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Molecular cloning and characterization of cDNAs for anionic and neutral peroxidases from suspensioncultured-cells of sweet potato and their differential expression in response to stress

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Abstract Two peroxidase (POD) cDNAs, swpa1 and swpn1, were isolated and characterized from suspension-cultured cells of sweet potato in order to understand the physiological function of POD isozymes. Sequence analysis showed that swpa1 encoded an anionic POD and swpn1 encoded a neutral POD. The swpa1 and swpn1 genes were both highly expressed in suspension-cultured cells in accordance with the high POD activity of these cells. Although both gene transcripts were detected in the stems of intact plants, their transcription levels were much lower than in suspension-cultured cells. During cell growth the pattern of mRNA accumulation of swpa1 differed from that of swpn1, suggesting that expression of these genes is differentially regulated by cell growth stage. In addition, the swpa1 and swpn1 genes responded differently to oxidative stress induced by chilling. The expression of swpa1 was weakly induced by 15° C acclimation and strongly induced by 4° C chilling, whereas the mRNA level of swpn1 was increased by 15° C acclimation and reduced by 4° C chilling. This indicates that the two isozymes encoded by swpa1 and swpn1 might contribute to protection against cold-induced oxidative stress through different signaling pathways. In leaves, both genes were induced by wounding with broadly similar expression patterns. Genomic analysis suggests that the two isozymes are encoded by different loci in the sweet potato genome.

Key words Chilling · Peroxidase cDNAs · Suspension-cultured cells · Sweet potato (*Ipomoea batatas*) · Wounding

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Introduction

Peroxidase (POD, EC 1.11.1.7) is a monomeric heme-containing enzyme of 32–45 kDa. POD has been of great interest because it plays a significant role in plant responses to stress. It is also used in various industrial applications (Krell 1991; Siers 1991). Most higher plants possess a number of POD isozymes, which are involved in many physiological processes, such as organogenesis, cross-linking of cell wall polysaccharides, lignification, suberization, and auxin metabolism (Fry 1986; Krell 1991; Lagrimini 1991; Castillo 1992; Lagrimini et al. 1993; Mohan et al. 1993; Zimmerlin et al. 1994). The expression of some of these POD isozymes is tissue specific and is regulated either developmentally or in response to external stimuli (Intapruk et al. 1993; Omann et al. 1993; Baga et al. 1995).

PODs are, in general, useful markers for environmental stress as POD activity in plants is affected by factors such as air pollution, low temperature, ozone, heavy metals, wounding, pathogens, salts, drought, and UV radiation (Castillo 1992). In all of these physiological processes the isozyme pattern of PODs is often complex. This complexity has caused problems in attempts to understand the specific function of these enzymes in vivo and their specific roles in plant growth and adaptation to the environment (Welinder 1992). Therefore, it is important to isolate the cDNAs and genes that encode each isozyme, and to analyze gene expression and the properties of the gene products. So far, plant POD cDNAs and genes have been isolated by molecular cloning from over 20 plants, including barley, wheat,

and rice (Johansson et al. 1992; Esnault and van Huystee 1994). However, molecular cloning of POD sequences from sweet potato has not been reported despite its importance as a food crop.

Plant PODs are divided into three subgroups based on their pI: anionic, neutral, or cationic. The anionic POD isozymes have been studied most extensively with respect to their expression in response to developmental and environmental factors, including their possible role in plant defense. cDNAs and genes encoding wound- and pathogen-induced anionic PODs have been isolated from wheat (Rebmann et al. 1991), tomato (Mohan et al. 1993), horseradish (Kawaoka et al. 1994), and potato (Lagrimini 1991). It is likely that these enzymes are involved in cross-linking and polymerization during construction of a physical barrier that prevents pathogen penetration of cell walls. However, the role of each isozyme still remains to be clarified. In contrast to anionic PODs, little is known about the biological role of neutral and cationic POD isozymes, although the physical and chemical properties of cationic PODs have been the subject of numerous studies. Analysis and comparison of POD sequences has shown that plant PODs are not related to any known animal heme-containing POD, whereas they are structurally homologous to yeast cytochrome *c* POD and bacterial catalase-POD (Welinder 1991, 1992).

Plant cells in culture are considered to be under conditions of high stress because the cells produce active oxygen species under both biotic and abiotic stress. As a result they produce large amounts of antioxidant enzymes to scavenge the oxygen species, which suggests that plant cell cultures are an efficient POD and SOD (superoxide dismutase) production system (Furusawa et al. 1984; Yamada et al. 1987; Kim et al. 1994; Kwak et al. 1994, 1995; Dey and Kar 1995; You et al. 1996). A sweet potato cell line with the highest yield of POD among 41 cell lines derived from 25 plant species has already been identified (Kwak et al. 1994). In suspension cultures of sweet potato 96% of the total POD activity was retained within the cells, whereas 4% of the activity was detected in the medium. In contrast, it has also been reported that most of the POD synthesized in cultured cells is secreted into the medium (Wink 1985; Mader and Walter 1986; Narita et al. 1995). Extracellular POD activity can be directly correlated with the growth kinetics and dry cell weights of cultured cells (Shetty et al. 1990).

