CSIRO PUBLISHING

Functional Plant Biology
http://dx.doi.org/10.1071/FP16324

Molecular insights into the functional role of nitric oxide (NO) as a signal for plant responses in chickpea

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Abstract. The molecular mechanisms and targets of nitric oxide (NO) are not fully known in plants. Our study reports the first large-scale quantitative proteomic analysis of NO donor responsive proteins in chickpea. Dose response studies carried out using NO donors, sodium nitroprusside (SNP), diethylamine NONOate (DETA) and S-nitrosoglutathione (GSNO) in chickpea genotype ICCV1882, revealed a dose dependent positive impact on seed germination and seedling growth. SNP at 0.1 mM concentration proved to be most appropriate following confirmation using four different chickpea genotypes. while SNP treatment enhanced the percentage of germination, chlorophyll and nitrogen contents in chickpea, addition of NO scavenger, cPTIO reverted its impact under abiotic stresses. Proteome profiling revealed 172 downregulated and 76 upregulated proteins, of which majority were involved in metabolic processes (118) by virtue of their catalytic (145) and binding (106) activity. A few crucial proteins such as S-adenosylmethionine synthase, dehydroascorbate reductase, pyruvate kinase fragment, 1-aminocyclopropane-1-carboxylic acid oxidase, 1-pyrroline-5-carboxylate synthetase were less abundant whereas Bowman-Birk type protease inhibitor, non-specific lipid transfer protein, chalcone synthase, ribulose-1-5-bisphosphate carboxylase oxygenase large subunit, PSII D2 protein were highly abundant in SNP treated samples. This study highlights the protein networks for a better understanding of possible NO induced regulatory mechanisms in plants.

Additional keywords: abiotic stress, gel-free proteomics, mass spectroscopy, sodium nitroprusside.

Received 20 September 2016, accepted 14 March 2017, published online 13 April 2017

Introduction

Nitric oxide (NO) has emerged as a central regulator of various growth and developmental events such as seed germination, root growth, floral transition, senescence and stress defences in plants (Beligni and Lamattina 2000; Beligni *et al*. 2002; He *et al*. 2004; Misra *et al*. 2011; Siddiqui *et al*. 2011). NO has been reported to exert a protective effect in response to drought (Garcia-Mata and Lamattina 2001; Santisree *et al*. 2015), salt stress (Bai *et al*. 2011), heat stress (Uchida *et al*. 2002; Bavita *et al.* 2012), heavy metal stress (Kopyra and Gwozdz 2003; Esim and Atici 2013; Yang *et al*. 2013; Kaur *et al.* 2015; Silveira *et al.* 2015), herbicides (Beligni and Lamattina 1999) and UV radiations (Tossi *et al*. 2011). Despite an increasing number of reports on the role of NO as an endogenous signalling molecule in plants, there is still a large knowledge gap about underlying molecular mechanisms of its action that can sense and transduce NO signals.

Unavailability of mutants for NO synthesis and signalling in many plant species and constrained knowledge on the molecular basis of the existing pleotropic mutations in model species makes further exploration at cellular level difficult. Similarly, transgenic efforts have been limited due to the dearth of molecular identities related NO synthesis and signalling in plants. So far most of the work focussed on the constitutive expression of rat and mammalian neural nitric oxide synthase (nNOS) genes in plants (Shi *et al*. 2014). Hence, a majority of the current research on NO in plants relies on exogenous application of NO-donors and inhibitors/scavengers. So far various NO donors have been deployed either to mimic an endogenous NO-related response or to substitute for an endogenous NO deficiency. Of these donors, SNP is the most widely used NO donor owing to its low cost, well documented application, continuous and long lasting NO release compared with others. Although numerous SNP-induced physiological and biochemical responses in plants have been observed (Santisree *et al*. 2015), information about actual molecular targets remained elusive till date.

In this context, the emerging genomics and transcriptomics studies utilising a range of platforms have been valuable in identifying various candidate genes associated with NO responses in plants (Besson-Bard *et al*. 2009; Yang *et al*. 2013; Begara-Morales *et al*. 2014) and NO-mediated post-transcriptional modifications (nitrosylation, nitration and carbonylation) influencing its actions under stress conditions (Astier and Lindermayr 2012). However, the identification and functional analysis of NO responsive proteins is still in its infancy in plants. Proteomic approaches have potential to provide valuable information that link transcriptomics and

metabolomics analyses, and thus can precisely explain the relevant phenotype. These are crucial to gain detailed insights into NO-mediated physiological changes at the cellular level, and for a deeper exploration for the mechanisms of plant processes to various environmental stimuli (Barkla *et al*. 2013). Reports on large-scale analysis of NO-mediated proteomics responses in plants have been very few (Meng *et al*. 2011; Fan *et al*. 2014). A pioneering study with cotton leaf profiles treated with SNP identified 166 differentially expressed proteins belonging to diverse pathways, and was followed by the identification of 167 phosphoproteins to be differentially phosphorylated in response to SNP (Meng *et al*. 2011); however, few other studies attempted to understand the proteomic basis of NO mediated stress tolerance (Bai *et al*. 2011; Sehrawat *et al*. 2013; Yang *et al*. 2013; Fan *et al*. 2014). Nevertheless, high-throughput proteomic signatures of NO-donors still need to be unfolded to further explore the complexity involved in their effects after exogenous applications.

In our study we investigated the molecular mechanisms of NO action in plants using chickpea as a crop model by a comparative gel-free proteome profiling. NO-induced changes in protein abundance were studied before and after 0.1 mM SNP treatment, which is the most commonly used dose in exogenous applications of various plants in various studies (see Table S1, available as Supplementary Material to this paper). Our results point at active metabolic adjustment and activation of several signal transduction pathways in response to nitric oxide in chickpea. To our knowledge, we present the first investigations on the effects of exogenous NO on a legume proteome.

Materials and methods

Plant material and growth conditions

Chickpea (*Cicer arietinum* L.) seeds of four different genotypes (ICCV1882 – drought sensitive, ICCV4958 – drought tolerant, ICC16374 – heat sensitive and JG-14 – heat tolerant) used in dose response studies were obtained from ICRISAT minicore collection. All subsequent experiments including proteome analysis were conducted with drought sensitive genotype ICCV1882. The seeds were sown in either ~23 cm pots containing 5.0 kg of black soil : sand : compost mixture in 3 : 2 : 1 ratio. The soil was prepared by sieving the vertisol through 1 cm wire mesh and mixing uniformly with diammonium phosphate (DAP) and muriate of potash at the rate of 0.3 g kg^{-1} and 0.2 g kg^{-1} soil, respectively, and pasteurised twice. Pots were initially saturated with 2 L of water. The seeds were treated with a fungicide (Thiram; Sudhama chemicals Pvt.Ltd) immediately before sowing. The plants were irrigated as required either with 500 mL of water or water containing 0.1 mM sodium nitroprusside (SNP, Sigma-Aldrich) every three days. All plants were grown in a glasshouse with 28/20°C day/night temperatures until the completion of experiment. The leaf tissue collected after anthesis (60 days after sowing) were flash frozen in liquid nitrogen and stored at −80°C until further use.

Physiological and biochemical estimations

Dose response assays

To examine the effect of NO donors on seed germination, the seeds were sown on agar (0.8%, w/v) in Petri plates (14 cm diameter) in the presence and absence of increasing concentrations of SNP, diethylamine NONOate sodium (DETA, Sigma-Aldrich) and S-nitrosoglutathione (GSNO, Sigma-Aldrich) ranging from 0.05 to 1 mM (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1 mM). The onset of seed germination was scored by visual observation of the emergence of radicle from the seed coat, 48 h after sowing. The seeds were also tested for germination under abiotic stress conditions (as described below) following treatment with either 0.1 mM SNP and 0.1 mM SNP + 1 mM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, Biomol Research Laboratory Inc.) for scoring germination under stress conditions. For NO dose response assay, surface-sterilised seeds of chickpea were germinated on two layers of germination papers soaked with distilled water in the dark for two days. After the emergence of the radicle, seeds were sown on the surface of moist soil (black soil and vermiculite in equal ratios), irrigated with a range of SNP, DETA and GSNO (0.1, 0.2, 0.3 and 0.5 mM; SRL Diagnostics) in growth chamber for 10 days. The root and shoot lengths were measured for the SNP-treated and untreated seedlings after 10 days of treatment. The water irrigated plants were used as control for comparisons. Germinated seeds were selected to avoid any discrepancies in length due to variation in germination period.

NO levels under abiotic stress

For measuring the endogenous NO levels in chickpea, ICCV1882 seedlings were grown in ~13 cm pots for 15 days. Subsequently at 15 days after sowing (DAS), the plants were subjected to different abiotic stress treatments (drought, salt, cold and heat). Drought stress was imposed by withholding water for 7 days followed by leaf sample collection, whereas the salinity stress was imposed by bringing the pots containing plants to field capacity with a 150 mM NaCl solution, followed by leaf sampling after 24 h of treatment. For cold and heat stress treatments, the seedlings were kept at 4 and 45°C, respectively, for 8 h before tissue sampling. The plants grown under normal conditions at 28 ± 1°C served as controls. Whole seedlings were used for NO assay using Griess reagent-based assay kit obtained from Alexis Biochemical as described by Negi *et al*. (2010).

Chlorophyll estimation

Freshly harvested leaf discs (1 g) from 2-month-old control and SNP-irrigated plants were washed, blotted dry and ground in 80% chilled acetone. The supernatant was taken and absorbance read at 663 nm, 645 nm and 480 nm for calculations according to Lichtenthaler (1987). For uniformity, leaf samples were collected from the same nodal position from five different untreated controls and 0.1 mM SNP treated plants.

Nitrogen content estimation

Total nitrogen content was estimated from leaves of five different chickpea plants after anthesis (two months old) using induced coupled plasma-optical emission spectrometry (ICP-OES) as described by Jukanti *et al*. (2012).

