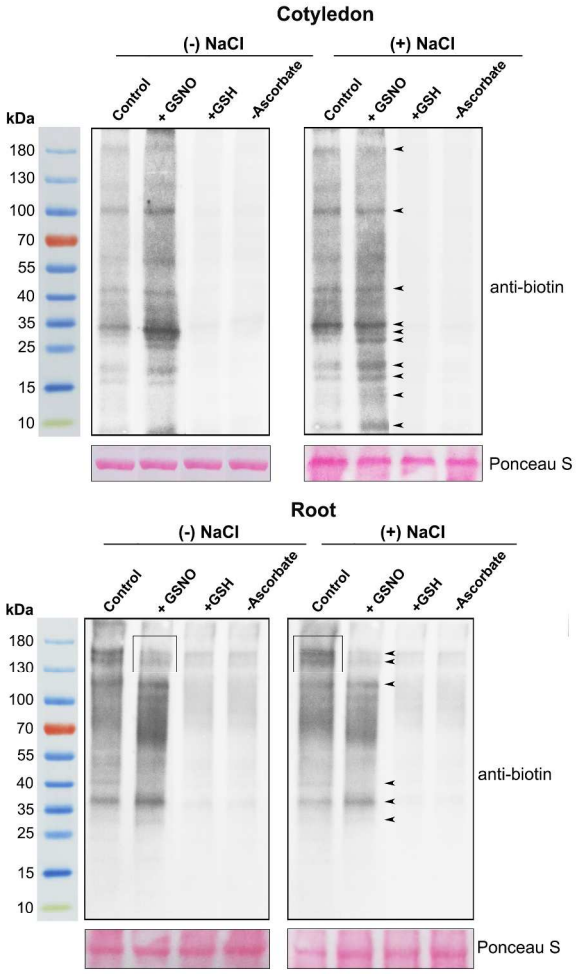

Fig. 3: Western blot analysis of S-nitrosylated proteins in cotyledons and roots of sunflower seedlings grown in the absence or presence of 120 mM NaCl. S-nitrosylted proteins were extracted by biotin-switch method and analyzed using anti-biotin antibody. Homogenate samples treated with 250 µM GSNO served as positive controls while those treated with 250 µM GSH and untreated with ascorbate (reducing agent) served as negative controls.

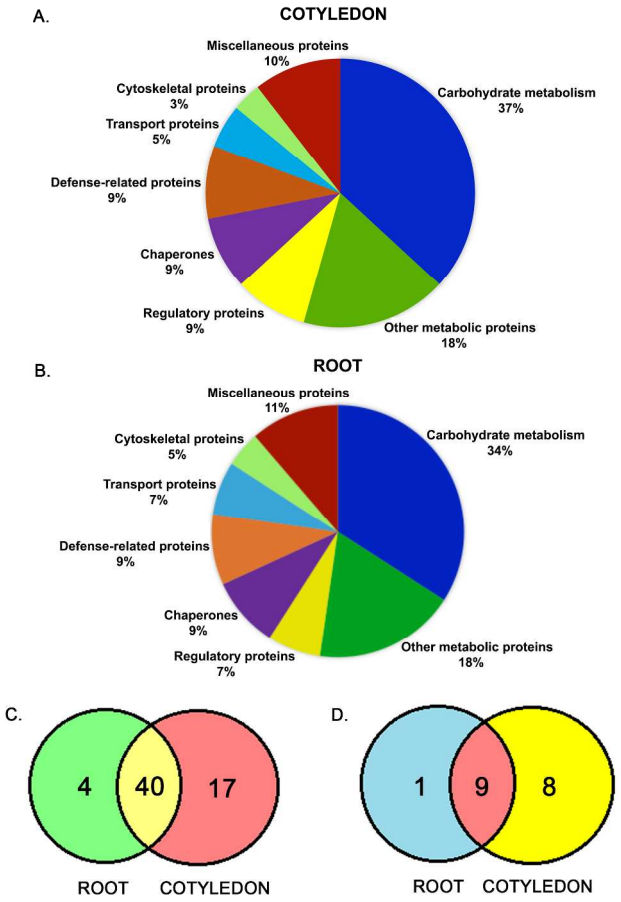

Fig. 4: Pie chart showing categories of proteins regulated by S-nitrosylation in the 10,000g supernatant from 2d old sunflower seedling cotyledons (A) and roots (B) in response to salt stress (120 mM NaCl). Venn diagram depicting the distribution of total (C) and novel (D) S-nitrosylated proteins in 2d 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.

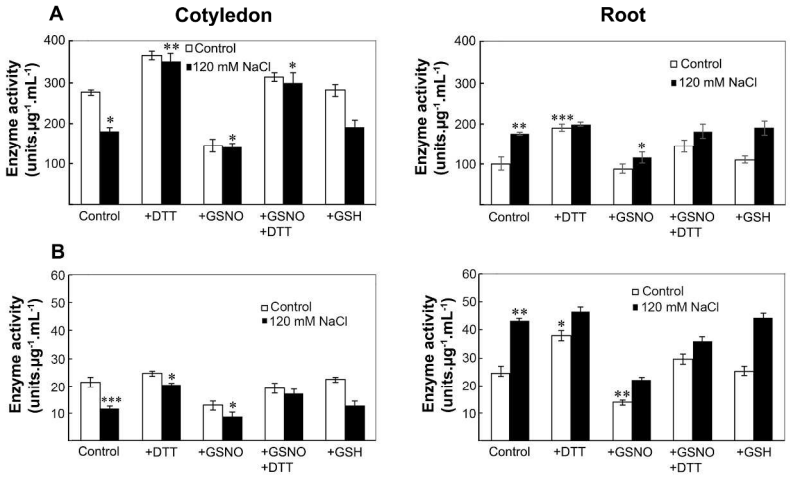

Fig. 5: Effect of GSNO and DTT (reducing agent) treatments on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) and monodehydroascorbate reductase (MDHAR) (B) activity in 10,000g supernatant obtained from cotyledons and roots of 2d old, dark-grown sunflower seedlings grown in the absence or presence of 120 mM 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 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

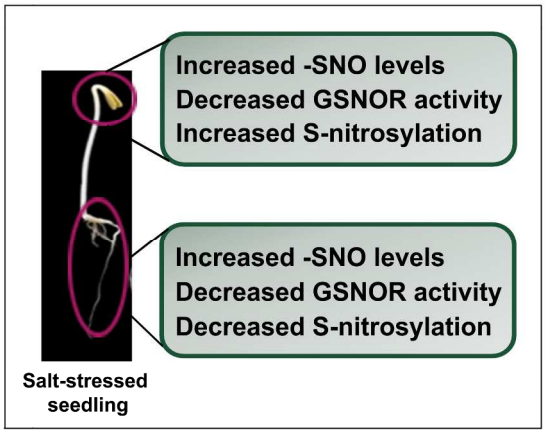

Fig. 6: S-nitrosylation status in sunflower seedling roots and coyledons