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Article

A Comparative Proteomics Survey of Proteins Responsive to Phosphorous Starvation in Roots of Hydroponically-grown Rice Seedlings

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Rice takes up phosphorous (P) as major nutrient source for its growth and development when grown under anaerobic water-logged soil conditions. To better understand the underlying mechanisms and to develop potential protein biomarkers of P-starvation, hydroponically-grown rice seedlings in the complete media and phosphorus absence (P-starvation) of phosphorous nutrient solutions were investigated for physiological and proteome changes. The P-starvation manifested significant reduction in root growth in three-week-old seedlings compared to respective complete media. Furthermore, P-starvation also showed increased activity of acid phosphatase in roots of one- and three-week-old seedlings, suggesting that experimental design is suitable for proteomics survey of P-starvation responsive proteins. Two-dimensional gel electrophoresis coupled with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis of total root protein from three-week-old seedlings identified 10 P-starvation responsive protein spots out of 140 high-quality protein spots. Identified 10 proteins were involved in metabolism and defense/stress response. Out of 10, 2 and 8 protein spots were found to be up- and down-regulated, respectively. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of corresponding genes of four randomly selected proteins, including putative glyceraldehydes-3-phophate dehydrogenase (G3PDH, spot R1), *S*-adenosyl-L-methionine synthetase (SAMS, spot R4), ATP synthase subunit alpha (spot R6), and root-specific pathogenesis-related protein 10 (PR-10, spot R8), showed that just as protein abundance, these proteins are also regulated at the transcript level. Results suggest identified P-starvation responsive proteins are involved in maintaining nutrient homeostasis and/or associated with changes in root physiology under the absence of P.

Key words: acid phosphatase, 2-dimensional electrophoresis, phosphorous starvation, proteomics, rice

Phosphorus (P) is one of the key macronutrients for crop production, playing an important role in energy metabolism, photosynthesis, respiration, and biosynthesis of nucleic acids and membrane [Raghothama, 1999; Vance *et al*., 2003]. Most of the inorganic Ps are present as phosphate esters or metal ion salts, which result in extremely low availability of applied P in soil to plants [Raghothama, 1999]. Phosphorus availability is estimated to be about 30-40% of arable lands [von Uexküll and Mutert, 1995]. Thus, to maintain sustainable P control in agriculture, a better understanding of the mechanism involved in enhanced ability of plants to absorb P is required [Vance *et al*., 2003].

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To overcome P-starvation, plants have evolved many adaptive strategies, which include dramatic changes in root growth and architecture, such as an increase of lateral roots as well as increases of root hair number and length, to enhance P usage [Bates and Lynch, 1996; Rubio *et al*., 2001]. Another strategy of P-deficient plants is the efficient use of P by secreting APase, RNase, and organic acids into the rhizosphere to improve P availability in the soil [Tadano *et al*., 1993; Duff *et al*., 1994; Green, 1994; Jones, 1998; Rao *et al*., 1999]. APase, a marker gene, induced by phosphate stress, plays an important role in the mobilization of immobile phosphate present in the soil or inside plant cells [Duff *et al*., 1994; Nanamori *et al*., 2004]. Studies showed RNase is probably involved in the overall turnover of RNA [Löffler *et al*., 1992 and is induced by P-starvation in cultured tomato cells and *Arabidopsi*s [Nürnberger *et al*., 1990; Bariola *et al*., 1994]. Although the role of phosphohydrolase induction during P-starvation has been widely studied, the response mechanisms of P-starvation are still unclear.

Underlying these adaptation processes, many genes involved in P-starvation responses have been examined by gene expression studies using DNA microarray in plants [Hammond *et al*., 2003; Uhde-Stone *et al*., 2003; Wasaki *et al*., 2003; Misson *et al*., 2005; Morcuende *et al*., 2007; Calderon-Vazquez *et al*., 2008]. Recent studies revealed differential protein expression using proteomic approaches under P-deficit condition in maize [Li *et al*., 2007; 2008]. These results indicate that P-starvation response is a complicated metabolic process controlled by complex regulation system. However, little is yet known about P-starvation responsive proteins in roots of rice seedlings [reviewed in Agrawal and Rakwal, 2006; Agrawal *et al*., 2009].

In the present study, integrated physiology, 2-dimensional gel electrophoresis (DGE)-based proteomics, and reverse transcription polymerase chain reaction (RT-PCR) approach were used to identify differentially expressed proteins responsive to P-starvation. Identified proteins were broadly related to metabolism and defense/stress response and were possibly associated with observed root physiology caused by P-starvation. Findings of the present study could help in better understanding the P-starvation mechanism in plants, and provide a basis for further characterization of function and regulation of P-starvation-responsive proteins.

