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ORIGINAL ARTICLE

Identification of flooding stress responsible cascades in root and hypocotyl of soybean using proteome analysis

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Abstract Flooding inducible proteins were analyzed using a proteomic technique to understand the mechanism of soybean response to immersion in water. Soybeans were germinated for 2 days, and then subjected to flooding for 2 days. Proteins were extracted from root and hypocotyl, separated by two-dimensional polyacrylamide gel electrophoresis, stained by Coomassie brilliant blue, and analyzed by protein sequencing and mass spectrometry. Out of 803 proteins, 21 proteins were significantly up-regulated, and seven proteins were down-regulated by flooding stress. Of the total, 11 up-regulated proteins were classified as related to protein destination/storage and three proteins to energy, while four down-regulated proteins were related to protein destination/storage and three proteins to disease/defense. The expression of 22 proteins significantly changed within 1 day after flooding stress. The effects of flooding, nitrogen substitution without flooding, or flooding with aeration were analyzed for 1–4 days. The expression of alcohol dehydrogenase increased remarkably by nitrogen substitution compared to flooding. The expression of many proteins that changed due to flooding showed the same tendencies observed for nitrogen substitution; however, the expression of proteins classified into protein destination/storage did not.

Keywords Flooding · Proteome · Soybean · Hypoxic response

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Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
MS	Mass spectrometry
CBB	Coomassie brilliant blue
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
pI	Isoelectric point

Introduction

Flooding stress is a widespread phenomenon in regions where the soil has an impermeable clay base or consists of cracking grey clays with slow drainage, where there is an extreme rainfall pattern, or where imperfect land planning has occurred. Higher plants are aerobic organisms that die when oxygen availability is limited due to soil flooding (Voesenek et al. 2006). Flooding leads to reduced gas exchange between the plant tissue and the atmosphere, because gases, particularly oxygen, diffuse 10,000 times more slowly in water than in air (Armstrong 1979). In addition, other changes affecting soil chemical characteristics during flooding include variations in soil pH (Probert and Keating 2000) and redox potential (Pezeshki 2001). Thus, oxygen deprivation by flooding may not only become the main limiting factor for normal plant development, but also is probably the primary signal triggering the response (Jackson and Colmer 2005). Gene expression studies on plants exposed to low oxygen revealed the up-regulation of genes coding for transcription factors (Liu et al. 2005), signal transduction components (Baxter-Burrell et al. 2002), nonsymbiotic hemoglobin (Dordas et al. 2004), ethylene signaling (Reggiani 2006), nitrogen

metabolism (Mattana et al. 1994), and cell wall loosening (Saab and Sachs 1996). However, research on changes at the protein level, which occur before observed functional changes, is still insufficient.

At the protein level, low oxygen selectively induces the synthesis of proteins known as anaerobic proteins, most of which are enzymes involved in sugar metabolism, glycolysis, and fermentation pathways (Huang et al. 2005). Most of these results have been studied using model plants such as the flood-intolerant species *Arabidopsis* or flood-tolerant rice. Despite knowledge of adaptive mechanisms and regulation at the molecular level, the understanding of the mechanisms behind plant response to flooding is very limited. Even *Arabidopsis* switches on many genes that are generally associated with responses to flooding (Gonzali et al. 2005; Loreti et al. 2005). These studies strongly suggest that the regulation of flooding tolerance in plants is far more complex than anticipated. Investigation of the mechanisms underlying the response to flooding stress using not only model plants but also other plants has been accumulating in order to generate flooding stress-tolerant plants. Since flooding is one of the environmental constraints for crop plants, more studies using flood-intolerant and flood-tolerant plants are required.

Soybean is a flood-intolerant plant. Flooding is a major problem that reduces soybean growth and grain yield in many areas of the world. Flooding injury of soybean seeds before radicle protrusion, namely during seed imbibition, was caused by physical disruption of the rapid uptake of water and can be alleviated by using seeds with high moisture content (Nakayama et al. 2004). The causes of flooding injury after radicle protrusion; however, have not been elucidated, although a physiological agent may be involved. Recently, Shi et al. (2008) reported that cytosolic ascorbate peroxidase 2 was involved in flooding stress responses in young soybean seedlings using a proteomic technique; however, the numbers of proteins analyzed were limited. Because the analysis of proteins using two-dimensional polyacrylamide gel electrophoresis (2-DE) is the most direct approach for defining gene function (Komatsu et al. 2003), this proteomic technique has been employed to analyze protein changes in response to environmental changes (Komatsu and Yano 2006). In this study, flooding inducible proteins were analyzed using a 2-DE to understand the mechanism by which soybean responds to immersion in water. Soybeans were also subjected to flooding, nitrogen substitution without flooding and flooding with aeration, then used to determine whether the flooding responses of soybean that are induced depend only on the hypoxic response.

Materials and methods

Plant growth and treatment

Seeds of soybean (*Glycine max* L.) cultivar Enrei, after sterilization by a sodium hypochlorite solution, were germinated on sand for 2 days and then flooded with water for 1, 2, 3 or 4 days under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25°C and 70% relative humidity in a growth chamber. Soybean seeds that had germinated on sand for 2 days were also treated with or without flooding, nitrogen substitution without flooding (density of oxygen: less than 3%), and flooding with aeration (density of oxygen: more than 15%) for 1, 2, 3 or 4 days. After treatment, physiological parameters including fresh weight and length of hypocotyl and root were measured, and protein analysis was carried out. The experiments were repeated 3 times.