Gene expression analysis

The leaves were collected from two months old glasshouse-grown control and 0.1 mM SNP irrigated chickpea plants after

anthesis, and flash frozen in liquid nitrogen. The frozen leaf tissue was homogenised to fine powder using a mortar and pestle. About 100 mg of the homogenised powder was used for the extraction of total RNA with the RNeasy kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was determined by measuring absorbance at A260 and A280 using a Nanodrop spectrophotometer. The integrity of RNA was verified by agarose gel electrophoresis. Reverse transcription was performed with 2 µg of total RNA in a total volume of 20 µL using a cDNA synthesis kit (Invitrogen). Gene-specific primers for qPCR were designed using PRIMER3 software based on published sequences of chickpea from NCBI, meeting following criteria: melting temperature of 59−61°C, primer length of 20–24 bp, 100–150 bp product size, with GC content of 45–60% and analyses were performed as described previously (Santisree *et al*. 2011). The nucleotide sequences of primers used in this study were provided in Table S2. qPCR was performed using cDNAs corresponding to 5 ng of total RNA in 20 µL reaction volumes using the SYBR Green PCR Master Mix (Takara) on a Realplex Real Time PCR system (Eppendorf). To determine relative fold differences for each sample in each experiment, the cycle threshold (Ct) values were normalised to glyceraldehyde 3-phosphate dehydrogenase (*CaGAPDH*) and elongation factor 4 (*CaIF4*) genes. Relative fold expression was calculated by $2^{-\Delta\Delta Ct}$ method (Santisree *et al*. 2011; Anbazhagan *et al*. 2015).

Protein analysis

Protein extraction

Total protein was extracted from the leaf sample of 60-day-old control and 0.1 mM SNP-irrigated ICCV1882 genotype of chickpea. Plants of control and SNP were irrigated just before the leaf collection. A sample of ~1 g was weighed and homogenised in liquid nitrogen. The crushed sample was collected and suspended in 7 mL of extraction buffer containing 0.7 M sucrose, 0.1 M KCl, 100 mM Tris buffer at pH 7.2, 50 mM EDTA, 50 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 µL of protease inhibitor cocktail (Sigma-Aldrich). Tris-saturated phenol was added in equal amounts and mixed thoroughly by shaking at 4°C for 30 min. The mixture was centrifuged at 20 000*g* for 30 min at 4°C. The upper phenolic phase was collected and re-extracted twice as described above. The proteins were precipitated by 0.1 M ammonium acetate containing 50 mM DTT ammonium acetate salt following centrifugation at 26200 g for 30 min at 4°C. The protein pellet was washed twice with methanol containing 10 mM DTT and once with acetone containing 10 mM DTT, and finally dissolved in 50 mM ammonium bicarbonate. The protein was quantified and normalised for equal concentration by polyacrylamide gel electrophoresis followed by trypsin digestion and subjected to further analysis (Sehrawat *et al*. 2013; Gupta *et al*. 2015). To ensure reproducibility and accuracy of results, three biological replicates and three experimental replicates were used in this study.

Trypsin digestion

The protein samples were subjected to denaturation using rapigest SF (Waters) and then treated with 10 mM DTT at 56°C for 30 min. Subsequently, the samples were treated with 25 mM iodoacetamide at room temperature for 45 min, bought to room temperature and incubated overnight at 37°C with 2 µg of sequencing grade porcine trypsin (Promega).

Mass spectrometry

The peptide amounts were quantified using a nanoAcquity nanoflow ultra performance liquid chromatography on-line connected to a SYNAPT QTOF G2 mass spectrometer (Waters). After digestion, the resulting peptide fragments were concentrated in a speed vacuum concentrator (Thermo Scientific) and reconstituted in 50 µL of 0.1% formic acid. Approximately 600 ng of each of the protein digests were separated on the NanoAcquity BEH C18 HPLC column (75 µm ×150 cm × 1.7 µm;Waters) connected to NanoUPLC system for 150 min with 50% gradient of water in 0.1% formic acid (v/v; mobile phase A) and acetonitrile in 0.1% formic acid (mobile phase B). The initial flow rate was 5 µL·min^{-1} of 97% mobile phase A for 3 min. Peptides were separated using a 1.7 µm BEH C18 75 µm × 100 mm column with a 60 min gradient. Mobile phase B was linearly ramped from 9 to 85% B over 90 min. The flow rate was 300 nL·min^{-1} and the column temperature was kept at 35°C. The NanoLC separated peptides were analysed for MS and MSMS fragmentation on SYNAPT G2 (Waters) nLC coupled QTOF mass spectrometer with ESI source on a positive mode. Data were acquired in resolution mode using the following settings: Da Range: 50–1990 Da; Scan Time: 0.6 Sec; Collision Energy: 1. Low Energy: Trap: 4V, Transfer:4V; 2. High Energy: Trap Collision Energy Ramp: 14–40 V; Transfer Collision Energy Ramp: OFF; Cone Voltage: 30V (Benjamini and Hochberg 1995; Weinhold *et al*. 2015).

Data analysis

Following data acquisition, the raw files were analysed for protein identification and expression using the Waters Protein Lynx Global Server (PLGS) ver. 4.1 software against chickpea sequences downloaded from UniProt database (http://www.uniprot.org, accessed 10 October 2015; The UniProt Consortium 2015). The following parameters were set for searches against target database: tryptic cleavage with a maximum of one missed cleavages; mass tolerance of 30 to 100 ppm; peptide mass tolerance of 0.1 to 1.0 D; a minimum of two peptides matching the protein; fixed and variable modifications like carbamidomethylation of Cys and Met oxidation respectively. The minimum number of fragment matches for proteins was set to five, with minimum number of fragment matches for peptides as two and minimum number of peptide matches for protein to two. Ion scores of greater than 44 were considered significant ($P < 0.05$). The false discovery rate for the statistically significant proteins was found to be 5% using the Benjamini and Hochberg (1995) method.

The quantitative analysis of protein abundance was carried out using PLGS as described by Shen *et al*. (2009) where the data was normalised based on inbuilt statistical analysis taking into account of the intensity of the many consistent qualitatively matched proteins (or peptides). All the processed protein hits were thus identified with a confidence of 95%. The peptides which are identical in three experimental

replicates per biological sample were clustered on the basis of mass precision of 5 mg kg^{-1} and a retention time tolerance of 0.25 min using PLGS 2.3. The significance of regulation level was specified as 30%. Proteins meeting the criteria of minimum two peptides expressing in at least two out of three biological replicates, 1.3-fold up- or downregulation and a *P*-value ≤ 0.05 were considered to be significant. The proteins showing a 2-fold change in expression were considered most significant. Keeping in view the possible post- translational modifications by SNP, the proteins with lesser expression ratio/fold-change were also captured.

Functional annotation

Functional annotations of the identified proteins were made using UniProt database. The identified proteins were assigned to Gene Ontology (GO) categories from the UniProt database. The proteins were mapped the identified to the reference canonical pathways of chickpea in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/tools, accessed 15 April 2016).

Statistical analysis

The data were analysed using Sigmaplot (ver. 11) based on the mean and s.e. values in all assays including dose response and biochemical studies. Statistically significant differences between treatments were determined by one-way ANOVA using the Student–Newman–Keuls method. The statistical analysis was conducted with at least three biological replicates.

Results

Effect of NO on plant growth

Nitric oxide can be both cytotoxic and protective to plant cells depending on its concentration and localisation in the plant cells (Santisree *et al*. 2015). To ascertain the effective and beneficial concentration of NO for exogenous application in chickpea, a dose response study was initially carried out using chickpea genotype, ICCV1882 using increasing concentrations (0.05–1 mM) of three NO donors including SNP, DETA and GSNO. Although a 2–5% increase in seed germination was observed after 48 h at 0.05 mM, a higher germination (10–20%) was recorded at 0.1 mM concentration. A reduction in the seed germination was observed with increasing concentrations to an extent of 80–90% inhibition in germination at 1.0 mM concentration (see Fig. S1*a*, available as Supplementary Material to this paper).

Similar to the seed germination response, NO donors caused a progressive dose-dependent reduction of hypocotyl and root lengths in chickpea seedlings. At higher concentrations i.e. >0.2 mM all NO donors significantly inhibited root and hypocotyl lengths in chickpea, whereas treatment at lower concentrations (up to 0.2 mM) enhanced the seedling growth. Seedlings were longer than the untreated controls at 0.1 mM concentration for all the three NO donors tested, whereas only a slight increase in the seedling growth was achieved at 0.2 mM concentration (Fig. S1*b*). Clearly, a similar overall trend of dose response results on both seed germination and seedling growth was achieved following DETA, GSNO and SNP treatments (Fig. S1*a*, *b*).

Since SNP has been widely used as exogenous donor of NO, the results of dose response assays were re-confirmed on four different chickpea genotypes (ICCV1882, JG-14, ICCV4958, ICC16374) using SNP at varying concentrations. SNP at 0.1 mM acted as growth stimulant in all the tested genotypes (Fig. S1*c*) and hence was chosen for subsequent experiments. Sixty-day-old ICCV1882 plants upon treatment with 0.1 mM SNP showed an enhanced total chlorophyll (chl) and nitrogen content in leaves when compared with the untreated controls (Fig. 1*a*, *b*). Although there was not much change in the chl *a* content of the treated leaf samples, chl *b* was enhanced by 20% resulting in an overall increment. The nitrogen content was enhanced 1.5-fold upon SNP treatment, which might have led to increased protein content in the treated chickpea plants.

