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Cytosolic ascorbate peroxidase 2 (cAPX 2) is involved in the soybean response to flooding

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Abstract

Proteomic analyses of soybean seedlings responding to flooding were conducted to identify key proteins involved. The seeds were germinated on a spongy matrix for two days, and then subjected to flooding for three days. After flooding, the total number of roots, the length of the main root, the lengths of the lateral and adventitious roots, and the fresh weight of the underground tissues of flooded soybean seedlings were significantly suppressed compared with nontreated plants. To identify the early flooding-responsive proteins, the seedling roots were used for preparing cytosolic and membrane fractions. After two-dimensional polyacrylamide gel electrophoresis and silver staining, 208 proteins were detected, and the levels of 44 were different from those of the control. The expression pattern of 10 proteins among the 44 from six different soybean cultivars confirmed that the 10 were flooding-responsive proteins. One of the 10 proteins was dominantly down-regulated under flooding conditions and was identified as cytosolic ascorbate peroxidase 2 (cAPX 2). Northern-hybridization showed that the abundance of cAPX 2 transcript decreased significantly after flooding, as did the enzymatic activity of APX. These results suggest that cAPX 2 is involved in flooding stress responses in young soybean seedlings.

Keywords: Soybean; Glycine max; Leguminosae; Ascorbate peroxidase 2; Flooding; Proteome; Root

1. Introduction

Flooding stress is a widespread phenomenon in regions where the soil has an impermeable clay base or consists of cracking gray clay with slow drainage, where the weather is characterized by extreme rainfall, or where imperfect land planning has taken place. Most major crops exhibit no tolerance to flooding stress, even though paddy or deepwater rice can tolerate some level of flooding. Many crops, such as canola and barley, experience serious yield losses when subjected to flooding stress (Dennis et al., 2000). The negative impact of flooding on plant growth and development is a consequence of the slow diffusion rates of gases in water compared to air and the relatively low solubility of oxygen in water (Voesenek et al., 2006). Fukao and Bailey-Serres (2004) reported that molecular oxygen was the terminal electron acceptor in the mitochondrial electron transport chain and was required by several enzymes, including those that produce reactive oxygen species (ROS) as signaling molecules. In plants, oxygen deficiency dramatically reduces the efficiency of cellular ATP production, which has diverse ramifications for cellular metabolism and developmental processes. The tolerance to oxygen deprivation depends on plant cell and tissue types,

Abbreviations: ROS, reactive oxygen species; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; APX, ascorbate peroxidase.

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developmental stage, and genotype of the species, as well as the severity and duration of the stress, light levels, and ambient temperatures. The response to environmentally induced oxygen deprivation has been studied in species that range from flooding sensitive to flooding-tolerant (Fukao and Bailey-Serres, 2004). Based on these studies, molecular mechanisms underlying plant responses to flooding stress have been explained.

To identify the stress-responsive components in plants, Klok et al. (2002) carried out global transcriptional profiling on hypoxia stress in *Arabidopsis* using an oligonucleotide array method. In rice, Agarwal and Grover (2005) performed a similar study using different plant materials by suppression subtraction hybridization. Both studies established that a common set of genes respond to anoxia stress. The candidate genes were involved in carbon and nitrogen metabolism, cell wall extension, signal transduction, and transcription regulation. To understand the structural characteristics of genes conferring tolerance on flooding, Xu et al. (2006) and Fukao et al. (2006) analyzed a special locus in the flooding-tolerant rice cultivar FR13A and its near-isogenic line. These studies indicated that the gene corresponding to flooding tolerance in rice was an ethylene-response-factor-like gene, suggesting that ethylene was involved in the response of rice to flooding stress. Although significant achievements have been made in understanding the genes in flooding tolerance, as well as the structural characteristics of individual genes, responding to flooding stress in *Arabidopsis* and rice, there is still much to be learned about other crop plants, such as wheat, barley, and soybeans. To extend our understanding of genes responding to flooding stress in soybeans, in this study we carried out proteomic analyses to discover proteins responding to flooding. One of the flooding-responsive proteins, cytosolic ascorbate peroxidase 2 (cAPX 2), was selected for a more detailed characterization.

2. Results and discussion

2.1. The growth of soybean seedlings is significantly suppressed after three days of flooding

Nakayama et al. (2004) reported that the flooding injury of soybean seeds before radical protrusion (seed inhibition) was caused by a physical disruption of the rapid uptake of water and can be alleviated by using seeds with high moisture contents. The causes of flooding injury in stages after radical protrusion, however, have not been explained, though a physiological agent may be involved (Nakayama et al., 2004). In this study, a proteomic approach was used to identify soybean seed proteins responding to flooding injury after radical protrusion.

