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RESEARCH ARTICLE

Nitrogen stress-induced alterations in the leaf proteome of two wheat varieties grown at different nitrogen levels

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Abstract Inorganic nitrogen (N) is a key limiting factor of the agricultural productivity. Nitrogen utilization efficiency has significant impact on crop growth and yield as well as on the reduction in production cost. The excessive nitrogen application is accompanied with severe negative impact on environment. Thus to reduce the environmental contamination, improving NUE is need of an hour. In our study we have deployed comparative proteome analysis using 2-DE to investigate the effect of the nitrogen nutrition on differential expression pattern of leaf proteins in low-N sensitive and low-N tolerant wheat (*Triticum aestivum* L.) varieties. Results showed a comprehensive picture of the post-transcriptional response to different nitrogen regimes administered which would be expected to serve as a basic platform for further characterization of gene function and regulation. We detected proteins related to photosynthesis, glycolysis, nitrogen metabolism, sulphur metabolism and defence. Our results provide new insights towards the altered protein pattern in response to N stress. Through this study we suggest that genes functioning in many physiological events coordinate the response to availability of nitrogen and also for the improvement of NUE of crops.

Keywords MALDI-TOF · Nitrogen deficiency · *Triticum aestivum* · Proteomics

Introduction

Nitrogen (N) is one of the most critical inputs that define cereal crops productivity and yield under field conditions, and must be supplemented to meet the food production demands (Liao et al. 2012). Global N fertilizer consumption is projected to reach 105 Tg N by 2030 and 135 Tg N by 2050 (F.A.O. 2010). Such a large-scale consumption of N fertilizer has resulted in a very significant alteration of the N cycle in air, land and water at local, regional and global scales (Guo et al. 2010) as the utilization of fertilizer-N by crops is less than 30–40 % of the applied N (Abrol et al. 2008). The unaccounted 67 % represents a $15.9 billion annual loss of N fertilizer (assuming fertilizer-soil equilibrium). Loss of fertilizer N results from gaseous plant emission, soil denitrification, surface runoff, volatilization ion and leaching. The reactive nitrogen (NO_x), which is emitted from the denitrification of the fertilizer N, has 300-times more global warming effect than CO_2 (Bates et al. 2008).

A number of studies have been undertaken by plant molecular physiologists to decipher the regulatory control mechanisms involved in nitrogen use efficiency (NUE) of crop plants (Shrawat and Good 2008). Plants have evolved an active, regulated and multiphasic transport system making their nitrate uptake scheme efficient enough to transport sufficient nitrate to satisfy total nitrogen demand of the plant in face of varying external nitrate concentrations (Abrol 1993). Nitrate is the most abundant form of nitrogen available to the plant roots in aerated soils. Root nitrate uptake is carried out by both high affinity and low affinity nitrate transporters that are encoded by a multigene family (Abrol et al. 1999; Forde 2002). The dynamics of nitrogen uptake can be quantitatively described using Michaelis-Menten equation (Tsay et al. 2007). Previously, Orsel et al. (2006) have also recognized kinetic parameters (K_m and V_{max}) as important factors that greatly affect the efficiency of nutrient uptake from soil, and suggested that these parameters can be useful to select the varieties having high nutrient-utilization efficiency.

Whole plant physiology, use of transgenic plants and QTL approaches have also been employed that suggest that the

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enzymes of secondary ammonia remobilization and regulatory processes that control N–C flux are better targets for manipulation, than the individual genes/enzymes of primary nitrate assimilation (Pathak et al. 2011; Andrews and Lea 2013). As N losses vary greatly between the growth conditions, therefore there is a great potential for increasing N uptake efficiency in crops (Abrol 1993). However, with the genetic selection procedures, time is a big constraint. Thus, the implementation of sensitive and rapid methods for protein identification is required. Two-dimensional gel electrophoresis (2-DE) have transformed the combination of genetics and proteomics techniques into a powerful tool for functional analysis. It is the most promising technique to identify proteins that are induced, repressed, or post-transcriptionally modified during a developmental process as complex as senescence (Agrawal and Rakwal 2006; Hirel et al. 2007; Amiour et al. 2012).

Recognizing the limitations of earlier approaches for improving NUE, we have identified low-N stress sensitive and low-N stress tolerant wheat varieties (Chandna et al. 2010; Chandna et al. 2012). Proteome analysis of these varieties was conducted in response to N stress, with the hope to advance understanding of the physiological and biochemical processes underlying the response to N stress. It is expected that information obtained in such a study will help the development of approaches for manipulating the genes for improving NUE of the wheat. From this study we get a fair idea of how different varieties behaves under a particular growth condition (N levels in our study), also helpful in identification of the underlying molecular components that help the plant to adapt or survive the stress/limiting factors.

Materials and methods

Plant culture

The seeds low-N stress tolerant (VL616) and low-N stress sensitive (UP 2382) varieties of wheat (*Triticum aestivum* L.) (Chandna et al. 2010) were germinated in moist Petri plates, after surface sterilization with 0.2 % mercuric chloride. The seedlings were grown with half-strength nutrient solution (Hirel et al. 2001) containing three levels of N viz., 1 mM (low nitrogen, control), 10 mM (moderate) and 25 mM (high). The N was supplied in the form of KNO_3. The plants were grown for 20 days in the growth chamber, maintained at day/night temperature (28 °C/22 °C), relative humidity (75 %), and photoperiod of 16/8 h (280–300 μmol m^{-2} s^{-1}). The nutrient solution was continuously bubbled with sterile air and changed every fourth day.

Protein extraction and 2D- electrophoresis

Proteins from the leaves of 20-day-old plants of VL616 and UP2382 varieties of wheat were extracted using method of Damerval et al. (1986). The leaf tissue was ground to a fine powder and suspended in chilled extraction buffer (containing Tris (hydroxymethyl) aminomethane hydrochloride, sodium dodecyl sulphate (SDS), glycerol, 2-mercaptoethanol and plant protease inhibitor). Homogenized mixture was centrifuged at low speed (500 × g) at 4 °C for 15 min. Cold acetone containing TCA and 2-mercaptoethanol were added to the supernatant, then, incubated at −20 °C for 1 h. The precipitated proteins were centrifuged at 15,000 × g at 4 °C for 45 min. Pellet was washed with acetone solution containing β-mercaptoethanol. The pellet was dried under nitrogen and re-suspended in the isoelectric focusing (IEF) extraction solution (urea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate, DTT and pH 4–7 ampholytes). After incubation for 2 h at 33 °C, the sample was centrifuged at 15,000xg at 4 °C for 30 min and the supernatant was subjected to IEF. The protein was quantified using 2D Bradford kit (BioRad, USA) with bovine serum albumin as the standard.

Two-dimensional electrophoresis (2-DE) of proteins was performed in accordance with the method of O' Farrell (1975) with some modifications. 500 μg leaf protein were applied on immobilized pH strips with highest pH range of 3–10, to determine the pH of expressed proteins. It was found that proteins expressed in the pH range of 4–7. Therefore for further work 17 cm, pH 4–7 gradient strips were used. After the application of protein on strip, the strip was covered with mineral oil. IEF was carried out with an IEF system (Protean I.E.F. Cell, BioRad, USA) applying the following conditions. For the rehydration step the voltage was maintained for 12 h at 30 V, then the proteins were focused for 1 h at 500 V, 1 h at 1, 000 V and 8 h and 20 min at 8,000 V. The temperature was maintained at 20 °C and the current was 50 μA per strip. After IEF, strips were equilibrated in DTT, followed by iodoacetamide as described by Chivasa et al. (2002) and then stored at −20 °C.

