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Proteome analysis of soybean roots under waterlogging stress at an early vegetative stage

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To gain better insight into how soybean roots respond to waterlogging stress, we carried out proteomic profiling combined with physiological analysis at two time points for soybean seedlings in their early vegetative stage. Seedlings at the V2 stage were subjected to 3 and 7 days of waterlogging treatments. Waterlogging stress resulted in a gradual increase of lipid peroxidation and *in vivo* H_2O_2 level in roots. Total proteins were extracted from root samples and separated by two-dimensional gel electrophoresis (2-DE). A total of 24 reproducibly resolved, differentially expressed protein spots visualized by Coomassie brilliant blue (CBB) staining were identified by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry or electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis. Of these, 14 proteins were upregulated; 5 proteins were decreased; and 5 were newly induced in waterlogged roots. The identified proteins include well-known classical anaerobically induced proteins as well as novel waterlogging-responsive proteins that were not known previously as being waterlogging responsive. The novel proteins are involved in several processes, i.e. signal transduction, programmed cell death, RNA processing, redox homeostasis and metabolisms of energy. An increase in abundance of several typical anaerobically induced proteins, such as glycolysis and fermentation pathway enzymes, suggests that plants meet energy requirement via the fermentation pathway due to lack of oxygen. Additionally, the impact of waterlogging on the several programmed cell death- and signal transduction-related proteins suggest that they have a role to play during stress. RNA gel blot analysis for three programmed cell death-related genes also revealed a differential mRNA level but did not correlate well with the protein level. These results demonstrate that the soybean plant can cope with waterlogging through the management of carbohydrate consumption and by regulating programmed cell death. The identification of novel proteins such as a translation initiation factor, apyrase, auxin-amidohydrolase and coproporphyrinogen oxidase in response to waterlogging stress may provide new insight into the molecular basis of the waterlogging-stress response of soybean.

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1. Introduction

Waterlogging is defined as prolonged soil saturation with water at least 20% higher than the field capacity (Aggarwal *et al.* 2006). It is a major problem of utmost importance, as it limits the growth and yield of many crops in humid areas. The inability of crops to withstand excess water in the rhizosphere adversely alters metabolism and leads to a major reduction

Keywords. Abiotic stress; programmed cell death; proteomics, soybean root; waterlogging

Abbreviations used: 2-DE, two-dimensional gel electrophoresis; ACN, acetonitrile; Adh, alcohol dehydrogenase; ANP, anaerobic polypeptide; CBB, Coomassie brilliant blue; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GS, glutamine synthetase; Hb, haemoglobin; IFR, isoflavone reductase; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; NO, nitrous oxide; PCD, programmed cell death; PFK, phosphofructokinase; PMF, peptide mass fingerprinting; PPi, inorganic pyrophosphate; ROS, reactive oxygen species; SAM, S-adenosyl-L-methionine synthetase; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substance; Ub, ubiquitin

Supplementary figure pertaining to this article is available on the *Journal of Biosciences* Website at *http://www.ias.ac.in/jbiosci/March2010/pp49-62/suppl.pdf*

in crop yield. Globally, approximately 10% of irrigated farmlands suffer from frequent waterlogging; however, values up to 20% occur in specific regions such as Eastern Europe and the Russian Federation (FAO 2002). Most soybean (*Glycine max*) acreages in East and Southeast Asia and the USA are vulnerable to frequent flooding. Intermittent downpour, especially after irrigation of poorly drained fields such as impermeable clay-based soils or converted paddy fields, can result in flooding and soil waterlogging (Araki 2006). Since gas diffusion is four orders of magnitude slower in water than in air, soil saturation causes a reduced supply of oxygen, which is crucial for the roots of the plant, especially those roots whose aerobic metabolism depends entirely on oxygen from the soil (Drew 1997). The deleterious effects associated with hypoxia and anoxia include a decrease in cellular energy charge, drop in cytoplasmic pH, and the accumulation of toxic metabolites and reactive oxygen species (ROS) which are responsible for the slowed growth and reduced yield of many agriculturally important crops (Subbaiah and Sachs 2003). Soybean is an important global crop that provides proteins and oils for human nutrition. Soybean cultivars are especially susceptible to waterlogging stress during germination, early vegetative and early reproductive growth (Githiri *et al.* 2006). Existing soybean cultivars may never fully recover from flooding injury; and seed yields are substantially reduced in response to the stress. The effect of waterlogging stress on physiological responses in soybean has been previously investigated (Oosterhuis *et al.* 1990; Boru *et al.* 2003; Amarante and Sodek 2006 and references therein).

Plants have evolved a number of adaptation mechanisms to cope with the anaerobiosis due to waterlogging. These mechanisms include metabolic adaptations such as the induction of fermentation pathway enzymes (ethanol, lactic acid and alanine fermentation) and structural modifications, such as aerenchyma and adventitious root formation (Drew 1997). Substantial progress has been made in understanding low oxygen stress response mechanisms in maize (Chang *et al.* 2000), rice (Dubey *et al,* 2003; Huang *et al.* 2005) and *Arabidopsis* (Klok *et al.* 2002; Liu *et al.* 2005) using proteomic and genomic approaches. Dramatic changes in protein synthesis have been reported in plant roots during anaerobiosis. Most of these anaerobic proteins (ANPs) have been identified as enzymes of glycolysis or sugar phosphate metabolism (Sachs *et al.* 1996; Ahsan *et al.* 2007a). ANPs that are part of other metabolic processes have also been reported (Chang *et al.* 2000; Shi *et al.* 2008). These reports strongly suggest that the regulation of low oxygen and flooding response in plants involves more than a simple adaptation of energy metabolism, and is far more complex than anticipated for many years.