Exogenous NO enhances stress tolerance in chickpea

To investigate the role of NO in regulation of various abiotic stress responses, ICCV1882 seeds germinated on 0.8% agar

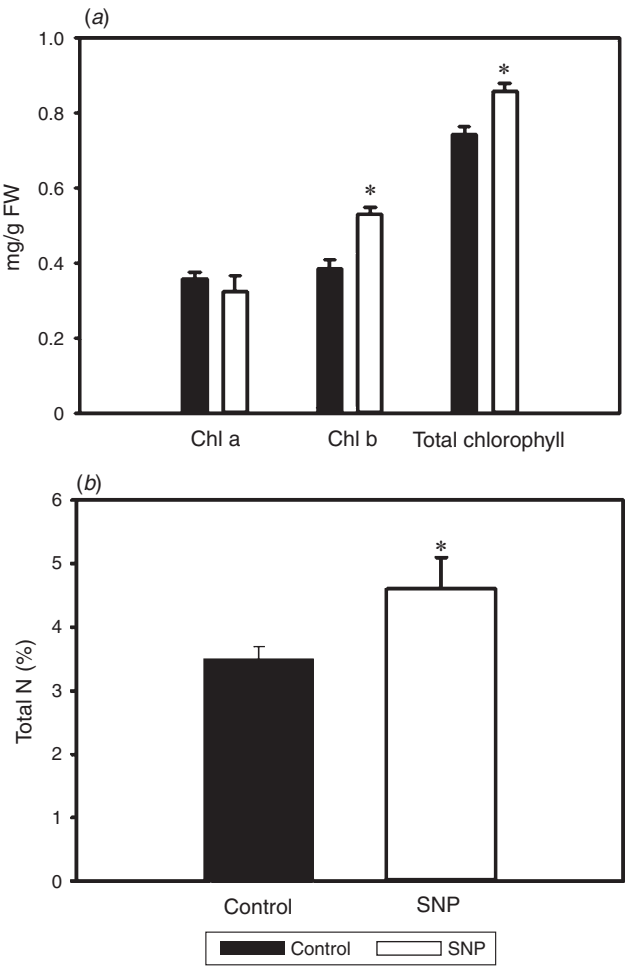

Fig. 1. Effect of 0.1 mM SNP treatment on chickpea leaf chlorophyll content (*a*), and leaf nitrogen content (*b*). Leaves were collected from two months old control and 0.1 mM SNP treated plants as described in methods. Results (±s.d.) are the means of three independent experiments. Statistically significant differences in response to SNP treatment are indicated: *, $P \leq 0.05$ with $n = 15$–20 per group.

plates were subjected to different stress treatments such as heat (40°C), cold (4°C) and salt (150 mM) as described in 'Materials and methods'. Abiotic stresses notably reduced seed germination compared with the unstressed controls, whereas addition of 0.1 mM SNP to the agar medium promoted the germination percentage in both stressed and unstressed condition. (Fig. S2*a*). Heat stress was most deleterious to seed germination followed by cold, salt and drought. Nevertheless SNP enhanced the seed germination to a greater extent (20–25%) under drought as compared with the increment of 5–15% under other stress conditions. The addition of NO scavenger, cPTIO (1 mM) along with SNP brought down the promoting effect of SNP on seed germination under both stress and non-stress conditions (Fig. S2*a*).

The endogenous NO levels in the seedlings grown under stress conditions with or without SNP treatment were measured, to attribute the observed stress amelioration by SNP is related to NO. As expected, 0.1 mM SNP enhanced NO levels in chickpea seedlings under both stress and non-stress conditions. The endogenous NO levels increased in response to the given abiotic stress conditions further supporting its role in stress signalling. Although SNP treatment elevated NO levels significantly under water deficit, salt and heat stress conditions; 1 mM cPTIO reverted this effect confirming the role of NO in abiotic stress amelioration in chickpea (Fig. S2*b*).

Effect of SNP on chickpea leaf proteome

To identify the molecular basis of the given SNP treatment, the protein profiles in leaves of control and SNP treated 60-day-old ICCV1882 line were compared using label-free quantitative proteomics based on nano LC-MS analysis (Fercha *et al*. 2013). A total of 3364 peptides from control and 2310 peptides from SNP-treated plants were used to identify protein matches and quantification from chickpea at UniProt database using Protein Lynx Global Server from Waters. After removing the redundant and invalid identifiers from individual samples, over 788 proteins in control and 765 proteins in SNP-treated sample were identified substantially across the UniProt database. Nevertheless, few proteins could not be identified by the UniProt database.

Identification of differentially expressed proteins (DEPs) by SNP treatment

Although, the comparative expression analysis indicated a set of 630 proteins common to both SNP- treated and untreated samples, only 248 proteins showed significant ($P \leq 0.05$) differential regulation by SNP. Of these 248 differentially expressed proteins, 76 proteins were increased in abundance whereas 172 proteins were reduced in abundance in response to SNP (Fig. 2*a*; Data sheet S1, available as Supplementary Material to this paper). Additionally, 19 proteins were specifically expressed only in SNP-treated samples and 64 proteins present in control samples were absent from SNP treated samples (Fig. 2*a*).

Protein classification and functional annotation

To obtain a general overview of 248 differentially expressed proteins by SNP (Data sheet S1), these were functionally classified into three principal ontologies based on molecular function, biological process and cellular component according to the UniProt gene ontology (Fig. 2*b*). However, an increase in the number of such proteins was observed due to the functional mapping of few proteins to more than one category. Ontology analysis grouped 209 proteins into nine categories based on molecular function, 74 proteins into 8 categories based on cellular component they belonged to, and 147 proteins into seven categories based on their involvement in the biological processes (Fig. 2*b*).

The proteins were classified into 10 different categories based on their predicted molecular function such as proteins having catalytic activity (145), binding (106), antioxidant (12), structural constituent of ribosome (eight), transporter activity (five), electron carriers (five), enzyme regulators (three), sequence specific DNA binding activity (two), and signalling receptor activity (one). Biological process analysis grouped them into seven categories covering a wide range of pathways in the following order; involved in metabolic process (118), cellular process (66), single-organism process (30), response to stimulus (26), biological regulation (12), localisation (10), and biogenesis (five) The detailed categorisation of the up- and downregulated proteins by SNP based on biological process was given in Fig. S3. Proteins that belonged to metabolic (34 and 83) and cellular processes (17 and 50) had most representation in both up- and downregulated proteins respectively.

Functional role of the most significant SNP responsive proteins in chickpea

For an ease of understanding, considering at least two biological replicates and a minimum of 2-fold change in the identified proteins, these numbers were further narrowed down to 67 most significant ($P \leq 0.05$) DEPs of which 45 proteins decreased and 22 proteins increased in abundance upon SNP treatment (Tables 1, 2). According to the ontological classification, the most represented proteins were related to metabolic processes with predicted catalytic activity (Fig. S4). The comparison of up- and downregulated proteins involved in biological processes are shown in Fig. 2*c*.

Proteins unique to, or absent from, SNP-treated samples

The SNP-treatment induced expression of 19 unique proteins – those were not observed in the controls– and silenced the expression of 64 proteins observed in the control leaf samples. A detailed list of these proteins is given in Table 3. With the purpose of investigating the significance of these proteins, the proteins unique to SNP-treatment were grouped into five classes based on biological process viz. metabolic process (eight), cellular process (seven), defence response (two), transporter activity (one), and single-organism process (one). We note that four unique proteins were localised in chloroplast suggesting their role in regulating photosynthesis-related activity. The most represented category based on molecular function, were proteins belonging to binding category (eight) that involve selective, non-covalent interaction of a molecule with one or more specific sites on another molecule, followed by catalytic activity category (five) (Fig. 3*a*). Likewise, out of 64 proteins absent from SNP-treated sample (Table 3), 18 proteins belongs to metabolic process and 23 proteins belongs to cellular

