BMB
reports

Molecular cloning of peroxidase cDNAs from dehydration-treated fibrous roots of sweetpotato and their differential expression in response to stress

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Three peroxidase (POD) cDNAs were isolated from dehydration-treated fibrous roots of sweetpotato (*Ipomoea batatas*) plant via the screening of a cDNA library, and their expressions were assessed to characterize functions of each POD in relation to environmental stress. Three PODs were divided into two groups, designated the basic PODs (*swpb4*, *swpb5*) and the anionic PODs (*swpa7*), on the basis of the pI values of mature proteins. Fluorescence microscope analysis indicated that three PODs are secreted into the extracellular space. RT-PCR analysis revealed that POD genes have diverse expression patterns in a variety of plant tissues. *Swpb4* was abundantly expressed in stem tissues, whereas the expression levels of *swpb5* and *swpa7* transcripts were high in fibrous and thick pigmented roots. *Swpb4* and *swpa7* showed abundant expression levels in suspension cultured cells. Three POD genes responded differently in the leaf and fibrous roots in response to a variety of stresses including dehydration, temperature stress, stress-associated chemicals, and pathogenic bacteria. [BMB reports 2008; 41(3): 259-265]

INTRODUCTION

Secretory class III peroxidase (POD, EC 1.11.1.7) catalyzes the reduction of H_2O_2 via the transport of electrons to a variety of donor molecules, including phenolic compounds and lignin precursors (1), and have been implicated in a broad range of physiological processes, including lignification, suberization, the cross-linking of cell wall proteins, defense against pathogenic attack, and oxidative stresses (1). Plant PODs are members of a large family of isoenzymes (2). The results of genomic sequence analyses have revealed that there are 73 POD-encoding genes in *Arabidopsis thaliana* and 138 genes in *Oryza sativa* (2, 3). The expression profiles of 23 POD genes in Arabidopsis and 21 POD genes in rice have been evaluated in a variety of tissues (1, 2). However, the specific functions of these POD genes in regard to plant growth and adaptations to environmental stresses remain to be elucidated.

Drought stress is one of the most salient environmental stresses affecting agricultural production (4). Sweetpotato plant is one of the most important root crops in the world. However, the sweetpotato has not been yet characterized on the molecular basis of its drought stress tolerance in terms of POD. Plant roots constitute the primary sensor of drought and salt stress (5). Plant PODs play important roles in defense mechanisms against environmental stresses, including drought stress.

In the previous studies, we established an efficient system for the production of PODs from suspension cultures of sweetpotato (*Ipomoea batatas*) (6, 7). Ten POD cDNAs were isolated from sweetpotato cell cultures, and their expression levels were characterized to understand functions of each POD in response to a variety of environmental stresses, including wounding, chilling, exposure to stress-related chemicals, air pollutants and pathogenic infection (8-13). In this study, in order to further study functions of POD genes in sweetpotato, we isolated three additional POD genes from the dehydrated fibrous roots of sweetpotato and analyzed expression profiles of each POD gene under various stress conditions.

RESULTS AND DISCUSSION

Cloning and sequence analysis of three POD cDNA

A cDNA library prepared with the RNA obtained from the dehydrated fibrous roots of sweetpotato plant was screened using two probes generated via PCR. Three representative clones, two basic PODs (*swpb4*, *swpb5*) and one anionic POD (*swpa7*) with a distinct 3'-UTR sequence, were selected for further characterization in terms of stress. The predicted protein sizes and pI values of the three isolated POD genes are listed

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Received 16 July 2007, Accepted 5 October 2007

Keywords: Dehydration, Environmental stress, Fibrous root, Peroxidase, Sweetpotato (*Ipomoea batatas*)