Changes in the total number of roots, the length of the main root, the length of lateral and adventitious roots, and the fresh weights of underground seedling tissues were measured after one, two, and three days of flooding. By the second day, the total number of roots and the length of lateral and adventitious roots were inhibited by flooding. The level of inhibition of root growth continued to the third day. A similar time-dependent inhibition pattern of change was observed for main root length and root fresh weight (data not shown). On the third day after flooding, the total number of roots, the length of lateral and adventitious roots, the length of the main root, and the fresh weight of the underground part showed significant growth suppression (Fig. 1). A reduction in root growth was one of the most commonly reported parameters during flooding

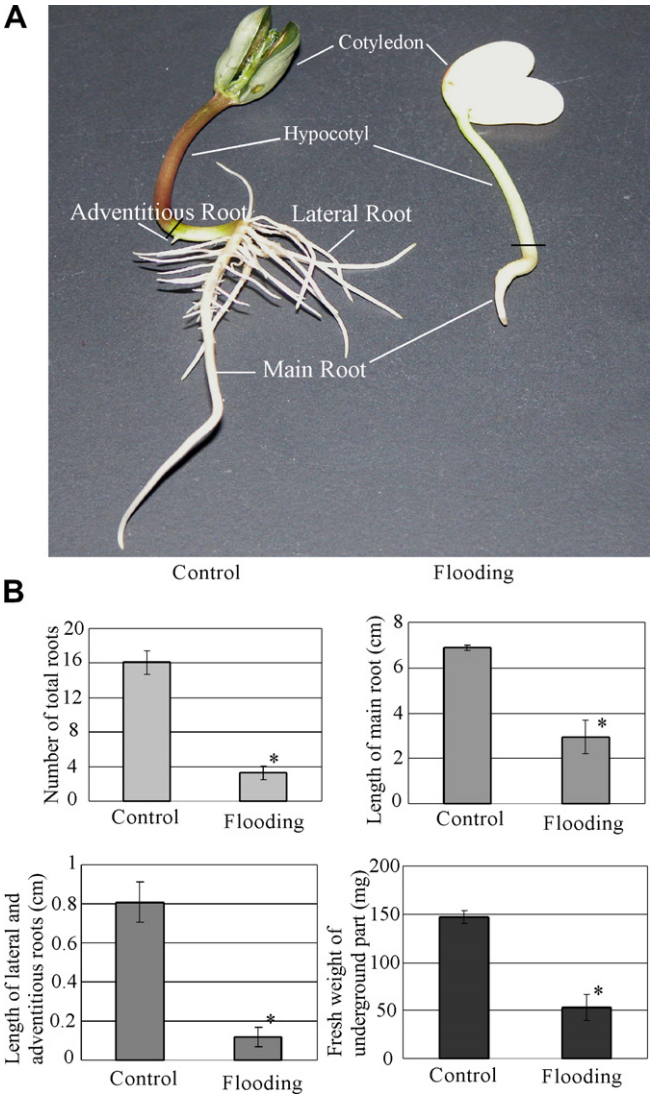

Fig. 1. Growth inhibition in soybean seedlings after three days of flooding. Soybean seeds were germinated on spongy media for two days and subjected to flooding for three days. (A) Photograph showing morphological differenced between control and submerged soybean seedlings. (B) Effect on the early growth stage of soybean seedlings exposed to flooding. The number of total roots, the length of the main root, the length of lateral and adventitious roots, and the fresh weight of underground parts data are the means and standard errors from four independent experiments. Asterisks showed the spots with significant differences of protein expression between nontreatment and flooding ($p < 0.05$). Adventitious roots, lateral roots, and the main root (A) were counted as the number of total roots (B).

(Wang and Jiang, 2007). Yield loss is a common consequence of the water-logging of the most sensitive crop plants. The growth suppression in the early developmental stages observed here was responsible for yield losses of soybeans that were grown in paddy fields.

2.2. Only 10 proteins are involved in the flooding tolerance of germinating soybean seeds after radical emergence

To identify flooding-responsive proteins, we used the roots of soybean seedlings after three days of flooding to prepare cytosolic and membrane proteins. After separation on 2D-PAGE and display by silver staining, 137 protein spots from the cytosolic fraction (Fig. 2A) and 71 protein spots from the membrane fraction (Fig. 2B) were detected. The p*I* value and M_r of these proteins ranged from 4.0 to 7.5 and 10.0–76.0 kDa, respectively. Because the number of detected proteins with p*I* values larger than 7.5 was negligible, only proteins ranging from p*I* 4.5 to 7.5 were identified in this study. Among these proteins, 44 showed reproducible expression changes in three independent experiments (Fig. 3). Ten out of 44 showed significant expression changes relative to nontreated plants (Fig. 3).

2.3. Seven proteins are directly responding to flooding

The expression pattern of the 10 candidate proteins were surveyed in six soybean cultivars that have different degrees of tolerance to flooding during germination: Enrei, Peking, Williams 82, Sachiyutaka, Tamahomare, and Nakasennari (Nakayama et al., 2004; Russell et al., 1990). The expression of all 10 proteins changed in these cultivars when submerged, confirming that they are involved in the flooding response (Fig. 4). Based on the expression pattern in response to flooding, these proteins can be separated into two groups. One included the cytosolic protein spots 1, 7, 10, 18, 21, 22, and 31, whose expression patterns were uniform even in different genetic backgrounds. The second group included cytosolic protein spot 26 and membrane protein spots 4 and 12 in which the expression pattern was affected by different genetic factors (Table 1). These results indicate that spots 1, 7, 10, 18, 21, 22, and 31 are proteins directly responding to flooding. Among the 10 proteins described above, spot 21 was prominently regulated by flooding. The expressed amount of this protein in submerged plants was reduced 67.3% relative to nontreated plants.