As a first step to understanding the physiological function of each POD isozyme, three anionic PODs were previously purified and characterized from suspension-cultured cells of sweet potato (*Ipomoea batatas*) (Kwak et al. 1995). In this study two cDNAs that encode POD from suspension-cultured cells of sweet potato were isolated and characterized (swpa1 and swpn1). The swpa1 and swpn1 cDNAs encode an anionic and a neutral POD, respectively. They are expressed in cultured cells at a high level; however, their expression patterns differ during cell growth. The induction of the two isozymes by wounding and cold stress was elucidated and the possible physiological role of each isozyme is discussed.

Materials and methods

Plant material

The cell line (SP-47) selected from suspension-cultured cells (*I. batatas* (L) Lam. cv. White Star) for a high yield of POD was used (Kim et al. 1994). One gram (fresh weight) of suspension-cultured cells subcultured at 20 day intervals was inoculated into 50 ml of MS basal medium (Murashige and Skoog 1962) supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid and 30 g/l sucrose and maintained at 25° C in darkness (100 rpm). The cells were collected at 0.5, 5, 11, 14, and 24 days after subculture to prepare the crude POD extracts and total RNA. Calli were maintained on agar plates containing MS medium as described above.

N-terminal amino acid sequencing

The POD isozymes were purified by DEAE-cellulose and Sephadex G-100 column chromatography from suspension-cultured cells of sweet potato as reported by Kwak et al. (1995). Their N-terminal sequence was analyzed by using Applied Biosystems 476A Model sequences.

Construction and screening of a cDNA library

A cDNA library of suspension-cultured cells of sweet potato was constructed with poly(A) + RNA in λ MOS*Slox* as described in the instruction manual supplied by Amersham. Two degenerate oligonucleotides were prepared based on the N-terminal amino acid sequences of PODs purified previously (Kwak et al. 1995) and from the highly conserved proximal histidine region reported for potato and tomato PODs (Intapruk et al. 1994). These oligonucleotides were used as primers to amplify POD cDNA by the polymerase chain reaction (PCR). The PCR product was used to screen a cDNA library in λ MOS*Slox* (Amersham). Hybridization was performed with ^{32}P-labeled probes by random priming (Pharmacia) according to the manufacturer's manual. After hybridization the membranes were rinsed once in 2 × SSC, 0.5% SDS at room temperature and then washed twice in 2 × SSC, 0.1% SDS.

Protein assay and enzyme activity

Cells were homogenized on ice with a Polytron homogenizer in 100 mM potassium phosphate buffer, pH 6.0. Protein was determined according to the method of Bradford (1976) using Bio-Rad protein assay reagents. The POD activity was assayed according to the method described by Kwak et al. (1995) using pyrogallol as a substrate. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0.

Southern blot analysis of genomic DNA

Genomic DNA of sweet potato was extracted from stem tissues containing relatively small amounts of polysaccharide by the method of Dellaporta et al. (1983). The genomic DNA was digested with several restriction enzymes, electrophoresed on a 0.8% agarose gel (15 μg in each lane), and blotted on a Hybond-N nylon membrane (Amersham). The blots were hybridized to a ^{32}P-labeled probe that was the 3′-untranslated sequence specific to their own cDNA. Hybridization was carried out in a solution of 6 × SSPE, 0.5% SDS, 10 × Denhardt's solution and sonicated

salmon sperm DNA (100 μg/ml) at 60° C. The membranes were rinsed once with 2 × SSC containing 0.5% SDS at room temperature and then washed twice with 0.1 × SSC containing 0.1% SDS at 60° C.

Wounding and chilling treatments

Sweet potato plants were grown in a greenhouse and the third leaves from the top were wounded by pressing with a needle puncher. Total RNA was extracted from wounded leaves at 0, 6, 12, 24, 48, and 72 h after wounding treatment.

To investigate induction of the swpa1 and swpn1 transcripts by acclimation and/or chilling, sweet potato plants were cultured in vitro on solid agar containing MS basal medium (Murashige and Skoog 1962) at 26° C (16:8 h light/dark). After the plants had been grown in vitro for 1 month, they were placed under chilling conditions (4° C) for 2 days with or without prior acclimation (15° C) for 2 days. Total RNA was extracted from leaves unchilled in vitro cultured sweet potato plants (Cont), plants acclimated at 15° C (Ac), plants acclimated and chilled at 4° C (AcCh), or plants chilled without acclimation (Ch). In all cases samples of leaf tissue were collected from four different plants.

Northern blot analysis

Total RNA was prepared from immature tuberous roots of sweet potato by the modified hot phenol/CsCl method (Sambrook et al. 1989; Podivinsky et al. 1994), and from leaf, stem, nontuberous root, calli and suspension-cultured cells by the guanidium thiocyanate/CsCl method (Sambrook et al. 1989). Tuberous roots were distinguished from nontuberous roots by their diameters: tuberous roots were 3–35 mm in diameter and nontuberous roots were less than 2 mm. Total RNA was fractionated on 1% agarose gels containing 0.67 M formaldehyde and blotted onto Hybond-N nylon membrane (Amersham).