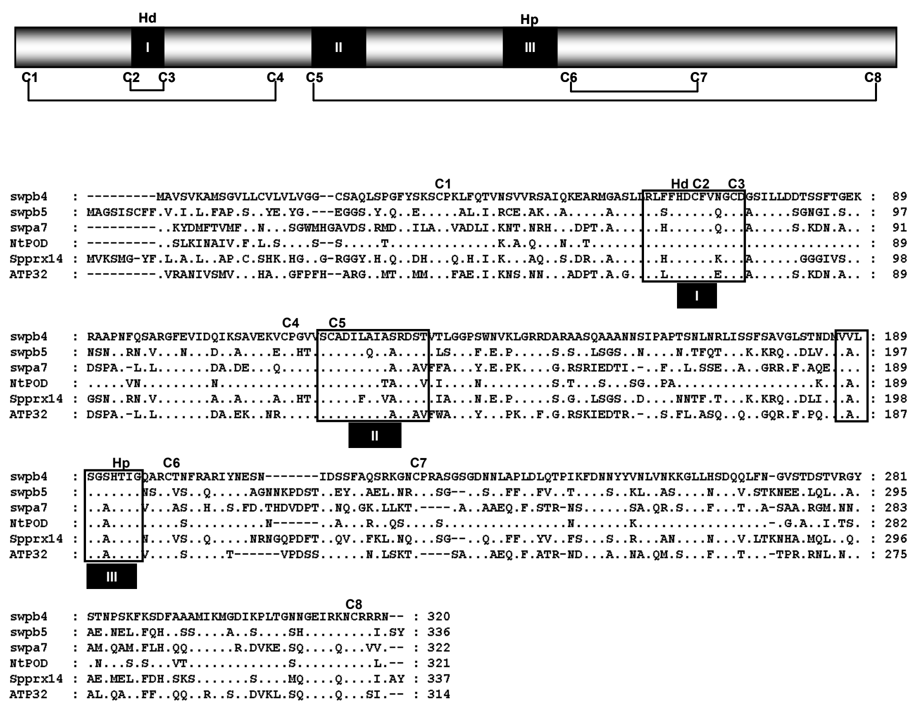

Fig. 1. Alignment of amino acid sequences of three PODs, two basic PODs (*swpb4* and *swpb5*) and one anionic POD (*swpa7*), from sweetpotato. Boxes represent the conserved distal heme-binding domain (I), the central conserved domain of unknown function (II), and the proximal heme-binding domain (III). The eight cysteines (C) that form disulfide bridges and the two histidines essential for catalysis, distal histidine (Hd) and proximal histidine (Hp), are indicated above the amino acid sequences. Dots represent amino acid residues that are identical to the *swpb4* sequence, and the dashes indicate gaps introduced to optimize the alignment. The GenBank accession numbers are as follows: *swpb4* (EF433455), *swpb5* (EF433456), *swpa7* (EF433454), *Nicotiana tabacum* (*NtPOD*, BAA82306), *Spinacia oleracea* (*Spprx14*, AAF63026) and *Arabidopsis thaliana* (*ATP32*, Q9SZB9).

in the Supplemental Table 2. *Swpb4* and *swpb5* encode basic PODs, whereas *swpa7* encodes an anionic POD. Database analysis indicates that three PODs harbor signal sequences for targeting to extracellular positions, including the cell wall.

The amino acid sequences of the three PODs are aligned as shown in Fig. 1. As had been expected, three PODs shared highly conserved I, II, and III helices, which are common to plant PODs and function in both catalysis and protein folding (14). They also harbor conserved structures, including the eight cysteines that form the disulfide bridges and two histidines that are essential for catalysis. Three PODs of swpb4, swpb5, and swpa7 exhibited 39-51 % similarity at the amino acid level. Among the thirteen PODs, swpb4 has the highest level of homology (98 %) with swpb3. Swpb5 showed a high homology (about 90 %) with swpb1 and swpb2. In contrast, we detected low levels of homology (51 %) between the two basic PODs (swpb4 and swpb5). Swpa7 exhibits low homology (below 40 %) with other sweetpotato POD genes, regardless of their pI values (Supplemental Table 3).

Genomic organization and subcellular localization of the three PODs

To elucidate the genomic organization of the three POD genes, the Southern blot analysis of genomic DNA digested with *Eco*RI, *Eco*RV, and *Hind*III is carried out (Fig. 2A). The number of hybridizing bands indicates the presence of multiple genes of three POD genes in the sweetpotato genome.

To investigate the subcellular localization of the three PODs, 35S-PODs::GFP fusion constructs were introduced into the onion epidermal cells by particle bombardment (Fig. 2B). The fluorescence signal of the 326-GFP control was detected within the cytosol. Extracellular localization of the three PODs were clarified in the presence of 10 % sucrose, which induces plasmolysis, pulling the cytosol away from the cell wall and thus allowing the two to be distinguished from one another. These results are consistent with the prediction data, which show that three PODs are secreted into the extracellular space, including the cell wall. Therefore, we suggest that three PODs are involved in plant growth and development by cell wall-related mechanisms, including the cross-linking of cell wall compounds, lignification, and suberization.