Recently, microarrays have allowed for the determination of steady-state mRNA levels in gene regulation under anaerobic conditions (Klok *et al.* 2002; Liu *et al.* 2005). However, post-transcriptional and translational processes have also been involved in the regulation of gene expression under low oxygen stress (Bailey-Serres and Freeling 1990; Dennis *et al.* 2000). So far, few proteomic studies have been conducted on hypoxia or anoxia stress responses in plants, e.g. in maize (Chang *et al.* 2000) and rice (Huang *et al.* 2005). These studies provide a clear indication that, in addition to the classical ANPs such as enzymes involved in sugar metabolism, glycolysis and fermentation, a wide range of genes are also involved in the anaerobic response, which provides new insight into the potential for enhanced flood tolerance. Although oxygen deprivation is the main consequence of waterlogging, soil may accumulate phytotoxic products, inorganic carbon and the gaseous plant hormone ethylene under conditions that could affect root growth and metabolism. Since waterlogging causes stratified oxygenation, unlike submergence, the shoots maintain normal oxygen levels; and most plants develop aerenchyma for survival. Therefore, to identify appropriate genes that are waterlogging responsive, it is imperative that the complete root proteome be determined under conditions of waterlogging stress (Dennis *et al.* 2000). Root proteome analysis of tomato seedling under waterlogging conditions showed modulation in a wide range of genes (Ahsan *et al.* 2007a). Recently, Sakata *et al.* (2009) catalogued a number of proteins from germinated soybean seedlings under submergence stress and constructed a proteome database. This study provides novel insight into the molecular basis of flooding stress on soybean seedling. To date, however, there is no report on proteomic studies on waterlogging stress in soybean plants at the early vegetative (V2) stage. An adequate evaluation of the root proteome exposed to waterlogging stress at different growth stages would help in comprehensively understanding the complexity of the networks involved in waterlogging stress, which might be advantageous in engineering waterlogging-tolerant plants. The main objective of this study was to investigate the proteome expression pattern and to identify the novel proteins and genes that are differentially regulated upon exposure to waterlogging stress. We found several novel proteins involved in different metabolic processes, which have not been previously known to be involved in response to waterlogging. Despite previous studies on the proteomics of hypoxia, anoxia and waterlogging, the identification of several proteins indicates that our proteomics study yielded mostly novel information. The possible functions of these identified proteins are discussed.

2. Materials and methods

2.1 *Plant growth and flooding treatment*

Soybean (*Glycine max* L. Merr. cv. Asoagari) seeds were planted in plastic pots containing horticulture nursery

medium (Biomedia, Korea) and grown in a growth chamber maintained at 25°C under white florescent light (480 µmol m^{-1} s^{-1}) with a 16 h photoperiod. Seedlings at the V2 stage (two weeks old) were subjected to waterlogging as described previously (Ahsan *et al.* 2007b). Briefly, to impose the treatments, seedling pots were moved to plastic containers 65 cm × 35 cm × 12 cm in size and filled with water so that the water level persisted up to 2 cm above the soil surface of the plant-containing pots. Control plants remained well watered (normal moisture conditions) during the period of the experiment. After treatment for 3 and 7 days, roots of the treated and control plants were excised in the root–shoot transition zone, washed with distilled water, immediately frozen in liquid nitrogen and kept at –80°C until use.

2.2 *Measurement of lipid peroxidation and hydrogen peroxide*

Lipid peroxidation was measured as the amount of thiobarbituric acid-reactive substance (TBARS) determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). The concentration of TBARS was calculated by using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. The H_2O_2 concentration in root samples from control and stress-treated plants was measured spectrophotometrically as previously described (Lee *et al.* 2007a). Three independent experiments were carried out, each consisting of at least three replicates.

2.3 *Protein extraction and 2-D PAGE*

Proteins were extracted from 3-day and 7-day treated root samples using a phenol extraction method as described earlier (Ahsan *et al.* 2007a). The acetone-precipitated protein samples were quantified using the method of Lowry *et al.* (1951) and subjected to two-dimensional gel electrophoresis (2-DE) following our previously described protocol (Lee *et al.* 2007a). The acetone-precipitated proteins were dissolved in a resuspension buffer (8 M of urea, 1% CHAPS, 0.5% [v/v] IPG buffer pH 4–7, 20 mM of DTT, and a trace of bromophenol blue). This sample was applied to the IPG dry strip pH 4–7 for 12 h, followed by focusing for 47 500 Volt-hours (Vh) using an IPGphor (Amersham Bioscience, Uppsala, Sweden). After isoelectric focusing, the IPG strips were equilibrated for 15 min in an equilibration buffer (50 mM of Tris-HCl, pH 8.8, 6 M of urea, 30% [v/v] glycerol, 2% [w/v] sodium dodecyl sulphate [SDS], and a trace of bromophenol blue) containing 10 mg/ml of DTT, followed by 15 min in an equilibration buffer containing 25 mg/ml of iodoacetamide. SDS-PAGE in the second dimension was carried out as described by Laemmli (1970) using a 12% polyacrylamide gel. The 2-DE gels were stained with Coomassie brilliant blue (CBB) as previously described (Ahsan *et al.* 2007a).

2.4 *Image acquisition and data analyses*

Images of CBB-stained gels were acquired by a high-resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad) and used for analysis. Spots were detected and quantified, and then matched with the Bio-Rad PDQuest software (Version 7.2; Bio-Rad, Hercules, CA, USA). To compensate for the variability in gel staining, the volume of each spot (spot abundance) was normalized as a relative volume. After automated detection and matching, manual editing was carried out. A minimum of three gels were generated for each sample. Only spots that showed significant and reproducible changes were considered as differentially expressed proteins. The standard error (SE) was calculated from three spots in replicated gels.

2.5 *In-gel digestion, MALDI-TOF MS, ESI-MS/MS and database search*

Selected protein spots were excised manually from the CBB-stained gels and washed with 50% (v/v) acetonitrile (ACN) in a 0.1 M NH_4HCO_3 solution and vacuum-dried. The gel fragments were reduced for 45 min at 55°C in 10 mM of DTT in 0.1 M NH_4HCO_3. After cooling, the DTT solution was immediately replaced with 55 mM of iodoacetamide in 0.1 M NH_4HCO_3. After washing with 50% ACN in 0.1 M NH_4HCO_3, the dried gel pieces were left to swell in a minimum volume of 10 μl digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/μl of trypsin (Promega, WI, USA) and incubated overnight at 37°C. Trypsin-digested peptides were extracted according to a previously described protocol (Kim *et al.* 2004). All the samples were analysed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Parent ion masses were measured in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.01%, and a delay time of 150 ns. A two-point internal standard for calibration was used with des-Arg1-Bradykinin (m/z 904.4681) and neurotensin (m/z 1672.9175). The software package PerSeptive-Grams was used for data processing. The peptide mass fingerprintings (PMFs) obtained from each digested protein were compared with PMFs in the non-redundant National Center for Biotechnology Information database (NCBInr) using the PROFOUND or MS-Fit interface. The search was performed within green plants (viridiplantae). A mass tolerance of 100 ppm and one incomplete cleavage was allowed; acetylation of the *N*-terminus, alkylation of cysteine by carbamidomethylation,

oxidation of methionine, and the pyroGlu formation of *N*-terminal Gln were considered as possible modifications. The estimated experimental M_r/p*I* was applied to increase the confidence of identification. ESI-MS/MS was performed as described previously (Lee *et al.* 2007b).

