

RESEARCH ARTICLE

A comparative proteome approach to decipher the mechanism of rice adaptation to phosphorous deficiency

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Mineral deficiency limits crop production in most soils and in Asia alone, about 50% of rice lands are phosphorous deficient. In an attempt to determine the mechanism of rice adaptation to phosphorous deficiency, changes in proteome patterns associated with phosphorous deficiency have been investigated. We analyzed the parental line Nipponbare in comparison to its near isogenic line (NIL6-4) carrying a major phosphorous uptake QTL (Pup1) on chromosome 12. Using 2-DE, the proteome pattern of roots grown under 1 and 100 μ M phosphorous were compared. Out of 669 proteins reproducibly detected on root 2-DE gels, 32 proteins showed significant changes in the two genotypes. Of them, 17 proteins showed different responses in two genotypes under stress condition. MS resulted in identification of 26 proteins involved in major phosphorous deficiency adaptation pathways including reactive oxygen scavenging, citric acid cycle, signal transduction, and plant defense responses as well as proteins with unknown function. Our results highlighted a coordinated response in NIL in response to phosphorous deficiency which may confer higher adaptation to nutrient deficiency.

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1 Introduction

Mineral deficiency limits crop production in all types of soil around the world. A given mineral element is considered as essential, when plants cannot complete their life cycle due to

its deficiency. Based on these criteria, 16 elements so far are considered as to be of great importance including: carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, manganese, zinc, copper, boron, molybdenum, and chlorine. Nutrient deficiency and stress in the plant occurs when a nutrient is insufficient in the growth medium and/or it cannot be absorbed and assimilated by the plants due to unfavorable environmental conditions. After nitrogen, phosphorus is the most important inorganic plant nutrient. Because of its tendency for tight binding in the soil, it is one of the least available nutrients. It is estimated that about 5.7 billion hectares and almost 50% of rice soils are currently phosphorus deficient [1, 2]. This deficiency can be compensated through fertilizer application but many resource-poor farmers may not have

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Abbreviations: ASR1, acid- and stress-inducible protein; IDH, isocitrate dehydrogenase; NIL6-4, near isogenic line; OsRMC, *Oryza sativa* root meander curling; RLK, receptor-like protein kinase; SSR, simple sequence repeats

access to phosphorus fertilizers. The cheap source of phosphates, such as phosphate rocks, will be depleted toward the end of the 21st century [3]. On the other hand, excess phosphorus added to soil can pollute watercourses and contribute to the process of eutrophication [4]. It is therefore, essential to minimize the application of phosphorus fertilizers through developing efficient cultivars capable of either acquiring phosphorus or using phosphorus more efficiently.

Plants have adapted to low phosphorus availability through developing several mechanisms that optimize phosphorus acquisition efficiency and internal phosphorus use efficiency or increase root interception of Pi through changes in root growth patterns [5]. However, the role of these mechanism in enhancing phosphorus-uptake in rice and molecular mechanisms underlying rice tolerance to phosphorus deficiency is yet to be revealed. Proteomics proved to be a powerful approach in elucidating molecular mechanisms involved in plant adaptation to stresses and mineral deficiency [6]. This approach has been applied to study the response of a maize genotype to phosphorus deficiency [7]. The authors identified several mechanisms involved in phosphorus sensing and the regulation of plant adaptation including phytohormone biosynthesis, carbon and energy metabolisms, protein synthesis and fate, signal transduction, cell cycle, cellular organization, defense, and secondary metabolism. Proteomics can be even more efficient in identifying stress tolerance genes and mechanisms if is applied to contrasting tolerant and susceptible genotypes. However, the major limitation with this approach is that we could spend a lot of time studying genes that related to different genetic background of two genotypes rather than their tolerance/sensitivity to stress. Using a near isogenic line (NIL) may offer a solution to this limitation.

For phosphorus-deficiency tolerance, two QTL mapping studies have been reported in rice. Wissuwa *et al.* [8] used a backcross inbred population with the recurrent parent Nipponbare (*japonica*, sensitive) and the landrace Kasalath (*indica*, tolerant). They detected a major QTL on chromosome 12 for phosphorus uptake, phosphorus-use efficiency, dry weight, and tiller number. Ni *et al.* [9], using RILs from the cross of IR20 (tolerant) with IR55178-3B-9-3 (sensitive), found a similarly strong QTL in the same location. NILs carrying the donor allele from Kasalath in a Nipponbare genetic background increased phosphorus uptake by three- to four-fold relative to the recurrent parent. This is because the NILs maintain relatively high root growth rates despite phosphorus deficiency, whereas low phosphorus availability severely reduced root growth in Nipponbare [10]. This QTL, subsequently designated *Pup1*, was found to explain close to 80% of the phenotypic variation in a secondary mapping population [11].

In order to elucidate the mechanism of rice adaptation to phosphorus deficiency and changes in protein synthesis associated with phosphorus deficiency, we analyzed the parental line Nipponbare in comparison to its NIL6-4 carrying a major phosphorus uptake QTL (*Pup1*) on chromosome 12.

Our results revealed a coordinated response in NIL6-4 response to phosphorus deficiency particularly in enzymes involved in reactive oxygen scavenging and organic acid production which may confer higher adaptation to nutrient deficiency.

2 Materials and methods

2.1 Plant growth and treatments

Experiments were conducted with genotypes Nipponbare (intolerant of phosphorus deficiency) and the derived NIL6-4 that is tolerant to phosphorus deficiency due to the presence of the phosphorus uptake QTL *Pup1* from donor parent “Kasalath” [8]. NIL6-4 was derived from a F₃ family of a NIL-*Pup1* × Nipponbare cross that had been used in substitution mapping of *Pup1* [11].