Protein extraction and 2-DE

A portion (200 mg) of root and hypocotyl was homogenized with 1 ml of phosphate-buffered saline (pH 7.6) containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl and 3 mM NaN_3 at 4°C using a glass mortar and pestle. The homogenate was centrifuged at 15,000×*g* for 10 min, and 50% trichloroacetic acid was added to the supernatant to a final concentration of 10%. The solution was kept for 30 min at 4°C and centrifuged at 15,000×*g* for 10 min (Zhong et al. 1997). The resultant precipitate was suspended in 300 μl of lysis buffer (O'Farrell 1975) containing 8 M urea, 2% Nonidet P-40, 0.8% Ampholine (pH 3.5-10, GE Healthcare, Piscataway, NJ, USA), 5% 2-mercapthoethanol and 5% polyvinylpyrrolidone-40, using a glass mortar and pestle. The homogenates were centrifuged twice at 15,000×*g* for 10 min each. The supernatant was used as protein extract.

Proteins (50 μl, 400 μg) were separated by 2-DE in the first dimension by an isoelectric focusing (IEF) tube gel (O'Farrell 1975) for the low pI range (pI 3.5–8.0) or an immobilized pH gradient (IPG) tube gel (Hirano et al. 2000) for the high pI range (pI 6.0–10.0) and in the second dimension by SDS-PAGE. The IEF gel consisted of 8 M urea, 3.5% acrylamide, 2% Nonidet P-40, 2% Ampholine (pH 3.5–10 and pH 5–8). Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. For IPG electrophoresis, proteins were directly applied to the acidic side of tube gels. Electrophoresis using IPG tube gels (pH 6.0–10.0) (Daiichi Kagaku, Tokyo, Japan) was carried out at 400 V for 1 h, followed by 1,000 V for 16 h and 2,000 V for 1 h. After IEF and IPG, SDS-PAGE was performed using 15% polyacrylamide

gels with 5% stacking gels. The gels were stained with Coomassie brilliant blue (CBB), and image analysis was performed.

Gel image analysis

2-DE images were synthesized and position of individual proteins on gels was evaluated automatically with Image Master 2D Elite software (version 3.01; GE Healthcare). The isoelectric point (pI) and molecular mass of each protein were determined using a 2-DE marker (Bio-Rad, Hercules, CA, USA). The amount of a protein spot was estimated using PDQuest software (Bio-Rad), and expressed as the volume of that spot, which was defined as the sum of the intensities of all the pixels that make up the spot. In order to correct the variability due to CBB staining and to reflect the quantitative variations in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume of all of the spots present in the gel.

Cleveland peptide mapping

Following separation by 2-DE, gel pieces containing protein spots were removed and the protein was electroeluted from the gel pieces using an electrophoretic concentrator (Nippon-Eido, Tokyo, Japan) at 2 W constant power for 2 h. After electroelution, the protein solution was dialyzed against deionized water for 2 days and lyophilized. The protein was dissolved in 20 µl of SDS sample buffer containing 0.5 M Tris–HCl (pH 6.8), 10% glycerol, 2.5% SDS and 5% 2-mercaptoethanol, and applied to a sample well in an SDS-PAGE gel. The sample solution was overlaid with 20 µl of a solution containing 10 µl of 0.1 µg μl^{-1} *Staphylococcus aureus* V8 protease (Pierce, Rockford, IL, USA) and 10 µl of SDS sample buffer. Electrophoresis was performed until the sample and protease were stacked in the stacking gel, interrupted for 30 min to allow digestion of the protein (Cleveland et al. 1977), and then continued until 5 mm from bottom of gel.

N-terminal and internal amino acid sequence analyses

To analyze N-terminal and internal amino acid sequences following separation using 2-DE or Cleveland peptide mapping, the proteins were electroblotted onto a polyvinylidene difluoride membrane (Pall, Port Washington, NY, USA) using a semidry transfer blotter (Nippon-Eido) and detected by CBB staining. The stained protein spots or bands were excised from the membrane and directly subjected to Edman degradation on a gas-phase protein sequencer (Procise cLC, Applied Biosystems, Foster City, CA, USA).

Analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Proteins were excised from the 2-DE gel stained by CBB, and then alkylation and protein digestion with trypsin were performed using a robotic system (DigestPro96, Intavis AG, Koeln, Germany). The degenerated peptides were purified using NuTip C-18 (Glygen, Columbia, MD, USA). The purified peptides were added to a α-cyano-4-hydroxycinnamic acid matrix and dried onto a plate for analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MS) (Voyager-DE RP, Applied Biosystems).

Calibration was external, and data were collected in the reflector mode. Data were searched on the internet using an in-house licensed MASCOT search engine (Mascot Version 2.2.18) software platform (Matrix Science, London, UK) against all entries in the soybean genome database version 4 (62,199 sequences), which was especially constructed for this research based on soybean genome preliminary sequences from the Department of Energy Joint Genome Institute and Soybean Genome Sequencing Consortium. Soybean genome sequences were downloaded from the DOE database (http://www.phytozome.net, release data 24 January 2008) and then were converted into FASTA format. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. In the case of peptides matching among multiple members of a protein family, the protein presented was selected based on the highest score and the highest number of the matching peptides.