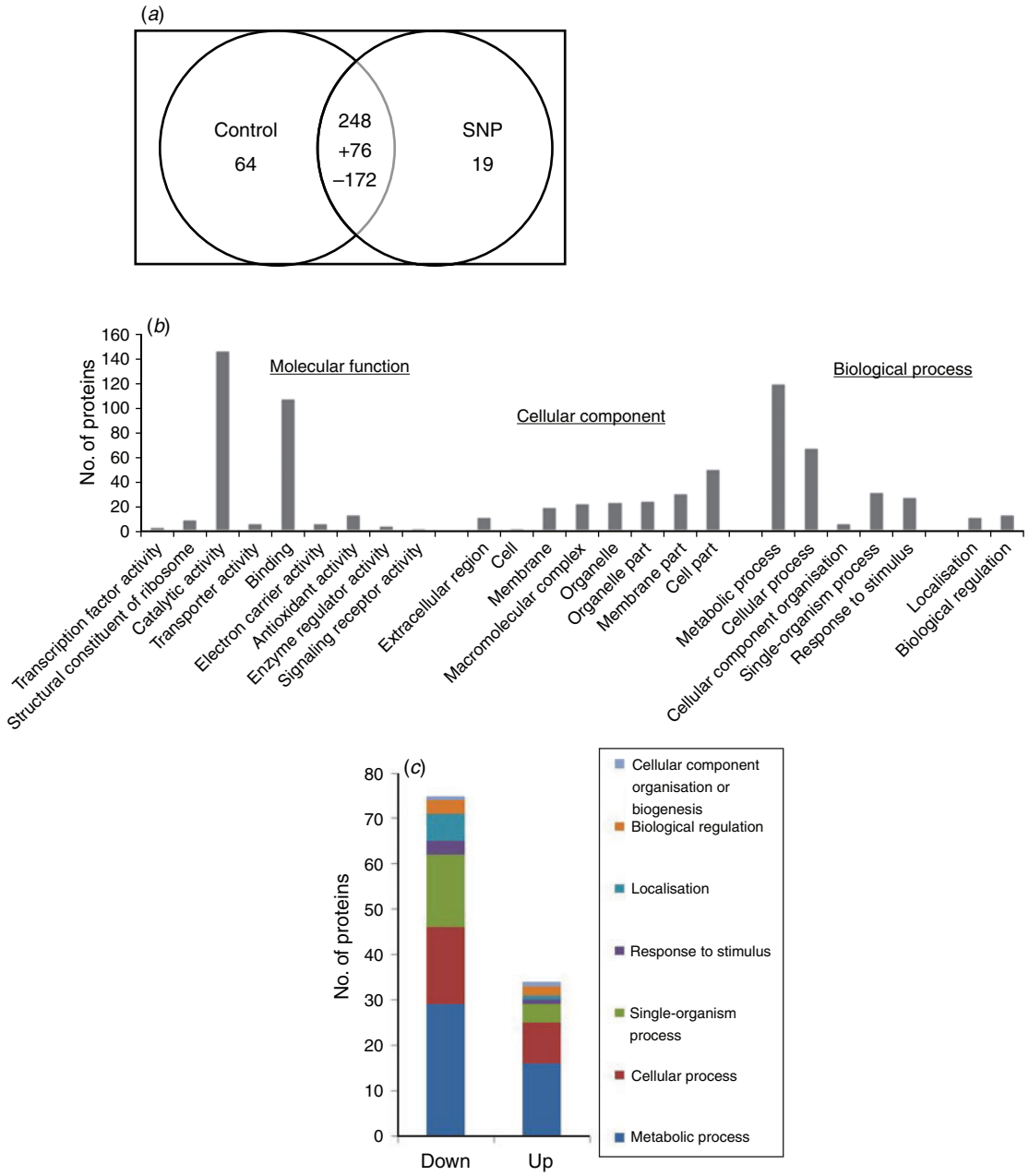

Fig. 2. Summary of the SNP responsive proteins identified in 60 days old chickpea leaves. (*a*) Venn diagram showing the number of proteins detected in 0.1 mM SNP treated and control samples. Numbers shared by two circles represent proteins shared by the two categories and up/ downregulation were denoted as +/−. (*b*) Histogram presentation of Gene Ontology classification of the differentially regulated proteins by SNP. The identified proteins are grouped into three main categories: biological process, cellular component, and molecular function. The *y*-axis indicates the number of proteins in a specific category. (*c*) Comparative analysis of most significantly (>2-fold; Tables 1, 2) altered proteins in response to SNP treatment based on involvement in various biosynthetic processes ($P < 0.05$). All the proteins were identified by gel-free quantitative proteome analysis as described in methods.

process. The detailed categorisation of these proteins based on molecular function was given as Fig. 3*b*.

Pathway classification of proteins

In an effort to map the total SNP responsive proteins to various known pathways in chickpea, over 144 proteins out of 331 (19 + 64 + 248) identifiers from UniProt were successfully mapped to 147 KEGG IDs that were assigned to various pathways. These proteins were well represented by metabolic pathways (36 proteins), ribosome metabolism (16), photosynthesis (14), biosynthesis of secondary metabolites (14), oxidative phosphorylation (10), phenylpropaniod biosynthesis (five), starch and sucrose metabolism (three),

Table 1. List of proteins significantly ($P < 0.05$) reduced in abundance at least by 2-fold in 0.1 mM sodium nitroprusside treated chickpea leaves
Fold change was calculated as the expression ratio of SNP/control identified by quantitative proteomics ($n = 3$)

Accession	Description	PLGS Score	SNP/Control
C3TS15	S adenosylmethionine synthase OS *Cicer arietinum* PE 2 SV 1	302.66	0.130
Q9SB99	Elongation factor 1 α Fragment OS *Cicer arietinum* GN ef1 a2 PE 2 SV 1	50.11	0.159
A0A067XU08	UDP glycosyltransferase OS *Cicer arietinum* GN UGT88E6 PE 3 SV 1	31.74	0.164
B5 LMR9	30S Ribosomal protein S15 chloroplastic OS *Cicer arietinum* GN rps15 PE 3 SV 1	77.76	0.336
A0A067XU03	UDP glycosyltransferase OS *Cicer arietinum* GN UGT79B23 PE 4 SV 1	139.44	0.346
B6SBM2	L myo inositol 1 phosphate synthase 2 OS *Cicer arietinum* PE 2 SV 1	41.19	0.416
Q9M3H6	Histone H2B OS *Cicer arietinum* PE 2 SV 1	140.82	0.419
Q9SXU6	Pyruvate kinase Fragment OS *Cicer arietinum* PE 2 SV 1	58.86	0.427
A0A067XTV7	UDP glycosyltransferase OS *Cicer arietinum* GN UGT88E10 PE 3 SV 1	56.47	0.432
Q8GTE2	Ribosomal protein RL5 OS *Cicer arietinum* GN rl5 PE 2 SV 1	91.19	0.432
Q00016	Isoflavone reductase OS *Cicer arietinum* GN IFR PE 1 SV 1	101.14	0.436
Q8VXR9	RGA D protein Fragment OS *Cicer arietinum* GN rga D PE 4 SV 1	154.53	0.436
A0A076 L212	Non-specific lipid transfer protein OS *Cicer arietinum* PE 3 SV 1	91.17	0.447
B5 LMM8	Adenosine triphosphate synthase subunit a chloroplastic OS *Cicer arietinum* GN atpI PE 3 SV 1	30.82	0.449
U5NFI7	Dehydroascorbate reductase OS *Cicer arietinum* PE 2 SV 1	63.37	0.452
B5 LMS7	Cytochrome c biogenesis protein CcsA OS *Cicer arietinum* GN ccsA PE 3 SV 1	34.43	0.455
A0A067XUD9	CC NBS LRR disease resistance protein OS *Cicer arietinum* PE 4 SV 1	110.72	0.460
A0A067XTY1	UDP glycosyltransferase OS *Cicer arietinum* GN UGT73K2 PE 3 SV 1	53.01	0.461
A0A076KWI1	Non-specific lipid transfer protein OS Cicer arietinum PE 3 SV 1	78.81	0.462
A0A076 L4T4	Peroxidase OS Cicer arietinum PE 3 SV 1	122.14	0.470
D0PWB2	NAC family transcription factor 3 OS *Cicer arietinum* GN NAC3 PE 4 SV 1	124.99	0.470
I7E3G7	DNA directed RNA polymerase Fragment OS *Cicer arietinum* GN rpoC1 PE 3 SV 1	227.07	0.472
A0A067XU50	UDP glycosyltransferase OS *Cicer arietinum* GN UGT79B24 PE 4 SV 1	135.71	0.477
V9PJ01	Uncharacterised protein OS *Cicer arietinum* PE 2 SV 1	148.69	0.477
Q84 L58	1 Aminocyclopropane 1 carboxylic acid oxidase OS *Cicer arietinum* GN aco PE 2 SV 1	82.8	0.479
Q9M445	Putative uncharacterised protein Fragment OS *Cicer arietinum* PE 2 SV 1	139.67	0.482
A0A067XNJ6	BZip OS *Cicer arietinum* GN bZIP PE 2 SV 1	143.28	0.487
Q9M6M9	Putative uncharacterised protein Fragment OS *Cicer arietinum* PE 4 SV 1	126.47	0.493
A0A067XUD0	CC NBS LRR disease resistance protein OS *Cicer arietinum* PE 4 SV 1	159.41	0.493
Q39449	Specific tissue protein 1 OS *Cicer arietinum* PE 2 SV 1	61.47	0.498
A0A076KXC8	Peroxidase OS *Cicer arietinum* PE 3 SV 1	111.66	0.499
Q9SMK8	Putative ABA responsive protein OS *Cicer arietinum* PE 2 SV 1	107.63	0.503
A0A067XU22	UDP glycosyltransferase OS *Cicer arietinum* GN UGT80B4 PE 4 SV 1	104.97	0.503
Q6IT03	Kunitz proteinase inhibitor 1 Fragment OS *Cicer arietinum* GN kpi1 PE 4 SV 1	78.53	0.503
Q9M451	Calmodulin binding protein Fragment OS *Cicer arietinum* PE 2 SV 1	30.19	0.503
Q8 L5Q4	Putative adenosine kinase Fragment OS *Cicer arietinum* GN adk PE 2 SV 1	107.4	0.504
B5 LMK9	Adenosine triphosphate synthase subunit β chloroplastic OS *Cicer arietinum* GN atpB PE 3 SV 1	192.05	0.504
A0A067XU01	UDP glycosyltransferase OS *Cicer arietinum* GN UGT92G3 PE 3 SV 1	55.36	0.504
A0A060A4A8	Aspartic proteinase OS *Cicer arietinum* GN AP27 PE 3 SV 1	147.98	0.505
A0A067XNC6	1 Pyrroline 5 carboxylate synthetase OS *Cicer arietinum* GN P5CS PE 2 SV 1	92.38	0.506
B5 LMS0	NAD P H quinone oxidoreductase subunit H chloroplastic OS *Cicer arietinum* GN ndhH PE 3 SV 1	118.81	0.506
Q1A7E2	NBS LRR type disease resistance protein Fragment OS *Cicer arietinum* PE 4 SV 1	73.6	0.508
A0A059XKX8	Serine protease OS *Cicer arietinum* PE 4 SV 1	70.15	0.509
O23759	Small ubiquitin related modifier OS *Cicer arietinum* PE 2 SV 1	50.01	0.510
M4I6P1	Protein L isoaspartate O methyltransferase OS *Cicer arietinum* GN PIMT2 PE 2 SV 1	67.47	0.510

amino sugar and nucleotide sugar metabolism (three) and various other pathways. Two representative KEGG maps of these SNP-responsive pathways are given in Figs S5 and S6.