The second-dimension separation of proteins was performed according to the method of Laemmli (1970) on a 12.5 % SDS polyacrylamide gel using Dodeca cell, electrophoresis unit (BioRad, USA). The electrophoresis was carried out at 25 °C and 17 W per gel. Following SDS polyacrylamide gel electrophoresis (PAGE), gels were stained with Coomassie brilliant blue R 250 in accordance with to the manufacture's manual (BioRad, USA).

A total of six gels were analysed, three gels for low-N stress sensitive varieties and three gels for low-N stress tolerant varieties, grown under various N treatments. Three gels were produced for each protein extract in order to take into account coloration effects. A total of 18 gels (2 varieties, 3 nitrogen doses, 3 repetitions for varieties) were considered. Molecular

weights of the proteins were referred to broad range protein marker (Bangalore Genei, India) of known molecular weights used.

Gel image and data analysis

The 2-DE gels were scanned with scanner. The image analysis was performed with PD Quest software version 8.0 (BioRad, USA). The optimized parameters were as follows: saliency 2.0, partial threshold 4, and minimum area 50. The intensity of the spots was normalized to that of landmark proteins used for internal standardization. Spots were quantified on the basis of their relative volume, which was determined by the ratio of the volume of a single spot to the whole set of spots.

Scoring methods and statistical analyses

Protein spots obtained were scanned for their density by a Bio-Rad GS 710 Calibrated Imaging Densitometer and quantified using the Gaussian method. The groups were defined after aligning and matching. PD Quest automatically computes the quantification values in % of volume. For each matched spot the % volume was calculated as its volume divided by the total volume of matched spots (referred to hereafter by intensity). Only those with significant (quantitative changes more than two-fold in abundance) and reproducible changes in three replicates were used for further analysis.

MALDI-TOF-MS analysis

The excised gel spots were destained completely. Then the gel spots were incubated with 200 mM ammonium bicarbonate (Sigma) for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min and then rehydrated with 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Sigma) on ice. After removal of solution, 50 mM ammonium bicarbonate was added and the digestion was performed overnight at 37 °C. The peptides were desalted and concentrated using C18 nanoscale (porus C18) column. For the analysis of MALDI-TOF-MS by peptide-mass fingerprinting (PMF) method, the peptides were eluted by matrix solution [70 % acetonitrile (Merck), 0.1 % TFA (Merck), 10 mg/ml alpha-cyano-4-hydroxycinamic acid (Sigma)]. The eluted peptides were spotted onto a stainless steel target plate. Masses of peptides were determined using MALDI-TOF-MS (Model MALDI-R; Micromass, Manchester, UK). Calibration was performed using internal mass of trypsin auto-digestion product (m/z 2211.105). To identify the protein, peptide masses from MALDI-TOF-MS were matched with the theoretical molecular weight of peptides for proteins in the NCBI database using MASCOT software (www.matrixscience.com). Significant differences linked to the factor N were analyzed through a heatmap methodology, using the software MultiExperiment Viewer version 4.6 as described by Saeed et al. (2003).

Results

Comparative proteomics

Results pertaining to N-induced expression of proteins in low N- tolerant (VL616) and low N- sensitive (UP2382) wheat varieties are presented (Fig. 1a and b). N-sensitive and N-tolerant wheat varieties were grown for 20 days with three levels of N viz., 1 mM, 10 mM and 25 mM. Differential expression of proteins in the leaves of the two varieties was analysed using 2D gel electrophoresis. Quantitative image analysis of the differentially expressed proteins was done by using the PD Quest ImageMaster software version 8.0. The intensity of the indicated spots were quantified, normalized and relatively expressed as arbitrary volume. This analysis revealed three patterns of changes in the expression of proteins, (1) increase in number of spots as N is supplied to the plants when compared with control, (2) a gradual decrease in spots number in UP2382 (low N- sensitive) at higher N levels and (3) a gradual increase in number of protein spots with increase in N supply in VL616 (low N- tolerant). In UP2382 and VL616, a total of 962 protein spots were observed in the leaves of plants. Among the observed protein spots, the intensities of 152 protein spots changed markedly in both the varieties by N treatments, when compared with control (low N condition). Variation of protein spot intensity due to the N nutrition level of two wheat varieties, VL616 and UP2382 is summarized in table 1.

Differential expression of proteins

From the 962 protein spots, 152 protein spots showed difference in their expression levels between cultivars. Out of 152 differentially expressed spots, 25 protein spots were excised manually from 2-D gels for tryptic digest and subjected to mass spectrometry. In order to verify their identity, protein spots were analysed from both the varieties, under three N treatments, and three biological replicates. The excised protein spots were analyzed by mass spectrometry (MS using MALDI-TOF) resulting in the identification of 25 proteins (Table 2). Most of the proteins identified were involved in energy production/regulation and metabolism as would be expected in plant leaf tissue. The proteins involved in energy production include those which play a role in glycolysis, respiration, electron transport and photosynthesis. Proteins involving in metabolism include those involved in metabolism of sugars, polysaccharides, sterols and cofactors. Five proteins identified belonged to the category of photosynthesis.