2.6 *RNA gel-blot analysis*

Total RNA was isolated from the root samples using a Plant RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Ten micrograms of RNA samples were separated on a 1.2% agarose gel containing formaldehyde. Hybridization probes were prepared by polymerase chain reaction (PCR) based on a soybean cDNA template with the following oligonucleotide PCR primers: Apyrase (forward primer: 5′−TGGATCAGTTCA AATGGCGT−3′ and reverse primer: 5′−CACCATTCCAA ATTCCACCA−3′); eIF 5A (forward primer: 5′−GCTGGT ACCATTCGCAAGAA−3′ and reverse primer: 5′−CTGA GCAAGCAATGCCTCAT−3′) and IFR1 (forward primer: 5′−AGACCCCATGCCAATGTTTT−3′ and reverse primer: 5′ −TGGGAGGAGTCTTTTGACGA−3′). Gene-specific PCR products were labelled with [α-^{32}P] dCTP by a random primer labelling kit (Amersham, Buckinghamshire, UK). Northern blot analysis followed, as previously described (Lee *et al.* 2007a).

2.7 *Statistical analysis*

Results of the physiological parameters were statistically analysed by ANOVA. Significant differences from control values were determined at $P<0.05$ levels. All the results were represented as means ± SE of at least three independent replications.

3. Results and discussion

3.1 *Physiological responses induced by waterlogging stress*

The generation of ROS is considered as one of the primary events under a variety of stress conditions. ROS are regarded as initiators of peroxidative cell damage. TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of cellular free radical generation. In order to determine the level of oxidative stress and membrane damage caused by waterlogging stress, we examined lipid peroxidation and *in vivo* H_2O_2 content. The TBARS content was increased in waterlogged root samples (figure 1A). Based on this observation, we also measured cellular H_2O_2 levels in the samples. The result showed that a higher amount of H_2O_2 had accumulated in waterlogged roots compared with the controls (figure 1B). An increased TBARS content correlated with the H_2O_2 levels. A significantly higher amount of TBARS was detected at the 7-day stage only. TBARS formation represents the terminal stage of lipid peroxidation, and, though lipid damage may be present at the 3-day stage, it is undetectable by TBARS assay. Accumulation of higher amounts of TBARS and H_2O_2

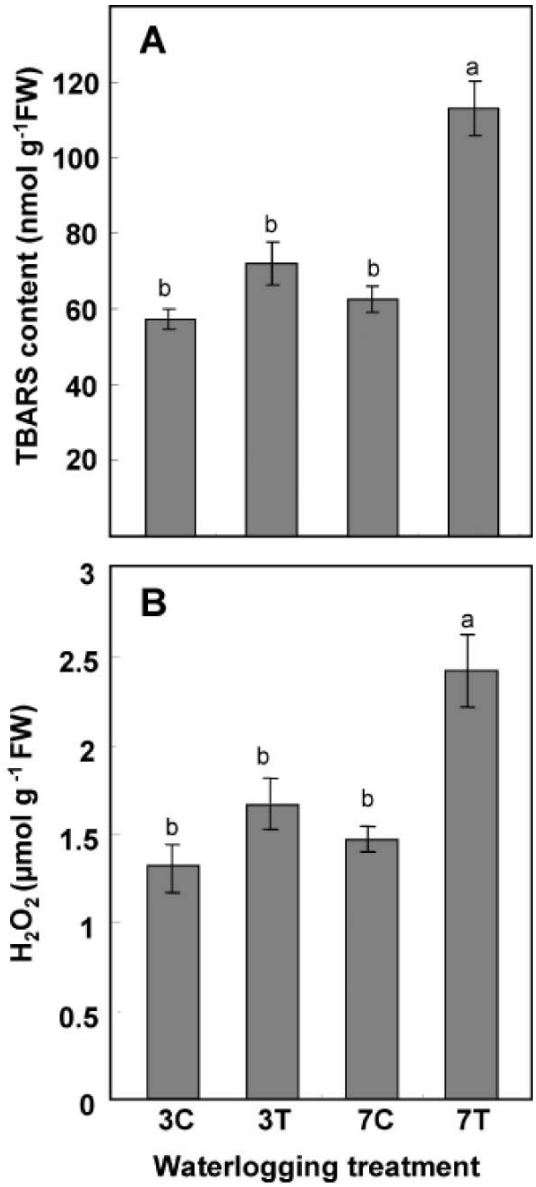

Figure 1. Physiological responses of soybean roots subjected to waterlogging. TBARS **(A)** and H_2O_2 **(B)** concentration in control (3C, normal moisture condition) of 3-day waterlogging treatment, 3-day treatment (3T), control for 7-day treatment (7C), or 7-day waterlogging treated (7T) soybean roots. Data represent the mean values and SE of three independent experiments. Different letters above the bars indicate a statistically significant difference (*P<0.05*).

compared with those observed in our previous study on tomato (Ahsan *et al.* 2007a) may be due to different growth stages and species. Hydrogen peroxide is the first stable compound among the ROS produced in the plant cell under normal conditions and as a result of various stresses. It has been reported that significant amounts of ROS such as O_2^- and H_2O_2 are generated by waterlogging stress, which can initiate the peroxidation of lipids with consequent membrane damage (Yan *et al.* 1996; Ahsan *et al.* 2007a,b). Our results indicate that plants subjected to waterlogging accumulate larger amounts of ROS, and undergo peroxidation of lipids and subsequent membrane damage.

3.2 *Alteration in root proteome under waterlogging stress*

To investigate the response of soybean root proteins under waterlogging stress, 3- and 7-day treated roots of V2 seedlings were used for proteome analysis. A high-resolution 2-DE gel pattern in the p*I* range between 4 and 7 was observed by CBB staining. After image analysis of the CBB-stained gels, about 900 protein spots were detected (figure 2). Using the PDQuest software, differences in the intensity of the protein spots between the control and waterlogging-treated samples were compared. About 42 protein spots were found to be expressed differentially in response to waterlogging treatment (figures 2 and 3). Among these, 24 reproducibly resolved protein spots were excised from the gels and digested with trypsin, and the peptides identified by PMF using MALDI-TOF MS or ESI-MS/MS. Among the 24 proteins identified, 5 spots were newly induced (spots 1, 7, 17, 18 and 20), 14 spots increased, and 5 spots decreased (figure 3). The identified proteins are listed in table 1. Transcriptome and proteome analyses have identified many oxygen deprivation stress-responsive genes in plants (Chang *et al.* 2000; Klok *et al.* 2002; Huang *et al.* 2005; Liu *et al.* 2005) and revealed the involvement of a complex network. The present proteomic study identified not only some well known oxygen deprivation-responsive proteins such as glycolytic and fermentation enzymes, but also several novel proteins such as translation initiation factor, apyrase, auxin-amidohydrolase and coproporphyrinogen oxidase. These identified proteins can be classified into the following categories (1) carbon metabolism and other energy-related proteins, (2) antioxidant- and nitrogen metabolism-related proteins, (3) signal transduction- and programmed cell death-related proteins, (4) secondary metabolism-related proteins, (5) DNA/RNA-binding proteins and (6) some proteins of unknown function. The possible roles of the identified proteins in relation to waterlogging are discussed in the following sections.