Seeds of Nipponbare and NIL6-4 were obtained from the International Rice Research Institute (Manila, Philippines). Surface sterilized seeds were soaked and pregerminated for 72 h and sown individually on holes of a Styrofoam board with a nylon net bottom (24 germinated seeds were sown in an area of 350 × 230 mm²). The Styrofoam board was then floated on rectangular plastic trays (36 × 24 × 25 cm³) filled with 18 L of complete strength Yoshida nutrient solution [12]. Trays were placed in a greenhouse under natural light. The pH of the solution was daily adjusted to 5.0. The nutrient solution was changed once a week and water loss due to evapotranspiration was compensated for by daily addition of deionized water. The experiment was conducted using a completely randomized design with three replicates and two phosphorus treatments: high phosphorus supply (100 μM phosphorus) and phosphorus deficiency (1 μM phosphorus). Plants can deplete phosphorus from such low concentration solutions within 24–48 h, therefore 1 μM phosphorus was added to culture solution of the 1 μM phosphorus treatment on days 3 and 5 of the weekly cycle to maintain a constant low level of phosphorus. The weekly phosphorus supply per 18 L tray was equivalent to 55.8 mg phosphorus in the 100 μM phosphorus treatment and 1.7 mg phosphorus in the 1 μM phosphorus treatment.

Number of tillers, root and shoot lengths and fresh weights were measured upon harvesting after an 80-day growth period. Roots and shoots were immediately frozen in liquid nitrogen and kept at –80°C until proteomics analysis. Root and shoot dry weights were determined after drying for 48 h in an oven at 60°C.

2.2 Protein extraction

Root samples (1 g) collected from three replications were pulverized to a fine powder with liquid nitrogen and a mortar and pestle. The powder was resuspended directly in 2.5 mL of Tris pH 8.8 buffered phenol and equal volume of extrac-

tion buffer containing 0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, and 0.9 M sucrose. The homogenate was mixed for 30 min at 4°C and centrifuged afterwards for 15 min at 5000 × g and 4°C. The phenol phase was removed and proteins were precipitated with five volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at –20°C for 16 h. Subsequently, the homogenate was centrifuged for 10 min as described above and the protein pellet was thoroughly washed twice in 20 mL of 0.1 M ammonium acetate in 100% methanol, followed by two washes in ice-cold 80% acetone and 10 mM DTT. The final pellet was lyophilized and the remaining powder was then solubilized in lysis buffer (9.5 M urea, 2% w/v CHAPS, 0.8% w/v Pharylyte pH 3–10, 1% w/v DTT). The protein concentration was determined by the Bradford assay (BioRad, Hercules, CA, USA) with BSA as the standard.

2.3 2-DE

IEF was carried out on IPG 24 cm pH 4–7 L strips on Multiphor II (GE Healthcare). For analytical and preparative gels, 150 µg and 1.5 mg of protein were loaded, respectively. The running condition was as follows: 500 V for 1 h, followed by 1000 V for 1 h, and finally 3500 V for 16 h. The focused strips were equilibrated twice for 15 min in 10 mL equilibration solution. The first equilibration was performed in a solution containing 6 M urea, 30% w/v glycerol, 2% w/v SDS, 1% w/v DTT, and 50 mM Tris-HCl buffer, pH 8.8. The second equilibration was performed in a solution modified by the replacement of DTT by 2.5% w/v iodoacetamide. Separation in the second dimension was performed by SDS-PAGE in a vertical slab of acrylamide (12% total monomer, with 2.6% crosslinker) using a Dodeca Cell (BioRad). The analytical and preparative 2-DE gels were stained with silver nitrate [13] and CBB G250 [14], respectively.

2.4 Image analysis

The silver stained gels were scanned at a resolution of 600 dpi on a GS-800 densitometer (BioRad). The scanned gels saved as TIF images for subsequent analysis. Spot quantitation was carried out using the Melanie 3 software (GeneBio, Geneva, Switzerland). After the image treatment, spot detection, protein quantification, and spot pairing were carried out based on Melanie-3 default settings. Then, spot pairs were investigated visually and the scatter plots between gels of each data point were displayed to estimate gel similarity and experimental errors. The molecular masses of proteins on gels were determined by coelectrophoresis of standard protein markers (Amersham Pharmacia Biotech) and *pI* of the proteins were determined by migration of the protein spots on 18 cm IPG (pH 4–7) strips. One 2-D gel *per* plant was run and percent volume of each spot was estimated and analyzed. Considering the two genotypes (Nipponbare and NIL6-4) and two treatments (normal and stressed), four genotype × treatment combinations were analyzed by one-

way ANOVA. Only the statistically significant differences ($p \leq 0.05$) which were consistently present in all three replications were considered for further analysis.

2.5 Protein identification by MS

Protein spots of interest were cut from the 2-DE gels and destained for 1 h at room temperature using a freshly prepared wash solution consisting of 100% ACN/50 mM ammonium bicarbonate (NH_4CHO_3) (50:50 v/v). Wash solution was removed and spots were left to dry for 30 min at 37°C. Proteins were digested using a trypsin solution containing 12 ng/µL (10 µL) trypsin in 50 mM ammonium bicarbonate solution. This reaction was left to proceed for 45 min at 4°C. Excess of trypsin solution was removed and 20 µL of 50 mM ammonium bicarbonate was added before gel pieces were placed in a 37°C incubator overnight.

All samples were desalted and concentrated with a 10 µL ZipTip_{C18} (PerfectPure C-18 Tip, Eppendorf), following the instructions provided by manufacturer. Peptide were eluted in a volume of 0.7 µL using a concentrated solution of CHCA (5 mg/mL) in 70% ACN and 0.1% TFA in water and deposited onto the MALDI target plate and left to dry in air. Peptide mixtures were then analyzed using MALDI-TOF/TOF-MS. Before each analysis, the instrument was calibrated with the Applied Biosystems 4700 Proteomics Analyzer Calibration Mixture. Data Interpretation was carried out using the GPS Explorer Software (Applied Biosystems) and automated database searching was carried out using the MASCOT program (Version 2.1, Matrix Science, London, UK). Combined MS-MS/MS searches were conducted with the selection of following criteria: NCBI nr database (release 20061019; 4 075 097 sequences; 1 404 825 948 residues), all entries, parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modification). The Probability score (95% confidence level) calculated by the software was used as criteria for correct identification.