Materials and Methods

Plant material and growth conditions. Mature rice seeds (cv. Jinheung) were obtained from the National Yeongnam Agricultural Experimentation Station (Miryang, Korea). Dehulled seeds were sterilized with 70% ethanol for 10 min, rinsed in distilled water, and their surface sterilized with 3% sodium hypochlorite for 30 min. Seeds were rinsed extensively with sterilized distilled water and imbibed in the same water for 3 days at 4°C to break dormancy. Seeds were then grown on a plastic supporting netting (mesh 1 mm^2) mounted in plastic containers at 28°C for 7 days. Seedlings thus obtained were transferred to plastic containers containing complete or P-starved nutrient solution, on which were plastic panels with 4 mesh (0.33 cm^2) containing one plantlet per mesh. The complete media nutrient solution contained: 1.07 mM NH_4NO_3, 0.03 mM $NaH_2PO_4 \cdot 2H_2O$, 0.39 mM K_2SO_4, 0.39 mM KCl, 1.25 mM $CaCl_2 \cdot 2H_2O$, 0.82 mM $MgSO_4 \cdot 7H_2O$, 35.8 μM $FeSO_4 \cdot 7H_2O$, 9.1 μM $MnSO_4 \cdot 4H_2O$, 46.3 μM H_3BO_3, 3.1 μM $ZnSO_4 \cdot 7H_2O$, 0.16 μM $CuSO_4 \cdot 5H_2O$, and 0.05 μM $Na_2MoO_4 \cdot 2H_2O$. The P-starved nutrient solution was the same as complete media but without 0.03 mM $NaH_2PO_4 \cdot 2H_2O$. The pH of solution was adjusted to 5.8. Plants were grown for 3 weeks at 28°C, during which nutrient solutions were replaced every week with freshly prepared nutrient solutions. Root samples were collected, washed with sterilized distilled water, removed excess water on paper towel, frozen immediately in liquid nitrogen, and stored at −80°C.

APase assay. Root surface phosphatase was determined by the hydrolysis of *p*-nitrophenyl phosphate as described by Boero and Thien [1979]. Briefly, the fresh roots were washed with distilled water, and then root tips (about 70 mm each in length) were placed directly into 5.0 mL of substrate solution [2.5% (w/v) *p*-nitrophenyl phosphate in 50 mM sodium citrate buffer (pH 5.6)] and incubated at 30°C for 30 min. To ensure linearity, 50 μL aliquot of the substrate reaction mixture was removed at 20 min interval for 1 h and added into 50 μL of 1.0 M NaOH, followed by spectrophotometric analysis at OD_{405} nm. Phosphatase activity was expressed as mg *p*-nitrophenol g^{-1} h^{-1}.

Analysis of physiological parameters of rice growth. One- and three-week-old rice seedlings were collected from complete media and P-starved nutrient solutions for physiological parameters analyses. Twenty seedlings were pooled together for each biological replication; each experiment included three biological replicates in which seedlings were collected from three independent containers. For each seedling, the shoot length and length of the three longest roots were measured. Differences between complete media and P-starved seedlings were assessed using Tukey tests ($p < 0.05$). SAS software ver. 9.3 (SAS Institute, Cary, NC) was used for all statistical analysis.

Protein preparation and 2-DGE analysis. Two grams

roots of three-week-old rice seedlings were used to extract total protein using Mg/NP-40 extraction buffer [0.5 M Tris-HCl, pH 8.3, 2% v/v NP-40, 20 mM $MgCl_2$, 2% v/v β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% w/v polyvinylpolypyrrolidone (PVPP), and 0.7 M sucrose] followed by phenol extraction method as described by Kim *et al*. [2001]. Total root protein was extracted from three independent biological samples. The 2-DGE and downstream 2-D gel analysis were performed as described previously [Kim *et al*., 2008b]. Briefly, a total of 250 μg protein was loaded on 18-cm immobilized pH gradient (IPG) strips (pH 4-7). The IPG strips were equilibrated with rehydration buffer [8 M (w/v) Urea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.002% (w/v) Bromophenol G, 20 mM DTT, and 0.5% (v/v) pharmalyte (pH 5-8)] for 12 h and focused at 50 V 8 h, 100 V 1 h, 500 V 1 h, 1000 V 1 h, 2000 V 1 h, 4000 V 2 h, 8000 V 5 h, 8000 V 3 h, 20 V 2 h, by IPGphore3 platform (GE healthcare, Waukesha, WI). A 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension was carried out as per the Laemmli method [1970]. Gels were stained with colloidal Coommassie brilliant blue G-250 (CBB) as described previously [Kim *et al*., 2008a]. Stained gels were scanned using a transmissive scanner (PowerLook III, UMAX, Fort Worth, TX). Pixel depth was 16 bit, resolution was 300 dpi; brightness and contrast were set to default. Gel images were exported as TIFF files from the scanner, followed by their image analyses with ImageMaster 2D Platinum software ver. 6 (Amersham Biosciences AB, Uppsala, Sweden).