Both the preparation of the swpa1- and swpn1-specific probes and hybridization were performed under the same conditions as for Southern blot analysis of genomic DNA. When full-length cDNAs were used as probes, the blotted membrane was hybridized and washed under the same conditions, except that the hybridization temperature was 55° C and filters were washed in 1×SSC containing 0.1% SDS at 55° C.

Results

Cloning of cDNAs for anionic and neutral PODs

A cDNA library of suspension-cultured cells of sweet potato was screened with a PCR product that had been amplified using two degenerate oligonucleotides. To design a primer for PCR, N-terminal amino acid sequences were determined from the three anionic POD isozymes A-1, A-2, and A-3 previously purified by Kwak et al. (1995). Of these, A-2 is the most abundant isozyme in suspension-cultured cells. These isozymes differed by one or three amino acids in a stretch of 13 amino acid residues (Fig. 1). When compared with other plant PODs, the sweet potato PODs showed the highest homology of amino acid sequence to anionic PODs from potato (Roberts et al. 1988) and tomato (Roberts and Kolattukudy 1989). Therefore, two degenerate primers were prepared, one based on the N-terminal sequence (SAVKEVV) of purified proteins and one from a well-conserved amino acid region (proximal heme ligand) from anionic PODs of both potato and tomato (AGAHTVG).

A-2	D	E	A	C	V	F	*S*	*A*	*V*	*K*	*E*	*V*	*V*
A-1	-	-	-	-	I	-	-	-	-	-	-	-	-
A-3	A	-	D	-	I	-	-	-	-	-	-	-	-

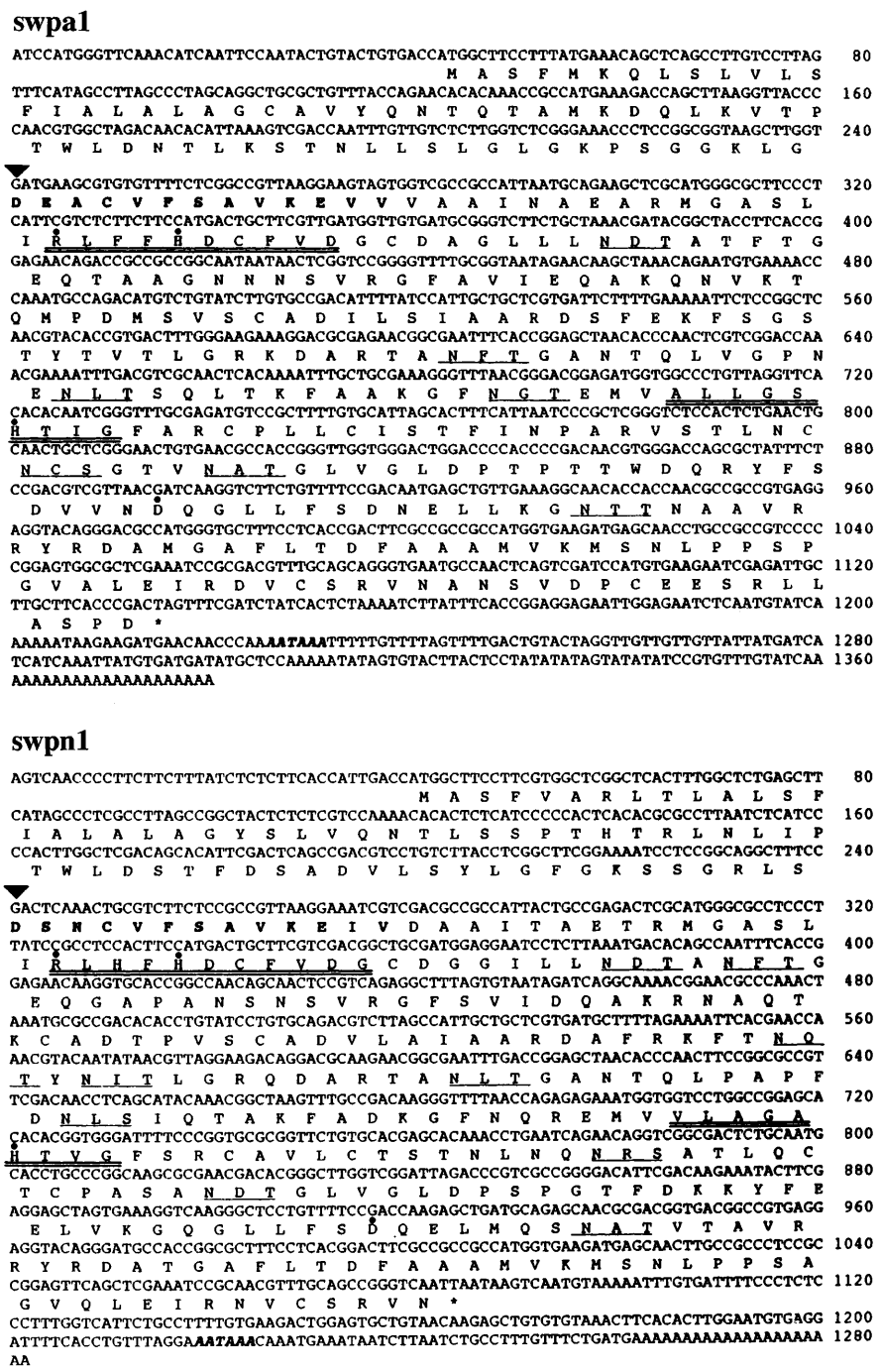
Fig. 1 The NH_2-terminal sequences of the three anionic peroxidase (POD) isozymes. N-terminal sequences were determined for the anionic PODs A-1, A-2 and A-3 (Kwak et al. 1995) purified previously. Of these, A-2 is the most abundant isozyme in cultured cells. The amino acid residues are shown in the one-letter code. Identical residues are indicated by *dashes*. The amino acid sequences used to design the degenerate oligonucleotide are indicated by *italic letters*