Functional validation of differentially regulated proteins for transcript abundance

To ascertain whether the changes in protein abundance agreed with the changes at the transcriptome, gene expression of some of the candidate proteins that are significantly more or less in abundance by SNP treatment was analysed using real time-PCR (Fig. 4). Results indicated that mRNA expression correlated with the protein abundance for most of the candidate proteins tested. *Bowman Birk type protease inhibitor* (*BBI*), *peroxidase*, *ribulose-1–5-bisphosphate carboxylase oxygenase* (*RuBisCO*) *large subunit*, *chalcone synthase* (*CHS*) increased in abundance under SNP treatment both at transcript and protein level whereas *S-adenosylmethionine synthase* (*SAMS*) and *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO1*) were downregulated at both transcript and protein level (Fig. 4). Apart from similar trend, the transcript and protein abundance correlated well for *RuBisCO large subunit* and *CHS*. The genes encoding *1-pyrroline 5 carboxylate synthetase* (*P5CS*) expressed in opposite manner to protein levels in response to SNP. This low

Table 2. List of proteins significantly ($n = 3$; $P < 0.05$) increased in abundance at least by 2-fold in 0.1 mM sodium nitroprusside treated chickpea leaves
Fold change was calculated as the expression ratio of SNP/control identified by quantitative proteomics

Accession	Description	PLGS Score	SNP/Control
A0A067XTH3	UDP glycosyltransferase OS *Cicer arietinum* GN UGT71S2 PE 3 SV 1	42.22	2.014
A0A089X1Y0	Glucanase OS *Cicer arietinum* PE 4 SV 1	140.25	2.014
Q9ZP08	Chlorophyll *a*/*b* binding protein OS *Cicer arietinum* PE 2 SV 1	76.95	2.034
A0A076 L4T8	Peroxidase OS *Cicer arietinum* PE 3 SV 1	66.37	2.054
B5 LMN9	PSII reaction centre protein L OS *Cicer arietinum* GN psbL PE 3 SV 1	232.62	2.075
G1K3R9	Lectin OS *Cicer arietinum* PE 1 SV 1	160.96	2.160
O81929	Glycine rich protein 1 Fragment OS *Cicer arietinum* GN grp1 PE 2 SV 2	540.05	2.160
Q708X1	RNA and export binding protein Fragment OS *Cicer arietinum* GN rrm PE 2 SV 1	49.6	2.248
Q9FSZ8	Putative uncharacterised protein Fragment OS *Cicer arietinum* PE 2 SV 1	44.27	2.270
Q9M6M8	Putative uncharacterised protein Fragment OS *Cicer arietinum* PE 4 SV 1	56.24	2.293
A0A067XT88	UDP glycosyltransferase OS *Cicer arietinum* GN UGT91M1 PE 4 SV 1	34.55	2.316
O81925	40S Ribosomal protein S6 Fragment OS *Cicer arietinum* PE 2 SV 1	48.15	2.316
Q9SML4	Chalcone synthase 1 OS *Cicer arietinum* GN CHS1 PE 2 SV 1	55.16	2.460
V5TH04	NBS LRR protein OS *Cicer arietinum* PE 4 SV 1	119.89	2.535
B5 LMM1	PSII D2 protein OS *Cicer arietinum* GN psbD PE 3 SV 1	71.6	2.560
B5 LMP7	50S Ribosomal protein L20 chloroplastic OS *Cicer arietinum* GN rpl20 PE 3 SV 1	163.49	2.560
Q8GUF3	Putative reverse transcriptase Fragment OS *Cicer arietinum* GN pol PE 4 SV 1	46.25	2.773
Q8WJD8	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit Fragment OS *Cicer arietinum* GN rbcL	133.69	2.858
Q0H2C5	Dehydration responsive element binding protein OS *Cicer arietinum* GN CAP2 PE 2 SV 1	18.61	2.915
A0A076KXC0	Non-specific lipid transfer protein OS *Cicer arietinum* PE 3 SV 1	59.28	3.743
B5 LMR7	30S Ribosomal protein S7 chloroplastic OS *Cicer arietinum* GN rps7 PE 3 SV 1	55.71	4.904
G9I2Q3	Bowman Birk type protease inhibitor OS *Cicer arietinum* PE 2 SV 1	99.53	35.874

correlation might be due to the difference in the half-life of transcripts and protein, post-translational modifications and technical difference in quantitative measurement of mRNA and protein.

Discussion

Nitric oxide is a key player in several biological cellular processes, acting either as a signalling or as a toxic molecule in plants (Mur *et al*. 2013; Yu *et al*. 2014). Exploring the importance of NO as a regulator of plant growth and stress defence has significantly increased in the past few years despite limited information on its mode of action and signalling (Siddiqui *et al*. 2011; Santisree *et al*. 2015). Therefore, the identification of NO target molecules in this study was considered essential for deeper insights into its functional role in plants.

In the present study SNP was chosen due to its high efficiency, slow and continuous NO production. Despite of the fact that SNP release cyanide and iron in addition to NO, it is the most widely used donor in plant NO studies so far (Table S1; Planchet and Kaiser. 2006; Schröder. 2006). The possibility of observed effects of SNP being mere artefacts or an effect of various reaction products of SNP was ruled out based on, data obtained in the confirmatory experiments using other NO donors, DETA and GSNO (Fig. S1). The effect of SNP has been reported in regulation of various plant processes in a wide range of plants (Farooq *et al*. 2009; Fan *et al*. 2012; Santisree *et al*. 2015). Nevertheless, there have been fewer efforts on exploring the molecular mechanism of SNP action in plants (Meng *et al*. 2011). Although there are reports on studies in chickpea using exogenous SNP applications (Sheokand *et al*. 2008; Chohan *et al*. 2012), most of these were confined to a particular biological response or a developmental event either at morphological or physiological level. Several reports have indicated H_2O_2 accumulation, accompanied by increased cellular damage levels due to nitrosative stress at higher NO concentrations (Groß *et al*. 2013), suggesting its cytotoxic role. On the contrary, plants treated with the lower NO concentrations accumulated lower H_2O_2 levels, consequently inhibiting the detrimental effects of membrane lipid peroxidation suggesting the protective role of NO (Hayat *et al*. 2011; Groß *et al*. 2013). Since it is fundamental to establish SNP dose to maximise the impact before elucidating its cellular targets, our study systematically dealt with the identification of right dose for exogenous application, followed by the efficacy of this particular concentration in modulating the physiological, defence and molecular responses in chickpea.

Four different genotypes of chickpea with varying stress tolerance for SNP dose response study were used to ensure the optimum concentration for achieving a positive impact. NO diminishes primary root growth and promotes lateral root development in many plants such as tomato and *Arabidopsis* (Negi *et al*. 2010; Liao *et al*. 2012). In our study, a dose dependent reduction in root and hypocotyl growth in chickpea was observed. The 0.1 mM concentration that emerged out of our experiments has also been used in many other plants for exogenous applications including chickpea (Table S1; Farooq *et al*. 2009; Hayat *et al*. 2011; Chohan *et al*. 2012; Santisree *et al*. 2015). Our study indicates a positive effect of SNP at 0.1 mM on seedling growth, chloroplast content, total nitrogen content and antioxidant levels (Figs 1, S1). These results also point chloroplast as one of the major sub-cellular targets of NO in chickpea, in line with previous reports on significantly enhanced chl content by NO (Jasid *et al*. 2006; Kumar *et al*. 2010; Procházková *et al*. 2013). Since chloroplast is the site of photosynthesis as well as reactive oxygen species production,

Table 3. List of proteins either present or absent specifically in 0.1 mM SNP treated leaf samples of chickpea

Entry	Protein name	Mass (Dalton)	PLGS Score	Gene ontology[A]
Present				
B5 LMN8	PSII reaction centre protein J	4089	105.08	Photosynthesis
B5 LMP5	50S Ribosomal protein L33, chloroplastic	7691	187.95	Translation
B5 LMR2	30S Ribosomal protein S19, chloroplastic	10 538	286.37	Translation
B5TJY7	Myb2	5765	252.85	DNA binding
B5TJY9	Myb4	8593	284.32	DNA binding
O65741	Transmembrane channel protein	12 302	318.69	Transporter activity
O65747	Cysteine synthase, O-acetyl-L-serine (Thiol)-lyase	27 911	212.48	Cysteine biosynthesis
O65756	Vegetative lectin	28 190	309.54	Carbohydrate binding
Q1A7E1	NBS-LRR type disease resistance protein	19 562	225.48	ADP binding
Q1A7E6	NBS-LRR type disease resistance protein	19 534	246.86	ADP binding
Q1A7F8	NBS-LRR type disease resistance protein	19 350	133.85	ADP binding
Q2I2W0	Defensin	8343	351.74	Defence response
Q5DU94	Reverse transcriptase	9070	202.36	RNA-directed DNA polymerase activity
Q6XW14	Defensin	8172	407.01	Defence response
Q84VU6	Reverse transcriptase	8228	286.34	RNA-directed DNA polymerase activity
Q8GUF1	Reverse transcriptase	3953	288.91	RNA-directed DNA polymerase activity
Q9M447	Putative uncharacterised protein	12 127	126.52	
Q9M6N0	Putative uncharacterised protein	19 293	115.71	ADP binding
Q9S8T2	Class III acidic chitinase	3261	326.1	Carbohydrate metabolism
Absent				
A0A059XKX3	Serine protease	27 832	261.45	serine-type endopeptidase activity
A0A067XTV5	Glycosyltransferase	50 030	308.88	Metabolic process
A0A068B7E6	Signal peptidase S24	14 881	344.79	Serine-type peptidase activity
A0A076KWH9	Protease inhibitor/seed storage	12 036	188.01	Peptidase activity
A0A076KXB3	Late embryogenesis abundant protein	20 116	375.93	–
A0A076 L209	Late embryogenesis abundant protein	20 193	271.95	Response to desiccation
A0A076 L2H9	Protease inhibitor	11 227	153.76	Peptidase activity
A0A076 L4T1	Protease inhibitor	19 409	285.87	Peptidase activity
B0 L629	Putative uncharacterised protein	12 141	173.11	ER to Golgi vesicle-mediated transport
B0 L630	3-Hydroxyisobutyryl-coenzyme A hydrolase	13 526	246.75	3-Hydroxyisobutyryl-CoA hydrolase activity
B0 L631	PR-4a protein	9133	185.98	Defence response
B0 L637	Putative uncharacterised protein	10 685	319.98	
B5 LMK5	30S Ribosomal protein S12, chloroplastic	13 706	609.49	Translation
B5 LML1	NAD(P)H-quinone oxidoreductase subunit 3	13 894	296.43	Transport
B5 LML7	PSI P700 chlorophyll a apoprotein	82 481	405.48	Photosynthesis
B5 LML8	30S Ribosomal protein S14, chloroplastic	11 690	401.67	Translation
B5 LMM2	PSII reaction centre protein M	3756	188.03	Photosynthesis
B5 LMN3	PSII reaction centre protein K	6865	283.04	Photosynthesis
B5 LMP6	30S Ribosomal protein S18, chloroplastic	12 728	334.72	Translation
B5 LMQ2	PSII reaction centre protein H	7772	249.88	Photosynthesis
B5 LMQ4	Cytochrome b6-f complex subunit 4	17 473	217.51	Photosynthesis
B5 LMQ8	30S Ribosomal protein S8, chloroplastic	15 304	166.61	Translation
B5 LMR0	50S Ribosomal protein L16, chloroplastic	15 753	193.96	Translation
B5 LMR4	50S Ribosomal protein L23, chloroplastic	10 677	199.61	Translation
B5 LMR6	NAD(P)H-quinone oxidoreductase subunit 2	56 657	374.77	Electron transport
B5 LMS4	NAD(P)H-quinone oxidoreductase subunit 4 L	11 267	385.97	Electron transport
B5TJZ0	Myb5	7892	418.26	DNA binding
G4XWZ0	Glycine decarboxylase complex subunit L	11 604	102.11	Cell redox homeostasis
G4XWZ4	Glycine decarboxylase complex subunit L	11 578	102.11	Cell redox homeostasis
M4I787	Protein-L-isoaspartate O-methyltransferase	31 173	607.47	Protein-L-isoaspartate (D-aspartate) O-methyltransferase activity
O65746	Putative uncharacterised protein	12 141	337.33	
O81926	Thaumatin-like protein PR-5b	25 948	283.74	
P83987	Cicerin	2257	150.87	Defence response
Q1A7D9	NBS-LRR type disease resistance protein	19 588	274.5	ADP binding