In-gel digestion and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Protein spots were carefully excised from Coommassie brilliant blue G-250 (CBB)-stained gels, digested with trypsin, and extracted peptides according to the method of Kim *et al*. [2008a]. Extracted peptide mixture was re-dissolved in one volume of a solution composed of distilled water, acetonitrile, and trifluoroacetic acid (93 : 5 : 2), bath-sonicated for 5 min, and centrifuged for 2 min at high speed (14,000 rpm). The matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) in acetone (40 mg/mL) and nitrocellulose in acetone (20 mg/mL). The α-cyano-4-hydroxycinnamic acid, nitrocellulose, and isopropanol solutions were mixed at 100 : 50 : 50, followed by addition of 2 μL this master matrix mixture to 2 μL of the prepared peptide sample. One microliter of the peptide and matrix mixture was spotted immediately onto a MALDI plate and left for 5 min. The MALDI plate was then washed with 0.1% (v/v) TFA. The gel spots were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.010%, and a delay time of 150 ns. Des-Arg1-bradykinin (*m*/*z* 904.4681) and angiotensin 1 (*m*/*z* 1296.6853) were used as a two-point internal standard for calibration. Peptides were selected in the mass range of 500-3,000 Da. Moverz program (http://bioinformatics.genomicsolutions.com) was used for data analysis. Database searches were performed using Mascot (http://www.matrixscience.com). To determine the confidence of the identification results, the following criteria were used: more than five matching peptides and sequence coverage greater than 15%.

Semi-quantitative RT-PCR analysis. Total RNA was extracted from complete media and P-starved roots of three-week-old seedlings using the SDS-phenol method [Kim *et al*., 2008c]. Total RNA samples (5 μg per reaction) were subjected to a cDNA synthesis system for RT-PCR according to the manufacturer's instructions (Invitrogen, Madison, WI). RT-PCR was performed with gene-specific primers corresponding to the identified proteins. Primers were designed to generate PCR products of 300-500 bp. Actin transcript was used as an internal control to normalize the concentration of cDNA in each sample. Primer pairs used for genes corresponding to protein spots: spot R1 (forward 5'-GTGCCAAGAAG GTCGTCATT-3' and reverse 5'-TGACGGATGAGGTCG ATCAC-3'); spot R4 (forward 5'-GTGCACGGCCACTT CACCAA-3' and reverse 5'-GATGATCTTGCGGCCGG TGA-3'); spot R6 (forward 5'-GCTCGACTGTGGCACA ATTA-3' and reverse 5'-GGATATAGGCCGATACGTCT-3'); spot R8 (forward 5'-CCGGCTTCATCGACGCCAT T-3' and reverse 5'-GCGACGAGGTAGTCCTCGAT-3'); and actin (forward 5'-TCCATCTTGGCATCTCTCAG-3' and reverse 5'-GTACCCGCATCAGGCATCTG-3').

Results and Discussion

Plant growth under P-starvation condition. In response to P-starvation, plants have developed physiological and biochemical mechanisms to acquire P from the external environment [Raghotham, 1999]. In the present study, to determine whether P-starvation in the P-starved nutrient solution has any effect on P-recycling enzyme, the APase activities were measured in roots of one- and three-week-old seedlings grown in complete media and P-starved nutrient solutions. The P-starvation caused significant increase in APase activities at both time points (Fig. 1). These results are consistent with

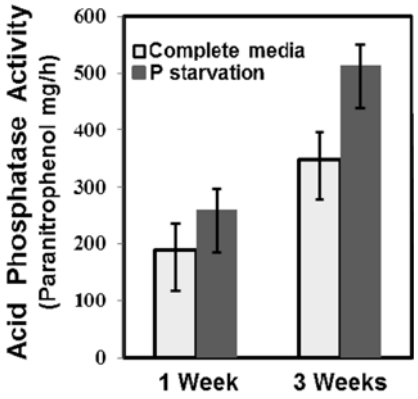

Fig. 1. APase activities in roots of one- and three-week-old seedlings were measured under P-starvation condition. APase activities were assayed in sodium citrate buffer (pH 5.6), using *p*-nitrophenylphosphate as a substrate. Results are shown as means with standard deviations.