2.6 RNA extraction and real-time PCR analysis

Total RNAs of each growth condition (normal and stressed) were extracted from similar samples to those used for proteome analysis in three independent extractions using Trizol reagent (Invitrogen, Life Technologies). Two microliters of each RNA sample was used for constructing cDNA using an iScript cDNA Synthesis kit (BioRad). Primer pairs were designed using Beacon Designer 2 software (Table 1). Gene expression was assayed using the iCycler iQ, Multicolor Real-Time PCR Detection System (BioRad) and iQ SYBR Green Supermix kit (BioRad). Reaction conditions (20 µL volumes) were optimized by changing the primer concentration and annealing temperature to minimize primer-dimer formation and to increase PCR efficiency. The following PCR profile

Table 1. The list of primers used for qRT-PCR

Spot no.	Gene symbol	Sense primer (5'-3')	Antisense primer (5'-3')
95, 58	Q5ZCQ9	GCC GAC ATT GTG ACA TAC C	CAG AGC GTC CAA AGA AAC C
63, 70	Q0JMY8	CCA TTG CCT TCA ACT ACA TCG	TCA GAG GAG CCC AGT TTA ATC
10	P93407	CCG ACG CCA CCA AGA AGG	GCC GTG AAG TCC AGG AGT AAG
291	Q0J0H4	ATG GGT GAA GAG GTG GGA GAG	CCA ACA GCA ATG CCA GTA AAC C
412	Q10S34	CTG GTG CTG AGT ACG GTA GTG G	CAA GGT TGC TGC GGT GAA TCC
218	P14654	ACT GCT ACA CGC CAC AAG G	ACT CCT GCT CAA TAC CGT ACC
273	Q6F361	CTA TCC TGC CAC TGT TCT CG	AAC CCT TTC CAG CCT TTG C
27	Q60EJ3	TGG CGG TCA AGG TCT ACG	CCC AGC ACC TCC TCT GAG
18S	Reference gene	ATG ATA ACT CGA CGG ATC GC	CTT GGA TGT GGT AGC CGT TT

was used: 2 min at 95°C, (10 s at 95°C, 45 s at 54°C, 10 s at 72°C) × 50, and 1 min at 72°C followed by recording of a melting curve. The lack of primer-dimer or nonspecific product accumulation was checked by melt-curve analysis. Each run included standard dilutions and negative reaction controls. The mRNA expression level of each gene of interest and of the ribosomal protein 18S rRNA, chosen as a housekeeping gene, was determined in parallel for each sample. Results were expressed as the normalized ratio of mRNA level of each gene of interest over housekeeping gene using the difference between threshold cycle values or $\Delta\Delta C_t$ method [15]. C_t values for individual target gene were calculated and the ΔC_t average for the housekeeping gene (18S rRNA) was treated as an arbitrary constant and used to calculate $\Delta\Delta C_t$ values for all samples. The induction fold resulted in three independent pools for each target gene was averaged and the SEM was calculated.

2.7 RFLP and simple sequence repeats (SSR) analyses

For RFLP analysis, total DNA was extracted from the leaf tissue of each selected individual according to the CTAB method [58]. The DNA was digested with eight restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RV, *Hind*III, *Apa*I, *Dra*I, *Eco*RI, and *Kpn*I. Electrophoresis and Southern blotting were performed according to Kurata *et al.* [16]. From the high-density linkage map we selected as probes 170 clones that were distributed evenly in the 12 chromosomes. Southern hybridization and detection were carried out according to the protocols of the ECL direct nucleic acid labeling and detection system (GE Healthcare).

NIL6-4 was further surveyed with 65 SSR markers located at putative Kasalath introgressed regions; amplifications of the sample DNA with SSR markers were performed using 40 ng of DNA. The PCR mix was prepared on 96-well polycarbonate plates and the thermocycling used follows the method described by Panaud *et al.* [17]. PCR amplification was performed using a PTC-100 Thermal Cycler (MJ Research, Waltham, MA, USA). Amplification reaction products were then loaded into an 8% w/v polyacrylamide gel for

size separation using a minivertical electrophoresis system (CBS Scientific). DNA fragments were stained with ethidium bromide and visualized with a UV transilluminator and documented using Quantity One computer program (Gel Doc EQ, BioRad) then the bands were scored manually.

3 Results and discussion

3.1 Physiological data analysis

In the present study, the response to phosphorus deficiency was investigated in the parental line Nipponbare and the derived line NIL6-4. Samples were grown under normal (100 μ M phosphorus) and phosphorous deficient condition (1 μ M phosphorus). Based on the analysis of variance, the overall effects of phosphorus deficiency were significant as determined by number of tillers and root dry matter (Fig. 1). The phosphorous deficiency resulted in markedly decreased plant root weight and tiller number. This reduction was more pronounced in Nipponbare compared to NIL6-4 (Fig. 1), supporting previous reports that Pup1-NILs maintained relatively high root growth rates under low phosphorus availability compared to Nipponbare.

3.2 Proteome response

Using a 2-DE based proteomics approach, 669 protein spots were reproducibly detected (Fig. 2). Considering the two genotypes (Nipponbare and NIL6-4) and two treatments (normal and stressed), the spots with significant differences were categorized in different groups including spots which showed differences at level of genotype (G), treatment (T), genotype × treatment (GT), G and T, G and GT (G/GT), T and GT (T/GT), or T and G and GT (T/G/GT).