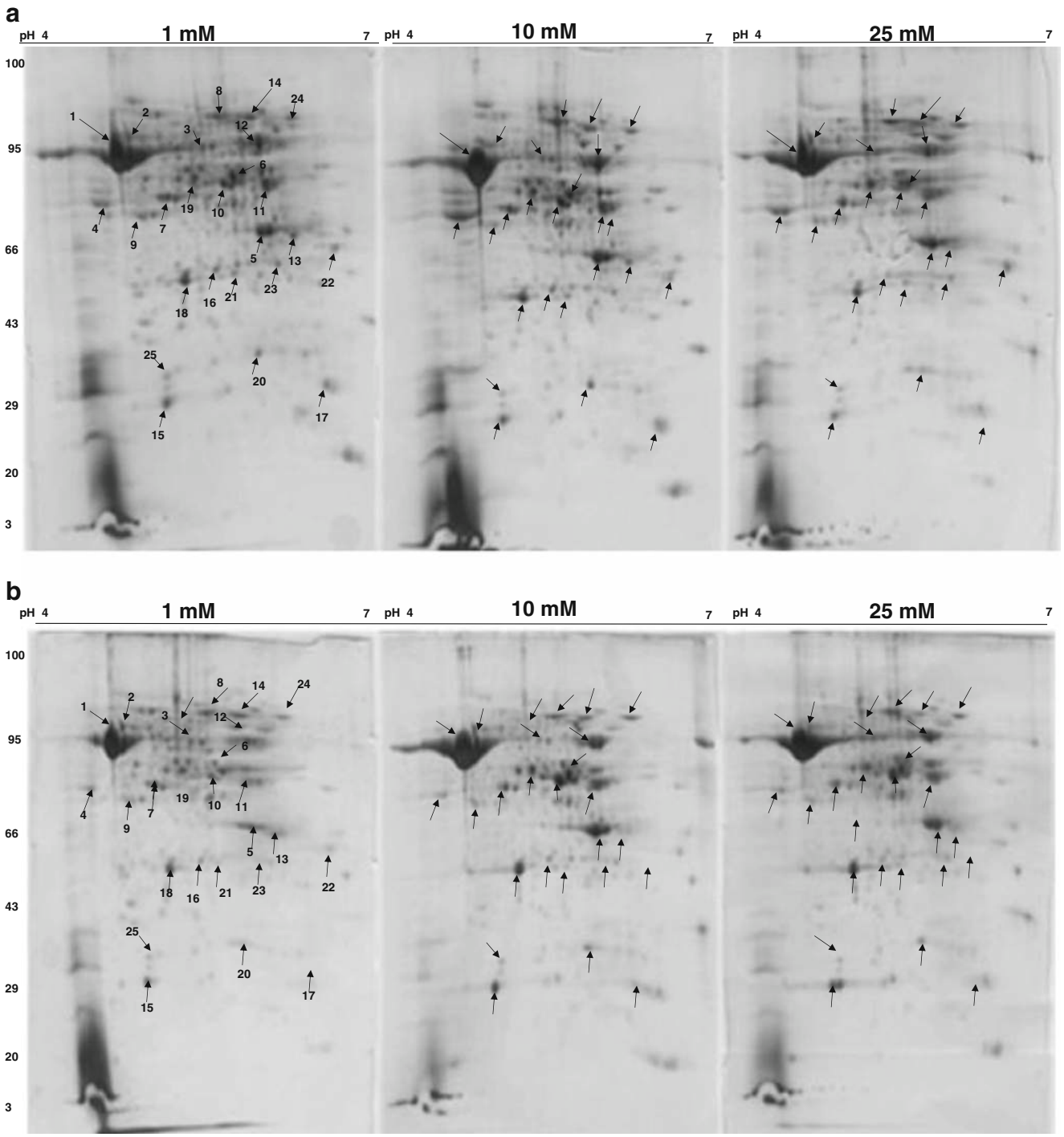


Fig. 1 **a** Representative 2-DE maps of *T. aestivum* cv. UP2382 leaf proteins at low (1 mM), moderate (10 mM) and high (25 mM) nitrogen treatments. Differentially accumulated protein spots which appeared in all time are selected for MALDI-TOF analysis and indicated by label in the map **b** Representative 2-DE maps of *T. aestivum* cv. VL616 leaf proteins at low (1 mM), moderate (10 mM) and high (25 mM) nitrogen treatments. Differentially accumulated protein spots which appeared in all time are selected for MALDI-TOF analysis and indicated by label in the map

Spot 1 was identified as Rubisco-large subunit (LSU) of *Oryza sativa*. The accession number is AAS46127. This enzyme acts as a carboxylase for mediating photosynthetic CO_2 assimilation, and as an oxygenase for catalyzing the first step of the photorespiratory pathway in plants. Spot 2 was identified as triosephosphate isomerases (accession number of XP_462797) of *Oryza sativa*, and is known to be a glycolytic enzyme and is involved in energy production. Spot 3 was identified as ATP synthetase complex (accession number NP_922436 of) *Oryza sativa*. This complex plays a central

Table 1 Differential expression of protein spots in VL616 and UP2382 wheat varieties under nitrogen treatments

Description	Number of spots
Increase in intensity when N increased	13
Decrease in intensity when N increased	10
Increase and then decrease in intensity when N increased	7
Decrease and then increase in intensity when N increased	4
Increase in 'VL616' and decrease in 'UP2382' when N increased	25
Appearance in two genotypes when N increased	13
Disappearance in two genotypes when N increased	11
Interaction between genotype and treatment	152

role in energy transduction in chloroplasts and mitochondria. Spot 4 showed high similarity with the photosystem II oxygen evolving complex protein 2 precursor of *Triticum aestivum* with accession number of S22763. It is involved in O_2 evolution and PSII stability. The protein identified in spot 5 has similarity with fructose bisphosphate aldolase of *Arabidopsis thaliana* with accession number of Q5XEU6_ARATH. It catalyzes the cleavage of fructose 1, 6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Protein spot 6 was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Oryza sativa*. The accession number is AAN59792. It catalyzes the conversion of glyceraldehyde-3-phosphate to glyceraldehye 1,3 bisphosphate. Protein spot 7 had the homology with cytochrome P450 of *Triticum aestivum* with accession number of AAR11387. This protein takes part in the electron transport pathways at the thylakoid membrane in chloroplasts. Spot 8 showed similarity with putative Elongation factor belonging to the GTP-binding elongation factor family of *Oryza sativa* with accession number of Q6H443_ORYSA. These proteins promote the GTP-dependent binding of aminoacyl-tRNA to the A site of ribosomes during protein biosynthesis, and are predicted to be localized to the chloroplast. Protein spot nine was identified as putative glycine dehydrogenase of *Triticum aestivum* with accession number of AAM92707. It is involved in photorespiration, and which has been suggested to be important for maintaining electron flow to prevent photoinhibition under stress condition. Spot 10 showed high similarity with Putative glyoxalase I of *Triticum aestivum*. The accession number is Q9XGF2. It plays role in glutathione synthesis, and is involved in the reaction of carbon-sulphur lyase. The isomerisation of hemithioacetal adducts which are formed in spontaneous reaction between glutathionol group and aldehyde. Spot 11 had homology with the glutamate synthetase of *Arabidopsis thaliana* with accession number of Q43127. It assimilates ammonium into amino acids, thus it is a key enzyme for nitrogen metabolism. Protein spot 12 was Quinone oxidoreductase like protein of *Arabidopsis thaliana* with accession number of Q8LEB8. It catalyses two electron reduction of quinones and quinonoid compounds to hydroxyquinones using either NADH or NADPH as electron donor. Spot 13 was similar to Chitinase 2 of *Triticum aestivum*. The accession number is Q8W428. Chitinase −2 is an enzyme involved in cell wall catabolic process. Protein of spot 14 belonged to porphobilinogen deaminase (fragment) of *Triticum aestivum* with accession number of Q8RYB1. It takes part in tetrapyrrole biosynthetic activity. Protein spot 15 was found to be similar to glutathione-S-transferase of *Arabidopsis thaliana* with accession number of CAA72973. It plays important role in defence as a detoxification enzyme of a variety of foreign bodies like oxidative stress. Protein spot 16 belonged to phosphatidylinositol-4-phosphokinase family protein of *Arabidopsis thaliana* with accession number of Q9LY23. They are involved in production of secondary messengers in response to stress. The protein of spot 17 was found to be flavonoid syn like protein of *Oryza sativa*, accession number AAM00948. This protein is involved in maintenance of the abundant plant quinines in the reduced state in the cell and play role in increasing plant resistance. Protein of spot 18 was recognized as nitrate reductase of *Arabidopsis thaliana* (accession number AAF19225). The nitrate reductase enzyme participates in nitrogen metabolism. The NR activity rate determines the inorganic nitrogen assimilation in plants. Protein spot 19 was identified as Stress induced protein Sti1-like protein of *Arabidopsis thaliana* with accession number CAB78283. Spot 20 has homolog with the salt-inducible protein of *Arabidopsis thaliana* with accession number of CAB78461. Spot 21 is an oxygen evolving enhancer protein of *Arabidopsis thaliana* with accession number of Q9S841. It is involved in maintaining cellular homeostatis and defense against oxidative stress. Protein spot 22 was identified as protein disulphide isomerase of *Triticum aestivum* (accession number CBK33653). It is involved in maturation of plasma membrane and storage proteins during protein synthesis. Protein spot 23 belonged to chromo domain containing protein of *Arabidopsis thaliana*, accession number Q946J8. Protein spot 24 was identified as SMC3 (*A. thaliana*), accession number AAS09910. It is known to be involved in chromosome dynamics. Protein spot 25 was identified as ccmFC (*T. aestivum*), accession number ADE08083. It played an important role in cytochrome biosynthesis.