previous findings on APase activity under P-starvation in rice [Lim *et al*., 2003] and suggest that the experimental condition is suitable for studying P-starvation responsive physiological and proteome changes in roots. Two physiological parameters were studied to determine the effect of P-starvation on rice growth and development compared to an appropriate control: (i) length of shoot and root and (ii) dry weight (DW) of shoot and root. The shoot and root growth of rice seedlings was comparable with that of control plants grown in a complete nutrient solution for 1 and 3 weeks after P was depleted, and dried weights of leaves and roots were also investigated for each time point. The means of three experiments were used. These measurements revealed that length of shoots and roots increased up to 6.5 and 27.7%, respectively. Dry weight of shoots increased up to 14.4%, and that of root slightly decreased compared to the complete nutrient solution at 1 week after P-starvation. However, the length of shoots and roots decreased by 3.9 and 14.2%, whereas the dried weight of shoots and roots decreased by 5.7 and 11.3% after 3 weeks P-starvation, respectively (Table 1). These data indicate nearly normal photosynthesis during the early period after P-starvation. However, at a later time and at a more severe P-starvation level, the radiation use efficiency was affected, although the exact mechanism involved is yet unclear, suggesting that the net photosynthesis per unit leaf area was reduced. These results are consistent with the findings of several studies on the effects of P-starvation on photosynthesis. On maize, Khamis *et al*. [1990] observed no effect of P deprivation on photosynthetic CO_2 fixation until 17 day after P-starvation. Although the P content in leaf was drastically reduced, the negative effect of P-starvation on CO_2 fixation was only observed later in their experiment. Similar results were also obtained by Usuda and Shimogawara [1991].

The 2-DGE comparative proteomics identified P-starvation responsive differentially expressed proteins in roots were mainly related to metabolism and defense/stress responses. The effect of P-starvation was more pronounced in roots of three-week-old seedlings; thus, total root protein from this stage was used for the comparative proteomic analysis. A total of 140 high-quality protein spots were detected in 2-D gels of both complete media and P-starved protein samples (Fig. 2) using the ImageMaster software. However, only 10 protein spots were found to be differentially expressed in response to P-starvation (Fig. 2B). Of these, 2 and 8 protein spots were up- and down-regulated, respectively. The MALDI-TOF-MS analysis of these spots identified them as: spot R1, putative glyceraldehydes-3-phosphate dehydrogenase; spot R2, putative UDP-glucose dehydrogenase; spot R3, reversibly glycosylated polypeptide; spot R4, *S*-adenosyl-L-methionine synthetase; spot R5, glutamine synthetase; spot R6, ATP synthase subunit alpha; spot R7, flavones O-methyltransferase; spot R8, pathogenesis-related protein 10 (PR-10); spot R9, ascorbate peroxidase; and spot R10, putative chitinase. Identified proteins belonged to two functional categories, metabolism (spots R1 to R7) and defense/stress response (spots R8 to R10). These results suggest that P-starvation influences the expression of the proteins primarily involved in metabolism, and these proteins could be responsible for the defense/stress response in roots.

Table 1. Length and dry weight (DW) of rice grown under P-starvation condition

Investigation	Treatments	Length (mm)			DW (mg per plant)		
		Shoot	Root	R/S	Shoot	Root	R/S
1 week	Complete media	137.4^{a}±5.9	57.4^{a}±5.1	0.42^{a}±0.03	10.4^{a}±0.84	5.57^{a}±0.40	0.56 ^{a}±0.07
	P-starvation	138.3^{a}±5.0	73.9^{b}±3.3	0.54^{a} ±0.03	11.9^{a}±0.50	5.43^{a}±0.52	0.47^{a}±0.06
3 weeks	Complete media	213.6^{a}±6.6	97.9^{a}±4.5	0.46^{a}±0.02	20.2^{a}±0.58	7.67^{a}±0.62	0.39^{a}±0.03
	P-starvation	205.6^{a}±9.9	85.7^{b}±3.0	0.42^{a}±0.02	19.1^{a}±1.73	6.89^{a}±0.55	0.36^{a}±0.04