(*continued next page*)

Table 3. (*continued*)

Entry	Protein name	Mass (Dalton)	PLGS Score	Gene ontology[A]
Q1A7E5	NBS-LRR type disease resistance protein	19 574	259.93	ADP binding
Q1A7F9	NBS-LRR type disease resistance protein	19 504	234.69	ADP binding
Q2 LGI7	Adenosine triphosphate synthase gamma subunit	11 395	407.16	Adenosine triphosphate Synthesis
Q39458	Metallothionein-like protein 1 (MT-1)	7611	219.06	Metal ion binding
Q5DUG8	Germin-like protein	19 538	220.19	Metal ion binding
Q5WM50	Putative uncharacterised protein	3572	303.59	–
Q8GTD8	Putative uncharacterised protein 275	9532	209.38	–
Q8 L5G4	Alpha-expansin 4	26 629	211.34	Cell wall organisation
Q8 L5G5	Alpha-expansin 3	26 602	231.54	Cell wall organisation
Q8 L5Q0	Putative invertase inhibitor	8202	422.02	Enzyme inhibitor activity
Q8 L5Q1	Putative epsilon subunit of mitochondrial F1-ATPase	7693	388.9	Adenosine triphosphate synthesis
Q8 L5Q3	Putative glycine-rich protein	10 840	207.56	–
Q8 LPE4	Putative nucleoid DNA-binding protein	15 350	198.05	Endopeptidase activity
Q949K8	Gag polyprotein	31 880	231.49	–
Q949 L2	Putative polyprotein	16 060	420.38	–
Q9FNT1	Alpha-expansin	26 460	153.23	Plant-type cell wall organisation
Q9FSY9	Putative extensin	4042	223.45	–
Q9M3Z0	60S Ribosomal protein L6	25 982	404.32	Translation
Q9M449	Putative uncharacterised protein	5627	183.43	–
Q9SMJ8	Putative water channel protein	25 078	808.66	Transporter activity
Q9SMJ9	Putative glycine-rich protein	12 779	292.46	–
Q9SMK0	Peroxidase	5179	182.62	Response to oxidative stress
Q9SXT1	Isoflavone reductase	10 149	334.45	–
Q9SXT4	DnaK-type molecular chaperone	11 753	203.56	Adenosine triphosphate binding
Q9SXT7	Rac-type small GTP-binding protein	21 699	330.94	GTPase mediated signal transduction
Q9SXU3	Fructose-bisphosphate aldolase	15 870	184.03	Glycolytic process
Q9ZRV2	Glycine-rich protein 2	19 489	260.39	–
Q9ZRV5	Basic blue copper protein	12 928	377.05	Electron carrier activity
Q9ZRW4	Putative uncharacterised protein	15 764	386.76	–
X5GE29	CenH3 (Centromeric histone 3)	14 276	267.68	DNA binding

[A]Ontology analysis was performed using the online www.uniprot.org link (accessed 20 April 2016).

NO can potentially affect and regulate a wide range of downstream signals through its effects on chloroplast (Lum *et al*. 2005). The observed increase in Chl content might be attributed to the activation of chl biosynthesis or/and its slow degradation or involvement of NO in the iron metabolism of plants. Indeed chl content and the chl *a*/*b* ratio are fundamental parameters for determination of photosynthetic activity and these parameters are often used as indicators of stress in plants (Kumar *et al*. 2010; Liao *et al*. 2012). The drought-induced reduction of *psbA* transcripts in NO donor treated wheat plants have reportedly led to enhanced activity of PSII and electron transfer rates during grain filling (Wang *et al*. 2011; Procházková *et al*. 2013). In the present study, several proteins involved in photosynthesis including PS-II D2 protein and PSII reaction centre protein L, needed for assembly and dimerisation of a stable PSII complex were also found to be NO responsive (Table 2). It is assumed that binding sites for NO existing within PSII between the primary and secondary quinone acceptors (Procházková *et al*. 2013). Other important proteins such as chl *a*/*b* binding protein, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, (PSII-L), one of the components of the core complex of PSII, NADPH-Quinone oxido reductase were also identified to be regulated by NO in chickpea (Table 2; Data sheet S1). Stimulation of antioxidant gene transcription in SNP-treated samples was also reported supporting its role in oxidative stress tolerance (Tossi *et al*. 2011; Wang *et al*. 2011; Yang *et al*. 2013; Santisree *et al*. 2015). A significant upregulation of chalcone synthase following SNP treatment was observed (Table 2), which could be primarily helpful in quenching singlet oxygen by isomerising into naringenin under various stress conditions. The increased abundance of this protein was also confirmed by the increased transcript accumulation (Fig. 4).

In this study, exogenous application of 0.1 mM SNP decreased the abiotic stress induced inhibition of seed germination. In addition to this, the applied SNP further enhanced the stress induced NO release in chickpea seedlings which got reversed by using NO scavenger, cPTIO, which is one of the most reliable NO scavenger for plants although at times have been reported to exhibit dual action as NO scavenger and N_2O_3 producer (Planchet and Kaiser. 2006). Nevertheless, in order to ensure quality data, and observe the scavenging effect of cPTIO, in our study we chose to use cPTIO at a higher concentration for a longer duration, since low concentration has been known to compromise its scavenging efficacy due to competitive reactions (D'Alessandro *et al*. 2013; Kováčik *et al*. 2014).

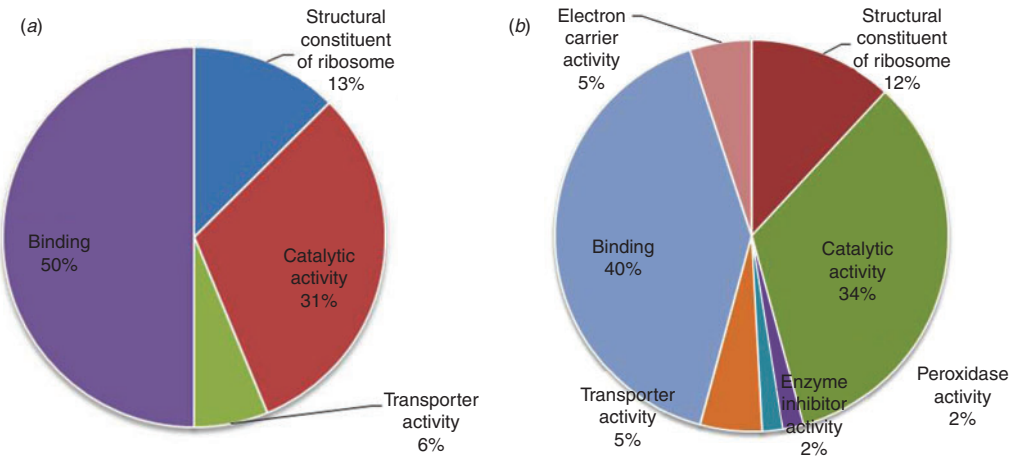

Fig. 3. Functional characterisation of chickpea proteins present (*a*) or absent (*b*) specifically in 0.1 mM SNP treated leaf samples based on molecular function. A list of proteins is given in Table 3.

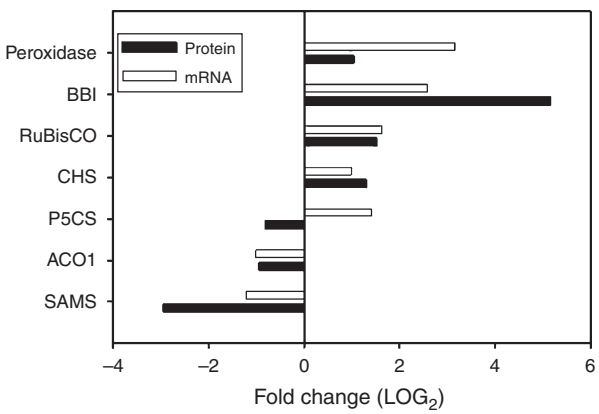

Fig. 4. The correlation of mRNA and protein expression levels of selected SNP-responsive proteins by quantitative real time-PCR. The logarithmic base-2 transformed fold change values of SNP treated vs control at protein and transcript level were plotted. Abbreviations: SMAS, S-adenosyl methionine synthase; CHS, chalcone synthase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase1; P5CS, 1 pyrroline-5-carboxylate synthetase; BBI, Bowman Birk type protease inhibitor.