Expression levels of identified proteins in wheat varieties

The results of our proteomics work in accordance to our aim to provide evidence of N regulation at molecular level suggested that 25 identified proteins were affected by various N treatments (Table 2, Fig. 1a and b). In VL616, it was observed that the protein spot of RUBISCO-LSU (spot 1) did not show any change in expression level with increase in N treatments

Table 2 Differentially expressed nitrogen-responsive proteins and their identification by MS

Spot No.	Protein name	Mr	PI	Mascot score	Accession No.	Functional category
1	Ribulose bisphosphate carboxylase-LSU	94,000	4.91	101	AAS46127	Photosynthesis
2	Putative Triosephosphate isomerase	95,500	5.18	101	BAD33340	Energy metabolism
3	ATPase alpha subunit	94,900	5.53	93	NP_922436	Photosynthesis
4	Photosystem II oxygen evolving complex protein 2 precursor-Wheat	72,000	4.34	157	S22763	Photosynthesis
5	Fructose bisphosphate aldolase-Arabidopsis thaliana	68,000	6.08	644	Q5XEU6_ARATH	Energy metabolism
6	Cytosolic Glyceraldehyde-3-phosphate dehydrogenase GAPDH	75,480	6.00	85	AAN59792	Energy metabolism
7	Cytochrome P450	87,000	5.39	70	AAR11387	Electron transport
8	Putative plastid specific elongation factor-Oryza sativa	98,000	5.98	101	Q6H443_ORYSA	Chromosome dynamics
9	Putative glycine dehydrogenase	66,000	4.96	76	AAM92707	Photosynthesis
10	Putative Glyoxalase I- Triticum aestivum	80,480	5.6	73	Q9XGF2_WHEAT	Sulphur metabolism
11	Glutamate Synthase	82,400	6.1	194	Q43127	N Metabolism
12	Quinone oxidoreductase like protein- Arabidopsis thaliana	96610.3	6.4	93	Q8LEB8_ARATH	Oxidoreductase Activity
13	Chitinase 2. wheat	64921.19	6.96	132	Q8W428_WHEAT	Chitin binding
14	Porphobilinogen deaminase (fragment)-wheat	97671.31	6.23	64	Q8RYB1_WHEAT	Tetrapyrrole biosynthetic activity
15	Glutathione S transferase	28,098	5.00	46	CAA72973_ARATH	Defence
16	Phosphatidylinositol-4- phosphokinase family protein	55,161	5.50	51	Q9LY23_ARATH	Defence
17	Flavonoid syn like protein	32,401	6.78	44	AAM00948_ORYSA	Plant sec metabolite
18	Nitrate reductase	47,311	5.30	51	AAF19225_ARATH	N Metaboism
19	Stress induced protein Sti1-like protein	81,280	5.5	31	CAB78283_ARATH	Defence
20	Salt inducible protein homolog	35,605	5.96	64	CAB78461_ARATH	Defence
21	Oxygen evolving enhancer protein	49,947	4.86	33	Q9S841_ARATH	Defence
22	Protein disulphide isomerase	60,396	6.89	49	CBK33653_WHEAT	Protein synthesis
23	Chromo domain containing protein	62,000	6.5	32	Q946J8_ARATH	Chromosome dynamics
24	SMC3 (*A. thaliana*)	96,000	6.67	32	AAS09910_ARATH	Chromosome dynamics
25	ccmFC (*T. aestivum*)	40,086	4.97	62	ADE08083_WHEAT	Organelle biogenesis

whereas a decline in expression of Photosystem II oxygen-evolving complex (spot 4) protein was found. However, ATP synthase (spot 3), glyceraldehyde-3-phospho dehydrogenase (spot 6), Glyoxalase I enzyme (spot 10), cytochrome P450 (spot 7), elongation factor protein-2 (spot 8), quinone oxido-reductase (spot 12) and Salt inducible protein homolog (spot 20) were found to be up-regulated by N treatments. Glutamate synthase (spot 11), triosephosphate isomerise (spot 2), fructose bis-phosphate (spot 5), Glutathione S transferase (spot 15), Phosphatidylinositol-4- phosphokinase family protein (spot 16), Nitrate reductase (spot 18), Stress induced protein Sti1-like protein (spot 19), Oxygen evolving enhancer protein (spot 21), Protein disulphide isomerase (spot 22), Chromo domain containing protein (spot 23), SMC3 (*A. thaliana*) (spot 24) and ccmFC (*T. aestivum*) (spot 25) showed no significant change in expression levels with increase in N level. For proteins Glycine dehydrogenase (spot 9), chitinase-2 (spot 13), Porphobilinogen deaminase (spot 14) and Flavonoid syn like protein (spot 17) we observed a low correlation between the changes in protein expression levels under moderate and high N treatments (Table 2).

In UP2382, all the proteins, except eight (Cytochrome P450 (spot 7), elongation factor protein (spot 8), chitinase-2 (spot 13), Glutathione S transferase (spot 15), Protein disulphide isomerase (spot 22), Chromo domain containing protein (spot 23), SMC3 (*A. thaliana*) (spot 24) and ccmFC (*T. aestivum*) (spot 25) were up-regulated by N treatments (Table 2). We observed an enhanced expression of glutamate synthase (GS) (spot 11) and nitrate reductase (spot 18), important enzymes of nitrogen metabolism, with increase in N levels.

Discussion

To raise food crops, application of inorganic nitrogenous fertilizers is one of the key inputs because the food crops fulfil

their demand for nitrogen (N) from fertilizers. It has been estimated that there has been a 420-fold increase in N fertilizer application in the last 50 years (Shrawat and Good 2008). As a consequence, the use of N fertilizers in agriculture has already shown a number of detrimental environmental impacts. The requirements for nitrogen fertilizer application will further increase to produce more and more food for a teeming population. Therefore, the need to reduce N fertilizer pollution strengthens the importance of improving the NUE of crop plants. The development of crop plants that take up and assimilate N more efficiently would reduce the need for N fertilizers and have a positive influence on the environment.

In this era of genetic engineering, the crop plants can be developed which can grow and yield well at low nitrogen application. Understanding how plant genes respond to varied N levels is essential for formulating approaches to manipulating genes for improving NUE. Most of the approaches, involving either whole plant physiology or the use of transgenic plants or mutants, have not contributed to an understanding of the physiological and genetic basis of NUE in a more integrated manner because these approaches studied the role of a single or limited number of enzymes. Since, after protein synthesis, they undergo post-translational modifications, such as phosphorylation or removal of a signal peptide, that alter/affect their activities as well as the location in the cell. In addition to this, because of the large differences in mRNA and protein turnover, the mRNA poorly reflects how much protein is present in the cell: a protein can be still abundant while the mRNA is no longer detectable because its synthesis has stopped. Finally, the molecular mechanisms that are involved in cell differentiation, development, adaptation to environment, etc., cannot be deduced only from nucleic acid sequences or quantity. To overcome these disadvantages, the proteome concept was introduced which is tightly linked to two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (Park 2004). 2-D PAGE allows the detection of variations which may be physiologically relevant.

In our present investigation the wheat varieties which showed contrasting N-efficiency was selected to investigate the underlying molecular components that help the wheat varieties to survive under limiting factors. The differential expressions of these protein spots are described in heatmap Fig. 2 and there relation to various metabolic processes is represented in Fig. 3.

Differential expression of protein in N-stress tolerant (VL616) and N- stress sensitive (UP2382) varieties of wheat was studied in response to various level of N by 2D gel electrophoresis. Two criteria were taken into account for choosing the protein spots to be sequenced: (i) sequencing of a maximum number of spots showing a significant variety x *N* level interaction; (ii) preference for spots whose variation in the analysis of variance was highly significant (1 % instead of 5 %). 25 protein spots (Table 2) were identified by MS/LC MS analyses. Groups of identified proteins involved in a particular process are described here.