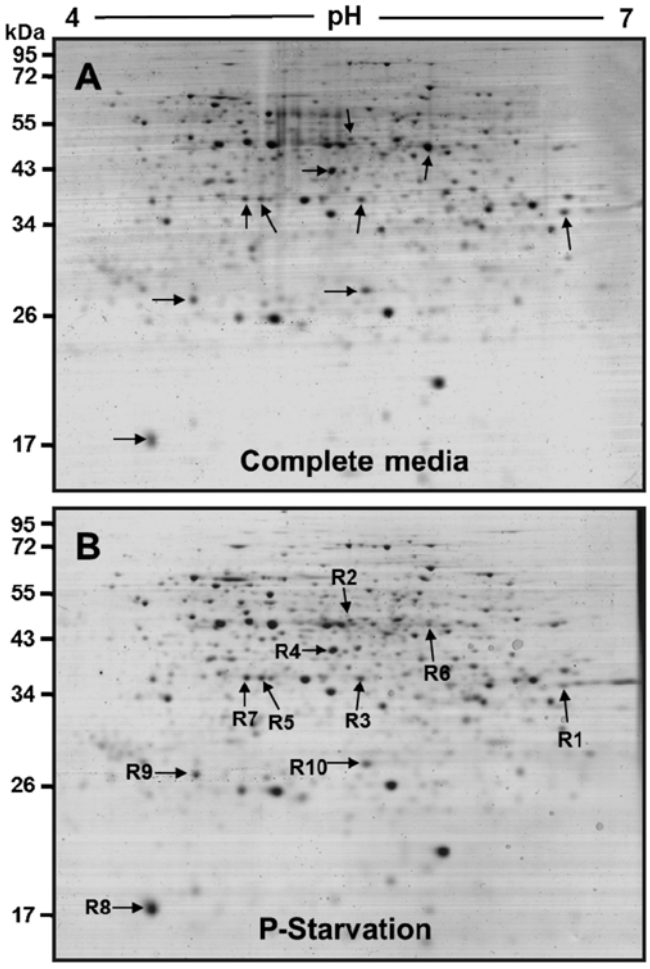

Fig. 2. 2-DGE analysis of differentially-expressed proteins in rice roots in response to P-starvation. Seedlings were grown for 3 weeks in the complete media and P-starvation micronutrient medium. Total root proteins were separated by 2-DGE (pH 4-7; 18-cm IPG strip), and gels were stained with colloidal CBB. (A) 2-DGE profile of proteins extracted from complete media-grown roots. (B) 2-DGE profile of proteins extracted from P-starvation-grown roots. Differentially-regulated root proteins by P-starvation are marked and numbered.

P-starvation responsive proteins involved in metabolism are mostly down-regulated and associated with changes in root physiology. Plant roots typically respond to P-starvation through allocation of carbon to roots and root morphological change for efficient P uptake [Raghotham, 1999], which influence carbohydrate and starch metabolism [Karkonen *et al*., 2005; Nanamori *et al*., 2004]. Theodorou and Plaxton [1993] reported that P-starvation induces glycolytic enzymes. Wasaki *et al*. [2003] found that transcriptional levels of several genes related to glycolysis increased in rice roots when subjected to P stress. In the present study, P-starvation was found to slightly down-regulate the putative glyceraldehydes-3-phosphate dehydrogenase (G3PDH, spot R1) protein (Fig. 3), which catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. During glycolysis, the cellular redundant is generated by two alternate pathways catalyzed by NAD-G3PDH and NADP-G3PDH, which is known as a P-starvation inducible bypass enzyme for NAD-G3PDH in *Brassica nigra* [Duff *et al*., 1989]. Li *et al*. [2007] showed that NADP-G3PDH was not significantly induced in maize and rice roots under P deficiency condition. Maize NAD-G3PDH was shown to increase in roots [Li *et al*., 2007]. Hence, the P-starvation responsive G3PDH (spot R1) protein in rice roots could be functionally closely related to NADP-G3PDH, and perhaps does not function as a glycolytic bypass.

The protein abundance of putative UDP-glucose dehydrogenase (UGDH, spot R2) was also down-regulated in response to P-starvation (Fig. 3). UGDH catalyzes the NAD^+-dependent oxidation of UDP-glucose into UDP-glucuronate in the starch degradation process. As UDP-glucuronate is a precursor of cell wall polysaccharide pectin, UGDH plays a role in the synthesis of cell wall polysaccharides [Karkonen *et al*., 2005]. Reversibly glycosylated polypeptide (RGP, spot R3), which was reversibly glycosylated using UDP-glucose and UDP-galactose as substrates, has been implicated in polysaccharide biosynthesis [Dhugga *et al*., 1997]. As in UGDH, the protein abundance of RGP was down-regulated in P-starved roots (Fig. 3). These data suggest that P-starvation slows down the overall process of starch degradation in roots, and enzymes involved therein could play a crucial role in adaptation mechanisms to P-deficiency.

Phytohormones such as ethylene are involved in primary root growth, promoting root hair, and lateral root formation [Davis, 1995]. P-starvation could enhance the sensitivity to exogenous ethylene [He *et al*., 1992]. In the present study, spot R4 was identified as the *S*-adenosyl-L-methionine synthetase (SAMS), a key enzyme in plant metabolism, catalyzing the conversion of ATP and L-methionine into *S*-adenosyl-L-methionine (SAM) (Table 2). SAM is a precursor for the biosynthesis of ethylene [Yang and Hoffman, 1984] and polyamines [Heby and Persson, 1990] and is involved in numerous transmethylation reactions [Tabor and Tabor, 1984]. The *SAMS* are considered "housekeeping genes" and expressed constitutively in most plant tissues [Gómez-Gómez and Carrasco, 1998]. The transcript level of *SAMS* is increased by various environmental stresses, including salt stress [Espartero *et al*., 1994; van Breusegem *et al*., 1994]. However, in the present study P-starvation was found to down-regulate SAMS. Because SAMS belongs to a protein family, it is highly likely that