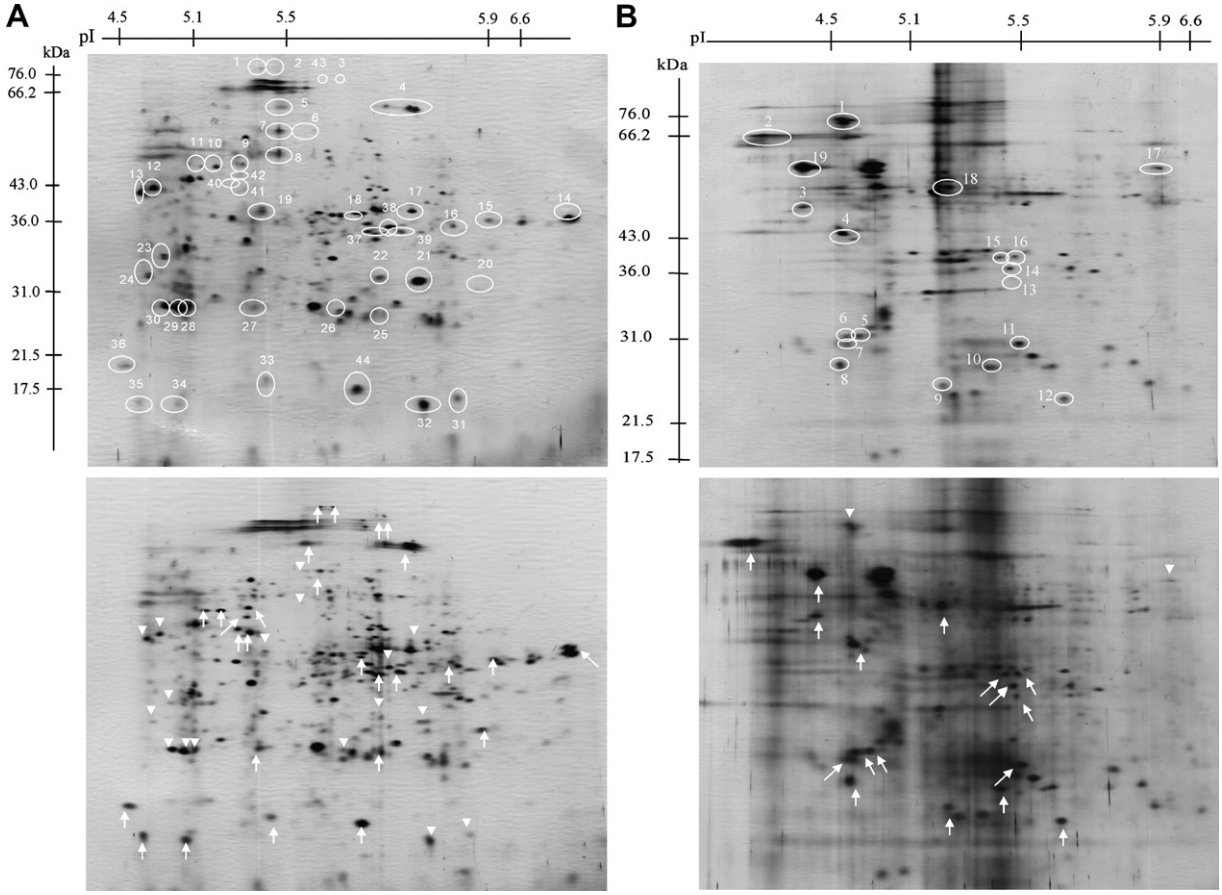

Fig. 2. Detection of flooding-responsive proteins by 2D-PAGE. Soybean seeds were germinated on sponge for two days and subjected to water flooding for three days (lower) and/or not as control (upper). The roots of seedlings on the third day after flooding were used to fractionate into cytosolic (A) and membrane (B) proteins. Proteins (25 μg) were separated on 2D-PAGE followed by silver staining. Upward arrows indicate the positions of up-regulated proteins, and downward arrows show the positions of down-regulated proteins; the circles represent the same proteins in control.