Photosynthesis

Rubisco-LSU, triosephosphate isomerases, ATP synthetase complex, oxygen evolving proteins (OEPs) of photosystem II, and glycine dehydrogenase were identified as the proteins involved in photosynthesis. Photosynthesis depends on (i) Light harvesting capacity, (ii) The rate at which NADPH and ATP can be regenerated; and (iii) The capacity for the carboxylation of RuBP by Rubisco. The N-containing components responsible for these processes are (i) chlorophyll-protein complexes (LHC) (ii) electron transport and photophosphorylation membrane complexes and (iii) photosynthetic carbon reduction (PCR) cycle, including ribulose-1, 5 bisphosphate carboxylase oxygenase (Rubisco). Rubisco accounts for about 12 % of total plant N in C-3 plants during vegetative growth (Portis and Parry 2007) and so has major implications for N-use efficiency of growth. Photorespiration, caused by the Rubisco oxygenase reaction, is intrinsically linked with nitrogen metabolism. Nitrogen (N) nutrition has been reported to play a crucial role in determining photosynthetic capacity of the plants in both natural and agricultural environments (Lawlor and Tezara 2009). It is well documented that approximately 60 % of the nitrogen in a plant leaf with C3 photosynthesis is invested in chloroplasts. Of the total leaf N, about 25 % is in Rubisco and about 25 % in the light harvesting and electron transport components (Portis and Parry 2007).

The expression of RuBisco-LSU increased in both the wheat varieties with N treatments. In N-stress tolerant variety and N-stress sensitive wheat variety, there was continuous increase in the expression level of this protein with the increase in N treatments (Fig. 2). The relationship between nitrogen supply and Rubisco content is complex. In crops, Rubisco content increases linearly (but not proportionately) with nitrogen uptake and leaf nitrogen (Nakano et al. 2006) and the proportion of nitrogen allocated to Rubisco is independent of nitrogen supply in trees seedlings and conifers (Baker et al. 1997).

Putative triosephosphate isomerases is a glycolytic enzyme that catalyses the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Triosephosphate plays an important role in several metabolic pathways and is essential for sensitive energy production (Caruso et al. 2008). Under N-deficiency condition (1 mM), the expression level of this protein is higher in VL616 (low N-tolerant) than UP2382 (low N-sensitive). The expression of this protein increases in both the varieties with N treatments (Fig. 2). At high N treatments, the expression of this protein was enhanced significantly in both the varieties. According to

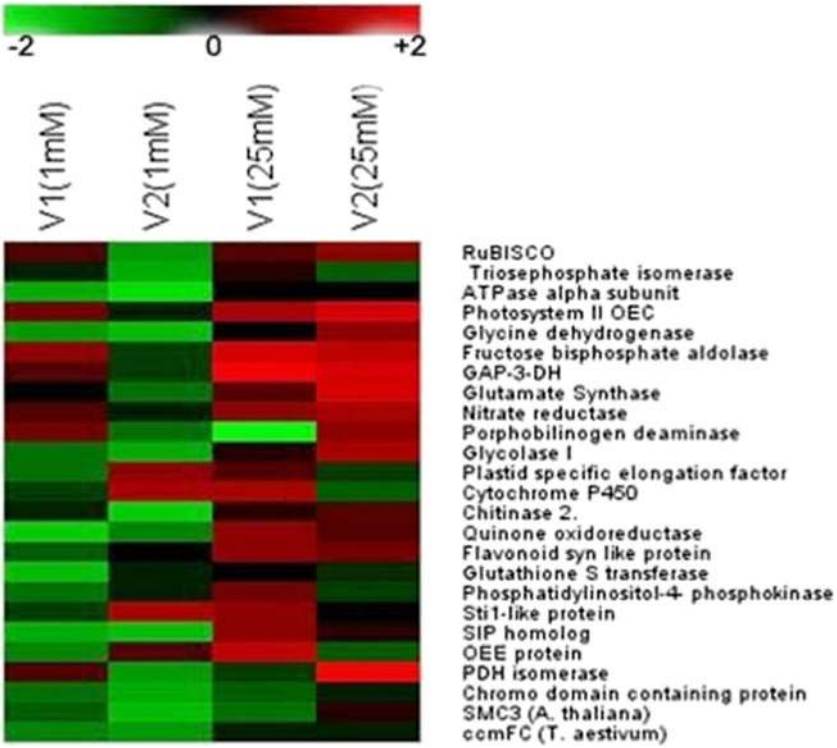


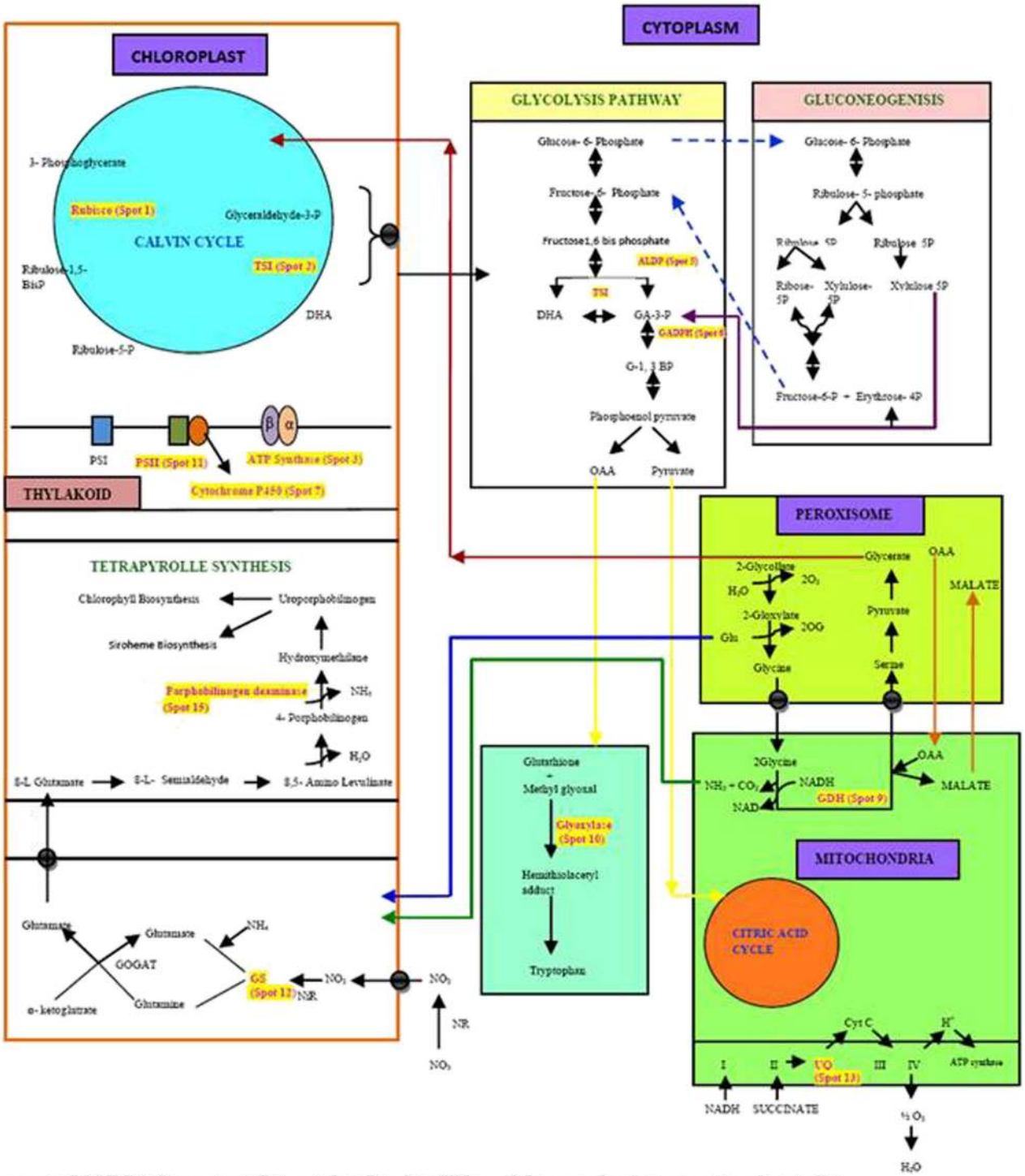
Fig. 2 Heatmap analysis of N treatments in the contrasting varieties VL616 (V1, N-tolerant) and UP2382 (V2, N-sensitive)