Expression patterns of sweetpotato POD genes in various tissues and dehydration conditions

Our RT-PCR analysis showed that three POD genes are differently expressed in various tissues of whoe plants and in cultured cells (Fig. 3A). In intact plant tissues, *swpb4* was expressed abundantly in stem tissues, whereas *swpb5* and *swpa7* were highly expressed in fibrous and thick pigmented roots. In suspension cell cultures, *swpa7* and *swpb4* were expressed 20 d after subculturing, whereas *swpb5* was not expressed at the same stages of the cell cultures. These results indicated that the expression of each POD gene might be regulated differentially in a variety of intact tissues and cultured cells.

The expression patterns of three POD genes were investigated after dehydration treatment (Fig. 3B). Two POD genes,

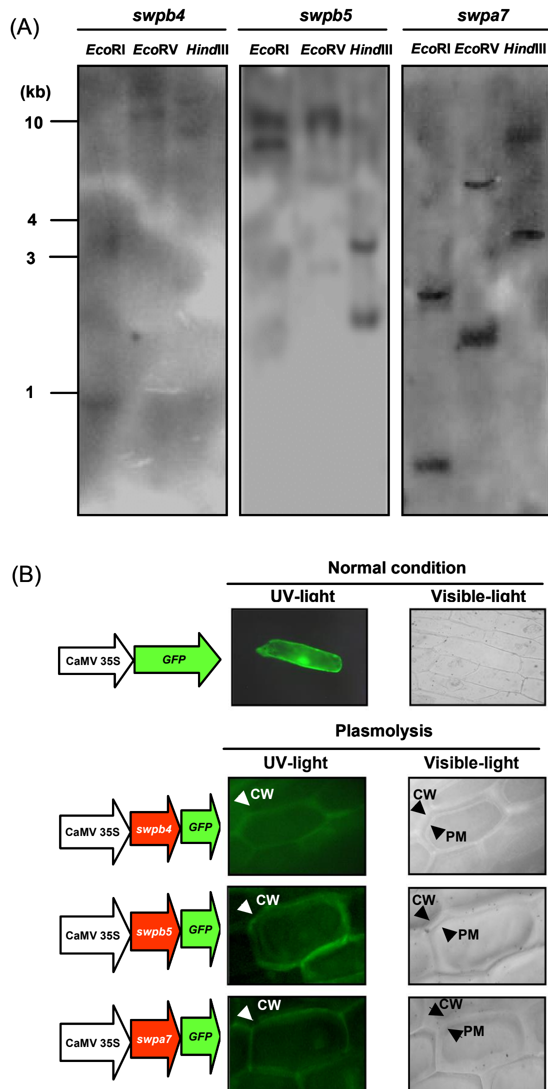

Fig. 2. Southern blot analysis and subcellular localization of three PODs. (A) Southern blot analysis of three POD genes from sweetpotato. (B) Subcellular localization of three 35S-POD::GFP proteins. UV-light (left) and visible light (right) images are shown. The expression of GFP image was assessed in the presence of 10 % sucrose. The arrow indicates cell wall (CW) or plasma membrane (PM).