Metabolic protein changes in response to SNP treatment

Although, the effects of exogenous application of SNP on various physiological and developmental processes are extensively studied, very few reports have dealt with the molecular aspects. In our study, a wide panel of 630 proteins proved to be differentially regulated, of which 45 were significantly reduced and 22 were significantly increased by 2-fold following SNP treatment in chickpea (Fig. 2). These chickpea proteins were distributed in a range of cellular compartments regulating wide range of biological processes. Previously, proteome profiling in cotton leaf identified 166 differentially regulated proteins by SNP treatment, of which 82 were downregulated and 47 were upregulated (Meng *et al*. 2011). In citrus the role of H_2O_2 and SNP was suggested in preventing the accumulation of a large number of NaCl-responsive proteins via specific proteome reprogramming (Tanou *et al*. 2009). Furthermore, Bai *et al*. (2011), reported the accumulation of G-protein-associated proteins and the induction of antioxidant enzymes as the master mechanisms through which *S*-nitroso-*N*-acetylpenicillamine (SNAP) treated maize seedlings alleviate salt stress, besides activating the defence proteins, energy metabolism, and cell structure/division remodelling. Our study is one of the first large-scale proteomic analyses of the NO response in chickpea, representing comprehensive and comparative analysis of proteins regulated by NO.

Nucleotide and lipid metabolism proteins

Several proteins involved in nucleotide metabolism such as 30S ribosomal protein S15, 40S ribosomal protein SA, required for the assembly and/or stability of the ribosomal subunits, elongation factor 1-α, were downregulated whereas replication factor C/activator 1 subunit, 50S ribosomal protein L20, putative reverse transcriptase, DNA-directed RNA polymerase subunit β were upregulated in chickpea by SNP treatment (Tables 1, 2). Besides regulating proteins involved in nucleotide metabolism, NO also seems to be involved in epigenetic regulation of plant chromatin by modifying key transcription factors such as NAC family transcription factors. NAC transcription factor proteins NAC 5 and 6 were reduced in abundance whereas NAC transcription factor protein 1 and 4 have been reported to be upregulated in response to SNP (Data sheet S1; Olsen *et al*. 2005). We also observed the downregulation of adenosine kinase, which is involved in phosphorylation of adenosine for its salvage and synthesis of various nucleic acids and nucleoside co-factors. This indicated the reduction in S-adenosyl methionine (SAM) and SAM-dependent methylation since adenosine kinase is also involved in maintaining methyl cycle that generates S-adenosyl methionine (Table 1; Mohannath *et al*. 2014). This evidently shows the potential of NO being involved in new synthesis and degradation at the molecular level. However, the absence of certain proteins such as PSII reaction centre components,

defence proteins such as protease inhibitor, PR proteins, CenH3 protein suggests the in-depth exploration of given concentration of SNP impacts in chickpea (Table 3).

Plant lipoxygenase are involved in several diverse aspects of plant physiology including growth and development, pest resistance, and senescence or responses to wounding. This study revealed that proteins involved in lipid metabolism L-myo inositol-1 phosphate synthase, acetyl-coenzyme A carboxylase and acyl carrier protein, involved in phospsholipid and oxylipin biosynthesis were responsive to SNP treatment in chickpea suggesting a possible role of NO in regulating membrane lipid quality and composition (Table 1; Data sheet S1).

Amino acid metabolism proteins

Proteins involved in amino acid metabolism formed the major class of metabolic process regulated by NO in our study with chickpea. Several proteins such as threonine dehydratase, S-adenosylmethionine decarboxylase proenzyme, SAM synthatase, serine acetyltransferase, showed reduction in our study. Similarly, SAM synthase (synthesises S-adenosyl-L-methionine from L-methionine), which is a precursor for many metabolites including glycine betaine, ethylene and polyamines, which is known to accumulate in response to a wide range of stresses got downregulated by SNP treatment in chickpea (Table 1). Our data suggested that NO can regulate ethylene biosynthesis as well as signalling pathway, in general agreement with the previous report in cotton (Meng *et al*. 2011; Manjunatha *et al*. 2012). Apart from SAM synthase, SNP treatment downregulated ACC oxidase protein abundance whereas increasing the abundance of one ethylene receptor like proteins (Table 1). A recent study also suggested the role of NO in phosphorylation of ETR1 and CTR1 while regulating ethylene response (Fan *et al*. 2014). Since SAM is also the methyl group donor in many important transfer reactions including DNA methylation, it is expected for NO to have a role in DNA methylation during the regulation of gene expression. Moreover, L-isoaspartate O-methyltransferase known to be part of methylation was also upregulated following SNP treatment (Data sheet S1). These findings are in line with the previous reports in *Dendrobium* (Fan *et al*. 2012).

Although the interaction between proline and NO has been a subject of major research interest (Wang *et al*. 2013), the synergistic or antagonistic relation between these two molecules is not yet clear. In our study we found lesser abundance of 1-pyrroline-5-carboxylate synthetase protein in SNP treated chickpea leaves suggesting a negative impact of SNP on proline biosynthesis (Table 1). Besides effect on proline biosynthesis, our data also suggested SNP induced differential accumulation of other amino acids possibly resulting from regulating their synthesis as well as catabolism. For example, enhanced abundance of glycine decarboxylase complex subunits and phenylalanine ammonia-lyase 2 suggested faster catabolism of glycine and L-phenylalanine respectively (Data sheet S1; Tossi *et al*. 2011). Similarly, reduced abundance of serine acetyltransferase, threonine dehydratase and methionine synthase suggests the reduction in isoleucine, cysteine and methionine in SNP treated samples, whereas increased abundance of adenosylhomocysteinase suggests the enhanced synthesis of L-homocysteine.

Protein folding, signalling and turnover

In a protein rich crop such as chickpea, the amino acid as well as protein content/quality are very important traits both under stress and non-stress conditions. NO not only regulates amino acid composition but also the degradation and folding of proteins (Fan *et al*. 2014). We observed that in chickpea, 28 proteins related to protein metabolic processes had an increased abundance and 60 proteins showed decrease in abundance in response to SNP treatment. SNP treatment downregulated various proteases such as subtilase, thiol protease, metalloprotease, serine protease, aspartic proteinase and various key kinases such as MAP kinase and Ser/Thr protein kinase that are involved in protein post-translational modifications (Data sheet S1). However, Bowman-Birk protease inhibitors were upregulated by SNP (Table 2). Besides, proteins such as Lon protease homologue which selectively degrades the misfolded, unfolded or damaged polypeptides, peptidyl-prolyl cis-trans isomerase, heat shock proteins and signal peptidases that help in protein targeting were also found to be responsive to SNP. Expression of HSP90, an ubiquitous molecular chaperone that is involved in modulating a multitude of cellular processes (Fan *et al*. 2014) was also known to be upregulated following SNP treatment. This points on the mechanism underlying the observed protein stability and cell survival promotion in SNP treated samples. We speculate that changes in the abundance of these proteins might reflect a coordinated activity of one or more kinase cascades involved in the NO-response process.

In addition to the proteases, NO also regulated good number of proteins involved in catabolic reactions. For instance, pyruvate kinase and chitinase involved in carbohydrate degradation and pectin esterase enzyme responsible for pectin degradation were reduced in abundance by SNP treatment (Data sheet S1).

Energy metabolism

Significant accumulation of malate dehydrogenase gene transcripts is related to plant and cell growth, as well as to tolerance to stress (Yao *et al*. 2011). FBP aldolase has been reported to stimulate glycolytic cycle and play an important role in signal transduction and gibberellin A (GA)-induced growth of rice roots (Konishi *et al*. 2005). However, in our study chickpea leaves subjected to SNP treatments did not induce the accumulation of malate dehydrogenase (MDH) and fructose-bisphosphate (FBP) aldolase to a significant level. Another glycolytic protein, sucrose-UDP glucosyltransferase 1 was upregulated upon SNP treatment similar to an earlier report in rice roots (Zhao *et al*. 2012). These proteins mediate the transfer of sugars to several acceptors including plant hormones and major classes of plant secondary metabolites, thus regulating their bio-activity and transfer. Moreover, glucose-1-phosphate adenyl transferase belonging to starch biosynthesis also found sensitive to exogenous SNP (Data sheet S1).

It is noteworthy that some key proteins involved in nodulation such as nodulation signalling pathway 2 proteins previously

showed reduced accumulation in chickpea following SNP treatment (Data sheet S1; Wienkoop *et al*. 2008). Further exploration of these protein targets may provide insight into previously unknown effectors of the NO signalling pathway during nodulation in legumes.

Proteins involved in response to stimulus

Application of SNP enhanced plant tolerance to various stress conditions is reportedly attributed to increased activities of antioxidant enzymes resulting in oxidative damage alleviation (Siddiqui *et al*. 2011). Our analyses are in accordance with these reports showing induction of several key proteins including peroxidase, glutathione peroxidase and ascorbate peroxidase at lower concentrations (Table 2; Data sheet S1). Interestingly catalase, few isoforms of peroxidase and superoxide dismutase were reduced following SNP treatment partially correlating with the gene expression. Similarly, other stress related proteins such as putative mitochondrial glyoxalase II involved in glutathione biosynthesis showed non-significant induction and dehydroascorbate involved in ascorbate metabolism also showed enhanced accumulation upon treatment. This data supports the role of NO in alleviation of oxidative stress, which is associated with induction of various reactive oxygen species (ROS)-scavenging enzyme activities during stress conditions.

We also observed an increased abundance of important secondary metabolite proteins such as chalcone synthase 1, β amyrin, flavoprotein WrbA-like, few isoforms of peroxidases and phenylalanine ammonia-lyase 2 in SNP treated chickpea leaves, possibly explaining the suggested role of NO in plant defence. These findings are consistent with a previous report in tomato, where NO enhanced resistance to *Botrytis cinerea* by enhancing the activities of polyphenol oxidase, chitinase, β-1, 3-glucanase, and phenylalanine ammonia-lyase (Zheng *et al*. 2014). Although PAL has previously been known to upregulate in the presence of SNP and cadmium, the varied expression of chitinase and glucanase both of which are pathogenesis related proteins suggest that the defence pathways can be activated by SNP treatment offering enhanced protection to the plants against stress (Data sheet S1; Tossi *et al*. 2011).