For analysis, four criteria were used to assign a positive match with a known protein: (1) the deviation between the experimental and theoretical peptide masses needed to be less than 50 ppm; (2) at least six different predicted peptide masses needed to match the observed masses for an identification to be considered valid; (3) the matching peptides needed to cover at least 30% of the known protein sequence; and (4) individual ions had to score more than 72 identity or extensive homology ($P < 0.05$).

Results

The growth of soybean seedlings is significantly suppressed after 1 day of flooding

In this study, before proteomics experiments, the physiological changes in an early stage after flooding stress were measured, to analyze the early effect of flooding stress. Soybean seeds were germinated on sand for 2 days, and

treated with or without flooding, nitrogen substitution without flooding, or flooding with aeration for 1, 2, 3 or 4 days (Fig. 1). Oxygen concentration was less than 3% for nitrogen substitution without flooding and more than 15% for flooding with aeration. The lengths of lateral and adventitious roots were inhibited by flooding, nitrogen substitution without flooding, and flooding with aeration after 1 day of treatment (Fig. 1). Changes in the length of hypocotyls and main root and fresh weight of hypocotyls and total roots were measured after 1, 2, 3 and 4 days of water flooding, nitrogen substitution without flooding, and flooding with aeration (Table 1). The elongation of hypocotyls was not completely inhibited by nitrogen substitution without flooding, although it was inhibited by flooding (Table 1). On the other hand, the length of the main root was shorter after 1 day of treatment by flooding, nitrogen substitution without flooding, and flooding with aeration compared to controls. The level of inhibition of root growth continued to 4 days (Table 1). A similar time-dependent pattern of inhibition was observed for fresh weight of hypocotyls and total roots (Table 1).

Twenty-eight proteins are involved in the flooding response of germinating soybean seeds

To investigate mechanisms regulated by flooding stress in soybean, flooding responsive proteins were identified by a proteome technique. Soybean seeds were germinated on sand for 2 days and subjected to flooding for 2 days. Proteins were extracted from roots and hypocotyls of seedlings, separated by 2-DE, stained by CBB (Fig. 2), and identified by protein sequencing and MS (Table 2). A total of 803 protein spots were detected, and the pI and molecular weight of these proteins ranged from 3.5 to 10.0 and 10.0–100.0 kDa (Fig. 2, upper panel). Among these protein spots, 28 protein spots changed by flooding: 21 protein spots were up-regulated and 7 protein spots were down-regulated (Fig. 2).

Up-regulated proteins were the 70-kDa heat shock protein (spot 1), protein phosphatase inhibitor 1 (spots 3 and 4), maturation polypeptide (spots 5, 6 and 7), embryonic abundant protein (spots 8 and 9), hemoglobin alpha-I chain (spot 10), UDP-glucose pyrophosphorylase (spot 11), fructose-bisphosphate aldolase (spot 12), alcohol dehydrogenase (spot 13), albumin-1 precursor (spots 14, 15 and 16), beta-conglycinin alpha subunit (spot 21), D-alanine-D-alanine ligase A (spot 22), V-type proton ATPase subunit D (spot 23) and glyceraldehyde-3-phosphate dehydrogenase (spot 28). Down-regulated proteins were protein disulfide isomerase (spot 2), lectin (spot 17), ascorbate peroxidase (spot 18), stem 31 kDa glycoprotein (spot 20) and stem 28 kDa glycoprotein (spot 25). Interestingly, one 1-Cys peroxiredoxin (spot 19) was down-regulated and another 1-Cys peroxiredoxin (spot 24) was up-regulated. Also, one Kunitz-type inhibitor B (spot 26) was down-regulated and another Kunitz-type inhibitor B (spot 27) was up-regulated. In total, 20 proteins were identified as unique proteins involved in flooding stress (Table 2).