3.2.1 *Altered carbohydrate metabolism favours the energy-conserving glycolysis and fermentation pathways:* Oxygen deprivation is the main consequence of waterlogging at the rhizosphere. Under low oxygen levels, fermentation is the predominant pathway for ATP production and NAD^+

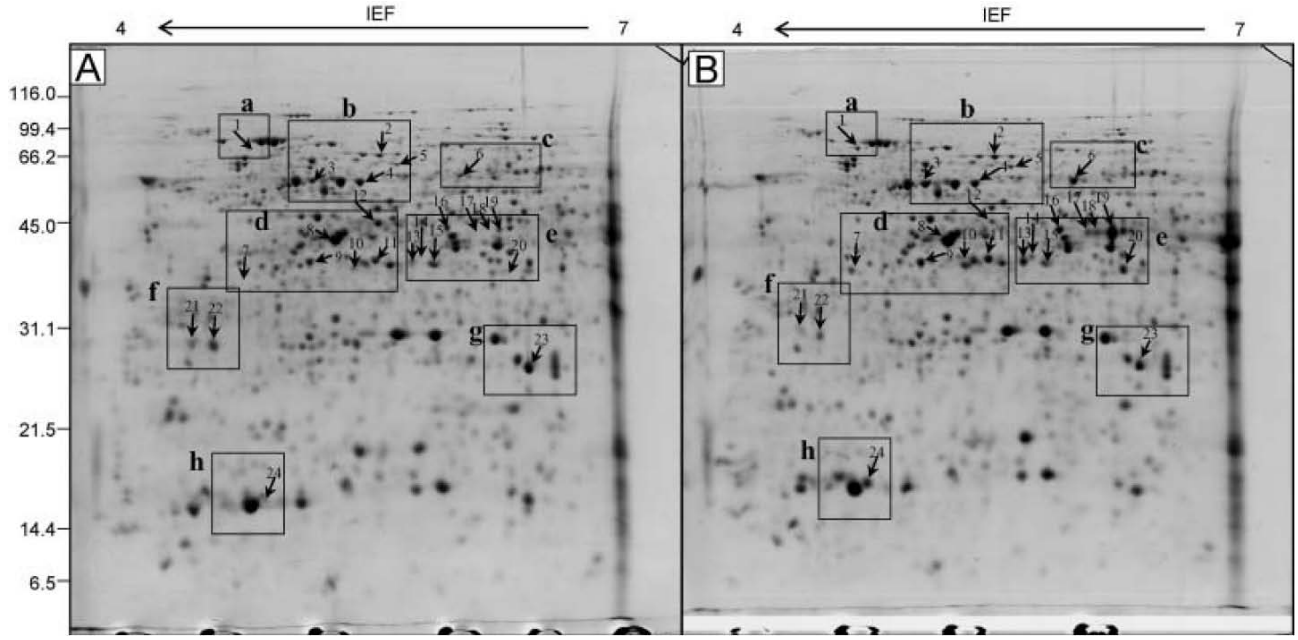

Figure 2. 2-DE analysis of soybean root proteins under waterlogging stress. A total of 350 μg protein was separated by two-dimensional gel electrophoresis (2-DGE) as described in Materials and Methods, and visualized with Coomassie brilliant blue (CBB) staining. **(A)** and **(B)** represent the 2-DE patterns of control and 7-day waterlogging-treated roots, respectively. The boxes (a–h) are enlarged in figure 3. Arrows indicate differentially expressed proteins identified in response to waterlogging stress.

regeneration. Therefore, plants show a higher glycolytic flux under waterlogging stress. In our investigation, UDP-glucose pyrophosphorylase (spot no. 3, UGPase, EC 2.7.7.9) and phosphofructokinase (spot no. 9, PFK, EC 2.7.1.11) were increased 2- to 3-fold under waterlogging stress (figure 3).

UGPase catalyses the reversible production of UDP glucose and pyrophosphate from glucose-1-P and UTP. PFK catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate and is a key regulatory enzyme in glycolysis. Besides using ATP as a phosphoryl donor, inorganic pyrophosphate (PPi) can also be used by PFK to phosphorylate fructose-1,6-bisphosphate. PPi-dependent phosphorylation is a reversible reaction and has been shown to be induced under anoxia (Mustroph *et al.* 2005).