Thirty-four proteins showed reproducible and statistically significant changes in response to phosphorus deficiency. Of them, 15 protein spots (spots 19, 27, 38, 39, 40, 58, 90, 95, 158, 169, 273, 274, 348, 412, and 660) belonged to T group whereas the expression of 19 responsive proteins

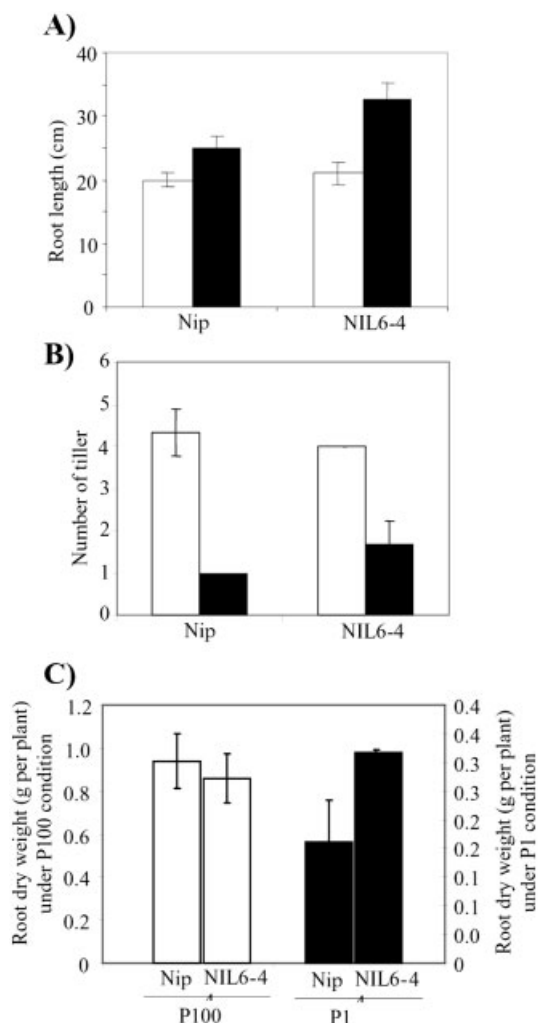


Figure 1. Changes in root length (A), number of tiller (B), and root dry matter (C) in Nipponbare and NIL6-4 in under normal (P100) and phosphorus deficiency (P1) conditions.

differed in NIL6-4 compared to Nipponbare (Fig. 3). Of them, two spots (spots 334 and 416) were in G group, three spots (spots 16, 103, and 488) were in T/G group, three spots (spots 226, 269, and 291) belonged to T/GT group, and 11 spots (spots 2, 8, 10, 14, 17, 59, 63, 70, 102, 218, 504) were in T/G/GT group.

MS analysis resulted in identification of 25 proteins (Table 2). The expression pattern of identified proteins was presented in Fig. 4. The identified proteins could be classified in different categories including oxygen scavenging, citric acid cycle, signal transduction, stress defense, phosphorus metabolism as well as proteins with unknown function demonstrating that not only a single pathway is responsible for the adaptation of limiting phosphorous availability but an interplay of different routes might control this phenomenon. The possible role of the identified proteins in NIL6-4 will be discussed in more details below.

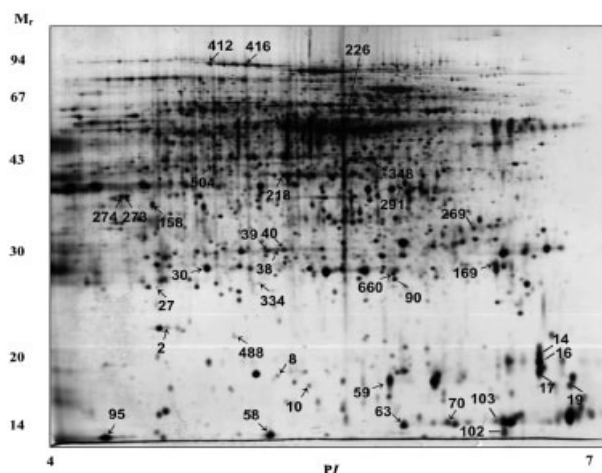


Figure 2. Silver stained 2-D gel of proteins extracted from root of Nipponbare. In the first dimension, 150 μ g of protein was loaded on an 18 cm IPG strip with a linear gradient of pH 4–7. In the second dimension, 12% SDS-PAGE gels were used. Thirty-two spots showed significant response to phosphorus deficiency. The arrows indicate proteins identified by MS. Spots 334 and 416 showed significant difference in two genotypes under normal condition but did not change in response to stress.

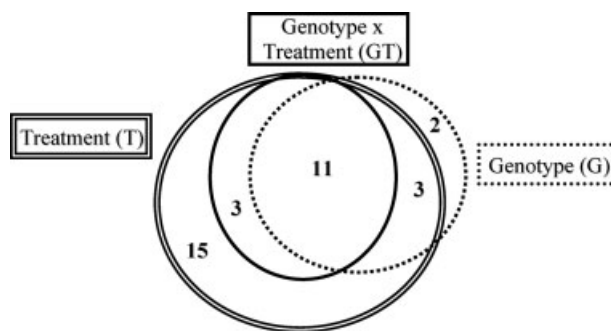


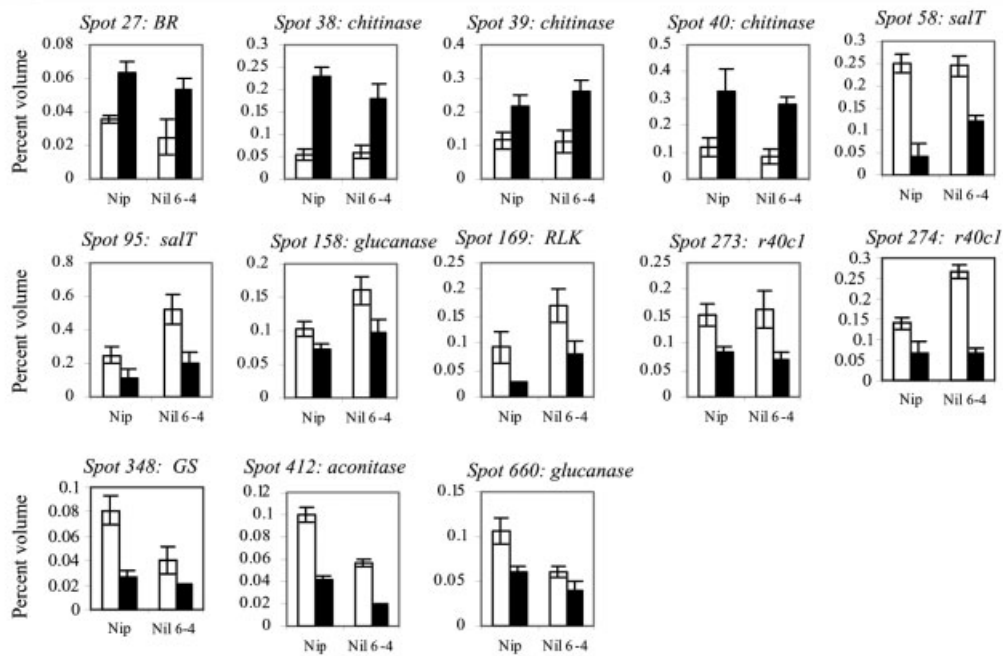
Figure 3. Venn diagram of protein spots showed significant differences at three levels including treatment (T), genotype (G), and genotype \times treatment (GT). Regions of overlap between circles indicate number of spots with significant differences at two (T/G, T/GT, G/GT) or three levels (T/G/GT). Regions that do not overlap between circles indicate number of spots significantly changed at one level (T, G, or GT) in response to stress.