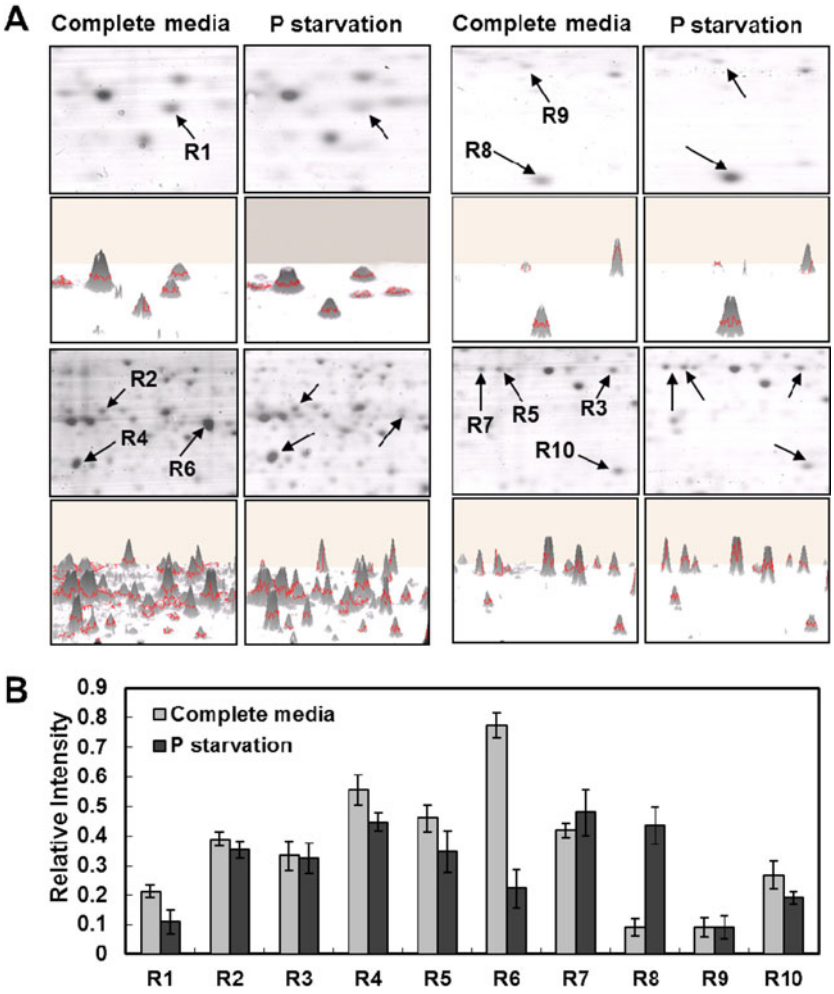

Fig. 3 Enlarged images of differentially-responsive protein spots. (A) 3-D images were generated by ImageMaster software. (B) Quantitative analysis of differential protein spots by ImageMaster software.

SAMS proteins behave differently under different stress conditions. Moreover, decrease in protein expression of this particular SAMS due to P-starvation could also be associated with the observed physiological changes in decreased length and dry weight of the root.

Glutamine synthetase (GS; spot 5), which catalyzes glutamate using ATP and NH_3 into glutamine, was identified as P-starvation responsive down-regulated protein (Fig. 3). This protein is known to be involved in photorespiration or amino acid metabolism [Cho *et al*., 2007]. Plants have a number of GS isozymes and, based on their subcellular location, they are classified as cytosolic-localized GS1 and plastid-localized GS2. In roots or other non-photosynthetic tissues, GS1 is predominant [Yan *et al*., 2005]. It has been reported that the GS1 synthetase-deficient plants manifest reduction in leaf blade elongation, plant height, panicle size, and grain filling [Tabuchi *et al*., 2005]. Therefore, the identified GS could be yet another component involved in root growth and adaptation to P-starvation.

Under P stress, the P movement from tonoplast to cytoplasm requires ATP to maintain P homeostasis in response to changing concentrations of P in cells [Raghothanma, 1999]. Moreover, transmembrane transport of P is an energy-mediated co-transport process driven by proton gradient, which is generated by ATPase in plants [Mimura *et al*., 1990; 1996]. The present study identified mitochondria-localized ATP synthase subunit alpha (spot R6) as a key component of ATP production and that it was down-regulated due to P-starvation in roots (Fig. 3). This result suggests that P-starvation may prevent efficient ATP production for cellular activities in rice roots.

A protein known to be involved in methylation of plant secondary metabolites, especially phenylpropanoid and flavonoid compounds, was identified. The flavone *O*-methyltransferase (FOMT, spot R7) was identified as one such down-regulated protein by P-starvation (Fig. 3), having 100% identical homology with caffeic acid 3-*O*-methyltransferase (COMT). The COMT catalyzes the

Table 2. Identification of spots differentially up/down-regulated by P-starvation in root using MALDI-TOF-MS