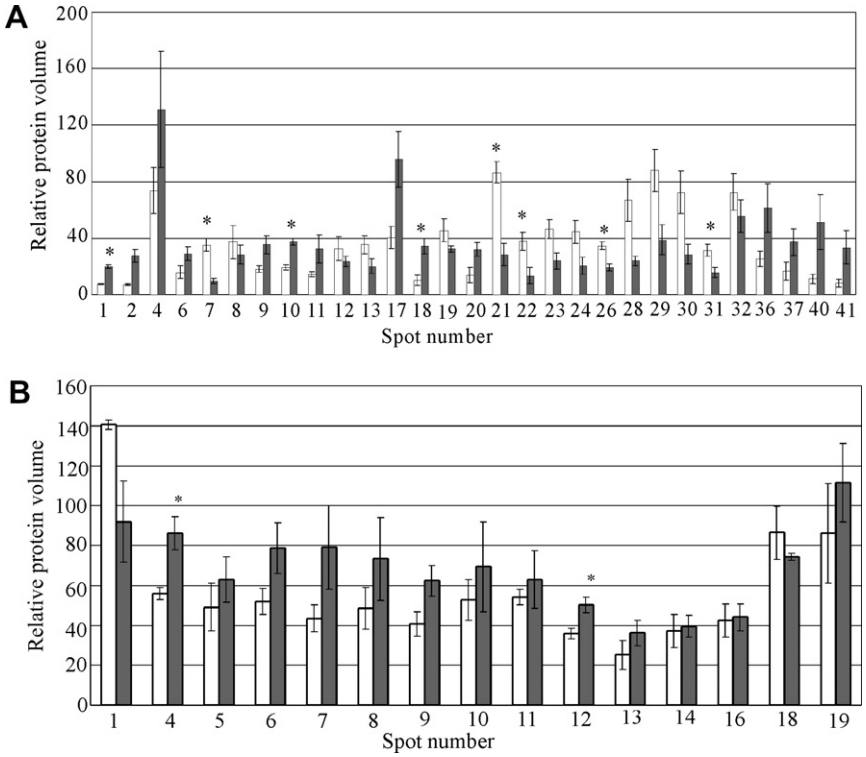

Fig. 3. Relative expression of changed proteins in response to flooding. The proteins from cytosolic fraction (A) and membrane fraction (B) were separated on 2D-PAGE. The changes in protein spots were calculated with 2D-Elite software and plotted as the relative intensities of 44 spots indicated in Fig. 2. The values are the mean ± SE of protein volumes of gels from three independent experiments. White and black bars represent control and flooding stress, respectively. Asterisks showed the spots with significant differences of protein expression between nontreatment and flooding ($p < 0.05$).

2.4. Cytosolic ascorbate peroxidase 2 is dominantly down-regulated by flooding

To identify spot 21, we used ESI-Q-TOF MS analysis. For this analysis, four criteria were used to assign a positive match with a known protein: (1) the deviation between experimental and theoretical peptide masses was less than 50 ppm; (2) eight different predicted peptide masses were matched to the observed masses for an identification to be considered valid; (3) the coverage of protein sequences by the matching peptides was 36%; and (4) the score that was obtained from the analysis with MASCOT software indicates the probability of a true positive identification, and it was 248 using the NCBInr database. One of the eight peptides was a unique sequence, which was "LAWHSAGTYDVSSK," for cAPX 2 compared to cAPX 1. By these criteria, this protein was identified as cAPX 2 (accession number gi 1336082). The M_r and p*I* of this protein from 2D-PAGE gel were 32.7 kDa and 5.7, respectively, values that are comparable with the theoretical values of M_r 27.2 and p*I* 5.65.

APX is an antioxidant enzyme that scavenges H_2O_2 and is distributed in at least four distinct cell compartments in higher plants: the stroma and thylakoid membrane of chloroplasts, the microsomes, and the cytosol (Yoshimura et al., 2000; Shigeoka et al., 2002). A fifth APX isoenzyme occurs in a mitochondrial membrane-bound form (Shigeoka et al., 2002). Each cellular compartment may contain either one or several APX isoforms (d'Arcy-Lameta et al., 2006). In *Arabidopsis*, eight types of APX have been described: three cytosolic (APX1, APX2, APX6), two chloroplastic types (stromal sAPX, thylakoid tAPX), and three microsomal (APX3, APX4, APX5) isoforms (Panchuk et al., 2002). A phylogenetic tree was created using a BLAST pairwise program, based on the predicted amino acid sequences of soybeans, *Arabidopsis*, rice, peas, and *Medicago* APXs (Fig. 5). The phylogenetic analysis based on amino acid sequences shows that soybean APX1 and 2 are closely related to pairs of rice APX1 and 2, and/or *Arabidopsis* APX1 and 2. The identities between soybean APX1 and APX2 were 95% (accession numbers Q67LA8 and Q76LA6). These two proteins are grouped together with the APX of *Medicago* and *Pisum* and are clearly separated from APX of rice and *Arabidopsis*. Therefore, APX1 and APX2 of soybean probably appeared in evolution after separation of the *Brassicaceae* and *Fabaceae* as recent gene duplication. Taking into account the tetraploid nature of soybean, it could be supposed that APX1 and APX2 represent two copies of the same gene, which accumulated mutations after polyploidization.

Shigeoka et al. (2002) investigated changes in APX transcript abundance in response to high light, drought, salt, and paraquat treatments and reported that the genes for chloroplastic APX and microsomal APX were constitutively expressed for the immediate and efficient detoxification of H_2O_2 under normal and stress conditions, and