the well known relationships existing between nitrogen and carbon metabolism, the changes in accumulation of this protein after nitrate addition are consistent with an increase of photosynthesis rate (Lawlor and Tezara 2009).

The terminal step in the main energy production of living organisms is the synthesis of ATP from ADP. It is catalyzed by the ATP synthetase complex located in mitochondrial or chloroplast membranes. The role of coupling factors in the mechanism of energy conversion has been extensively investigated in respiratory as well as in photosynthetic systems. Electron transport pathways are necessary to uncouple respiratory precursor synthesis from ATP production. The present analysis emphasizes the metabolic value of ATP produced during N-linked respiration, with cellular ATP supply being tailored to ATP demand (Noctor and Foyer 2000). Under N-deficiency condition, the intensity of this spot in higher in VL616 than UP2382 in our study. Under stress conditions, this protein complex plays a crucial role in maintaining the function of the chloroplast and the whole cells (Ali and Komatsu 2006). It is expected that higher expression of this protein in UP2382 varieties provides tolerance to N-deficiency (−N). Expression of this enzyme complex increased in both the varieties of wheat by N treatments (Fig. 2). Nitrogen nutrition has been reported to initiate respiratory chain and enables ATP synthase enzyme to produce ATP for energy production (Crawford 1995).

Oxygen (O_2) evolving proteins (OEPs) of photosystem II (PSII) is involved in O_2 evolution and PSII stability (Sugihara et al. 2000). Expression of this protein increased in both the varieties with the increase in N treatments (Fig. 2). An increase of its activity is correlated to photosynthetic activity (Bahrman et al. 2005). This protein has been previously shown to be induced by salt stress (Kim et al. 2005) but reduced by ozone and sulphur dioxide fumigation (Rakwal et al. 2003).

Putative glycine dehydrogenase (GDC) also named glycine-cleavage-system or glycine dehydrogenase) is a multi-protein complex that occurs in all organisms, prokaryotes and eukaryotes. GDC, together with serine hydroxyl methyltransferase (SHMT), is responsible for the interconversion of glycine and serine, an essential and ubiquitous step of primary metabolism. The expression of this protein spot increased in UP2382 wheat varieties with increase in N treatments. In VL616 wheat variety, the expression of this protein decreased with increase in N levels (Fig. 2). GDC, under unstressed conditions, represents the sole source of photorespiratory CO_2 and NH_3 and functions as an important link between photorespiration and other metabolic pathways such as nitrate and ammonia assimilation. The photosynthetic enzyme activities located in chloroplasts increase at higher ratio than leaf-nitrogen content in contrast to the change in the mitochondrial protein, due to relatively greater increase in the photorespiratory enzyme components of the mitochondria, such as glycine decarboxylase.. Thus, allocation of nitrogen between respiratory and photorespiratory functions in leaf mitochondria may not be uniform in response to nitrogen supply (Douce et al. 2001).



The enzymes highlighted represent the proteins showing differential expression in our proteomics results.
GADPH: Glyceraldehyde-3-phosphate dehydrogenase
ALDP: Aldolase or Fructose 1,6 bisphosphatase
TSI: Triosephosphate isomerases
PSII: Photosystem II Oxygen evolving complex protein
UQ: Ubiquinone (Quinone oxido reductase)
GDH: Glycine Dehydrogenase
Rubisco: Ribulose bis phosphate carboxylase
GS: Glutamine synthase

Fig. 3 Graphical representation of putative function of the differentially expressed protein in wheat varieties

Glycolysis

The metabolism of carbon- and nitrogen-containing compounds is fundamental to all forms of life. To cope with changing environmental conditions, organisms have to sense the nutrient supply and adapt their metabolism accordingly. It is known that nitrate reductase activity is coordinated with the rate of photosynthesis and the availability of C skeletons by both transcriptional and post-translational controls (Wang et al. 2003). In our study, three proteins showed differential expression in the leaves of wheat varieties in response to nitrogen treatments (Fig. 2). These results show that the effect of nitrate on gene expression is substantial (affecting almost 10 % of the genes with detectable mRNA levels) yet selective and affects many genes involved in carbon and nutrient metabolism.

Fructose-bisphosphate aldolase class-I enzyme is a key enzyme of the glycolytic pathway catalyzing fructose 1, 6-bisphosphate to glyceraldehyde 3-phosphate and hydroxyacetone phosphate. Presence of an EST of fructose-bisphosphate aldolase was detected in a nitrogen starvation experiment, and this gene was upregulated by nitrate in tomato roots (Wang et al. 2001). Expression level of this protein spot in our experiment was almost similar in both the varieties under N deficiency (1 mM). Nitrogen treatments increased the expression level of this protein in both the varieties. However, there was continuous enhancement in the expression of this protein in UP2382 with the increase in the level of N treatments. Contrary to this, the expression of this protein was constant at all levels of N treatments in VL616 wheat variety (Fig. 2). The strong response of the nitrogen supply affects and increases the activities of fructose 1, 6-bisphosphate in leaves since the carbohydrate metabolism is controlled by this enzyme and strongly responding to carbohydrate demand (Takahashi et al. 2009).

Expression of cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was observed to be higher in VL616 than UP2382 variety of wheat under N-deficiency condition. Nitrogen treatments enhanced the expression of this protein in both the varieties. Increase in the gene expression of glyceraldehyde-3-phosphate dehydrogenase has been reported earlier in *Lycopersion esculentum* roots with increased nitrate supply (Wang et al. 2001). Expression of the protein itself was increased in grain of *Triticum aestivum* which received an increased supply of ammonium nitrate (Flæte et al. 2005). However, there was marked variation in the expression level of this protein by N treatments in our study. In VL616, there was continuous increase in the expression of this protein with the increase in the level of N treatments. Contrary to this, the expression of this protein in UP2382 increased by 2-fold at 10 mM N, and there was no further increase in the expression level with the increase in N treatment upto 25 mM (Fig. 2).

Nitrogen metabolism

Nitrate reductase (NR, EC 1.6.6.1–3) enzyme identified in our study is the first and the most important enzyme in the pathway of nitrate reduction to nitrite and then to ammonium (Ahmad and Abdin 1999; Rosales et al. 2011). The NR activity determines the rate of inorganic nitrogen assimilation in plants and has a crucial influence on nitrogen metabolism, with protein being the main product used for organogenesis and plant growth. The expression of NR enzyme in our study was reported to increase with increased application of N treatments in VL616. Whereas, in UP2382 no significant change in the expression of this protein spot was observed even at higher N treatment (Fig. 2). Studies by Debouba et al. (2007), Mokhele et al. (2012), Averinaa et al. (2014) have shown that higher NR activity is related to enhancement of some mechanisms towards salt tolerance. Contrary, no change in the expression of NR enzyme in UP2382 with the increase in N treatments does show the normal requirement of nitrate levels for this variety.