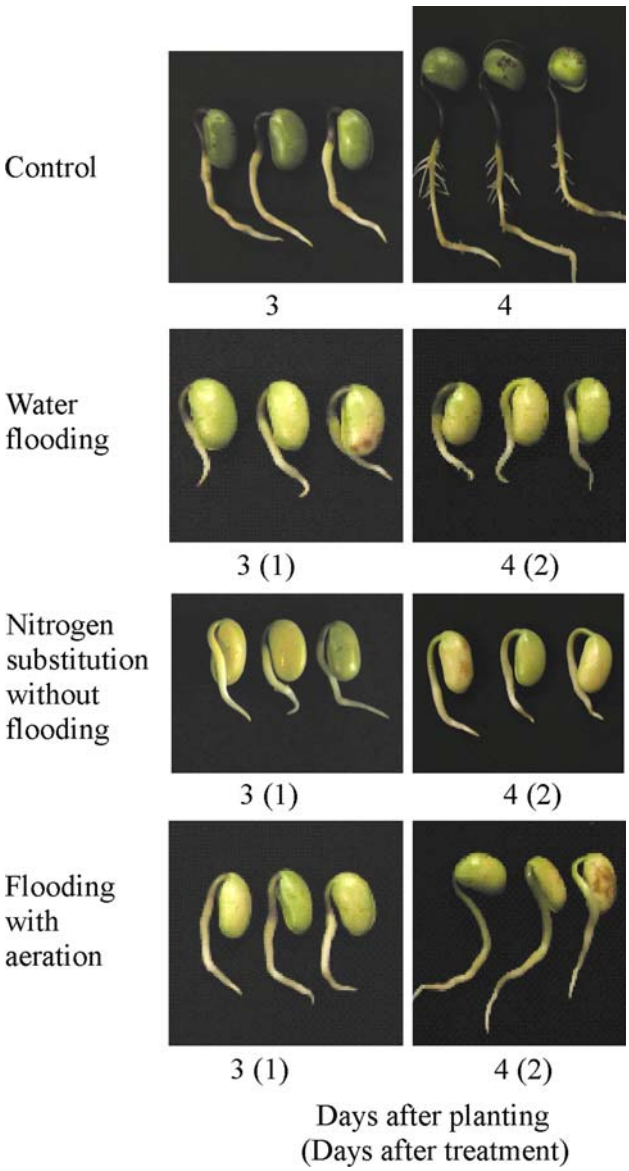

Fig. 1 Growth suppression in soybean seedlings after flooding. Soybean seeds were germinated on sand for 2 days, and treated with or without flooding, nitrogen substitution without flooding or flooding with aeration for 1, 2, 3, or 4 days. Photographs show the physiological differences among the plants

To determine which types of proteins are involved in the flooding stress responses of soybean, the proteins were categorized in seven functional groups on the basis of their physiological roles in plants (Bevan et al. 1998; Tanaka et al. 2004) (Table 2). Among 21 up-regulated proteins, 11 proteins were classified in categories related to protein destination/storage, three proteins in energy, two proteins

Table 1 Growth suppression in soybean seedlings after flooding

	Days after planting (days after treatment)					
	1	2	3 (1)	4 (2)	5 (3)	6 (4)
Length of hypocotyl (mm)						
Control	–	7.42 ± 0.63	12.00 ± 0.96	20.43 ± 1.43	25.86 ± 2.51	39.50 ± 0.96
Flooding	–	–	8.90 ± 0.46	10.00 ± 0.63**	9.57 ± 0.65**	12.38 ± 0.70**
N2	–	–	6.77 ± 0.69*	15.31 ± 0.61	19.36 ± 1.88*	22.50 ± 2.00**
A	–	–	9.10 ± 0.80	16.00 ± 0.80	–	–
Length of main root (mm)						
Control	5.20 ± 0.39	18.33 ± 1.88	29.44 ± 12.33	40.00 ± 2.60	59.00 ± 2.90	71.75 ± 4.81
Flooding	–	–	9.63 ± 1.27**	12.80 ± 0.86**	17.44 ± 1.46**	17.65 ± 0.65**
N2	–	–	12.33 ± 0.94**	16.62 ± 1.22**	16.20 ± 1.15**	17.50 ± 1.48**
A	–	–	14.00 ± 1.00**	18.00 ± 1.00**	–	–
Fresh weight of hypocotyl and total roots (mg)						
Control	16.60 ± 1.18	102.20 ± 5.55	193.00 ± 14.84	252.17 ± 13.41	330.60 ± 21.69	517.40 ± 16.42
Flooding	–	–	86.29 ± 7.81**	128.40 ± 9.07**	136.60 ± 11.89**	130.83 ± 7.74**
N2	–	–	90.63 ± 9.82**	119.38 ± 7.13**	131.27 ± 11.34**	154.00 ± 9.73**
A	–	–	95.00 ± 8.00**	120.00 ± 8.00**	–	–

Soybean seeds were germinated on sand for 2 days, and treated with or without flooding, nitrogen substitution without flooding or flooding with aeration for 1, 2, 3, or 4 days. The lengths of hypocotyls and main root, and fresh weight of hypocotyls and total roots were measured after treatment without (Control) or with water flooding (Flooding), nitrogen substitution without flooding (N2), and flooding with aeration (A) for 1, 2, 3 and 4 days. Values are mean ± SE from 3 independent experiments. For each experiment, 10 soybean plants were used. *Asterisks* indicate significant differences between control and each treatment (*$P < 0.05$; **$P < 0.01$)

in primary metabolism and two proteins in signal transduction. On the other hand, among seven down-regulated proteins, four proteins were functionally distributed in protein destination/storage and three proteins in disease/defense. These results indicated that proteins related to protein destination/storage, such as the 70-kDa heat shock protein, protein disulfide isomerase, maturation polypeptide, embryonic abundant protein, albmin-1 precursor, stem 31 kDa/21 kDa glycoprotein precursor, beta-conglycinin alpha subunit and Kunitz trypsin protease inhibitor might have abundant role with up- and down-regulation under flooding stress of soybean.

Protein expression patterns over time after water flooding are clustered in 5 patterns

To analyze the time dependence of expression of 28 proteins after flooding, soybean seeds were germinated on sand for 2 days, and treated with or without water flooding for 1, 2, 3 or 4 days. The quantitative expression changes of 28 protein spots indicated in Fig. 2 were compared for control and flooding treatments using PDQuest software (Table 2). They were classified into five groups as "Group A: Protein that increased remarkably at 1 and 2 days after flooding treatment", "Group B: Protein that increased remarkably at 1 or 2 days after flooding treatment", "Group C: Protein that increased within 2 days by flooding treatment", "Group D: Protein that kept increasing remarkably by flood treatment" and "Group E: Protein that increased during growth but decreased by flood treatment" (Table 2). The main two groups were "Protein group that increased remarkably at 1 and 2 days after flooding treatment" and "Protein group that increased during growth, but decreased by flood treatment".