A PCR product (approximately 0.5 kb) was amplified using the two degenerate oligonucleotides and used as a probe for screening the cDNA library. Twenty positive clones with inserts of more than 1 kb were isolated. Among these, five clones, which contained inserts of different lengths, were sequenced. The nucleotide sequences of the cDNA clones showed that they fall into two distinct groups: one group encoding anionic POD isozymes and the other group encoding neutral POD isozymes. Three cDNA clones, encoding the anionic PODs swpa1, swpa2, and swpa3, are 96% identical in their coding region at the nucleotide level. Two other cDNA clones, encoding the neutral PODs swpn1 and swpn2, are 99% identical in their coding region at the nucleotide level (data not shown). Of these, swpa1 and swpn1 were further analyzed.

Analysis of the deduced amino acid sequences of swpa1 and swpn1

The swpa1 cDNA is 1380 bp and the swpn1 cDNA is 1282 bp long (Fig. 2). The swpa1 and swpn1 cDNAs contained open reading frames of 364 and 348 amino acids, respectively (cf. EMBL Nucleotide Sequence Database accession numbers Z84472 for swpa1 and Z84473 for swpn1). They showed 71% and 66% identity at the nucleotide and amino acid levels, respectively. Both cDNAs have a poly(A) tail and a putative polyadenylation signal, AAUAAA (Joshi 1987), which was present 155 and 64 bp upstream of the polyadenylation site of swpa1 and swpn1, respectively. The N-terminal amino acid sequences of PODs purified from suspension-cultured cells of sweet potato suggested that the leader peptides of swpa1 and swpn1 contained 66 and 67 amino acid residues, respectively. The predicted isoelectric points (pI) of the preprotein (mol. wt. 38.7 kDa) and the mature protein (mol. wt. 31.7 kDa) of swpa1 were 5.3 and 4.7, respectively, indicating that swpa1 encodes an anionic POD. swpn1 encodes a preprotein of 37.0 kDa with a pI of 7.0, and the mature protein (mol. wt. 30.0 kDa) has a pI of 6.3, which indicates that swpn1 encodes a neutral POD. The mature proteins of swpa1

Fig. 2 Nucleotide and deduced amino acid sequences of sweet potato POD cDNAs (swpa1 and swnp1). The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. The N-termini of the mature proteins are indicated by the *arrowheads* at amino acid residues 67 and 68 for swpa1 and swpn1, respectively. The amino acid sequence corresponding to the N-terminus of A-2 POD, purified from suspension-cultured cells (Kwak et al. 1995), is indicated in *bold letters*. *Underlining* represents putative glycosylation sites. The distal and proximal histidine regions are indicated by *double underlining*. The four residues presumably essential for catalysis of hydroperoxide oxidation by POD are indicated by *closed circles* above the amino acid sequence. *Bold italic letters* represent potential poly(A) signal sites



and swpn1 have seven and nine putative glycosylation sites (Asn-Xxx-Thr/Ser), respectively.

The N-terminal amino acid sequence of the A-2 isozyme (DEACVFSAVKEVV) was the same as the sequence of POD encoded by swpa1. The A-2 isozyme showed three sequence differences from swpn1 (DSNCVFSAVKEIN). Sequence comparisons with other plant PODs did not reveal identities greater than 55% in the coding region. However, the two isozymes encoded by swpa1 and swpn1 are highly conserved at heme contact surfaces, around the heme-bound proximal region, and near the distal region that catalyzes the heterolytic cleavage of hydrogen peroxide (Fig. 2). Arg95, His99, and His228 of swpa1, and Arg96, His100, and His226 of swpn1 are thought to be involved in activation of the hydroperoxide during catalysis.

Genomic organization of the two POD genes

To elucidate the genomic organization of the two isozyme genes, genomic DNA was prepared from sweet

potato stem, digested with *Eco*RI, *Hin*cII and *Hin*dIII, and then subjected to Southern blot analysis (Fig. 3). Isozyme-specific probes were prepared by PCR amplification of the 3′-untranslated regions of swpa1 and swpn1. In this region they showed only 56% identity, which indicates that they are specific for their respective genes. In a preliminary experiment these isozyme-specific probes did not cross-hybridize with each other at high stringency. The hybridization bands detected by the swpa1- and swpn1-specific probes were different in size and number, suggesting that the genes corresponding to the two isozymes are located at different loci on the chromosome. Several bands were detected by each probe, implying that there is more than one gene corresponding to swpa1 and swpn1 in sweet potato.