Glutamine synthetase (GS; EC 6.3.1.2) enzyme identified in our study was found to be a key enzyme in nitrogen metabolism (Cai et al. 2009). GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate (Glu) to yield glutamine (Gln); GOGAT transfers the amide group of Gln to a-ketoglutarate (2-OG) to subsequently produce Glu (Ireland and Lea 1999). Gln then serves as one of nitrogen donors for the biosynthesis of organic nitrogenous compounds, such as amino acids, nucleotides, and chlorophyll. Thus, the GS enzyme is likely to be a major check point controlling plant nitrogen assimilation (Coque and Gallais 2006; Kichey et al. 2006). In our study we reported that UP2382 (low N-stress sensitive) wheat variety showed enhanced expression of GS in response to increased levels of N treatments. This enhanced GS expression provides enough amounts of GS to incorporate NH_4^+ into organic compounds (Wallsgrove et al. 1987). In VL616 (low N-stress sensitive) variety of wheat, no significant change in the expression of this protein spot, however, was observed in response to increased levels of N treatments (Fig. 2). This showed constitutive nature of GS in this variety. The constitutive nature of the enzyme whatever the N nutrition was also highlighted by the identification of the N-responsive chromosomal region following recurrent selection (Coque and Gallais 2006). The finding in both maize and wheat (Kichey et al. 2006) suggested that GS enzyme activity is representative of the plant N status.

Expression of porphobilinogen deaminase was upregulated with the increase in the level of N treatments in UP2382 wheat variety, whereas in VL616 the expression level decreased continuously with the increase in N levels, reaching to non-detectable level at 25 mM N treatment. Porphobilinogen (PBG) deaminase catalyzes the polymerization of four PBG monopyrrole

units into the linear tetrapyrrole hydroxymethylbilane, leading to tetrapyrrole biosynthesis (Moulin and Smith 2005). Higher plants contain four classes of tetrapyrroles, namely, chlorophyll, heme, siroheme, and phytochromobilin. Siroheme is a prosthetic group of nitrite and sulphite reductase that plays central roles in nitrogen and sulphur assimilation, respectively. Nitrogen supply has been reported to affect the tetrapyrolle synthesis (Tandeau de Marsac and Houmard 1993). The role of sirohaem in nitrite reductases means that it is responsible for the assimilation of nitrogen in the biosphere. Decrease in the level of expression of this protein in N-stress sensitive varieties (Fig. 3) under N treatments may be due to the fact that being high nitrogen sensitive, this varieties contains adequate amount of required nitrate reductase and nitrite reductase enzymes for sensitive nitrogen assimilation.

Sulphur metabolism

Putative Glyoxalase 1 enzyme, also known as lactoylglutathione lyase, belonged to this functional group. It catalyses a key limiting step in the production of glutathione, which is a thiol implicated in resistance to various environmental stresses. An increase in its expression was observed with increase in N treatments in both VL616 and UP2382 varieties (Fig. 2). This result showed an interaction between the varieties and the N treatment levels, indicating the importance of N nutrition for assimilation of sulphur assimilation (Grove et al. 2009). There are reports showing that sulphur and nitrogen interact with each other in such a way that lack of one reduces the uptake and assimilation of the other. The interaction of N and S has been shown to affect the yield and productivity of the crop. A constructive response of N and S interaction on leaf area index, rate of photosynthesis and biomass production have also been observed. In wheat and canola, highest dry matter yields were achieved where the ratios of available N:S in soil and plant tissue ranged between 5 and 13, respectively (Qian and Schoenau 2007).

Oxidative defence system

Cytochrome P450 proteins, named for the absorption band at 450 nm of their carbon-monoxide bound form, are one of the largest superfamilies of enzyme proteins. They contribute to vital processes such as carbon source assimilation, biosynthesis of hormones and of structural components of living organisms, and also carcinogenesis and degradation of xenobiotics. In plants, chemical defence seems to be a major reason for P450 diversification. Oxidative detoxification of a number of herbicides in plant tissues is also achieved by a cytochrome P450-dependent monooxygenase system (Sandermann 1992). Expression of this protein in UP2382 variety increased at low N treatments, but decreased at high N treatments. Contrary to this, the expression of this protein was increased by all levels of N treatments in VL616 variety (Fig. 2). During sorghum seedling development, application of nitrate increased the activities of cytochrome P450s and this activity was correlated well with the corresponding mRNA levels. This indicates that the activity of this protein in plants is controlled at the transcriptional level and most likely by common transcriptional control mechanisms by nitrogen (Busk and Møller 2002).

Elongation factor belongs to the GTP-binding elongation factor family. The protein EF-Tu is highly conserved and plays a role in polypeptide elongation during protein synthesis. It is a GTP binding protein (Young and Bernlohr 1991) and functions in the binding and transport of codon specific tRNAs to aminoacyl site on the ribosome. However EF-Tu has been shown to have functions other than its role in polypeptide elongation (Young and Bernlohr 1991). EF-Tu has been found to function in the refolding of denatured proteins. Kudlicki et al. (1997) reported a chaperone like property of bacterial Ef-Tu in the refolding of denatured rhodanese. Ef-Tu interacts with unfolded and denatured proteins and forms stable complexes. EF-Tu was also found to protect citrate synthase and α- glucosidase from thermal aggregation, and the chaperone activity of the EF-Tu was shown to occur at very low levels (20- fold lower than cellular concentrations) (Cladas et al. 1998).

Quinone oxidoreductase and flavonoid synthesis like protein, are the flavo protein involved in electron transport system and stress protecting agents. Flavonoids occur extensively in plants and are a biologically important and chemically diverse group of secondary metabolites (Treutter 2005). Quinone oxidoreductase and flavonoid synthesis like proteins might be involved in maintenance of the abundant plant quinines in the reduced state in the cell. It has been speculated that the quinone/quinol equilibrium may function in cells as a means of modulating secondary metabolism, host-parasite interactions and growth-regulating mechanisms (Spitsberg and Coscia 2005). They are also beneficial for the plant itself as physiologically active compounds, antimicrobial, antiherbivory, protectant against UV, in general, play a significant role in plant resistance (Dixon and Pasinetti 2010).

Increasing level of these proteins with increase in N conditions in both the wheat varieties (Fig. 2) suggests its importance in sensing and counteracting the generated oxidative stress via the increased photorespiration and energy generation mechanisms.

Chitinase-2 also plays role in cell defence. Since the majority of chitinases are induced by stress factors, mainly by infection, and some isoforms show antifungal properties in in vitro assays, the role of chitinases is usually considered to be an active or passive defence mechanism against pathogens (Regalado et al. 2000). Developmentally-regulated

induction of these enzymes in healthy tissues, e.g., in germinating seeds or ripening fruits, has been interpreted as a plant defence mechanism against possible pathogen attacks on sensitive or mechanically-unprotected organs. However, it is now supposed that chitinases in healthy plant tissues can play a role other than that in defence functions. Chitinases may regulate processes of growth and development by generating or degrading signal molecules, as during the nodulation process, where the bacterial lipochitooligosaccharides (Nod factors) are degraded by chitinases (van der Holst et al. 2001).