The protein group that increased remarkably at 1 and 2 days after flooding treatment contained protein phosphatase inhibitor 1 (spots 3 and 4), maturation polypeptide (spots 5, 6 and 7), embryo abundant protein (spots 8 and 9), 1-Cys peroxiredoxin (spot 24), 70 kDa heat shock protein (spot 1), albumin-1 precursor A1 (spot 14, 15 and 16), D-alanine-D-alanine ligase A (spot 22), beta-conglycinin alpha subunit (spot 21), Kunitz-type trypsin inhibitor B (spot 27) and V-type proton ATPase subunit D (spot 23). The protein group that increased remarkably at 1 or 2 days after flooding treatment contained UDP-glucose pyrophosphorylase (spot 11) or alcohol dehydrogenase (spot 13), respectively. The protein group that increased within 2 days by flooding treatment contained hemoglobin alpha-I chain (spot 10) and glyceraldehyde-3-phosphate dehydrogenase (spot 28). The protein group that kept increasing remarkably by flood treatment contained only fructose-bisphosphate aldolase (spot 12). On the other hand, the protein group that increased during growth but decreased by flood treatment contained protein disulfide isomerase

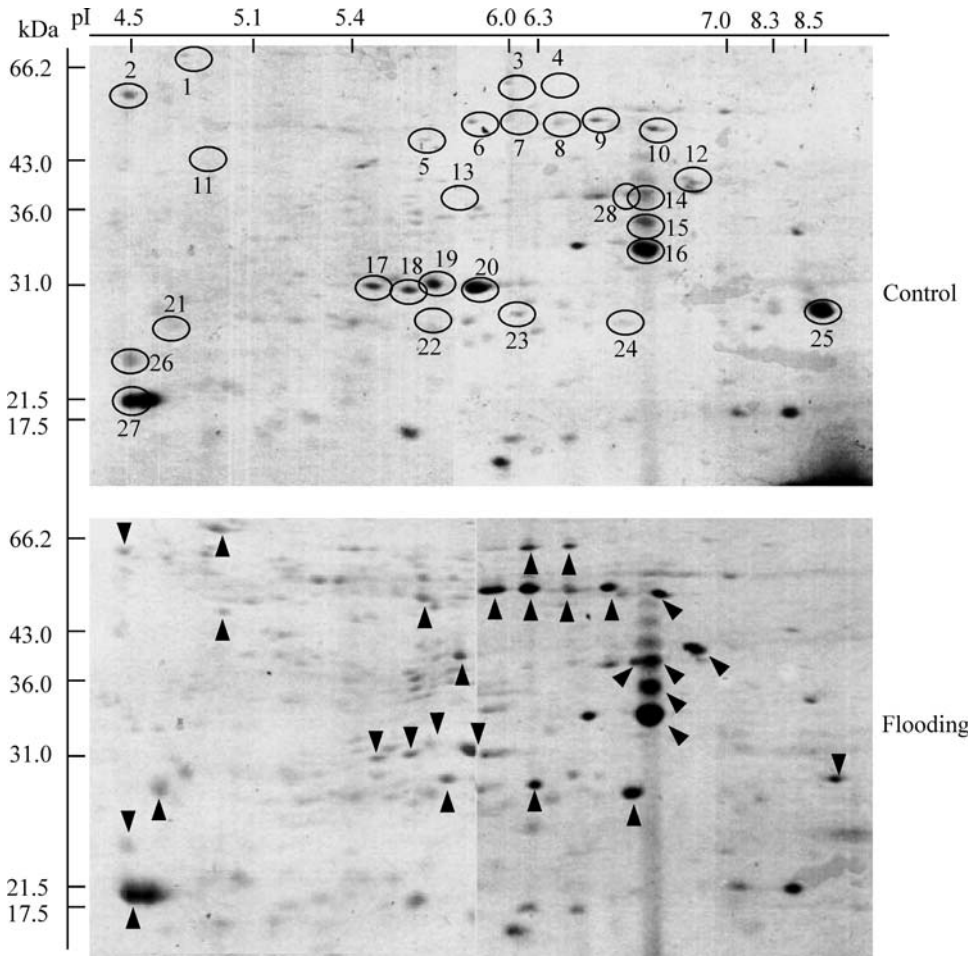

Fig. 2 Detection of flooding responsive proteins by 2D-PAGE. Soybean seeds were germinated on sand for 2 days and subjected to flooding for 2 days. Proteins (400 μg) were extracted from root and hypocotyls, then separated on 2-DE followed by CBB staining. For the first dimension, isoelectric focusing (low pI range) and immobilized pH gradient (high pI range) were used and overlapped at around pI 5.8. The pI and relative molecular weight of each protein were determined using a 2D-marker (Bio-Rad). Open circles show the spots that changed in protein expression, and arrowheads show up-regulated spots and down-regulated spots. Spot numbers are the same as Table 1

(spot 2), ascorbate peroxidase (spot 18), 1-Cys peroxiredoxin (spot19), lectin (spot 17) Kunitz-type trypsin inhibitor B (spot 26) and 31 kDa/28 kDa glycoprotein precursors (spots 20 and 25).