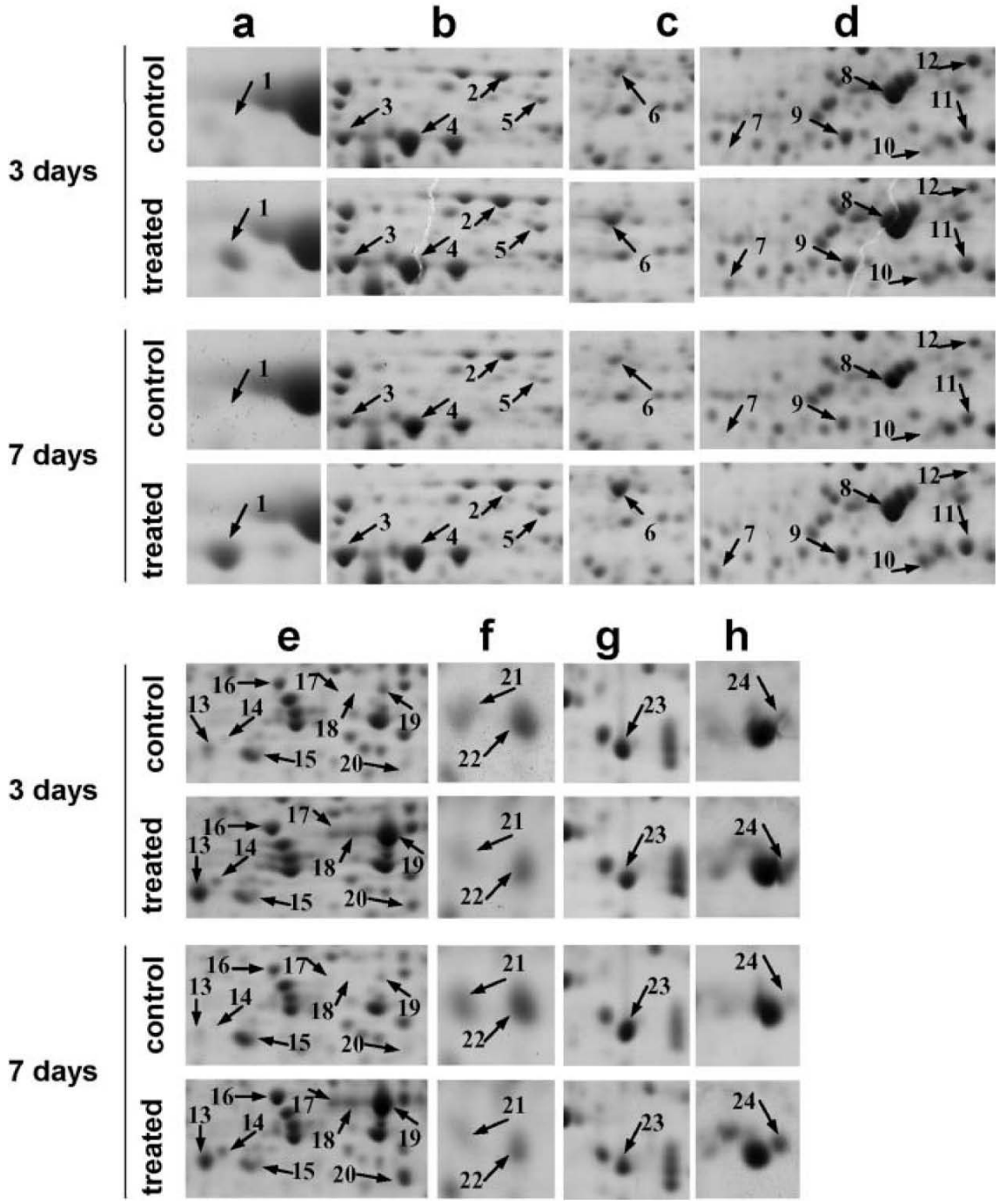

Figure 3. Close-up views of Coomassie brilliant blue (CBB)-stained gels of the differentially expressed proteins marked in figure 2 (a–h). Arrows indicate the waterlogging stress-responsive identified proteins.

Table 1. Waterlogging-induced differentially expressed proteins in soybean root identified by MALDI-TOF or ESI-MS/MS analysis. Proteins up (↑)- or down (↓)-regulated or (N) newly induced in response to waterlogging

Spot	Protein	Organism	Accession[a]	Mr/PI		SC[b] (%)	PM[c]	Expectation	Fold change[d]	
				Theoretical	Observed				3T/3C	7T/7C
1N	Putative auxin-amidohydrolase precursor	*Populus alba x Populus tremula*	CAG32960.1	47.97/5.8	65/5.0	16	4	4.6e-2	new	new
2↑	RING-H2 finger protein ATL1C precursor	*Arabidopsis thaliana*	Q9SK92	42.23/6.0	60/5.5	23	4	294[†]	1.90±0.25	1.97±0.09
3↑	UDP-glucose pyrophosphorylase	*Amorpha fruticosa*	AAL33919.1	51.68/6.1	50/5.1	23	9	9.4e-9	2.04±0.06	2.98±0.50
4↑	Enolase	*Glycine max*	42521309	47.99/5.3	50/5.5	36	11	2.7e-7	1.65±0.08	1.61±0.03
5↑	Alcohol dehydrogenase	*Barbarea vulgaris*	6684374	41.76/5.9	60/5.7	17	4	2.5e-3	2.04±0.04	2.43±0.24
6↑	Enolase 1	*Zea may*	55296986	48.27/5.2	57/6.0	23	8	9.1e-3	2.53±0.96	3.26±1.12
7N	ARR15 (RESPONSE REGULATOR 15); transcription regulator	*Arabidopsis thaliana*	NP_177627.1	22.50/5.8	33/4.9	39	5	2.8e-2	new	new
8↑	Cytosolic glutamine synthetase GS beta1	*Glycine max*	AF301590_1	39.14/5.5	38/5.3	31	8	7.2e-3	1.78±0.14	1.84±0.03
9↑	Pfkb-type carbohydrate kinase family protein	*Arabidopsis thaliana*	15224669	35.43/5.3	35/5.1	28	7	2.9e-3	1.93±0.18	2.14±0.03
10↑	Apyrase 2	*Solanum tuberosum*	AAQ10658.1	31.41/5.5	34/5.4	17	5	4.3e-4	6.62±0.38	7.20±0.76
11↑	Hypothetical protein OsJ_024153	*Oryza sativa*	EAZ40670.1	35.81/5.3	35/5.5	14	4	137[†]	1.71±0.07	2.08±0.06
12	MTO3 (S-adenosylmethionine synthase 3); methionine adenosyltransferase	*Arabidopsis thaliana*	NP_188365.1	43.18/5.5	44/5.5	24	6	1.5e-3	0.50±0.03	0.62±0.04
13↑	S-receptor kinase 13-29	*Arabidopsis lyrata*	27545496	20.19/4.7	34/5.6	31	5	3.7e-2	9.90±1.68	8.63±0.96
14↑	Coproporphyrinogen III oxidase precursor	*Glycine max*	P35055.1	43.60/6.7	38/6.0	29	9	6.3e-4	4.33±0.69	8.36±1.44

Table 1. *Continued*

15↓	Receptor-like kinase extracellular domain precursor RLK10A2	*Avena sativa*	AF237550_1	32.60/5.7	33/6.1	21	4	5.1e-6	0.66±0.09	0.41±0.05
16↑	Cytosolic phosphoglycerate kinase	*Pisum sativum*	AF275639_1	42.27/5.7	42/5.7	20	7	1.3e-3	2.13±0.25	2.54±0.46
17N	Kelch repeat containing F-box family protein	*Arabidopsis thaliana*	NP_173623.1	53.14/6.3	42/6.0	17	4	4.5e-3	new	new
18N	Alcohol-dehydrogenase	*Glycine max*	AAC97495.1	37.05/6.1	40/6.2	36	10	3.8e-6	new	new
19↑	Alcohol dehydrogenase	*Glycine max*	AAC97495.1	37.05/6.1	40/6.2	27	8	9.6e-5	9.95±0.99	16.48±2.0
20N	Isoflavone reductase homologue 1*	*Glycine max*	6573169						new	new
21↓	Putative apoptosis antagonizing transcription factor	*Oryza sativa*	BAB91998.1	48.11/4.6	30/4.6	21	4	1.4e-2	0.34±0.07	0.35±0.05
22↓	NUDIX/mutT hydrolase family protein	*Arabidopsis thaliana*	NP_178525	24.39/7.0	28/4.7	24	4	792†	0.42±0.06	0.31±0.09
23↓	Peroxidase precursor	*Glycine max*	4204759	38.63/6.9	27/6.6	18	5	375†	0.80±0.05	0.44±0.03
24↑	Eukaryotic translation initiation factor 5A-1 (eIF-5A-1) (eIF-4D)	*Medicago sativa*	P26564	17.87/5.5	16/4.9	23	4	2.5e-2	2.37±0.34	11.78±0.78