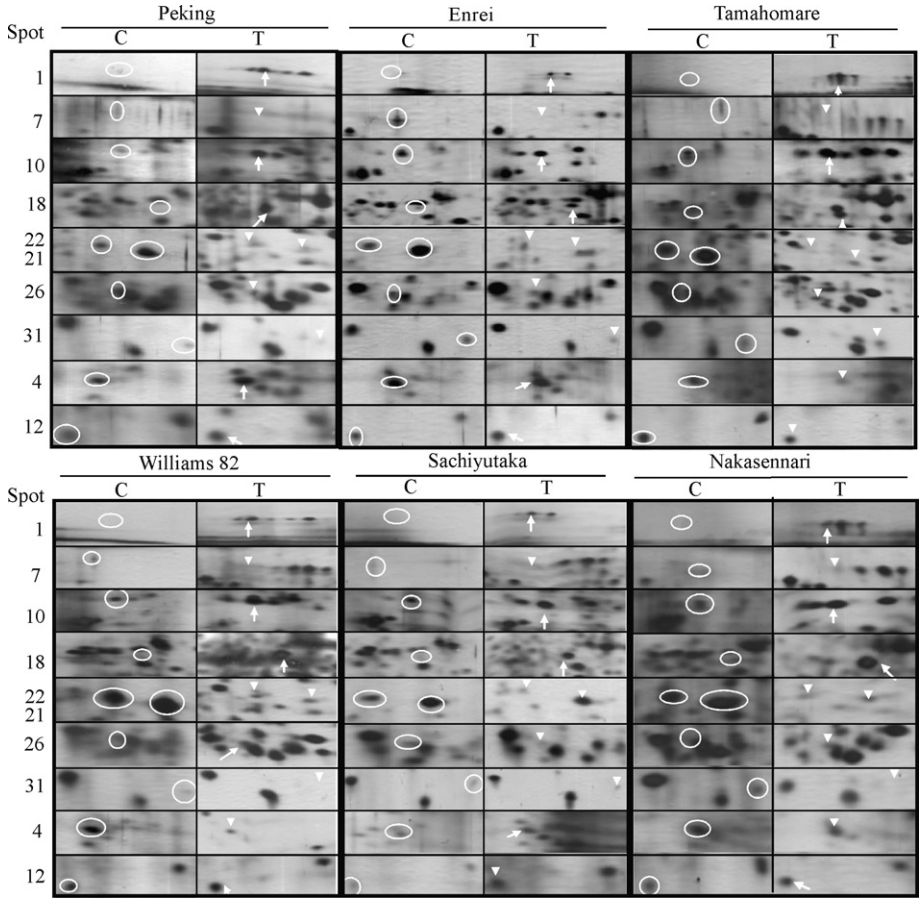

Fig. 4. Partial 2D-PAGE showing the difference on expression pattern responding to flooding for 10 proteins in 6 soybean cultivars. The seeds of 6 soybean cultivars, Peking, Williams 82, Sachiyutaka, Tamahomare, and Nakasennari, as well as Enrei were germinated on spongy matrix for two days and submerged in water for three days. Cytosolic and membrane proteins were extracted from the roots of untreated and submerged soybean seedlings and separated on 2D-PAGE followed by silver staining. For each cultivar, an expression pattern of nontreatment was shown in the left lane (C) and that of flooding in the right lane (T). Spots 1, 7, 10, 18, 21, 22, 26, and 31 were cytosolic proteins, and spots 4 and 12 were membrane proteins.

Table 1
Expression patterns for 10 proteins showing significant expression changes in cultivar Enrei were surveyed in five other cultivars

Spots	Cultivars					
	High[a]		Medium[a]		Low[a]	
	Peking	Williams82	Enrei	Sachiyutaka	Tamahomare	Nakasennari
Cytosolic						
1	Up	Up	Up	Up	Up	Up
7	Down	Down	Down	down	Down	Down
10	Up	Up	Up	Up	Up	Up
18	Up	Up	Up	Up	Up	Up
21	Down	Down	Down	Down	Down	Down
22	Down	Down	Down	Down	Down	Down
26	Down	Up	Down	Down	Down	Down
31	D	Down	Down	Down	Down	Down
Membrane						
4	Up	Down	Up	Up	Down	Down
12	Up	Up	Up	Up	Down	Up

[a] High, medium, and low indicate the difference in tolerance to flooding injury when the seeds contain the same moisture content during the germination period (inhibition stage). Up: the relative volume of the specific spot increased after flooding. Down: the relative volume of the specific spot decreased after flooding.

that the gene expression for cAPX was responsive to environmental changes. Furthermore, APX2 in *Arabidopsis* is really induced upon stress-treatment, but the transcript level remains 5–10 times lower than the level of APX1 (Volkov et al., 2006). APX1 and APX2 demonstrated different transcriptional patterns: APX1 is expressed at level in the