3.2.1 Proteins with different response in two genotypes

3.2.1.1 Coordinated expression of proteins involved in citric acid cycle in NIL6-4

Production of organic acids in root and their excretion to soil is a well-known mechanism by which plants cope with nutrient deficiencies particularly Pi deficiency. It has been shown that the excretion of organic acids into the rhizosphere led to an increased phosphorus solubilization and thus to an enhanced phosphorus uptake by rice from a phosphorus-deficient soil [18].

Proteins significantly changed in response to P deficiency without significant difference in two genotypes (T*)



Protein spots significantly changed in response to P deficiency with significant difference in two genotypes and significant interaction between genotype and P levels (T*/G*/GT*)

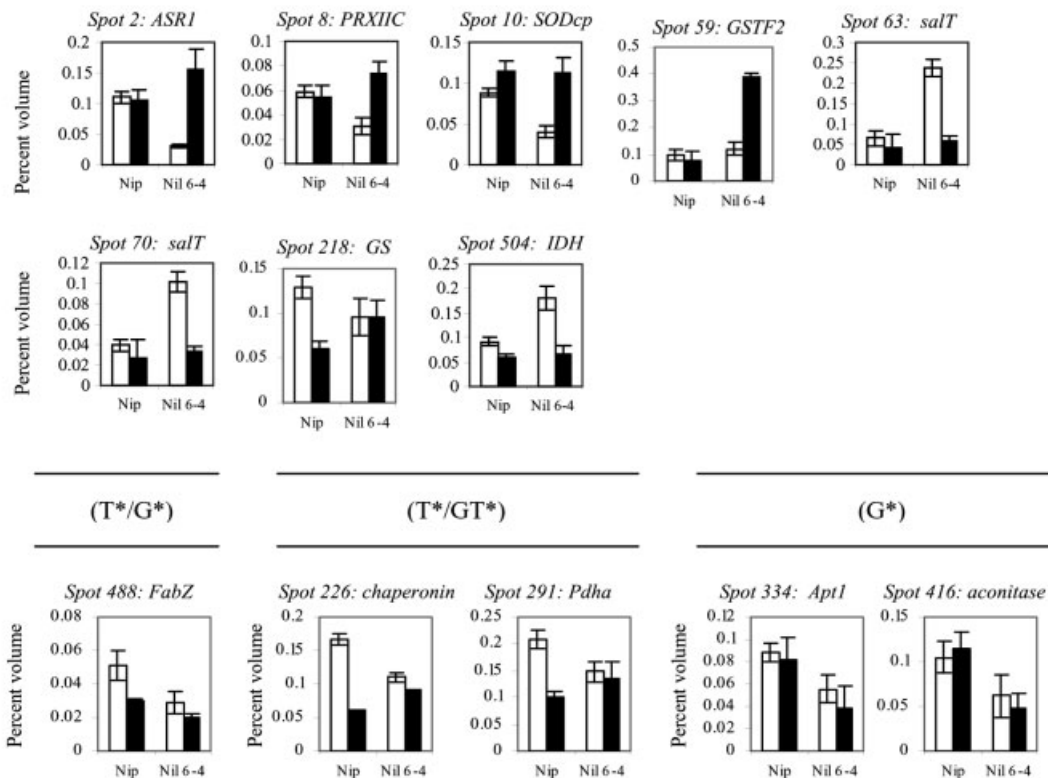


Figure 4. The abundance (percent volume) of 25 identified phosphorus deficiency responsive proteins in Nipponbare (and NIL6-4) under normal (open bar) and stress conditions (solid bar).

Table 2. Proteins identified by MS (MALDI TOF/TOF)