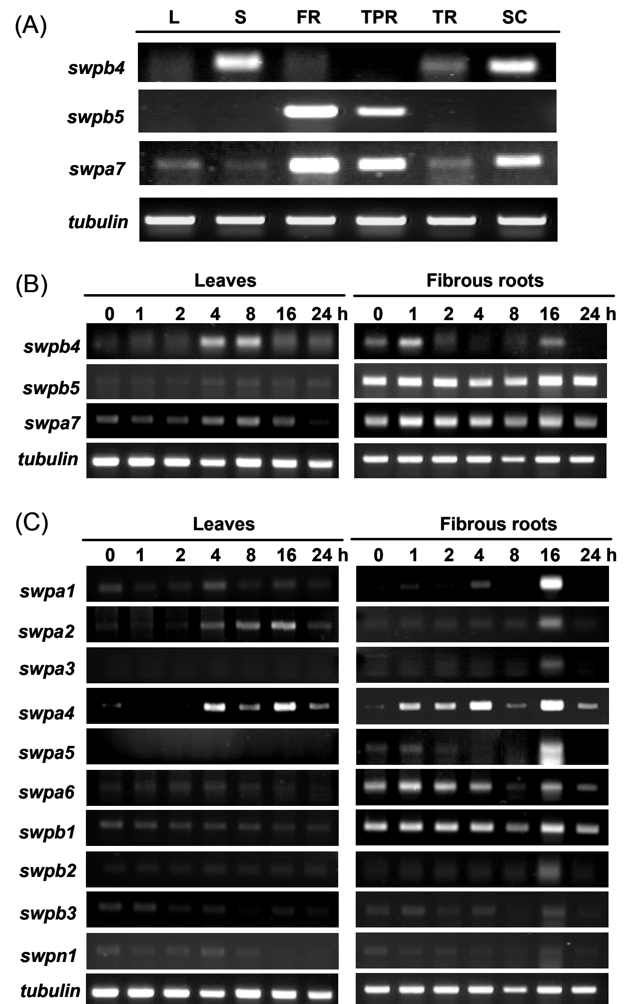

Fig. 3. Expression of sweetpotato POD genes in various tissues and dehydration conditions. (A) Expression of three POD genes in various tissues. Total RNAs were extracted from leaf (L), stem (S), fibrous root (FR), thick pigmented root (TPR), tuberous root (TR), and suspension-cultured cells (SC). (B) Expression patterns of three POD genes in sweetpotato plants under dehydration conditions. (C) Expression patterns of ten POD genes from suspension cell cultures in sweetpotato under dehydration conditions. *Tubulin* was utilized as a control for equal loading.

swpb4 and *swpa7*, strongly responded to dehydration, which showed the highest expression levels at 4 and 8 h in the leaves. In the fibrous roots, three genes were expressed under normal conditions (0 h). High expression levels of *swpb5* and *swpa7* transcripts in the fibrous roots were constitutively maintained for 24 h. Interestingly, the expression levels of the *swpb4* and *swpa7* in fibrous roots appeared twice, at 1 and 16 h. The responses to drought stress of ten POD genes were not characterized. Therefore, the expression patterns of ten POD genes were evaluated in sweetpotato plants after dehydration treatment (Fig. 3C). In the leaves, *swpa1*, *swpa2*, and *swpa4* expression was induced slightly by dehydration, whereas the transcripts of ten POD genes were increased upon dehydration in the fibrous roots. It has been shown that drought imposes different stresses, including water deficiency, nutrient limitation, salinity, and oxidative stress (15). In the Norway spruce, the expressions of several POD isoenzymes were shown to correlate with lignifications in response to drought stresses and

pathogenic infection (16). In the white clover, elevated POD activity and isoenzyme patterns were also correlated with the increased lignin contents under drought stress conditions (17). Moreover, it was suggested that lignification by POD operates as a physical barrier against pathogenic infection (1). The relationship between dehydration-responsive POD genes and pathogenic infection remains to be determined.

Differential expression of three POD genes in response to a variety of stresses

Sweetpotato plants were subjected to temperatures of 42°C, 15°C, and 4°C for 16 h to determine the effects of temperature stress on the levels of POD expression (Fig. 4A). The *swpb4* gene was expressed strongly in leaves at 42°C and 15°C, and in the fibrous roots at 15°C. However, the transcript of *swpb4* was down-regulated at 4°C in both the leaves and fibrous roots. *Swpb5* was not expressed in leaves under any of the temperature conditions, whereas it was expressed in fibrous roots at temperatures of 15°C and 4°C. *Swpa7* expression was reduced slightly at temperatures of 15°C and 4°C in leaves. Interestingly, *swpa7* was expressed predominantly in fibrous roots at 4°C. Extreme temperatures induce increases in reactive oxygen species (ROS) levels in plants and induce a variety of antioxidant enzymes to overcome oxidative stress (18). In our previous studies, among ten POD genes from suspension culture cells, *swpa5*, *swpb1*, *swpb2*, and *swpb3* were expressed abundantly in leaves of sweetpotato after treatment at 37°C (13). In addition, *swpa1*, *swpa2*, *swpa3*, *swpa4* and *swpn1* were expressed in leaves of sweetpotato after treatment with 15°C or 4°C (8, 10, 13). This indicates that the expression of each POD gene was regulated differentially by ROS accumulation under different temperature conditions.