Change in POD activity and differential expression of the swpa1 and swpn1 genes during cell growth

The rate of growth of suspension-cultured cells of sweet potato was maximal at 11 days after subculture (DAS). Cells showed a typical sigmoidal growth curve consisting of an initial lag period (up to 5 DAS) followed by an exponential rise (Fig. 4). POD activity per gram dry cell weight was measured throughout cell growth. The level of POD activity in cells increased during the initial lag period (0.5–5 DAS), then decreased during the exponential growth phase. Thereafter, POD activity increased linearly up to 20 DAS, in the stationary phase. This pattern of POD activity during the growth cycle is the same as the pattern of specific POD activity (units per unit protein) (data not shown).

The expression profiles of the swpa1 and swpn1 genes during cell culture were examined with the swpa1- and swpn1-specific probes. swpa1 was highly expressed at 0.5 DAS (Fig. 5). Expression of swpa1 was not detected at 5 DAS (cells entering the exponential phase), then expression was highly induced during the stationary phase (11–20 DAS) (Fig. 5A). In contrast, the swpn1 gene showed a very low transcription level at 0.5 DAS (Fig. 5B), suggesting that the expression of swpa1 and swpn1 is differentially regulated in suspension-cultured cells.

To investigate whether the swpa1 and/or swpn1 transcripts encode major POD isozymes expressed in suspension-cultured cells, Northern blot analysis was performed with full-length swpa1 and swpn1 cDNAs (Fig. 5C). Other POD transcripts could also hybridize with the probe because it contained a highly conserved region and Northern hybridization and washing were performed at a low stringency. The overall expression pattern observed for POD transcripts in suspension-cultured cells was similar to the expression pattern of swpa1, whereas it was different from that of swpn1. When the expression profile of swpa1 was compared with the pattern of POD activity during cell growth, swpa1 expression preceded POD activity (Figs. 4, 5C).

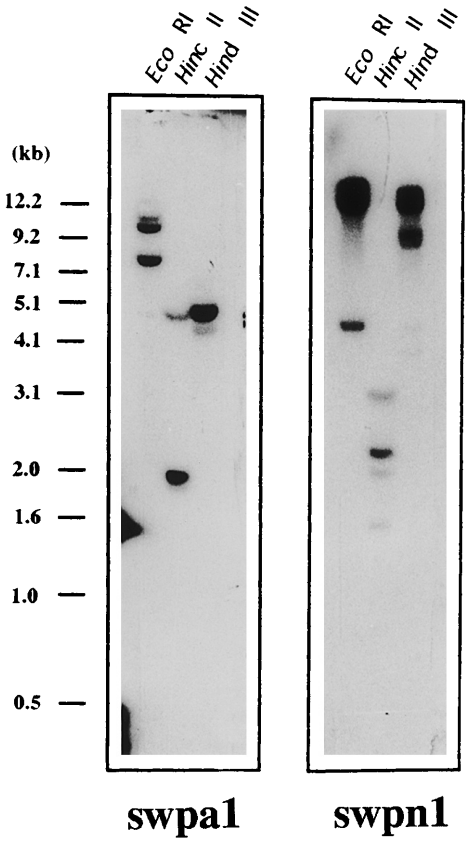


Fig. 3 Southern blot analysis of genomic DNA. Genomic DNA was prepared from the stem of sweet potato, digested with *Eco*RI, *Hin*cII, and *Hin*dIII, and hybridized with DNA fragments specific to swpa1 (213 bp) or swpn1 (195 bp) as probes. Size markers are shown on the left

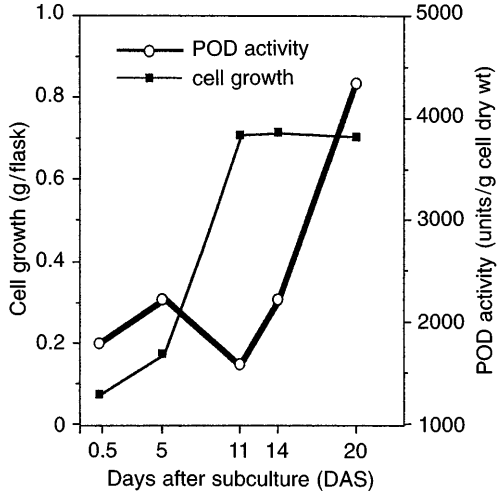


Fig. 4 Change in POD activity during growth of suspension cultures of sweet potato cells. The POD activity was determined by the Sigma method using pyrogallol as a substrate according to the method described by Kwak et al. (1995)