Glutathione S-transferases (GSTs; EC 2.5.1.8) represents a family of diverse group of enzymes that plays role in catalization of reduced tripeptide glutathione (GSH; g-Glu-Cys-Gly) into a wide variety of hydrophobic and electrophilic substrates (Dixon and Edwards 2010; Liu et al. 2014). Act as a detoxification enzymes of a variety of xenobiotic substrates like pathogen attack, oxidative stress, and heavy-metal toxicity. Dixon et al. (2009) characterized the GSTs in *Arabidopsis thaliana* and found it is related to high tolerance to abiotic stress. Yu et al. (2003) showed the transgenic tobacco plants that were overexpressing cotton glutathione were resistant to methyl viologen. Liu et al. (2014) identified 37 GSTs from *Physcomitrella* and showed their diverse functional traits in response to abiotic stress. In our study an increase in the enzyme expression with increase in N treatments in VL616 shows the importance of the enzyme in combacting the growing oxidative stress in the plant. In UP2382, no significant change was observed in GST expression with increase in N treatments (Fig. 2).

Phosphatidylinositol-4- phosphokinase (PL-4-PK) family protein has been identified as an important signaling molecule in plants. Unlike structural lipids signaling lipids are also present. They respond rapidly to certain stimuli. They trigger the production of second messengers like Ca_2^+ and cAMP in response to stress factors (Wang 2004; Testerink and Munnik 2005). In our study the PL-4-PK enzyme was found to increase in VL616 whereas no significant change in the expression was observed in UP2382 with increase in N treatments (Fig. 2).

STI1 (Stress-inducible protein 1) also known as HOP (heat shock protein 70/90 organising protein) are best characterized as Hsp70/Hsp90 co-chaperones in their organisms. Hsp act as molecular chaperones, responsible for protein synthesis, targeting, maturation and degradation. Under normal growth conditions, they contribute to cellular homeostasis, but under unfavourable environmental conditions, they work in direction for inducing adaptive mechanism in plants (Kotak et al. 2007). Chen et al. (2010) showed that Hop/Sti1 and Hsp90 are present at the plasma membrane. Also that the Hop/Sti1-Hsp90 chaperone complex plays a vital role in the maturation and transport of pattern recognition receptors. PRRs represents a critical first step of innate defence in plants.

Salt inducer proteins (SIP) and its homologs have functional relevance towards abiotic stress and production of reactive oxygen species. Functional characterization of SIP by Kumar et al. (2007) showed that after silencing of SIP genes in *Nicotiana benthamiana*, there was enhanced membrane damage and chlorophyll degradation under stress compared with wild-type plants. They also stated that these SIP homolog were likely to be a protein kinase and is found to have 77 % nucleotide identity with one of a membrane associated protein induced under salt in tobacco (TIGR gene ID no. NP917780). These are also known to have an enhanced expression during salt stress.

Oxygen evolving enhancer (OEE) protein /complex is related to PSII complex and photosynthetic oxygen evolution in higher plants (Mayfield et al. 1987). Murota et al. (1994) demonstrated the significance of OEE2 in salt tolerant in photoautotrophically cultured green tobacco cells. It was observed that no dissociation of OEE2 from thylakoid membranes in NaCl-adapted cells occurred, whereas, this dissociation of the OEE2 was observed in unadapted cells under salt stress, reflecting the strong association of OEE2 in salt tolerant cells. Study by Tada and Kashimura (2009) in *Bruguiera gymnorrhiza* also showed upregulation of OEE2 in response to salt stress, suggesting its role in repair the injury of the PSII complex and to maintain the oxygen evolution reaction. In our study, increase in the expression of OEE was observed in VL616 with the increase in N treatments, whereas, no change in the expression was observed in UP2382 (Fig. 2).

Protein synthesis

Protein disulphide isomerise (PDI) are involved in synthesis, folding, and sorting of proteins in the endoplasmic reticulum (ER). They play important roles in the maturation of plasma membrane and storage proteins. Adverse environments conditions disturb the ER physiological processes leading to unfolding or misfolding of proteins. Thus, PDI proteins are essential to assist folding of nascent polypeptides to form specific functional proteins. These functional proteins regulate metabolic activities and results in an increase in plant resistance (Zhu et al. 2014). In our case, PDI showed no change in the expression in VL616 with increase in N treatments whereas increase in the expression of PDI was observed in UP2382 (Fig. 2), suggesting the requirement of high levels of N for carrying out processes in N-sensitive genoptye, UP2382.

Chromosome dynamics

Structural maintenance of chromosome protein (SMC3) participates in generating dynmic tension between microtubules and sister chromatids (Tanaka et al. 2000). Mengiste et al. (1999) study, carried out in *Arabidopsis thaliana* under genotoxic stress, showed the evidences for the involvement

of an SMC protein in recombinational DNA repair. Chromo domain containing protein initiate DNA methylation *de novo* at sites with certain histone modifications and target silenced transposons and heterochromatin during replication (Garg et al. 2014). Mitochondrial cytochrome C maturation (ccmFC) is involved in cytochrome biogenesis. It is involved in the crucial and final step of cytochrome c maturation, i.e., covalent attachment of heme to apo-cytochrome C (Giege et al. 2008). In case of our study, the above proteins showed no change in their expression with increase in N treatments in VL616. Contrary to this, the protein expression increased with increase in N treatments in UP2382 (N-sensitive) genotype suggesting the requirement of more nitrogen for biological systems to work properly (Fig. 2).

In our study, we observed a low correlation between the changes in gene and protein expression levels, as also noted by Yan et al. (2006) in rice for glutamine synthetase (GS) which has maintained a constant expression under low, moderate and high N treatments. It is observed in higher plants that inorganic nitrogen from the soil is initially converted into organic nitrogen by two enzymes, glutamine synthetase and glutamate synthase. GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate (Glu) to yield glutamine (Gln); GOGAT transfers the amide group of Gln to a-ketoglutarate (2-OG) to subsequently produce Glu (Ireland and Lea 1999). Glutamine then serves as one of nitrogen donors for the biosynthesis of organic nitrogenous compounds, such as amino acids, nucleotides and chlorophylls. Thus, the GS enzyme is likely to be a key factor, controlling plant nitrogen assimilation. The constitutive nature of the GS was also highlighted by the identification of the N-responsive chromosomal region following recurrent selection (Coque and Gallais 2006). Earlier findings in maize (Hirel et al. 2005) and wheat (Kichey et al. 2006) have suggested that GS enzyme activity is representative of the plant N status. In maize, Hirel et al. (2001) have highlighted the putative role of glutamine synthetase (GS) in kernel productivity using a quantitative genetic approach, since QTLs for the leaf enzyme activity have been shown to coincide with QTLs for yield. One QTL for thousand kernels weight was coincident with a GS (*Gln1-4*) locus, and two QTLs for thousand kernel weight and yield were coincident with another GS (*Gln1-3*) locus. Such strong coincidences are consistent with the positive correlation observed between kernel yield and GS activity (Gallais and Hirel 2004).

Conclusion

Constructing 2-D gel reference map for use in comparative proteomics among cultivars for N response will be necessary for precise identification of potential molecular protein markers to assist the breeders for screening N-sensitive varieties and help in understanding how wheat adapts to low N availability. Glutamine synthetase might act as suitable targets for genetic manipulation for improving nitrogen use efficiency of crop plants. The study presented here is a novel approach for coupling the genetic-based selection (high nitrogen sensitive and low nitrogen sensitive) of N-related varieties with proteomics to unravel the molecular differences in wheat plants grown under different N levels.

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