Spot ^{a)} ID	Protein name	Entry ^{b)}	Score/% coverage ^{c)}	pI/MW Theo. ^{d)}	pI/MW Exp. ^{e)}	PMF/ MS-MS ^{f)}	Chr. location
2	Abcisic ASR1	Q53JF7	139/70	6.20/15	6.31/24	14/0	11
8	Peroxiredoxin-2C (PRXIIC)	Q9FR35	195/91	5.58/17	5.76/20	18/1	1
10	Superoxide dismutase (Cu–Zn), chloroplast precursor (SODcp)	P93407	75/46	5.79/20	5.61/19	12/0	8
27	Putative 1,4-benzoquinone reductase (BR)	Q60EJ3	76/58	6.30/22	6.36/27	13/0	5
38	Putative chitinase	Q5WMX0	117/37	6.08/32	5.76/32	14/0	5
39	Putative chitinase	Q5WMX0	114/44	6.08/32	5.81/32	14/0	5
40	Putative chitinase	Q5WMX0	159/30	6.08/32	5.73/32	14/0	5
58	Putative salT (salT)	Q5ZCQ9	137/39	7.72/28	5.79/15	6/2	1
59	Glutathione S-transferase (GSTF2)	O82451	80/51	5.77/24	5.00/18	10/0	1
63	Salt stress-induced protein (salT)	Q0JMY8	98/83	5.00/15	5.09/16	10/1	1
70	Salt stress-induced protein (salT)	Q0JMY8	82/71	5.00/15	4.88/16	7/0	1
95	Putative salT	Q5ZCQ9	120/46	7.72/28	6.61/15	7/2	1
158	β-1,3-Glucanase	Q7F354	112/57	7.01/36	6.38/38	16/1	1
169	Putative receptor-like protein kinase	Q8S3P3	230/59	5.01/28	4.65/30	12/3	4
218	Glutamine synthetase root isozyme (GLN1-2)	P14654	196/57	5.73/39	5.73/42	17/0	3
226	Mitochondrial chaperonin-60	Q8H903	77/29	5.71/61	5.39/65	8/0	10
273	Putative r40c1 protein	Q8H7M3	99/47	8.22/35	6.51/39	14/0	3
274	Putative r40c1 protein	Q8H7M3	208/42	8.22/35	6.54/39	27/2	3
291	PDH E1 β subunit isoform 3 (Pdha)	Q0J0H4	70/23	5.36/40	5.10/42	10/0	9
334	Adenine phosphoribosyltransferase 1 (APT1)	Q2QMV8	119/43	9.26/26	5.81/28	11/0	12
348	Glutamine synthetase (GS)	Q0J9E0	69/28	5.96/47	5.26/45	7/0	4
412	Aconitate hydratase, cytoplasmic	Q10S34	118/24	6.45/106	6.09/98	16/0	3
416	Putative aconitate hydratase, cytoplasmic	Q6YZX6	75/26	5.67/98	5.89/99	18/0	2
488	β-Hydroxyacyl-ACP dehydratase precursor (FabZ)	Q0J770	79/46	8.24/22	5.98/23	9/2	8
504	NAD-dependent IDH	Q7XK23	177/43	5.77/37	6.08/45	18/3	4
660	Putative β-1,3-glucanase	Q6YVU4	100/24	5.11/61	5.15/29	14/4	7

a) The numbering corresponds to the 2-D gel in Fig. 2.

b) Accession number in Swiss-Prot.

c) MASCOT score/sequence percentage coverage.

d) Theoretical pI and molecular weight.

e) Experimental pI and molecular weight.

f) Number of peptides identified by MS and MS/MS.

Our results showed that the expression of several citric acid cycle proteins changed in response to Pi deficiency. These changes were in favor of increasing organic acids in Nil compare to Nipponbare. Two citric acid cycle proteins, isocitrate dehydrogenase (IDH) (spot 504), and aconitase (spot 412), were identified in our study. Both of them were down-regulated in response to phosphorus deficiency which might be resulted in citrate accumulation. Citrate is usually converted to 2-oxoglutarate by a combined activity of aconitase and IDH. The expression of both enzymes decreased markedly in NIL6-4 compared to Nipponbare. Citrate accumulation has been demonstrated in the cluster root of white lupine when the activity of aconitase decreased [19, 20]. The differential expression of key enzymes in citrate metabolism may contribute to higher citrate accumulation in NIL6-4.

Additional, the expression level of pyruvate dehydrogenase (PDH) decreased in Nipponbare but not in NIL6-4 under phosphorus-deprived condition illustrating that a

limitation of substrate, pyruvate may lead to a reduction of the activity of citrate synthase and thus to an accumulation of citrate.

3.2.1.2 Up-regulation of antioxidant scavenging enzymes

A large number of enzymes involved in oxidative stress defense have been described in various stress proteome studies (for review, see Salekdeh and Komatsu [6]). Abiotic stresses can elevate the production of ROS resulting from photosynthesis, respiration, and NADPH oxidase [21]. Several of the genes transiently up-regulated after withdrawing phosphorus are involved in cell rescue and defense. These proteins include chloroplastic peroxiredoxin-2C (spot 8), superoxide dismutase (spot 10), putative 1,4-benzoquinone reductase (spot 27), and GST (spot 59). Our results showed that the up-regulations of oxidative stress defense enzymes

were more pronounced in NIL6-4 compared to Nipponbare under phosphorus shortage suggesting a more efficient ROS detoxification in Nil which may play a role in rice adaptation to phosphorus deficiency.

One of the major ROS detoxification pathways of plants include SOD, found in almost all cellular compartments. SOD converts superoxide to less toxic hydrogen peroxide molecules. In response to phosphorus deficiency, the abundance of the chloroplast Cu–Zn SOD increased by about three-fold in NIL6-4 but only by 30% in Nipponbare.

The detoxification of H₂O₂ is accomplished by several enzymes including 2-Cys peroxiredoxins. These enzymes constitute a ubiquitous group of peroxidases that reduce H₂O₂ and alkyl hydroperoxide [22]. It has been shown that 2-Cys peroxiredoxin-dependent water/water cycle may be an important alternative to detoxify H₂O₂ under oxidative stress conditions [23]. In response to phosphorus deficiency, the abundance of 2-Cys peroxiredoxin increased three-fold in NIL6-4, whereas no significant change was observed in Nipponbare.

The up-regulation of 1,4-benzoquinone reductase in response to phosphorus deficiency may be considered as another mechanism evoked by plant cells for defense against oxidative stress caused by stress. This enzyme, also known as quinone-oxidoreductase catalyzes the two-electron reduction of several quinines and other electron acceptors [24].

GST identified in this study showed similarity to Phi GST family. GSTs are cytosolic dimeric proteins involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH) with a wide range of endogenous and xenobiotic alkylating agents. The inducibility of phi GSTs following exposure of plants to biotic and abiotic stresses is a characteristic feature of these genes [25] and may increase plant tolerance to abiotic stresses [26]. GSTs may also play a role in stress tolerance by involvement in cell signaling [27]. Under phosphorus-deficient condition, the abundance of GST increased up to four-fold, but with no significant change in Nipponbare.