Next, the expression patterns of three sweetpotato POD genes were analyzed after treatments with NaCl, MV and ABA (Fig. 4B). The expression levels of *swpb4* and *swpa7* transcripts were increased after MV, NaCl and ABA treatment, whereas *swpb5* was down-regulated in the fibrous roots. MV is a well-known ROS generating chemical which causes a massive, light-mediated accumulation of superoxide radicals in plant tissues (19). Six POD genes from the suspension cultured cells showed increased expression by MV treatment (13). In the rice plant, certain POD genes expressed in roots were also induced by MV-mediated oxidative stress (20). Thus, MV-inducible POD genes, including *swpb4* and *swpa7*, would be involved in the detoxification of H_2O_2 which accumulates as the result of MV. High salinity induces both hyperionic and hyperosmotic stresses, and can also result in plant cell death via oxidative stress (21). Furthermore, exposure to dehydration and salt stress triggers many common reactions in plants (15). Tomato cell wall POD (*TPX2*) overexpressed in transgenic tobacco plants increased the germination rate under osmotic stresses, including mannitol and NaCl (22). This indicates that two cell wall POD genes, *swpb4* and *swpa7*, may be associated with osmotic protective functions in root tissues under

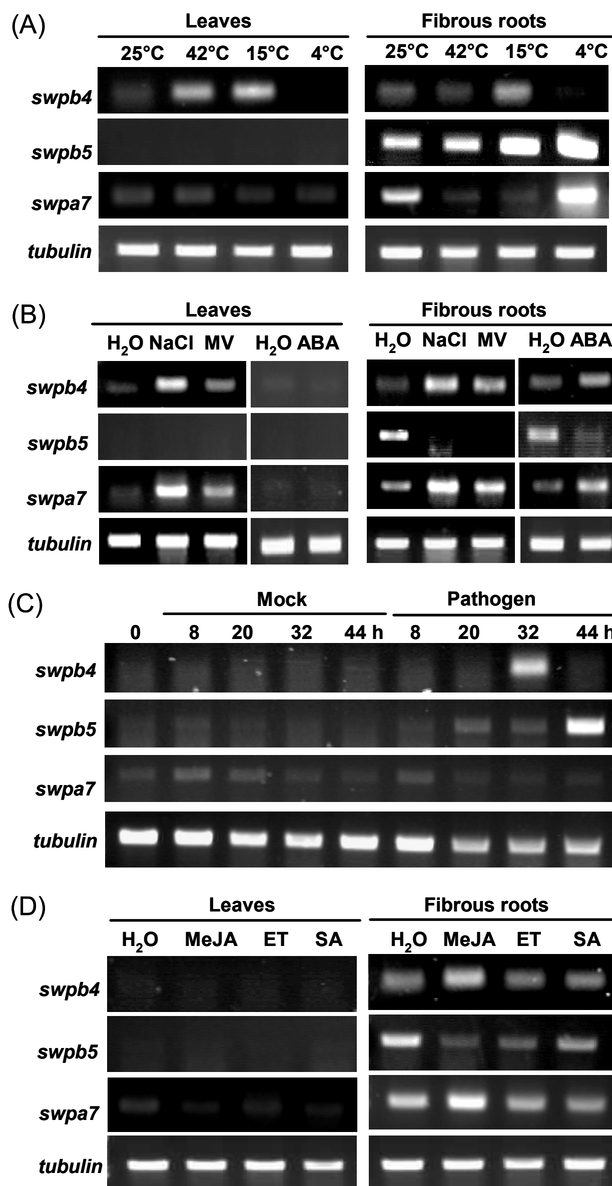

Fig. 4. Expression of three POD genes under a variety of stress conditions. (A) Different temperature conditions. (B-D) Various stress conditions. Plants are subjected to NaCl (100 mM) and MV (0.05 mM) for 24 h, or ABA (0.1 mM), MeJA (0.1 mM), ET (0.1 mM), and SA (0.1 mM) for 48 h. Control plants were maintained in distilled water. Bacterial pathogen (*P. chrysanthemi*) was infectied to leaves of plants. Mock represents treatment with 10 mM $MgCl_2$. Tubulin was used as an equal loading control.

drought and salt stress conditions. ABA regulates interacting signaling pathways involved in the responses of plants to several abiotic stresses (15). Recently, some reports have demonstrated a relationship between the signal transduction path-

ways for ABA and ROS production (23). In particular, plasma membrane NADPH-oxidase and cell wall POD are reported to be involved in ABA-induced ROS generation (24, 25). Therefore, our findings indicate that under several abiotic stresses including dehydration, salt, and ROS, *swpb4* and *swpa7* may be linked to the ABA signaling pathway.