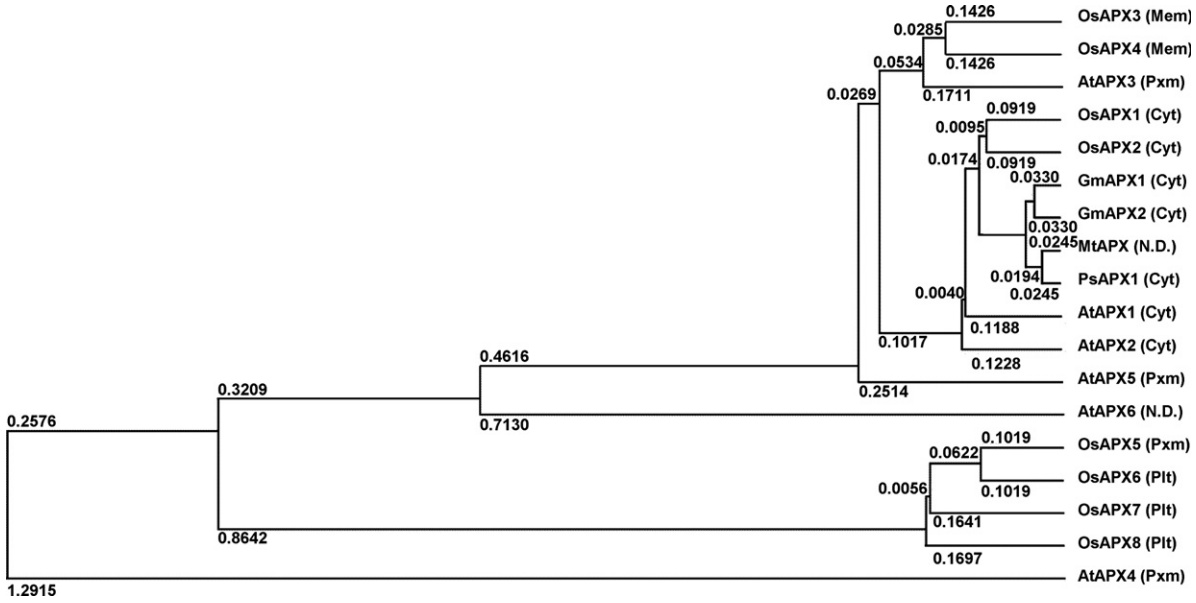

Fig. 5. Phylogenetic relationships among soybean, *Arabidopsis*, rice, peas, and *Medicago* APXs. A phylogenetic tree was created using a BLAST pairwise program, based on the predicted amino acid sequences of soybeans, *Arabidopsis*, rice, peas, and *Medicago* APXs. The numbers in the figure show scale for genetic distances. Cytosolic, membrane, peroxisome membrane, and plastid are marked as Cyt, Mem, Pxm, and Plt, respectively. "N.D." shows "Not determined". AtAPX1 (Q05431), AtAPX2 (Q1PER6), AtAPX3 (Q42564), AtAPX4 (P82281), AtAPX5 (Q7XZP5), AtAPX6 (Q8GY91), OsAPX1 (P93404), OsAPX2 (Q9FE01), OsAPX3 (Q6TY83), OsAPX4 (Q6ZJJ1), OsAPX5 (P0C0L0), OsAPX6 (P0C0L1), OsAPX7 (Q7XJ02), OsAPX8 (Q69SV0), PsAPX1 (P48534), MtAPX (Q1SDP6), GmAPX1 (Q76LA8), GmAPX2 (Q76LA6).

absence of stress-treatment and is additionally induced after stress, and APX2 is a typical stress gene which is expressed only after stress-treatment (Karpinski et al., 1997; Panchuk et al., 2002). These results suggest that transcriptional profiles of APX2 of *Arabidopsis* and soybean are very different indicating different functions in stress response. Mittler and Zilinskas (1994) reported that expression of APX1, which is one of the genes encoding cAPX, increased during drought stress. On the other hand, Wang and Jiang (2007) reported that the activity of APX in creeping bent-grass roots significantly decreased by 46% and 40% during water-logging at 1 cm and 15 cm below the soil surface. In this study, the APX protein levels decreased in soybean cultivars after flooding for three days. These studies suggest that APX may have different physiological responses to different stresses.

2.5. The Apx 2 transcript level decreases significantly with flooding

Northern hybridization was performed using total RNAs either isolated from the roots of soybean seedlings flooded for three days or from controls to determine if transcript levels of APX2 changed with flooding. The probe used for Northern-hybridization was sufficiently specific in order to discriminate individual mRNA. The abundance of APX2 transcripts in nontreated plants was 13-fold higher than that of three-day flooded plants (Fig. 6). This result indicated that the decreased APX protein levels resulting from flooding is the consequence of low transcript levels. Many research groups have reported that the expression of the APX gene, APX protein, and APX activity can be altered by many biotic and abiotic stresses (Mittler and Zilinskas, 1994; d'Arcy-Lameta et al., 2006; Wang and Jiang, 2007). In parallel with the cAPX 2 protein amount decrease, the transcript level was also reduced, suggesting that the cAPX 2 expression level depends on decreasing the transcriptional level.

2.6. The enzymatic activity of APX is reduced in parallel with the decreased amount of protein

Because changes in gene expression do not necessarily reflect changes in the enzyme catalysis (Vacca et al., 2004), the activity of APX was measured in the roots of nontreated and flooded soybean seedlings in this study. In parallel with the decrease in protein amounts and the levels of APX 2 transcript, the activity of APX was significantly reduced after flooding for three days ($p < 0.01$) (Fig. 7). In general, plants show a two-phase response when subject to stress (Huynh le et al., 2005). The first phase is an early response to stress, and the second is an acclimation to it. Gene transcription and the enzyme activity of APX changed by flooding should follow this two-phase response pattern.

3. Concluding remarks

A reduction of APX activity could provoke a large increase in ROS and induce oxidative damage that would impair plant development. Alternatively, ROS could be