3.2.1.3 Up-regulation of abscisic acid- and stress-inducible protein (ASR1)

Protein spot 2 was up-regulated up to 5.2 times under phosphorus deficient condition in NIL6-4 but not in Nipponbare (Fig. 4). The protein showed sequence homology to an abscisic ASR. The function of the ASR1 proteins is not well understood but there are three different hypotheses concerning ASR function. ASR1 protein is located in the nucleus [28] and has DNA-binding activity [29]. These findings may support this hypothesis that ASR1 may be involved in the protection of DNA structure during water loss or in gene regulation upon stress by changing DNA topology. The up-regulation of ASR1 has also been reported in response to several abiotic stresses including drought [30, 31] and salt stress [32]. The overexpression of ASR1 resulted in an increased salt tolerance [33]. These findings suggest that it

acts as downstream components of a common signal transduction pathway. In addition, Cakir *et al.* [34] provided the first clue toward the possible involvement of an ASR protein in signal transduction cascades of sugar metabolism.

It has been proposed that these functions can be complementary and ASR could conceivably act as DNA binding proteins involved in protection against a range of stress signals by modulating cell sugar traffic [35]. Our results suggest that ASR1 may act as downstream components of a common signal transduction pathway. The use of reverse genetic approaches might provide further insight into the precise function of ASR1 in response to phosphorus deficiency.

3.2.1.4 Down-regulation of salt stress induced proteins (*SalTs*)

Four of the proteins that were down-regulated in response to phosphorus deficiency were identified as *SalT* homologs (spots 58, 63, 70, and 95). Of them, spot 58 showed similar response in the two genotypes. Spots 63, 70, and 95 with remarkably higher abundance in NIL6-4 under normal condition, showed a dramatic down-regulation in response to stress only in NIL6-4.

The *SalT* gene was first isolated and characterized from the roots of rice plants treated with salt [36]. Several studies showed that *SalT* protein participates in developmental and global responses such as those to salts, dehydration stress, phytohormones, and defense [36–39]. However, none of these studies had identified the biochemical functions of this protein. It is necessary to understand the function of *SalT* before we can rationalize the importance of this protein in response to phosphorus-deficiency stresses.

3.2.1.5 Integration of Kasalath in different chromosomal locations of NIL6-4

Pup1 was fine-mapped to within 0.6 cM on Chromosome 12, however, NIL6-4 contained several other not Pup1-related Kasalath inserts on chromosomes 1, 7, 10, and 12 (Fig. 5). Based on positions of flanking markers it is estimated that Kasalath inserts amount to approximately 20 Mb or about 5% of the total rice genome. We observed different proteome responses between NIL6-4 and its parental line, Nipponbare. These differences could be due to the genes residing in the Pup1 region and their interactions with genes located in other regions. None of the proteins identified in current study were within the introgressed regions and there were not regulatory genes. Taking into consideration that stress responsive genes/proteins have been located on several chromosomes (Table 2), we cannot rule out the contribution of other Kasalath chromosomal segments integrated in NIL6-4 in its response to stress, which may be responsible for different expression pattern of NIL6-4 compared to Nipponbare.

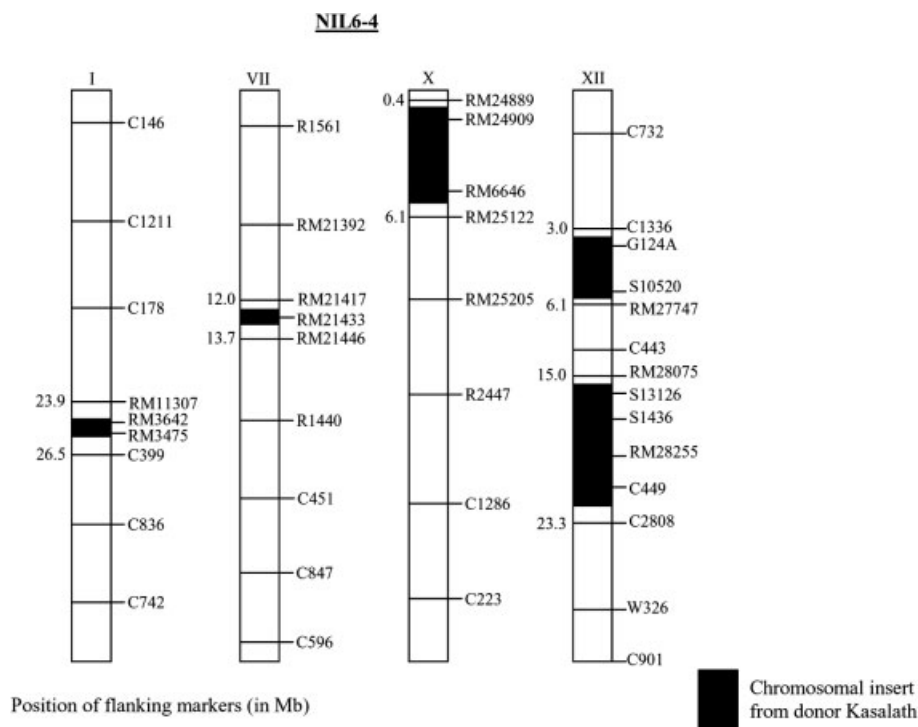


Figure 5. Graphical genotype of NIL6.4. NIL6-4 was derived from a NIL-*Pup1* × Nipponbare backcross. NIL-*Pup1* had been characterized by 170 RFLP markers that revealed the presence of *Pup1* donor (Kasalath) inserts on chromosomes 1, 8, 10, and 12. The genotype of NIL6-4 was originally determined with 46 RFLP markers located at known Kasalath inserts. Subsequent genotyping with 65 SSR markers revealed the presence of one small additional Kasalath insert in chromosome 7.

3.2.2 Proteins with similar response in two genotypes

3.2.2.1 Up-regulation of chitinases

In response to phosphorus deficiency, the abundance of three chitinases (spots 38, 39, and 40) increased from two- to four-fold in both genotypes. Changes in the expression of chitinase in response to phosphorus deficiency have also been observed in *Arabidopsis* [40]. Wasaki *et al.* [41] suggested that chitinase may be released from cluster roots of white lupine into rhizosphere. Chitinases produced by plant roots may play a role in both antifungal plant interactions and in various developmental activities, such as cell division, differentiation, and development [41]. Chitinase is a catalytic enzyme responsible for the hydrolysis of the chitin polymer found in the fungal cell wall, insects, a variety of crustaceans, and nematode eggs [42].