Flooding stress induces the hypoxic response, but the expression of proteins classified into protein destination/storage doses not

Soybeans were subjected to flooding, nitrogen substitution without flooding, and flooding with aeration, and used for analyses to determine whether the induction of flooding responses of soybean is only dependent on the hypoxic response. Soybean seeds were germinated on sand for 2 days, and treated with or without flooding, flooding with aeration or nitrogen substitution without flooding for 2 days. Proteins from the root and hypocotyl of seedlings were separated by 2-DE followed by CBB staining. The changes in protein spots among the treatments were calculated with PDQuest software and plotted as relative protein volume of spots, indicated in Fig. 2. They were classified as "Group A: Protein that increased after flooding treatment" and "Group B: Protein that decreased by flood treatment" (Table 2).

Alcohol dehydrogenase (spot 13) increased remarkably by nitrogen substitution compared to up-regulation by flooding. The expression of the majority of proteins that changed in response to flooding showed the same tendencies as those changing in response to nitrogen substitution. However, the expression of proteins classified into protein destination/storage (spots 1, 5, 6, 7, 14, 15, 16, 21, and 27), which were 70-kDa heat shock protein, maturation polypeptide, albumin-1 precursor, beta-conglycinin alpha subunit and Kunitz-type trypsin inhibitor B, did not show the same tendency in the case of the nitrogen substitution.

Discussion

Shi et al. (2008) reported that the total number of roots, the length of the main root, the length of the lateral and adventitious roots, and the fresh weight of the roots of flooded soybean seedlings were significantly suppressed compared with untreated plants after 3 days of flooding

Table 2 Identification of proteins increased and decreased by flooding in root containing hypocotyl of soybean

No[a]	MW[b]	pI[b]	Amino acid sequences[c]	Homologues proteins	Identity (%) (similar %)[d]	Ac No[e]	U or D[f]	Time dependency[g]	Oxygen dependency[h]
1	65.7	4.7	I-EKVVGIDLGT	70 kDa heat shock protein	90 (100)	P31082	↑ (Ps)	Group A	Group A
2	61.9	4.6	I-EESSEKEFVL	Protein disulfide isomerase	100 (100)	PX0084	↓ (Ps)	Group E	Group B
3	61.5	6.2	I-EHQLDQQKAG	Protein phosphatase inhibitor 1	80 (100)	Q9ERT9	↑ (St)	Group A	ND
4	61.7	6.5	I-EHQLDQQKAG	Protein phosphatase inhibitor 1	80 (100)	Q9ERT9	↑ (St)	Group A	ND
5	52.6	5.9	N-blocked (MS)	Maturation polypeptide	121* (42*)	AAA33985	↑ (Ps)	Group A	Group A
6	56.4	6.1	N-blocked (MS)	Maturation polypeptide	78* (32*)	AAA33985	↑ (Ps)	Group A	Group A
7	56.4	6.2	N-blocked (MS)	Maturation polypeptide	82* (34*)	AAA33985	↑ (Ps)	Group A	Group A
8	55.6	6.7	I-STTNKVSDYA	Embryonic abundant protein	100 (100)	S61428	↑ (Ps)	Group A	ND
9	55.6	6.7	I-STTNKVSDYA	Embryonic abundant protein	100 (100)	S61428	↑ (Ps)	Group A	ND
10	54.2	7.0	N-TSVAHMD	Hemoglobin alpha-I chain	85.7 (100)	P41330	↑ (Pm)	Group C	ND
11	56.4	4.7	N-blocked (MS)	UDP-glucose pyrophosphorylase	92* (38*)	AAL33919	↑ (Pm)	Group B	Group A
12	47.7	7.2	I-GTLLKPNMVT	Fructose-bisphosphate aldolase	100 (100)	P91759	↑ (E)	Group D	Group A
13	55.2	5.9	I-YTVVHAGXVA	Alcohol dehydrogenase	100 (100)	S51937	↑ (E)	Group B	Group A
14	43.0	6.6	N-ADXNGAXSPF	Albumin-1 precursor (A1)	80 (100)	Q9FRT8	↑ (Ps)	Group A	Group A
15	40.0	6.6	N-ADXNGAXSPF	Albumin-1 precursor (A1)	80 (100)	Q9FRT8	↑ (Ps)	Group A	Group A
16	37.0	6.6	N-ADXNGAXSPF	Albumin-1 precursor (A1)	80 (100)	Q9FRT8	↑ (Ps)	Group A	Group A
17	29.8	5.5	I-KDTVSFTFN	Lectin	100 (100)	DQ235094	↓(Dd)	Group E	Group B
18	34.2	5.7	N-GKSYPTVSAD	Ascorbate peroxidase	100 (100)	AB082932	↓ (Dd)	Group E	Group B
19	35.8	5.9	N-PGLTIGDTIP	1-Cys peroxiredoxin	100 (100)	Q6E2Z6	↓ (Dd)	Group E	Group B
20	35.1	6.1	N-ERSSEVKXAS	Stem 31 kDa glycoprotein precursor	90 (100)	P10743	↓ (Ps)	Group E	Group B
21	30.9	4.3	N-HKNKPFLFG	Beta-conglycinin alpha subunit	90 (100)	P52572	↑ (Ps)	Group A	Group A
22	28.5	5.9	I-APIAVDVVP	D-alanine-D-alanine ligase A	87.5 (100)	Q89GA7	↑ (Cs)	Group A	ND
23	31.8	6.3	I-AKAAGAVDEF	V-type proton ATPase subunit D	70 (90)	P87220	↑ (T)	Group A	ND
24	29.4	6.8	N-PGLTIGDTIP	1-Cys peroxiredoxin	100 (100)	Q6E2Z6	↑ (Dd)	Group A	Group A
25	30.5	8.0	N-ARTPEVKXAS	Stem 28 kDa glycoprotein precursor	90 (100)	P15490	↓ (Ps)	Group E	Group B
26	23.8	4.1	N-AEPEPVVD	Kunitz trypsin protease inhibitor	100 (100)	EU444601	↓ (Ps)	Group E	ND
27	16.7	4.1	N-DFVLDNEGNP	Kunitz-type trypsin inhibitor B	100 (100)	P01071	↑ (Ps)	Group A	Group A