The expression patterns of three POD genes were investigated in the leaves after infection of pathogenic bacterial (*P. chrysanthemi*) (Fig. 4C). The *swpb4* transcript was profoundly induced by bacterial infection at 32 h and *swpb5* expression was highly expressed at 44 h. However, *swpa7* was only weakly expressed in the early stages. Pathogenesis-related (PR)-9 genes has been identified as a specific type of POD that might operate in cell wall reinforcement via the catalysis of lignification (1). In the defense of cotton against bacterial pathogens, POD activity is significantly increased in the infected region 12 h after treatment, and is localized principally in the apoplast and proximal to the bacterial infection site (26). Therefore, it has been suggested that two basic POD genes are involved in pathogen defense via cell wall-related mechanisms, including the cross-linking of cell wall proteins and lignification.

MeJA, ET, and SA are the primary endogenous low molecular weight signal molecules that are involved in the regulation of defense responses in plants under various stress conditions (27). Therefore, MeJA, ET, and SA were applied to sweetpotato plants for 48 h (Fig. 4D). The expression levels of *swpb4* and *swpa7* were enhanced in fibrous roots after MeJA treatment, whereas *swpb5* expression was down-regulated after the application of ET and MeJA. MeJA has been associated with responses to abiotic and biotic stresses, including wounding and pathogens (28). The expressions of the fungus-inducible rice POD genes were induced only by JA treatment (29). Pathogen-inducible ten wheat POD genes showed a profound induction by MeJA rather than SA (30). It is likely MeJA, rather than SA, which performs the most important role as a pathogen-responsive POD signal in wheat and rice. This indicates that three POD genes may be associated with the MeJA-dependent or independent signaling pathway under pathogen infection conditions. Therefore, the expression patterns of three POD genes are likely to be regulated by different signal molecules, including ABA, MeJA and ROS, under a variety of abiotic stresses and pathogen infections.

In conclusion, our data regarding three sweetpotato POD genes showed a variety of environmental stress-induced expression profiles of each of the POD genes. Among the three POD genes, *swpb4* and *swpa7* responded to a variety of abiotic stresses, including dehydration, NaCl, MV, ABA, and MeJA treatment, with similar expression patterns. Interestingly, the expression levels of *swpb4* and *swpb5* transcripts were induced upon infection with pathogenic bacteria. All observations in this study indicate that each POD may be differentially or cooperatively involved in defense mechanisms that can overcome oxidative stresses under a variety of environmental stress conditions. For a better understanding of the roles of individual PODs, further experiments utilizing transgenic plants with the suppression or enhancement of the expression of each POD gene will be necessary. In addition, we anticipate that root-specific promoters from *swpb5* and *swpa7* will be studied with regard to the development and production of useful components in the tuber using transgenic sweetpotato plants.

MATERIALS AND METHODS

Plant materials

Sweetpotato (*Ipomoea batata* L. *Lam.* cv. White Star) plants were grown for 50 d in a growth chamber. One gram (fresh weight) of cultured cells subcultured at 14 d intervals were inoculated into 50 ml of MS (31) basal medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l) and sucrose (30 g/l), then incubated in darkness at 25°C (100 rpm). The cells were collected 20 d after subculturing.

Preparation of POD probes

Two primers (5'-CACTTCCACGACTGCTTCGT-3', 5'-ACGA-AGCAGTCGTGGAAGTG-3') designed from the conserved regions of previously reported POD sequences were employed (8, 10, 13).

Construction and screening of a cDNA library

For the construction of the cDNA library, total RNA was isolated from the fibrous roots of sweetpotato plant at 0, 0.5, 2, 6, and 10 h after dehydration, respectively. Poly(A)+ RNAs were purified with a Poly(A) Trac mRNA isolation system (Promega, USA). The cDNA was synthesized from poly(A)+-enriched RNA with the SMART™ cDNA Library Construction Kit, according to the manufacturer's instructions (Clontech, Japan). The ligated DNA was packaged using a lambda packaging kit, and the library was amplified in the XL-1 Blue *E. coli* strain (Clontech) prior to screening. The plaques were then transferred to Hybond-N nylon membranes (Amersham, Sweden), UV cross-linked, and screened for hybridization with radio-labeled probes. The phagemids were excised from the phage according to the manufacturer's instructions (Clontech).