†Searched in MS Fit interface and the values are the MOWSE score, others have been searched in Profound interface. A Profound expectation value of <5e-2 is considered a significant protein match (confidence >95%).
(a) Accession number in NCBI database
(b) SC, sequence coverage by PMF using MALDI-TOF MS (c) PM, number of peptides matched
(d) Increased or decreased compared with control. 3T/3C and 7T/7C are the changes in abundance ratios under 3- and 7-day waterlogging treatment, respectively.
* Identified by ESI-MS/MS, sequence: QVDVVISTVGRAQLSDQVK; Score 36

Such preferential utilization of PPi-driven reactions may substitute for those requiring ATP to support metabolic and cellular functions under low oxygen. Other downstream enzymes of glycolysis and fermentation pathways (so-called ANPs), including phosphoglycerate kinase (spot 16), enolase (spots 4, 6) and alcohol dehydrogenase (spots 5, 18, 19), were upregulated under waterlogging stress. Cytosolic phosphoglycerate kinase (spot 16), which catalyses the formation of 3-phosphoglycerate from 1,3-bisphosphoglycerate, was upregulated by more than 2.5-fold. Adenosine triphosphate (ATP) is generated by transferring a phosphate group to adenosine biphosphate (ADP) in this reaction. The products of the reaction are strongly favoured due to the presence of the unstable high-energy anhydride bond of 1,3-bisphosphoglycerate (Dennis *et al.* 2000). Enolase (2-phospho-d-glycerate hydratase; EC 4.2.1.11), an integral enzyme in glycolysis, catalyses the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate. The phosphate attached to the 2-position of pyruvate also has a high negative free energy of hydrolysis, allowing the transfer of the phosphate group to ADP in the subsequent reaction. The anaerobic expression of enolase has been revealed previously (Ahsan *et al.* 2007a). Highly upregulated (9–16-fold) alcohol dehydrogenase (Adh) reduces acetaldehyde to ethanol with concomitant reoxidation of NAD^+, which is essential for continuing glycolysis. In this shift, plants keep the redox status unaltered for a prolonged period, which is important for plant survival under low oxygen stress. Increasing ethanol fermentation by overexpressing pyruvate decarboxylase was helpful for survival under moderately severe oxygen limitation in *Arabidopsis*, whereas overexpression of Adh did not show promising results (Ismond *et al.* 2003). The multiple protein spots identified as enolase and Adh are probably different isoforms.

3.2.2 *Alteration in other energy-related proteins:* Accumulated evidence suggests that a metabolic pathway involving nitrous oxide (NO) and haemoglobin (Hb) provides an alternative type of respiration to mitochondrial electron transport under limited oxygen (Sairam *et al.* 2008). The enzyme coproporphyrinogen oxidase (spot 14) catalyses the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX in the haem and chlorophyll biosynthesis pathway(s). In our experiment, this protein level was increased 4–8-fold under waterlogging conditions. Previous workers have also shown that coproporphyrinogen oxidase activity became rate-limiting for haem production when its substrate oxygen was limiting. Cells responded to oxygen limitation by increasing the amount of the enzyme (Amillet *et al.* 1996; Blanco *et al.* 2005). Thus, an increased level of coproporphyrinogen oxidase may play a crucial role in linking haem synthesis to the oxygen/haem-dependent control of gene expression during waterlogging. It is noteworthy that Hb levels may be elevated by an increased level of auxins (Watts *et al.* 2001) and may directly affect ethylene signalling (Manac'h-Little *et al.* 2005). We also identified an auxin amidohydrolase that can potentially release auxin from a conjugated storage pool.

3.2.3 *Modulation in antioxidant- and nitrogen metabolism-related proteins:* To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes (SOD, catalase, peroxidases) that scavenge ROS. Increased peroxidase activity has been seen due to flooding stress and was suggested to assist in the reduction of ROS (Lin *et al.* 2004). However, in this study, peroxidase (spot 23) was downregulated during the waterlogging period. Decreased peroxidase levels can cause an increase in ROS accumulation. Our result is consistent with that of Shi *et al.* (2008), who showed a drastic decrease in cytosolic ascorbate peroxidase activity under flooding stress in germinated soybean seedlings.

Glutamine synthetase (GS); EC 6.3.1.2 (spot 8), the key enzyme involved in ammonia assimilation in plants, catalyses the ATP-dependent condensation of ammonia with glutamate to produce glutamine. It has been suggested that the assimilation of nitrate or nitrite as amino acids may serve as an alternative electron acceptor to oxygen in disposing of reducing power generated by glycolysis during anoxia (Weger and Turpin 1989). Reggiani *et al.* (2000) observed higher GS and glutamate synthase (GOGAT) activity during the anaerobic response in rice root. Chemical inhibition of GS and GOGAT reduced the anaerobic concentration of total amino acid as well as alanine and gamma-aminobutyric acid (GABA), suggesting an important role of the GS–GOGAT cycle in amino acid metabolism under anoxia. Along with the previous evidence, upregulation of GS might play an important role in waterlogged soybean root by contributing to H^+ homeostasis and maintaining root osmotic potential.

3.2.4 *Upregulation in signal transduction- and programmed cell death-related proteins:* When soil lacks oxygen due to soil waterlogging, aerenchyma – tissue comprising a high proportion of gas-filled spaces or lacunae – provides the plant with an alternative strategy for obtaining O_2. The interconnected lacunae, extending from below the ground up into the stems and leaves, make up an internal aeration system. Lysigenous aerenchyma provides not only an internal pathway for O_2 transfer, but also simultaneously reduces the number of O_2-consuming cells, a feature that might assist in low O_2 environments. The molecular mechanism of aerenchyma formation was largely unknown; however, it occurs as a consequence of programmed cell death (PCD) and cell wall autolysis. In this study, we found several proteins related to PCD, which may be involved in aerenchyma formation and/or minimizing stress injuries. Eukaryotic translation initiation factor 5A-1 (eIF-5A-1; spot 24) was proposed to function as a biomodular