Spot	Protein name	AC	Score	MP	SC	Ex Mr	Th Mr	Ex pl	Th pI	Up/Down
R1	Putative glyceraldehyde-3-phosphate dehydrogenase	gi|115447137	82	7	24	37.57	36.54	6.9	7.68	↓
R2	Putative UDP-glucose dehydrogenase	gi|125545780	70	8	21	52.14	51.31	5.6	5.67	↓
R3	Reversibly glycosylated polypeptide	gi|115454033	85	8	23	41.27	41.32	5.74	5.82	↓
R4	*S*-adenosyl-L-methionine synthetase	gi|125570164	69	7	27	43.2	41.2	5.5	5.97	↓
R5	Glutamine synthetase	gi|115448531	67	6	16	41.58	39.17	5.7	5.51	↓
R6	ATP synthase subunit alpha, mitochondrial	gi|148886790	175	16	36	51.48	55.33	6.17	5.85	↓
R7	Flavone *O*-methyltransferase	Q19BJ6	54	7	23	41.6	39.7	5.6	5.41	↑
R8	Root specific pathogenesis-related protein 10	gil77556750	96	7	44	18.12	16.89	4.17	4.88	↑
R9	Ascorbate peroxidase	gi|115474285	88	7	40	30.17	27.1	5.34	5.21	↓
R10	Putative chitinase	gi|54291729	176	14	42	30.57	32.52	5.98	6.08	↓

Abbreviations; MP: matched peptide; Ex Mr: experimental molecular weight; Ex pI: experimental isoelectric point; SC: sequence coverage; Th: threological; ↑: Up-regulated; ↓: Down-regulated.

multi-step methylation reactions of the hydroxylated monomeric lignin precursors, and is believed to occupy a pivotal position in the lignin biosynthetic pathway [Kim *et al*., 2006]. This result suggests that COMT is involved in not only lignin biosynthesis but also in the production of a wide range of metabolites derived from phenylpropanoid pathway.

Stress- and defense-related P-starvation responsive proteins. Plants are known to secret proteins to overcome P-deficiency, such as APase and RNase [Tadano *et al*., 1993; Duff *et al*., 1994; Green, 1994]. In the present study, PR-10 (spot R8) with increased accumulation in roots due to P-starvation was identified (Table 2; Fig. 3). The PR-10 family proteins, generally known to have RNase activity, have been identified in most plants, and, in most cases, are involved in defense responses as well as in plant development [Dixon and Lamb, 1990; Green, 1994; Kim *et al*., 2008d]. PR-10 protein plays yet another role under P-starvation as stress response and/or adaptation to P-deficiency. Two of the well-characterized senescence-associated RNase genes (*RNS1* and *RNS2*) from *Arabidopsis* were shown to be induced under P-starvation [Bariola *et al*., 1994; Green, 1994]. Such genes have been cloned and characterized in tomato and tobacco and are also inducible by P-starvation [Kock *et al*., 1995; Dodds *et al*., 1996]. Muramoto *et al*. [1999] suggested that the over-expression of ribonuclease gene in salt-stressed barley leaves could play a role in remobilization of nucleotides or phosphate for the benefit of fresh organs. Recently, Kim *et al*. [2008d] also showed that JIOsPR10 possessing RNase activity may function in a remobilization of nucleotides or phosphates in the vascular bundle when exposed to biotic/abiotic stresses in rice. These RNases may participate in the hydrolysis of nucleic acids and nucleotides under P-starvation condition in rice.

In general, abiotic stress causes accumulation of reactive oxygen species (ROS) in plant tissues or organs [Bartosz, 1997; Torres and Dangl, 2005; Kim *et al*., 2008b]. Excess/deficiency of micronutrients also influences ROS production [Cakmak, 1994; Caro and Puntarulo, 1996]. One of the proteins involved in scavenging H_2O_2 was identified as ascorbate peroxidase (spot R9; Fig. 3 and Table 2). This result was consistent with protein activities during phosphate deficiency in roots of bean plants (*Phaseolus vulgaris* L.) but in phosphate-starved bean roots, activities of catalase and total peroxidase were higher than control roots [Juszczuk *et al*., 2001].

Another identified defense/stress related protein was chitinase (spot R10; Table 2). Chitinase also belongs to a multiprotein family and is involved in plant defense responses to fungal pathogen infection as well as responses to abiotic stresses including salinity, drought, wounding, and ozone [Chen *et al*., 1994; Hong and Hwang, 2006]. Chitinase is capable of digesting chitin, the main component of cell wall. The protein abundance of identified chitinase was down-regulated by P-starvation (Fig. 3). The transcriptional expressions of chitinases in response to phosphorus starvation have also been shown to be regulated in *Arabidopsis* [Hammond *et al*., 2003]. Wasaki *et al*. [2005] suggested that chitinase is released from cluster roots of white lupine into rhizosphere. Furthermore, 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.