Southern blot analysis

The genomic DNA of sweetpotato plant was extracted from leaves in accordance with the method described by (32), digested with *Eco*RI, *Eco*RV, and *Hind*III (Roche, Germany), electrophoresed on 0.8 % agarose gel, and blotted onto Zeta-probe GT membranes (Bio-Rad, USA).

Subcellular localization of three PODs using GFP

The *swpb4*, *swpb5*, and *swpa7* DNAs were generated via PCR using the following primer sets with the *Bam*HI restriction enzyme sites (underlined): for *swpb4*, 5'-AGGATCCATGGCGG-TTTCTGTTAAGGCTAT-3' and 5'-TGGATCCAGTTCCGTCTC-

CTGCAGTTCTTC-3'; for *swpb5*, 5'-AGGATCCATGGCTGGC-TCAATCAGCTGTTT-3' and 5'-GGGATCCAATAAGAGTTGA-TTTTCCTGCAA-3'; and for *swpa7*, 5'-GGATCCATGGCTAA-GTATGATATGTTCAC-3' and 5'-TCGGATCCAGTTGACCACA-CGGCAAT-3'. The amplified DNAs were cloned into the *Bam* HI sites of the TOPO vector (Invitrogen, USA) to fuse 326-GFP in-frame to the C-termini of the DNA products. The resultant 35S-POD::GFP constructs were introduced into onion epidermal cells with a helium biolistic particle delivery system (Bio-Rad) as previously described by (33). After 12 to 48 h of incubation at 25°C, the subcellular distribution of the GFP fusion proteins was assessed under a fluorescence microscope (AXIOSKOP, Zeiss, Germany).

Stress treatment

For stress treatments, 50 days old sweetpotato plants were used. For dehydration treatment, the plants were carefully pulled out, transferred onto filter papers and allowed to dry. For the temperature stress treatment, the plants were exposed to temperatures of 42°C, 15°C, 4°C, and 25°C for 16 h, respectively. For treatment with NaCl (100 mM) and methyl viologen (MV, 0.05 mM), sweetpotato plants were incubated for 24 h in glass bottles containing 400 ml of each chemical solution. For treatments of abscisic acid (ABA, 0.1 mM), methyl jasmonate (MeJA, 0.1 mM), ethephon (ET, 0.1 mM) and salicylic acid (SA, 0.1 mM), sweetpotato plants were incubated for 48 h. Sterile water was utilized as a control for the stress-related chemical treatments. For bacterial treatment, *Pectobacterium chrysanthemi* (*Erwinia chrysanthemi*, KCTC 2569) was treated in accordance with the method described by (9).

RT-PCR analysis

For RT-PCR analysis, first-strand cDNA was synthesized using MMLV Reverse Transcriptase (Clontech) from 1 μg of total RNA. PCR amplification reactions were incubated initially for 5 min at 94°C, followed by 26-30 cycles at 94°C for 30 s, 56-59°C (depending on the melting temperature of each gene-specific primer) for 30 s, and 72°C for 45 s using each of the gene-specific primers (Supplemental Table 1). The total synthesized cDNA was also utilized to amplify the tubulin gene as an internal standard, using tubulin gene-specific primers (5'-CAACTACCAGCCACCAACTGT-3', 5'-CAAGATCCT-CACGAGCTTCAC-3').

DNA and protein sequence analysis

The sequence identities were determined using the BLAST program of the NCBI web-server, and multiple sequence alignment was determined using the Clustal X and GeneDoc programs. In order to predict the isoelectric point (pI), molecular weight, and signal peptides of the deduced proteins, ExPasy (http://www.expasy.org/tools), PSORT (http://psort.ims.u-tokyo.ac.jp), and SoftBerry (http://www.softberry.com) programs were used.

Acknowledgments

This work was supported by the Environmental Biotechnology National Core Research Center, Korea Science & Engineering Foundation (KOSEF), by the Korea Foundation for International Cooperation of Science & Technology (KICOS), and by KRIBB initiative program.

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