Proteins associated with biological regulation

Being a molecular messenger, NO can trigger the variations in gene expression and activation. As mentioned before, SAM and protein-L-isoaspartate O-methyltransferase, methyl group donors in many important transfer reactions including DNA methylation for regulation of gene expression have been known to be part of NO mediated gene regulation. Similarly, our studies revealed that few late embryogenesis abundant (LEA) protein, reduced in abundance by SNP treatment in chickpea (Data sheet S1). This is in contrast with the observations in wheat seedlings, where SNP was reported to upregulate LEA transcription especially under drought (Garcia-Mata and Lamattina 2001), suggesting that specific stresses might modify SNP-specific protein changes in plants. Besides, a good number of transcription factors and proteases such as kunitz proteinase inhibitor-1, NAC transcription factors, CAP2, bZIP were found responsive to SNP indicating the biological regulation of various cellular processes by endogenous NO (Table 2; Data sheet S1; Shukla *et al*. 2006). This study is the first indication on the upregulation of Legumin (alpha-amylase inhibitor), a seed storage protein that inhibits α-amylase in chickpea leaves (Data sheet S1). These observations are aligned to reports in barley, where phylogenetically distinct legumin were responsive to exogenous SNP having different physiological function in both seed and vegetative tissues (Julián *et al*. 2013). We noted that downregulation of cytochrome c biogenesis protein, CcsA required during chloroplast c-type cytochromes biogenesis needs further exploration for conclusiveness (Table 1).

Proteins associated with signal transduction and biogenesis

Fifteen proteins involved in signal transduction were differentially accumulated in response to SNP treatment in chickpea, of which four increased and 11 decreased in abundance (Data sheet S1). Ethylene receptor like-protein and non-specific serine/threonine protein kinase were identified as the major signal transduction proteins in chickpea responsive to SNP.

Nitric oxide is a key player in several biological cellular processes, acting either as a signalling or as a toxic molecule in plants (Mur *et al*. 2013; Yu *et al*. 2014). Exploring the importance of NO as a regulator of plant growth and stress defence has significantly increased in the past few years despite limited information on its mode of action and signalling (Siddiqui *et al*. 2011; Santisree *et al*. 2015;). Therefore, the identification of NO target molecules in this study was considered essential for deeper insights into its functional role in plants.

In the present study SNP was chosen due to its high efficiency, slow and continuous NO production. Despite of the fact that SNP release cyanide and iron in addition to NO, it is the most widely used donor in plant NO studies so far (Table S1; Planchet and Kaiser. 2006; Schröder. 2006). The possibility of observed effects of SNP being mere artefacts or an effect of various reaction products of SNP was ruled out based on, data obtained in the confirmatory experiments using other NO donors, DETA and GSNO (Fig. S1). The effect of SNP has been reported in regulation of various plant processes in a wide range of plants (Farooq *et al*. 2009; Fan *et al*. 2012; Santisree *et al*. 2015). Nevertheless, there have been fewer efforts on exploring the molecular mechanism of SNP action in plants (Meng *et al*. 2011). Although there are reports on studies in chickpea using exogenous SNP applications (Sheokand *et al*. 2008; Chohan *et al*. 2012), most of these were confined to a particular biological response or a developmental event either at morphological or physiological level. Several reports have indicated H_2O_2 accumulation, accompanied by increased cellular damage levels due to nitrosative stress at higher NO concentrations (Groß *et al*. 2013), suggesting its cytotoxic role. On the contrary, plants treated with the lower NO concentrations accumulated lower H_2O_2 levels, consequently inhibiting the detrimental effects of membrane lipid peroxidation suggesting the protective role of NO (Hayat *et al*. 2011; Groß *et al*. 2013). Since it is fundamental to establish SNP dose to maximise the impact before elucidating its cellular targets, our study

systematically dealt with the identification of right dose for exogenous application, followed by the efficacy of this particular concentration in modulating the physiological, defence and molecular responses in chickpea.

Four different genotypes of chickpea with varying stress tolerance for SNP dose response study were used to ensure the optimum concentration for achieving a positive impact. NO diminishes primary root growth and promotes lateral root development in many plants such as tomato and *Arabidopsis* (Negi *et al*. 2010; Liao *et al*. 2012). In our study, a dose dependent reduction in root and hypocotyl growth in chickpea was observed. The 0.1 mM concentration that emerged out of our experiments has also been used in many other plants for exogenous applications including chickpea (Table S1; Farooq *et al*. 2009; Hayat *et al*. 2011; Chohan *et al*. 2012; Santisree *et al*. 2015). Our study indicates a positive effect of SNP at 0.1 mM on seedling growth, chloroplast content, total nitrogen content and antioxidant levels (Figs 1, S1). These results also point chloroplast as one of the major subcellular targets of NO in chickpea, in line with previous reports on significantly enhanced chl content by NO (Kumar *et al*. 2010; Procházková *et al*. 2013). Since chloroplast is the site of photosynthesis as well as reactive oxygen species production, NO can potentially affect and regulate a wide range of downstream signals through its effects on chloroplast (Lum *et al*. 2005). The observed increase in chl content might be attributed to the activation of chl biosynthesis or/and its slow degradation or involvement of NO in the iron metabolism of plants. Indeed chl content and the chl *a*/*b* ratio are fundamental parameters for determination of photosynthetic activity and these parameters are often used as indicators of stress in plants (Kumar *et al*. 2010; Liao *et al*. 2012). The drought-induced reduction of *psbA* transcripts in NO donor treated wheat plants have reportedly led to enhanced activity of PSII and electron transfer rates during grain filling (Wang *et al*. 2011; Procházková *et al*. 2013). In the present study, several proteins involved in photosynthesis including PS-II D2 protein and PSII reaction centre protein L, needed for assembly and dimerisation of a stable PSII complex were also found to be NO responsive (Table 2). It is assumed that binding sites for NO existing within PSII between the primary and secondary quinone acceptors (Procházková *et al*. 2013). Other important proteins such as chl *a*/*b* binding protein, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, (PSII-L), one of the components of the core complex of PSII, NADPH-Quinone oxido reductase were also identified to be regulated by NO in chickpea (Table 2; Data sheet S1). Stimulation of antioxidant gene transcription in SNP-treated samples was also reported supporting its role in oxidative stress tolerance (Tossi *et al*. 2011; Wang *et al*. 2011; Yang *et al*. 2013; Santisree *et al*. 2015). A significant upregulation of chalcone synthase following SNP treatment was observed (Table 2), which could be primarily helpful in quenching singlet oxygen by isomerising into naringenin under various stress conditions. The increased abundance of this protein was also confirmed by the increased transcript accumulation (Fig. 4).

In this study, exogenous application of 0.1 mM SNP decreased the abiotic stress induced inhibition of seed germination. In addition to this, the applied SNP further enhanced the stress induced NO release in chickpea seedlings that got reversed by using NO scavenger, cPTIO, which is one of the most reliable NO scavenger for plants although at times have been reported to exhibit dual action as NO scavenger and N_2O_3 producer (Planchet and Kaiser. 2006). Nevertheless, in order to ensure quality data, and observe the scavenging effect of cPTIO, in our study we chose to use cPTIO at a higher concentration for a longer duration, since low concentration has been known to compromise its scavenging efficacy due to competitive reactions (D'Alessandro *et al*. 2013; Kováčik *et al*. 2014).

Although, the effects of exogenous application of SNP on various physiological and developmental processes are extensively studied, very few reports dealt with the molecular aspects. In our study a wide panel of 630 proteins proved to be differentially regulated of which the abundance of 45 was significantly reduced and 22 was significantly increased by 2-fold following SNP treatment in chickpea (Fig. 2). These chickpea proteins are distributed in a range of cellular compartments regulating wide range of biological processes. Previously, proteome profiling in cotton leaf identified 166 differentially regulated proteins by SNP treatment of which 82 were downregulated and 47 were upregulated (Meng *et al*. 2011). In citrus the role of H_2O_2 and SNP was suggested in preventing the accumulation of a large number of NaCl-responsive proteins via specific proteome reprogramming (Tanou *et al*. 2009). Furthermore, Bai *et al*. (2011) reported the accumulation of G-protein-associated proteins and the induction of antioxidant enzymes as the master mechanisms through which *S*-nitroso-*N*-acetylpenicillamine (SNAP) treated maize seedlings alleviate salt stress, besides activating the defence proteins, energy metabolism, and cell structure/division remodelling. Our study is one of the first large-scale proteomic analyses of the NO response in chickpea, representing comprehensive and comparative analysis of proteins regulated by NO.

Conclusions

The biggest challenge in NO research concerns the identification and the localisation of its sub-cellular targets, which has remained elusive till date. Although a number of studies have established the role of NO in plant growth and stress responses at the physiological level, the lack of in-depth molecular studies has created a huge knowledge gap. In the present study, a gel-free proteomic technique was used for the quantitative analysis of global protein profiles in the leaves of chickpea plants exposed to SNP. The identified proteins revealed involvement in various metabolic pathways and processes partially explaining the role of nitric oxide in plant growth. Although previous researches have suggested that several subcellular organelles including mitochondria, nucleus, and peroxisomes are predominantly involved in NO signalling in plants, a closer look at our study indicates that many of these NO responsive proteins are chloroplastic. This study provides a deeper insight into the molecular targets of SNP not previously observed in plants, allowing an understanding of NO signalling in plants in general and chickpea in particular. In conclusion, we have provided evidence that the exogenously applied NO donor can modulate the proteome of chickpea, revealing

potential candidates that attribute the reported functions of NO in plants. This altered protein expression in SNP-treated chickpea plants indicates a potential role in growth as well as for priming defence responses for ensuing stress episodes. Nevertheless, further detailed explorations of how these proteins fit into the context of NO signalling and their relationships with other well-known receptor and protein kinases will be useful.

Acknowledgements

PS thanks ICRISAT for hosting and providing research facilities. This work was undertaken as part of the CGIAR Research Program on Grain Legumes. This work was supported by financial support to PS through the INSPIRE Faculty Award (IFA12-LSPA-08) from the Department of Science and Technology, Government of India and partial funding from the CGIAR Research Program on Grain Legumes to KKS.

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