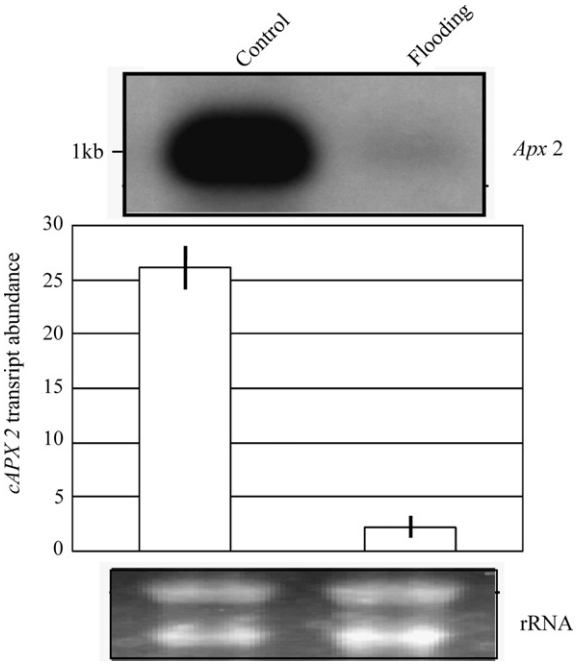

Fig. 6. Abundance analysis of APX2 transcript by Northern hybridization. The total RNAs were isolated from the roots of soybean seedlings that were germinated on wet spongy matrix for two days and sequentially submerged for three days or not submerged. For Northern hybridization, 6.5 μg of total RNAs were loaded. An antisense RNA probe was prepared with PCR amplified cAPX 2. The blot was subjected to immunological detection using antidigoxigenin antibody conjugated to alkaline phosphatase. Hybridization signals were visualized by developing X-ray film. The size of band was calculated based on the mobility of rRNA. The data were shown for three independent surveys as average ± SE. A double asterisk showed the significance at $p < 0.01$.

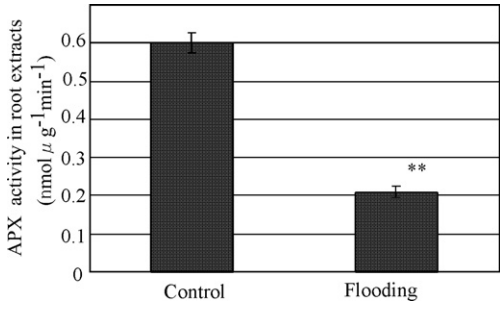

Fig. 7. The effect of flooding on the APX activity of extracts from soybean cultivar Enrei. The roots of soybean seedlings submerged in water for three days after being germinated on sponge for two days were used to prepare extracts. APX activity was determined by monitoring the oxidation of ascorbic acid at 290 nm (absorption coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) and expressed in nmol μg^{-1}protein min^{-1}. The data were shown for two independent surveys as average ± S.E. A double asterisk showed the significance at $p < 0.01$.

involved in signaling events related to the slowdown in growth observed in flooded plants. The introduction of exogenous genes and the enhancement of endogenous genes encoding antioxidant enzymes in soybeans have reportedly resulted in increased chilling tolerance (Sato et al., 2001). On the other hand, Funatsuki et al. (2003) showed that the genotypes lacking a cAPX isozyme exhibit relatively high chilling tolerance. Furthermore, Wang and Jiang (2007) reported that the activity of APX in creeping bent-grass roots significantly decreased by 46% and 40% during water-logging at 1 cm and 15 cm below the soil surface. These reports indicate that the decrease of APX associates with stress-response and/or tolerance. Panchuk et al. (2002) suggested that AtAPX 2 is a novel heat shock gene and that the enzymatic activity of APX2/APXs is required to compensate the heat–stress-dependent decline of APX 1 activity in the cytosol. This report shows that APX 2 has a new function in stress-response. Although it remains to be elucidated how the deficiency of cAPX 2 associates with flooding stress, our findings are informative for further genetical and physiological studies on soybean flooding tolerance.

4. Experimental

4.1. Plant growth and treatment

Seeds of soybean (*Glycine max* [L.] Merr.) cultivar Enrei after sterilization by sodium NaOCl were germinated on spongy media for two days and then submerged into distilled H_2O for three 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. To survey the difference on an expression pattern of flooding-responsive proteins among soybean cultivars, Peking, Williams 82, Sachiyutaka, Tamahomare, and Nakasennari, and also Enrei, were used. This treatment was carried out with five independent experiments.

4.2. Preparation of protein fractions

The roots of soybean seedlings after three days of flooding were used to prepare cytosolic and membrane fraction. A portion (100 mg) of soybean roots were homogenized with 300 μl homogenization buffer containing sucrose, 20 mM Tris–HCl (pH 7.5), 10 mM EGTA, 1 mM DL-dithiothreitol (DTT), and 1 mM phenylmethyl sulfonyl fluoride. The homogenates were centrifuged at 3000*g* at 4 °C for 5 min, followed by the ultracentrifugation of the supernatant at 259,000*g* at 4 °C for 15 min (Beckman, Palo Alto, CA, USA). Cytosolic fraction was obtained by collecting the supernatant (Komatsu and Hirano, 1993). The pellets were washed with 500 μl of homogenization buffer followed by ultracentrifugation, as stated above. It was then solubilized with buffer containing 1% Triton X-100, 20 mM Tris–HCl (pH 7.5), EDTA, and 50 mM 2-mercaptoethanol and kept on ice for 30 min. The membrane fraction was obtained by collecting the supernatant after ultracentrifugation at 259,000*g* at 4 °C for 8 min. For two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), lysis buffer with 1:1 ratio was added to the cytosolic and membrane fractions before loading.

4.3. 2-dimensional polyacrylamide gel electrophoresis

Prepared protein (25 μg) was separated in the first dimension by isoelectric focusing (IEF) tube gel and in the second dimension by SDS–PAGE (O' Farrell, 1975). An IEF tube gel 11 cm long and 3 cm in diameter was prepared, which consisted of 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate, and tetramethylethylenediamine. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 18 h and 600 V for 1 h. After IEF electrophoresis, SDS–PAGE was carried out using 15% polyacrylamide gel with 5% stacking gels. Proteins separated on 2D-PAGE were visualized by silver staining (Nakarai, Kyoto, Japan). 2D-PAGE images were captured, and the positions of individual proteins on gels were automatically evaluated using ImageMaster 2D-Elite software (GE Healthcare, Piscataway, TX, USA). The isoelectric point (p*I*) and relative molecular mass (M_r) of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA).