protein capable of binding both RNA and protein, and therefore involved in multiple aspects of cellular signalling activities (Jao and Chen 2006). Among the three isoforms found in *Arabidopsis*, AteIF-5A1 is expressed only in senescing tissue (Thompson *et al.* 2004). In this study, we identified this protein to be highly upregulated in response to waterlogging stress. Activation of eIF-5A requires hypusination, a post-translational modification formed by deoxyhypusine synthase (DHS) (Thompson *et al.* 2004). Wang *et al.* (2001) showed a parallel increase in DHS and eIF-5A transcripts together with their cognate proteins in senescing tomato flowers, tomato cotyledons, tomato fruit and environmentally stressed tomato leaves exhibiting symptoms of PCD. These results suggest that eIF5A may play a vital role in PCD due to waterlogging stress in soybean roots. Apyrase (spot 10) is a calcium-activated plasma membrane-bound enzyme (magnesium can also activate it) (EC 3.6.1.5) that catalyses the hydrolysis of ATP to yield adenosine monophosphate (AMP) and inorganic phosphate. The optimal level of extracellular ATP (eATP) is predominantly regulated by apyrase, since a reduced or excess level inhibits growth (Roux and Steinebrunner 2007). Despite the consequence of normal growth and development, plant cells also release ATP in response to various abiotic stresses, such as osmotic stress, cold stress and mechanical stimulation (Jeter *et al.* 2004). However, apyrase protein has not been reported during hypoxia or waterlogging in plant cells. The destruction or overaccumulation of eATP can lead to diminished growth and induce PCD in several plant species (Chivasa *et al.* 2005; Kim *et al.* 2006). These data suggest a possible role of apyrase in regulating PCD by adjusting the extracellular ATP levels, which might have a possible role in waterlogging tolerance.

Apoptosis-antagonizing transcription factor (AATF; spot 21) is a leucine zipper domain-containing protein that has anti-apoptotic properties. The role of AATF has not been well studied in plants. AATF may participate in inhibition of proapoptotic pathways and/or activation of antiapoptotic pathways. Overexpression of AATF results in significant anti-apoptotic activity, whereas a knockdown of AATF by small interference RNA led to exacerbated cell death after hypoxic stress in animal cells (Xie and Guo 2006). In our study, downregulation of this gene might involve a reduction in apoptosis due to waterlogging stress. S-adenosyl-L-methionine synthetase (SAM synthetase, spot 12) was also downregulated during waterlogging stress. SAM is the key enzyme in the synthesis of S-adenosyl-L-methionine, which is an important metabolic component in many cellular processes, including the biosynthesis of ethylene. The downregulation of SAM is probably associated with reduced ethylene production in the root upon prolonged waterlogging treatment, as we have observed earlier (Ahsan *et al.* 2007a).

Spot 1 is a putative auxin-amidohydrolase newly induced upon waterlogging stress. Auxins are ubiquitous plant hormones involved in the regulation of many aspects of plant growth and development including regulation of gene expression. Auxins are commonly used to induce rooting. Induction of adventitious root at the base of the shoot is an important adaptation to flooded conditions and takes place soon after flooding; this may require increased auxin release from the conjugated form. Auxins are present as a conjugate storage pool from which the active hormone can be released by selective classes of enzymes, such as amino acid amidohydrolases, providing a means for the regulation of auxin levels in plants (Woodward and Bartel 2005). Thus, a significant induction of auxin-amidohydrolase might help the plant to release auxin for induction of adventitious roots to adapt to the waterlogged condition.

Spots 12 and 15 were matched with receptor kinase-like proteins. ARABIDOPSIS RESPONSE REGULATORS (ARRs; spot 7) are transcription regulators involved in cytokinin response. Roots are the major site of cytokinin synthesis. Cytokinin delays senescence induced by flooding stress in *Arabidopsis* plants (Huynh *et al.* 2005). In our study, the cytokinin signalling protein was newly induced upon waterlogging which might be involved in increased cytokinin sensitivity when cytokinin synthesis is depressed.

Two receptor kinase-like proteins (spots 13, 15) were modulated under waterlogging stress. S locus receptor kinase (SRK) encodes a plasma membrane-spanning receptor serine-threonine kinase known to be involved in self-incompatibility responses in *Brassica*. Based on its homology to lectins, the agglutinin motif may bind α-mannose, while the PAN motif has been implicated in mediating protein–protein and protein–carbohydrate interaction (Haffani *et al.* 2004). Although several members of the plant receptor kinase gene family have been implicated in playing key roles in diverse developmental pathways, there is also evidence that others play important roles in the defence response (Torii and Clark 2000). Therefore, these proteins might also have a role in waterlogging-induced signal transduction.

Spot 20 was a newly induced protein under waterlogging stress, which was identified as isoflavone reductase homologue 1 by ESI-MS/MS. Isoflavone reductase (IFR) is a key enzyme in the isoflavonoid biosynthesis pathway. Isoflavonoids serve important roles in plants during growth, development, nodulation and in defence against microorganisms, pests and abiotic stresses. Specifically, IFR and homologues isolated from various plant species have been implicated in responses to various biotic or abiotic stresses (Dakora and Phillips 1996; Petrucco *et al.* 1996; Lers *et al.* 1998). Previously, we have identified flavanone 3-hydroxylase, another protein of the flavonoid biosynthesis pathway in tomato root subjected to waterlogging (Ahsan

et al. 2007a). Taken together, the induction of IFR in response to waterlogging clearly indicates the distinct involvement of isoflavonoid pathways in survival and PCD in plants exposed to waterlogging stress.

3.2.5 *Miscellaneous and unknown proteins:* Spot 22 was identified as a NUDIX/mutT hydrolase family protein, which was downregulated. The proteins of this family are characterized as enzymes that catalyse the hydrolysis of nucleoside diphosphates, while linked to some other moiety x. Increased Nudix hydrolase expression due to oxidative stress has been reported in *Arabidopsis* (Jambunathan and Mahalingam 2006). Despite their originally proposed house-cleaning roles, activities such as RNA processing, Ca^{2+} channel gating, and regulation of ERK signalling show that the Nudix fold and motif have been adapted for the binding and hydrolysis of a wide range of nucleosides and other pyrophosphates for a much greater diversity of purpose. Therefore, an overall role for the Nudix family cannot easily be defined (McLennan 2006). In our proteomic analysis, a Nudix family protein was decreased; however, the precise function of this protein in response to waterlogging stress response is not clear.

The ATL proteins are likely to be single-subunit E3 ubiquitin ligases (Stone *et al.* 2005). Experimental evidence suggests the involvement of some of its members in the growth-regulator response, response to biotic stress and plant development. In addition, several ATLs may participate in defence responses in plants (Serrano *et al.* 2006). Thus, upregulation of an ATL-type RING finger protein (spot 2) might involve defence, directing proteolysis, or modifying protein trafficking machinery under waterlogging stress. In plants, as in other living organisms, protein turnover is a key regulatory mechanism in many cellular processes, including developmental pathways, stress responses and various signal transduction pathways. The ubiquitin (Ub)/26S proteasome pathway is responsible for the selective degradation of most intracellular proteins in eukaryotes (Nandi *et al.* 2006). The F-box protein performs the crucial role of conferring specificity of activated Ub for the appropriate targets by acting as E3 ligases in plant. It has been reported that their expression is influenced by light and abiotic stresses (Jain *et al.* 2007). Taken together, the identification of a Kelch repeat containing F-box protein (spot 17) might suggest involvement in targeting the degradation of proteins which downregulate the hypoxic response.