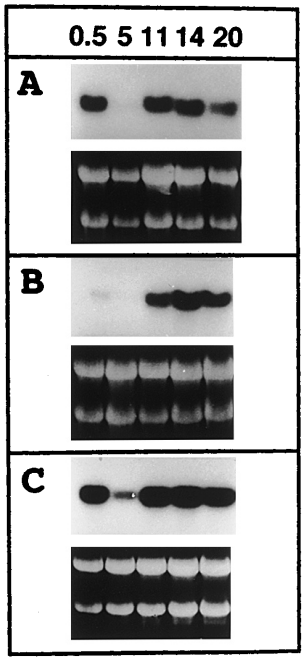


Fig. 5A–C Transcription of swpa1 and swpn1 in suspension-cultured cells of sweet potato during cell growth. RNA was extracted at 0.5, 5, 11, 14 and 20 days after subculture. Equal amounts (10 μg) of each sample were loaded in each lane. Swpa1 (**A**), an swpn1-specific DNA fragment (**B**), or full-length cDNAs of swpa1 and swpn1 (**C**) were used as probes. The lower part of each panel shows an ethidium bromide-stained gel

Expression level of swpa1 and swpn1 in cultured cells and in various tissues of whole plants

The expression patterns of the two isozymes in cultured cells and various tissues of whole plants were examined by Northern blot analysis (Fig. 6). Total RNAs from calli, suspension cells, leaf, stem, nontuberous root, and tuberous root were extracted and hybridized with isozyme-specific probes. The accumulation of swpa1 and swpn1 transcripts was weakly detected in stem, but not in leaf, nontuberous root and tuberous root. However, transcript levels were much lower in intact stem than in suspension-cultured cells. The level of the swpa1 transcript was similar in calli and suspension-cultured cells, whereas the level of the swpn1 transcript was lower in calli than in suspension-cultured cells.

Induction of swpa1 and swpn1 transcripts in sweet potato leaves by wounding

The nucleotide sequences of swpa1 and swpn1 are homologous to the sequence of a tomato POD that is induced by wounding (Lagrimini et al. 1993; Mohan et al. 1993). This suggested that expression of swpa1 and swpn1 might be induced by wounding. The effects of wounding on expression of the swpa1 and swpn1 genes were tested by Northern hybridization. Transcription of both swpa1 and swpn1, as shown in Fig. 7, was induced by wounding, with similar expression patterns. The levels of both transcripts increased between 0–12 and 24–72 h; however both genes appear to be expressed at the same level at 0 and 24 h. The highest expression level of the swpa1 and swpn1 genes was observed 72 h postwounding. This suggests that the two POD isozymes participate in physiological processes that are enhanced following mechanical damage.

Differential expression of the swpa1 and swpn1 genes by chilling stress

Antioxidant enzymes constitute a cellular defense against oxidative stress associated with low temperature (Murata et al. 1992). Some POD isozymes in maize seedlings are induced by chilling acclimation (Prasad

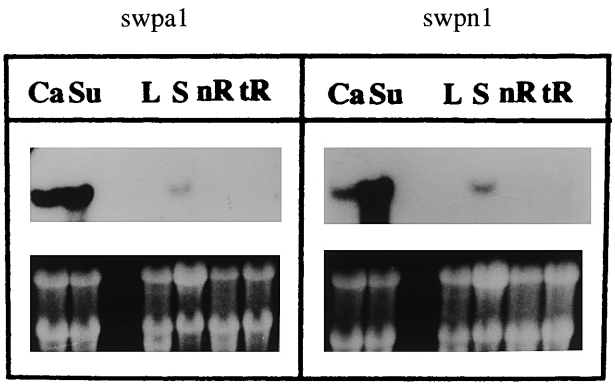


Fig. 6 Expression of swpa1 and swpn1 in cultured cells and various tissues of sweet potato. RNA was extracted from calli (*Ca*), suspension-cultured cells (*Su*), leaf (*L*), stem (*S*), tuberous root (*tR*), and nontuberous root (*nR*). swpa1- or swpn1-specific DNA fragments were used as probes. The lower part of each panel shows an ethidium bromide stained gel

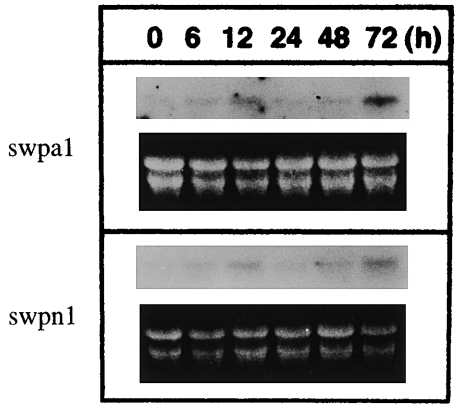


Fig. 7 Induction of swpa1 and swpn1 transcripts in response to wounding of sweet potato leaves. Total RNA was isolated from wounded leaves at 0, 6, 12, 24, 48 and 72 h postwounding. An swpa1- or swpn1-specific DNA fragment was used as a probe. The lower part of each panel shows an ethidium bromide-stained gel

et al. 1994; Anderson et al. 1995). Therefore, the response of expression of swpa1 and swpn1 to oxidative stress resulting from chilling acclimation or chilling in sweet potato was investigated. Total RNA was extracted from leaves of unchilled in vitro cultured sweet potato plants (Cont), plants acclimated at 15° C (Ac), plants acclimated and chilled at 4° C (AcCh), or plants chilled without acclimation (Ch). This RNA was used for Northern blot analysis. As shown in Fig. 8, the swpa1 and swpn1 genes responded differently to acclimation and chilling. Transcription of swpa1 was induced weakly by acclimation, whereas it was induced strongly by chilling treatment (AcCh and Ch). In contrast, transcription of swpn1 was induced by acclimation and repressed by chilling. This suggests that the two isozymes play distinct roles in physiological processes that induce tolerance to chilling damage. The mRNA found in small amounts in the leaves of control plants is probably due to stress following in vitro culture for 1 month.