Table 2 continued

No[a]	MW[b]	pI[b]	Amino acid sequences[c]	Homologues proteins	Identity (%) (similar %)[d]	Ac No[e]	U or D[f]	Time dependency[g]	Oxygen dependency[h]
28	46.3	6.8	I-LDIVSNASXT	Glyceraldehyde-3-phosphate dehydrogenase	90 (100)	AB106523	↑ (E)	Group C	Group A

[a] Spot numbers are the same as Fig. 2

[b] Relative molecular weight (MW) and isoelectric point (pI) were calculated using 2D-marker (Bio-Rad) and 2D-PAGE gel in Fig. 2

[c] N-terminal (N-) and internal (I-) amino acid sequences were determined by protein sequencer. N-blocked (MS) means that the proteins were N-terminaly blocked and identified by MS

[d] Identity (Similar) is percentage of amino acid overlapped in 10 amino acid. *The number shows score, and % in brackets shows coverage in the MS analysis

[e] "Ac No" shows accession number in database of UNIPROT-SPROT and NCBI

[f] "U or D" shows the proteins increased and decreased by flooding stress in root containing hypocotyl of soybean. Abbreviations in brackets show *Ps* protein destination/storage, St Signal transduction, Pm: Primary metabolism, E: Energy, Dd: Disease/defense, Cs: Cell structure and T: Transporters

[g] Dependence of relative levels of protein expression of root and hypocotyl of soybean on time after flooding. Proteins were classified as "Group A: Protein that increased remarkably at 1 and 2 days after flooding treatment", "Group B: Protein that increased remarkably at 1 or 2 days after flooding treatment", "Group C: Protein that increased within 2 days by flooding treatment", "Group D: Protein that kept increasing remarkably during flood treatment" and "Group E: Protein that increased during growth but decreased by flood treatment"

[h] Effect of oxygen concentration on protein expression of root and hypocotyl of soybean. Proteins were classified with "Group A: Protein that increased after flooding treatment" and "Group B: Protein that decreased by flood treatment"

stress. A reduction in root growth is one of the most commonly reported parameters during flooding (Wang and Jiang 2007). Yield loss is a common consequence of water logging of the most sensitive crop plants. In this study, the growth of roots of soybean seedlings under flooding was significantly suppressed as much as that of seedlings treated by nitrogen substitution, but that of hypocotyls under flooding did not show the same tendency as under nitrogen substitution.

In rice, low oxygen selectively induces the synthesis of proteins and production of metabolites, most of which are enzymes and metabolites involved in sugar metabolism, glycolysis, and fermentation pathways (Huang et al. 2005; Bailey-Serres and Voesenek 2008). However, there are few reports on effects of flooding at the molecular level in flood-intolerant crops such as soybean. In this study, to understand the mechanism by which soybean responds to flooding stress and whether flooding responses of soybean are induced only depending on hypoxic response, flooding inducible proteins were analyzed using a proteomic technique.

Expression of proteins involved in glycolysis and fermentation pathways, such as UDP-glucose pyrophosphorylase (spot 11), fructose-bisphosphate aldolase (spot 12), glyceraldehyde-3-phosphate dehydrogenase (spot 28) and alcohol dehydrogenase (spot 13) were altered in response to flooding stress, suggesting that flooding stress includes stress from oxygen limitation. The up-regulation of fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase accelerates the glycolysis pathway. However, the shift to alternative pathways of energy generation is crucial for plants to survive flooding stress because photosynthesis cannot be carried out under flooding because of scant light and low CO_2 availability (Bailey-Serres and Voesenek 2008). Thus, the alcohol dehydrogenase was activated in fermentation pathway (Table 2), but alcohol dehydrogenase's expression decreased within 2 days after flooding stress (Table 2), perhaps because of a limiting concentration of pyruvic acid in the plant. Among the identified glycolysis-related proteins, UDP-glucose pyrophosphorylase was up-regulated by flooding stress, indicating that soybean seedlings can adjust to flooding. Coleman et al. (2007) reported using overexpressed UDP-glucose pyrophosphorylase in poplar that while it was possible to alter the allocation of carbon in favor of cellulose biosynthesis, whole plant changes resulted in unexpected decreases in growth and an increase in defense metabolites, which also supports our results.