In addition to the proteins described here, one protein (spot 11) was identified as a protein with unknown function. We were unable to correlate the role of this protein in relation to oxygen deprivation or waterlogging stress. Further studies are needed to address its possible role in relation to waterlogging stress.

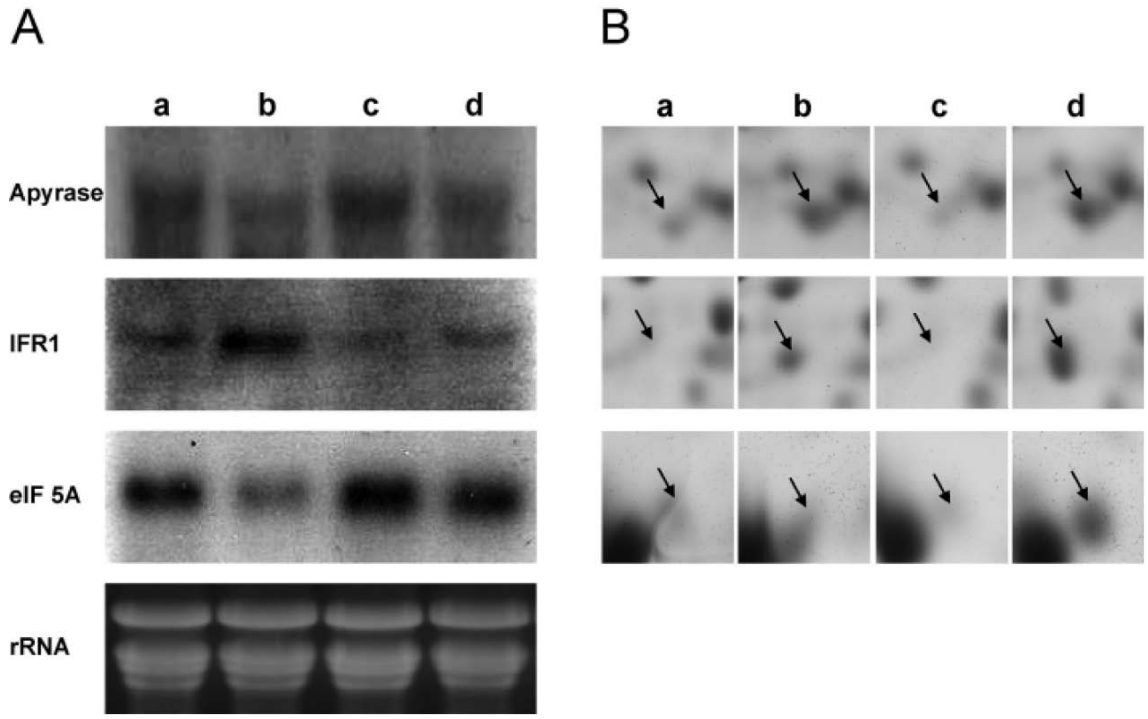

Figure 4. Northern blot analysis of three selected protein genes expressed in response to waterlogging stress. **(A)** Total RNA was extracted from 3-day control **(a)**, 3-day treated **(b)**, 7-day control **(c)**, and 7-day treated **(d)** root samples. Total RNA (10 μg) was separated by electrophoresis on an agarose gel, blotted on a nylon membrane and hybridized with ^{32}P-labelled gene-specific cDNA probes. **(B)** represents the proteomic expression of the corresponding genes.

3.3 *Gene expression at the mRNA level*

Among the novel products identified in our proteomic analysis, regulation of three PCD-related proteins modulated by waterlogging was analysed at the mRNA level using northern blot analysis (figure 4). The mRNA levels in the control and treatment groups were compared. The mRNA expression levels of eukaryotic translation initiation factor 5A-1 (eIF 5A) and apyrase were decreased due to waterlogging compared with the control; however, it was slightly increased on 7-day treatment compared with 3-day treatment. The isoflavone reductase homologue 1 (IFR1) mRNA level was increased in 3-day waterlogging, followed by a decrease, but it was still higher than in the control. The results of the present investigation support the well-known phenomenon that transcription patterns do not always provide pertinent information on protein expression levels. Post-transcriptional regulation plays an important role in stress-responsive gene expression. Proteins can also undergo several modifications following translation by proteolytic cleavage of amino acid residues, as well as chemical derivatizations of their side chains including acetylation, glycosylation, hydroxylation, methylation, acylation, phosphorylation, ubiquitination and sulphation (Jensen 2004). Moreover, a further range of modifications occurs including carbonyl formation, disulphide formation, S-nitrosylation and attachment of lipid aldehydes during oxidative stress (Ito *et al.* 2007). These wide ranges of modifications lead to a lack of correlation between transcript and protein abundance, which can have important consequences during the stress response.

4. Conclusion

The molecular responses to waterlogging stress were investigated at the protein level in soybean seedling at the early vegetative stage. Among the differentially expressed proteins, 24 were identified by MS analysis. These proteins were involved in several processes that might work cooperatively to establish a new homeostasis under waterlogging stress. The initial non-specific step in the waterlogging stress response is the generation of ROS, a phenomenon that has been shown to be the primary event in a large number of stresses. Imposition of waterlogging stress results in increased glycolytic flux to meet ATP requirements by upregulating anaerobic energy metabolism. Since the terminal electron acceptor of ETC (oxygen) is unavailable, the intermediate electron carriers become reduced. This process in turn affects the redox characteristics of the cell (i.e. $NADH/NAD^+$ ratio); and the upregulation of GS might help to maintain this. Auxin-amidohydrolase might help plants to release auxin for adventitious root induction while cytokinin signalling protein may be involved in reducing senescence. Hypoxic stress increases the levels of Hb, which may be affected by auxins that directly affect ethylene signalling. Although the signalling mechanism leading to waterlogging-specific metabolic responses is not clearly understood, it appears that oxygen deprivation stress may initiate the signalling cascade leading to PCD. The originality of the present work is our attempt to link waterlogging stress and PCD in actively growing soybean roots. Our results provide new insight into waterlogging stress and provide genes of interest for transgenic research. However, a comparative study between contrasting cultivars would be an avenue for further elucidation of the responsive pathway for this stress.

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