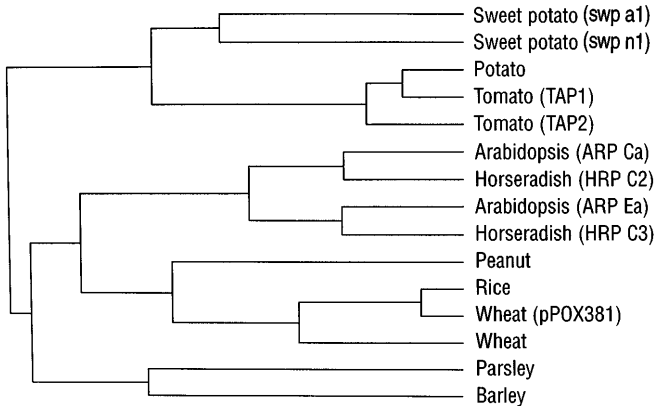


Fig. 9 Amino acid similarity in mature POD isozymes. The dendrogram was generated by Clustal W (Thompson et al. 1994). Sequence data are from the following sources: potato (Roberts et al. 1988), tomato (TAP1 and TAP2 Roberts and Kolattukudy 1989), ARP Ca and ARP Ea (Intapruk et al. 1991), HRP C2 and HRP C3 (Fujiyama et al. 1990), peanut (Buffard et al. 1990), rice (unpublished data), wheat (Cecilia et al. 1991), pPOX381 (Gabriela et al. 1991), parsley (Kawalleck et al. 1995), and barley (Johansson et al. 1992)

Discussion

Two cDNA clones, swpa1, which encodes an anionic POD, and swpn1, which encodes a neutral POD, were isolated from suspension-cultured cells of sweet potato. They are 71% and 66% identical at the nucleotide and the amino acid sequence levels, respectively. Figure 9 shows a dendrogram of the overall similarity relationships among the mature forms of the 14 sequenced POD isozymes. The mature POD proteins encoded by swpa1 and swpn1 are homologous (58–62%) to the anionic PODs expressed in suberizing potato tubers and tomato fruit (TAP1 and TAP2) (Roberts et al. 1988), whereas they showed relatively low homology (30–42% identity) to other plant PODs. The dendrogram in Fig. 9 shows that the isozymes of different dicot family members are less similar than isozymes from the same family. Parsley POD (dicot; Kawalleck et al. 1995) is homologous to barley POD (monocot; Johansson et al. 1992), and peanut POD (dicot; Buffard et al. 1990) is homologous to rice and wheat PODs (monocot; Cecilia et al. 1991). This suggests that POD isozymes evolved before the evolutionary separation of monocots and dicots.

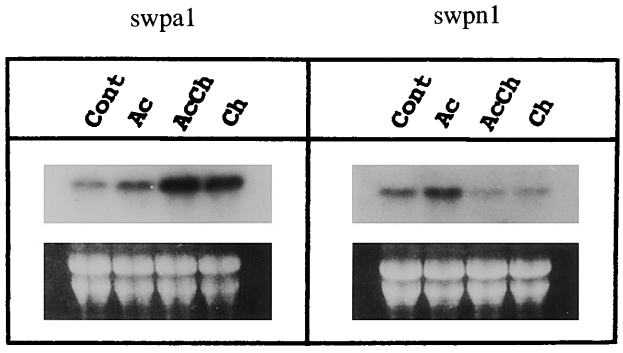


Fig. 8 Transcript levels of swpa1 and swpn1 in leaves of unchilled plants (*Cont*), acclimated at 15° C for 2 days (*Ac*), acclimated and chilled at 4° C for 2 days (*AcCh*), or chilled without acclimation (*Ch*). DNA fragments specific for swpa1 or swpn1 were used as probes. The lower part of each panel shows an ethidium bromide-stained gel

While acidic PODs are secreted into the cell wall and slightly acidic PODs are bound to pectin-rich areas of the cell wall, basic PODs are secreted into vacuoles (Schloss et al. 1987). PODs are also located in the cytoplasm, and they can break down H_2O_2 and oxidatively degrade indole acetic acid. N-terminal sequencing of the mature swpa1 and swpn1 proteins revealed leader peptides of 66 and 67 amino acid residues, respectively (Fig. 2). The N-terminus of mature swpa1 and swpn1 PODs is aspartic acid, whereas other plant PODs have an N-terminal glutamine. The putative transit peptides of both isozymes show different levels of hydrophilicity. The hydropathy index (Kyte and Doolittle 1982) of swpa1 was 2.9 while swpn1 showed stronger hydrophilicity with an index of 5.0. According to the criteria of Kyte and Doolittle (1982), the anionic POD isozyme encoded by swpA1 is classified as an integral protein, whereas the neutral POD isozyme encoded by swpN1 is not.