Differential expression of few selected proteins correspond well with their transcript Levels. To determine whether the 2-DE-derived protein expression profile of identified proteins correspond with their

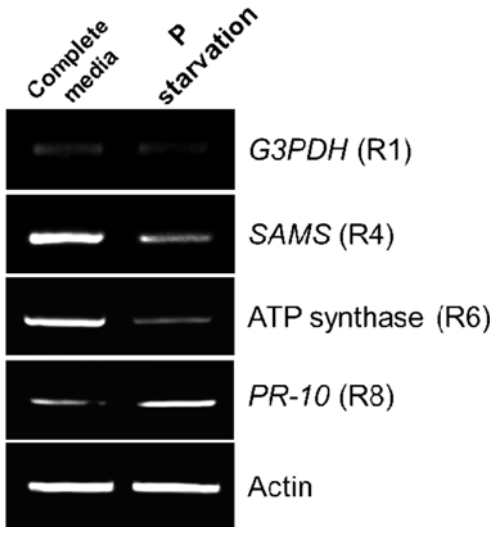

Fig. 4 RT-PCR analysis of genes corresponding to proteins responsive to P-starvation.

transcript levels, a semi-quantitative RT-PCR analysis was performed on same samples using gene-specific probes from cDNAs encoding four randomly selected proteins, including G3PDH (spot R1), SAMS (spot R4), ATP synthase (spot R6), and PR-10 (spot R8) (Fig. 4). Transcript level of *PR-10* (spot R8) was up-regulated, whereas those of *G3PDH* (spot R1), *SAMS* (spot R4), and *ATP synthase* (spot R6) were down-regulated by P-starvation compared to the complete media (Fig. 4). RT-PCR data were reproducibly detected through at least two replicates. These results show that these proteins, regulated by P-starvation are also similarly regulated at the transcript level.

Integration and mapping of identified proteins on cellular events in response to P-starvation. The effect of P-starvation was studied in rice roots at morphological, physiological, biochemical, and molecular levels to obtain a better insight into its underlying adaptation mechanisms. Results obtained in above sections were integrated, and identified proteins were mapped on to cellular events (Fig. 5). As depicted, proteomics approach coupled with physiological and biochemical approaches extend the previous finding, suggesting that P-starvation influences multiple cellular processes in roots. Most of these processes are down-regulated including the ROS scavenger, defense, metabolic networks, phytohormone, energy production, and photorespiration. Abundance of identified P-starvation responsive proteins was down-regulated except for PR-10 and FOMT (marked by upward arrows). Majority of proteins were associated with metabolism of carbohydrate and starch (G3PDH, UGDH, and RGP). Saturating the root proteome responsive to P-starvation will be required to identify most of the cellular events involved in P-starvation.

In conclusion, the 2-DGE-based proteomics approach resulted in cataloging the P-starvation-responsive proteins of rice roots. These proteins were mainly involved in metabolism and defense/stress responses, and correlated well with the root physiology and biochemical changes. Protein responsiveness to P-starvation was also in line with the transcript level for some of the randomly selected proteins. Integrated data provided a view on cellular events regulated by P-starvation in rice roots,

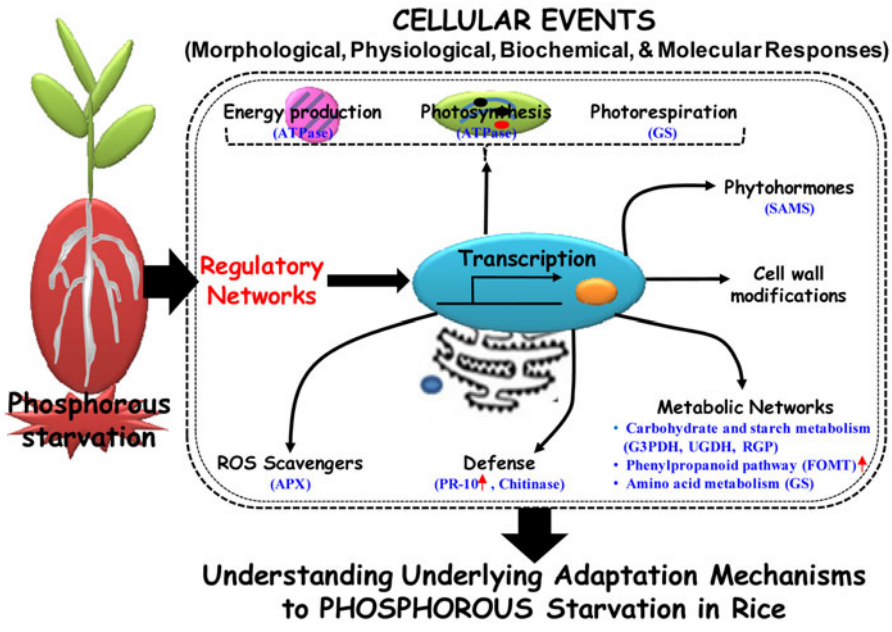

Fig. 5 An integrated view of cellular events responsive to P-starvation in rice roots. P-starvation affects the cellular processes and responses at various levels. Details are in the text.

which involve changes in protein abundance of multiple cellular processes. These findings provide new insight into the root biology. Although the obtained information is a step forward in understanding of the underlying adaptation mechanism of root to P-starvation, developing a complete list of root proteins responsive to P-starvation will be required using multiple complementary proteomics approaches.

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