Hemoglobin alpha-I chain (spot 10) which is an oxygen binding protein, was also up-regulated during flooding stress (Table 2). Overexpression of class 1 hemoglobin 1 protects *Arabidopsis thaliana* plants from severe hypoxia (Hunt et al. 2002). Hunt et al. also reported that the

constitutive expression of hemoglobin 1 results in a reduced number of root hairs and increased number of laterals in the root system. These results suggest that hemoglobin 1 might play an important role to rescue a plant from flooding stress in the early stage of growth.

Reactive oxygen species scavengers as 1-Cys peroxiredoxin (spots 19 and 24) and ascorbate peroxidase (spot 18) were found to be flooding responsive proteins. Reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide act in plants as toxic products of normal cell metabolism and as regulatory molecules in stress perception and signal transduction (Navrot et al. 2006), as well as substances to prevent oxidative damage in light- or heat-stressed plants (Sato et al. 2001). Ascorbate peroxidase was discovered as a down-regulated protein in soybean seedlings subjected to flooding stress (Shi et al. 2008). In this study, ascorbate peroxidase and acidic 1-Cys peroxiredoxin were also down-regulated by flooding stress, though basic 1-Cys peroxiredoxin was up-regulated (Table 2 and Fig. 2). Pulido et al. (2008) reported that the oxidation status of this protein was analyzed in extracts from developing seeds and from aleurone cells dissected from germinating seeds, and part of the 1-Cys peroxiredoxin was displaced to the acidic part of the 2-DE gel, indicating that 1-Cys peroxiredoxin is affected by overoxidation. In this study, the basic form (spot 24) is significantly lower in molecular weight compared with acidic form (spot 19), suggesting that it may be a breakdown product.

V-type proton ATPase subunit D (spot 23) was up-regulated within 2 days by flooding stress (Table 2). Increased V-type proton ATPase subunit D activity could be required for a homeostatic mechanism to maintain the cytoplasmic pH near neutrality (Matsumoto 1998). Stem elongation under anoxia results from the cell expansion that occurs in the absence of an adjustment in cytosolic pH and appears to be maintained by tight constraints on ATP production and consumption (Dixon et al. 2006). D-alanine-D-alanine ligase A (spot 22), which is known to be involved in cell wall biosynthesis, was also up-regulated within 2 days by flooding stress (Fig. 2). Increases in acid-induced cell wall extension upon submergence have been observed in rice (Cho and Kende 1997). Cell wall extensibility is associated with cell wall loosening proteins such as expansins and xyloglucan endotransglycosylase/hydrolase (Darley et al. 2001). These results suggest that D-alanine-D-alanine ligase A and V-type proton ATPase subunit D might be involved as cell wall loosening proteins at the early stage of flooding stress in soybean.

Up-regulation of protein phosphatase inhibitor 1 (spots 3 and 4) by flooding stress has not previously been reported in plants. This protein is a major inhibitor of protein phosphatase 1, which regulates signal transduction in many eukaryotic cellular events. Protein phosphatase inhibitor 1 has been postulated to act as a protective molecule by inhibiting protein aggregation and guarding *E. coli* against various stresses (Kim 2006). Lectin (spot 17), which belongs to the defense system, was down-regulated by flooding stress. Most lectins play a role in defense against different kinds of organisms (Peumans and van Damme 1995). Lectin in soybean has been also down-regulated by salt stress (Aghaei et al. 2009).

Although the expression of proteins categorized in protein destination/storage such as protein disulfide isomerase (spot 2), stem 31 kDa/28 kDa glycoproteins (spots 20 and 25), and Kunitz-type trypsin inhibitor B (spot 26), were slightly down-regulated, they appeared under the low oxygen condition. Protein disulfide isomerase family proteins play important roles in the folding of nascent polypeptides and the formation of disulfide bonds in the endoplasmic reticulum. These proteins are involved in the proper folding or quality control of such storage proteins as molecular chaperones (Kamauchi et al. 2008). These results suggest that the down-regulation of protein disulfide isomerase induces the misfolding of proteins and results in down-regulation of glycoproteins such as stem 31 kDa/28 kDa glycoproteins, Kunitz-type trypsin inhibitor B and lectin.

However, the up-regulation of proteins in the same destination/storage category, such as maturation polypeptide (spots 5, 6 and 7) and embryonic abundant protein (spots 8 and 9), albumin-1 precursor (spots 14, 15, and 16), Kunitz-type trypsin inhibitor B (spot 27), beta-conglycinin alpha-subunit (spot 21) and 70-kDa heat shock protein (spot 1) did not respond to low oxygen conditions in the same way (Table 2). This is because these proteins are basically necessary for germination and disappear afterwards. However, they continue to be present because growth is delayed by flooding for several days. Therefore, these proteins are not affected or controlled by low oxygen stress. On the other hand, some proteins (spots 12, 24 and 28) were induced even more by flooding with aeration than by flooding alone (Table 2). Fructose-bisphosphate aldolase (spot 12) and glyceraldehyde-3-phosphate dehydrogenase (spot 28) involved in glycolysis were not controlled by additional oxygen though these proteins were affected by low oxygen stress. This result indicates that a direct effect of flooding stress might exist for which the low oxygen is not used. The up-regulation by flooding with aeration of these proteins observed in this study suggests new areas for future studies on the improvement of flooding tolerance in soybean.

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