As shown in Figs. 4 and 5, POD activity and the transcript levels of the swpa1 and swpn1 genes changed during cell growth. The high level of POD activity and the expression of swpa1 during both the early initial lag period and the post-exponential growth period seemed to be associated with fresh medium stress (dilution effect), medium depletion, and cell aging. In contrast to swpa1, the accumulation of the swpn1 transcript was much lower at 0.5 DAS, in the early initial lag period. The different biochemical characteristics and the differential expression of the two isozymes suggest that their functions differ during cell growth of suspension cultures.

It has been reported that anionic PODs are associated with lignification in tobacco (Lagrimini et al. 1987) and suberization in tomato and potato (Roberts et al. 1988). Expression of swpa1 and swpn1 occurred only in stems of intact plant tissues, although transcript levels were much lower than in cultured cells (Fig. 6). Stem-specific expression in intact plants, even at low levels, has also been described for the lignin-forming POD from non-stressed tobacco plants (Lagrimini et al. 1987). However, the deduced amino acid sequences of swpa1 and swpn1 show low homology (33% and 35% identity, respectively) to that of the lignin-forming POD of tobacco. Preferential expression is probably associated with cell wall formation when, under normal conditions, the anionic isozyme participates in lignin synthesis (Lagrimini et al. 1990). At present, it is not known whether the isozymes encoded by swpa1 and swpn1 participate in lignin synthesis in the stem.

A highly anionic POD is primarily involved in polymerizing phenolic monomers to generate the aromatic matrix of suberin. Plants resort to suberization whenever physiological or developmental changes or stress factors require the erection of a diffusion barrier. Transcripts of various POD isozymes accumulate in response to wounding, chilling, abscisic acid, pathogens, citrus exocortis viroid, and fungal elicitor in tobacco, tomato, potato, and barley (Roberts et al. 1988; Roberts and Kolattukudy 1989; Lagrimini 1991; Rebman et al. 1991; Vera et al. 1993). swpa1 and swpn1 exhibited 53–55% identity in the coding region to the anionic PODs induced in potato tubers and tomato fruits during wound-healing (Roberts et al. 1988). Tomato POD plays a role in defense against bacterial infection, as well as in suberization (Roberts and Kolattukudy 1989). The anionic nature of the swpa1 protein and its homology to the suberization-associated anionic PODs of potato and tomato suggest that this protein has a role similar to that of these PODs. Wounding of sweet potato leaves induced the expression of both swpn1 and swpa1 (Fig. 7). This indicates that both isozymes are probably involved in wound-inducible suberization, helping to form a water-tight barrier over the wound.

Chilling leads to elevated levels of active oxygen species, resulting in oxidative stress, such as damage to cellular components and severely disrupted metabolic function (Omran 1980; Elstner 1987; Wise and Naylor 1987; Prasad et al. 1994). Chilling injury also causes changes in membrane properties, solute leakage, reduced transport across the plasmalemma, and malfunction of mitochondrial respiration (Lyons and Ralson, 1970; Goldberg et al. 1986; Guy 1990). The production of active oxygen can exceed the capacity of the scavenging systems of plants, resulting in oxidative damage (Elstner 1987). Thus, the ability of a plant to improve its active oxygen-scavenging capacity might be a key element in stress tolerance. In maize seedlings, the levels of catalase transcript and POD activity were elevated during cold-induced oxidative stress, whereas SOD activity was not effected (Prasad et al. 1994). Anderson et al. (1995) reported that in maize seedlings nine of the most prominent POD isozymes are induced by acclimation, one of which is strongly induced and two of which are located in the cell wall. However, there are no reports about chilling-induced expression of POD genes. Figure 8 shows that the swpn1 transcript was induced by 15° C acclimation and repressed by 4° C chilling, whereas the expression of swpa1 was weakly enhanced by acclimation and strongly induced by chilling. In maize seedlings one of the POD isozymes is induced by acclimation and repressed by chilling (Anderson et al. 1995), which is reminiscent of the expression pattern of swpn1. This suggests that the two POD isozymes contribute to protection from chilling-induced oxidative stress through different signaling pathways.

The transcripts of swpa1 and swpn1 accumulated differentially during growth of suspension-cultured cells and in response to chilling stress, which indicates that the anionic and neutral PODs encoded by the two genes have different biological functions. However, the fact that both anionic and neutral PODs showed wound-induced expression suggests that these proteins play a role in plant defense. In order to understand the function of each isozyme in detail, transgenic plants overproducing the anionic and neutral PODs encoded by swpa1 and swpn1 have been generated and are currently being analyzed. The results of this study should help in understanding the physiological roles of the two isozymes and might lead to development of transgenic plants with resistance to various environmental stress, including oxidative stress. Future experiments will include a test of this possibility.

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