Earlier studies of the role of the chitinase revealed its involvement in plant defense responses to fungal pathogen infection [43]. However, chitinase is also induced in response to other biotic stresses including infection with viruses, bacteria, and oomycetes [44] as well as abiotic stresses such as salinity, drought, wounding, and ozone [45, 46]. Although the overexpression chitinases conferred resistance to pathogens and increase tolerance to abiotic stresses in plants [47, 48], the diverse biological functions of chitinase in the abiotic stress signaling and other cellular adaptation remain poorly understood. It is obvious that abiotic stress-signaling path-

ways also share common elements with biotic stresses that are in some cases considered as possible crosstalk points [49, 50].

Further studies of the chitinases expression and their interactions with its molecular targets are necessary to clarify the mode of their regulation by external stimuli in plant cells and their involvement in plant adaptation to stresses.

3.2.2.2 Down-regulation of a putative receptor-like protein kinase (RLK)

The expression level of a putative RLK (osRLK, Q8S3P3) decreased in response to phosphorus deficiency in both genotypes. Receptor protein kinases are involved in signaling processes regulating growth and development [51]. These proteins have been grouped into 15 subfamilies on the basis of extracellular domains [52]. Putative RLK identified in this study belongs to unknown function 26 (DUF26) (cysteine-rich repeat (CRR)) family. This domain is found in plants but with no known function. In *Arabidopsis*, members of DUF26 family were induced by pathogen attack, oxidative stress, and salicylic acid [53–55]. These studies revealed that the proteins of DUF26 might be involved in biotic and abiotic stress signaling. Jiang *et al.* (2007) [56] showed that osRLK (Q8S3P3) was expressed largely in roots and preferentially localized on the plasma membrane. Due to the phenotype of root meander curling on germination in transgenic plants, these genes were designated as *Oryza sativa* root meander curling (OsRMC). They showed enhanced JA-sensitive response and

increased root coiling in *OsRMC* knockdown transgenic rice plants, which suggest that *OsRMC* is probably involved in JA signal transduction that mediates root development and negatively regulates root curling in rice.

3.3 Poor correlation between mRNA and protein levels

Changes in gene expression at the mRNA level of eight stress responsive genes were investigated using qRT-PCR in three independent biological replicates. The list of genes and the sequence of forward and reverse primers are summarized in Table 1. We observed low correlation between the changes in gene and protein expression levels under normal and stress conditions except for SalT (Q5ZCQ9) and glutamine synthetase (P14654) (Fig. 6). Transcriptomics techniques, such as qRT-PCR, were proved to be powerful approaches in profiling mRNA expression. An important issue is the extent to which the changing expression patterns of mRNAs reflect corresponding changes in their cognate proteins. Our results confirmed that the mRNA levels do not correlate with the protein levels. Disparity between the relative expression levels of mRNA and their corresponding proteins have also been shown elsewhere [57]. The lack of correspondence between mRNA and protein levels might be due to the fact that mRNA levels usually peak before protein increments. Post-

transcriptional and -translational modifications as well as differential mRNA and protein degradation rates may also contribute to these discrepancies.

4 Concluding remarks

Our study provided evidence that phosphorus deficiency causes significant changes in several proteins and adds insights to our understanding of the response of rice to phosphorus deficiency. By comparing a parental line, Nipponbare, with its NIL carrying a major QTL (Pup1) on chromosome 12 with major effects on phosphorus uptake, several proteins emerged as key participants in phosphorus response. These proteins are involved in oxidative stress pathways and citric acid cycle. In addition to the involvement of identified proteins in phosphorus stress responsive pathways, the use of these proteins might be extended to applications in breeding for enhanced tolerance. The fact that none of the genes encoding the proteins differentially expressed mapped in the introgressed fragments from NIL6-4 raises the question about the nature of the QTL Pup1 and its effects on NIL6-4 proteome response. This may be due to interaction of genes residing in Kasalath genome inserts integrated in NIL6-4, including PUP1, with genes in other regions. Specific areas would be worthy of further study including (i) identification and characterization of genes located in Pup1 region and their possible contribution in controlling the expression of proteins identified in this study and (ii) measuring citric acid and ROS production in the two lines to decipher hypotheses generated by current proteome study.

The 2-DE databases of responsive proteins contains clickable 2-DE gel images and descriptive textual information such as protein name, M_r/pI values, MS score, and sequence coverage and other information is available for public access at <http://www.proteome.ir>.

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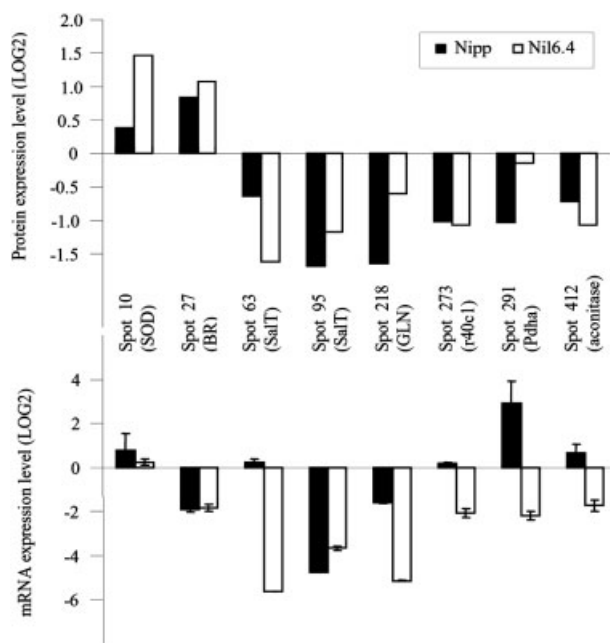


Figure 6. Comparison of eight genes at the level of mRNA and protein in Nipponbare and NIL6-4 under normal and stress conditions. The expression level is expressed as average abundance of protein/mRNA in two genotypes under stress condition divided by average abundance of protein/mRNA in two genotypes under normal condition. The fold changes in down-regulated proteins and mRNA have been shown as negative values.

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