4.4. Image acquisition and data analysis

Three independent experiments were done with proteins obtained from seedlings from different experiments. The images of silver-stained gels were captured with ImageMaster 2D-Elite software (version 3.01, GE Healthcare). Spot detection, spot measurement, background subtraction, and spot matching were performed. Following capture, the gel images were carefully edited. The gel image from a nontreated sample was selected as a reference, and one from a submerged sample was subjected to automatical spot matching. The amount of a protein spot was expressed as the volume of the spot, which was defined as the sum of the intensities of all the pixels that make up the spot. To correct the variability because of silver staining and to reflect the quantitative variations in the intensity of protein spots, the spot volumes were normalized as the percentages of the total volume in all of the spots present in the gel. Standard error was calculated from spots of the gels from three independent experiments and used as error bars.

4.5. Mass spectrometry analysis and database search

Protein spots stained with coomassie brilliant blue (CBB) were excised from gels, washed with 25% methanol and 7% acetic acid for 12 h, and destained with 50 mM NH_4HCO_3 in 50% methanol for 1 h at 40 °C. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry, then rehydrated in 100 mM NH_4HCO_3 with 1 pM trypsin (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C overnight. The digested peptides were extracted from the gel grains with 0.1% trifluoroacetic acid in 50% acetonitrile/water three times. The peptide solution thus obtained was dried and reconcentrated with 30 μl of 0.1% trifluoroacetic acid in 50% acetonitrile/water and desalted with NuTip C-18 pipette tips (Glygen, Columbia, MD, USA). The above peptide solution was analyzed by electrospray ionization-quadrupole-time-of-flight mass spectrometry (ESI-Q-TOF MS, Q-TOF micro, Micromass, Manchester, UK). The mass spectra were subjected to a sequence database search using MASCOT software (Matrix Science, London, UK).

4.6. RNA isolation and Northern hybridization

Total RNAs were isolated from the roots of soybean seedlings that were germinated on a wet sponge for two days and sequentially submerged for three days, using an RNeasy Plant Minikit (Qiagen, Valencia, CA, USA). cDNA was generated from a portion of isolated total TNA by a ReverTra Plus RT PCR kit (Toyobo, Tokyo, Japan). The PCR amplification was conducted for 30 cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 1 min. The primer pair for amplifying APX 2 is 5′AACTTACGACGTGAGCTCGAAG3′ and 5′GAGCCTCAGCGTAATCAGCGAAG3′. The PCR product was electrophoresed in 1% agarose mini gel, and the amplified DNA fragment was extracted by QIA quick PCR purification kit (Qiagen). The purified DNA fragment was cloned into the pGEM-T Easy vector and used as a template for a generation of antisense RNA probe (Roche Diagnostics, Mannheim, Germany). The reaction for generating antisense digoxigenin-labeled RNA probe was carried out in a 20 μl solution containing 100–200 ng template, 2 μl 10 × RNA polymerase transcription buffer, 2 μl digoxigenin–RNA labeling mix, 20 U RNase inhibitor, and 20 U T7 RNA polymerase by incubating at 37 °C for 2 h. Following the degradation of template DNA by DNase, the RNA probe was precipitated and resuspended with distilled water.

A 6.5 μg aliquot of total RNA was denatured at 65°C for 10 min in 10 μl of loading buffer containing 20% formaldehyde, 60% formamide, and 20 mM MOPS (pH 7.0). The denatured RNA was separated on 1.2% agarose gels containing 0.66 M formaldehyde and transferred to a nylon membrane (Roche Diagnostics) by downward blotting. After checking the transfer, the membrane was baked for 1 h at 80 °C and hybridized at 65 °C for 12–18 h with digoxigenin-labeled RNA probes. The membrane was washed in 2× SSC with 0.1% SDS for 30 min and in 0.2× SSC with 0.1% SDS for 45 min twice at 65 °C. The blots were subjected to immunological detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and CDP-Star (Roche Diagnostics). Hybridization signals were visualized by developing X-ray film (Yamamoto et al., 2007).

4.7. Assay of APX activity

APX activity was determined by monitoring the oxidation of ascorbic acid at 290 nm (absorption coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$), as described by Nakano and Asada

(1987). A total of 250 mg of soybean seedling roots submerged in water for three days after being germinated on spongy media for two days were homogenized with 2.5 ml of 25 mM potassium phosphate buffer (pH 7.8) containing 2% polyvinylpolypyrrolidone, 0.4 mM EDTA-4H, and 1 mM ascorbic acid. After centrifugation at 15,000*g*, 4 °C for 20 min, the soluble fraction was filtered by a layer of miracloth, and the eluted solution was an enzyme crude extract. Protein contents in crude extract were measured by Bradford assay (Bradford, 1976), using bovine serum albumin as a standard. For a measurement of APX activity, the assay medium was comprised of 25 mM potassium phosphate buffer (pH 7.0), 0.25 mM ascorbic acid, 0.4 mM EDTA-4H, and 0.1 mM H_2O_2.

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