ORIGINAL PAPER

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Molecular cloning of a cytosolic ascorbate peroxidase cDNA from cell cultures of sweetpotato and its expression in response to stress

Abstract A cDNA encoding a cytosolic ascorbate peroxidase (APX), *swAPX1* , was isolated from cell cultures of sweetpotato (*Ipomoea batatas*) by cDNA library screening, and its expression in the context of various environmental stresses was investigated. *swAPX1* contains an ORF of 250 amino acids (27.5 kDa) encoding a protein with a pI value of 5.32. The *swAPX1* ORF does not code for a transit peptide, suggesting that the product is a cytosolic isoform. RNA blot analysis showed that *swAPX1* gene is expressed in cultured cells and mature leaves, but not in stems, non-storage or storage roots of sweetpotato. The level of *swAPX1* RNA progressively increased during cell growth in suspension cultures. In leaf tissues, the gene responded differentially to various abiotic stresses, as revealed by RT-PCR analysis. *swAPX1* was highly induced in leaves by wounding, and treatment with methyl viologen (50 µM), hydrogen peroxide (440 mM), abscisic acid (ABA; 100 µM) or exposure to high temperature (37°C). In addition, the gene was strongly induced in the leaves following inoculation with a bacterial pathogen (*Pectobacterium chrysanthemi*). These results indicate that *swAPX1* may be involved in hydrogen peroxide-detoxification and thus help to overcome the oxidative stress induced by abiotic and biotic stresses.

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Introduction

Molecular oxygen is essential for the existence of aerobic organisms including plants. However, toxic reactive oxygen species (ROS), which include the superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are formed in all aerobic cells as by-products of normal metabolic processes (Asada 1992). In particular, ROS are overproduced in plant cells under environmental stress. Indeed, such oxidative stress is one of the major causes of damage to cells in plants exposed to environmental stress. Plants possess the ability to cope with oxidative stress by inducing the synthesis of ROS-scavenging enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and low-molecular-weight antioxidants including ascorbic acid (ascorbate), glutathione and phenolic compounds (Noctor and Foyer 1998).

Ascorbate peroxidase (EC 1.11.1.11, APX) has been found in higher plants, algae (Shigeoka et al. 1980), and some cyanobacteria (Tel-Or et al. 1986), but not in animals. Ascorbate peroxidases are hydrogen peroxide-scavenging enzymes with high specificity for ascorbate as the electron acceptor. It is clear that a high level of endogenous ascorbate is essential to maintain the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses (Shigeoka et al. 2002). APX in higher plants exists in four isoforms: cytosolic, stromal, glyoxysomal and thylakoid membrane-bound (Chen and Asada 1989; Tanaka et al. 1991; Miyake and Asada 1992). APX activity in higher plants has been shown to increase in response to various stress conditions such as drought, ozone, chemicals, salt, heat and microbial infection (Smirnoff and Colombe 1988; Mittler and Zilinskas 1994, Lopez et al. 1996).

Several cDNAs for ascorbate peroxidases have been isolated from various plant species such as pea, *Arabidopsis* and bell pepper (Mittler and Zilinskas 1994; Kubo et al. 1992; Schantz et al. 1995). Sequencing of the genome of *Arabidopsis thaliana* has revealed the presence of nine APX genes (The Arabidopsis Genome Initiative 2000). The specific functions of each of these APX genes in the context of growth and development remain to be determined.

Plant cell cultures are very important in the field of plant biotechnology for the development of transgenic plants, for mass propagation and for the biosynthesis of useful components. But there have so far been no reports on the isolation of APX genes from plant cell cultures. Plant cells in culture are considered to be subject to high levels of oxidative stress, suggesting that they are good models for the study of antioxidative mechanisms and the production of useful antioxidants. In previous studies, we have investigated the levels of antioxidant enzymes such as SODs and PODs, and low-molecular-weight antioxidants such as ascorbate and glutathione in 100 cell lines derived from various plant species (Kim et al. 1994; You et al. 1996; Ahn et al. 1999; Lee et al. 1999a). Plant cells growing in culture were found to have much higher levels of antioxidant enzymes such as SODs and PODs than differentiated plant tissues. In particular, one cell line of sweetpotato (*Ipomoea batatas*) produced a very high level of POD (Kwak et al. 1995). We have isolated several POD cDNAs from suspension cultures of sweetpotato and characterized their expression in response to oxidative stress (Huh et al. 1997; Kim et al. 1999). In the course of isolation of new POD genes from sweetpotato (Park et al. 2003), a cDNA encoding a cytosolic APX was cloned from cell cultures. In this study, we describe the full-length APX cDNA, *swAPX1* , from suspension cultures of sweetpotato, and its expression pattern in response to various environmental stresses, and inoculation with the bacterial pathogen *Pectobacterium chrysanthemi*.

Materials and methods

Plant materials

The sweetpotato (*Ipomoea batata* L. *Lam.* cv. White Star) cell line selected for these studies expresses a high level of POD activity (Kim et al. 1994; Kwak et al. 1995). One-gram (fresh weight) samples of cells, subcultured at 14-day intervals, were inoculated into 50 ml of MS (Murashige and Skoog 1962) basal medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l) and sucrose (30 g/l), and incubated at 25°C in darkness (100 rpm). The cells were collected 0.5, 5, 11, 14, and 20 days after subculture (DAS). Sweetpotato plants were grown in the greenhouse for 1 month, and the third leaves from the tops of plants were used for the stress treatments. The first un-expanded leaf from the top (an immature leaf) and fully expanded mature leaves were also sampled. Sweetpotato plants were also grown in vitro on solid MS basal medium at 25°C for 1 month for chilling and heating treatments. These plants were subcultured every month by implanting the excised apical shoot including 2 or 3 leaves in new medium in an in vitro vent container (Duchefa Biochemie BV, Haarlem, The Netherands). The plants reached a height of approximately 12 cm with 8–10 leaves.

Preparation of gene probes

Two primers (5′-CACTTCCACGACTGCTTCGT-3′and 5′-ACGAAGCAGTCGTGGAAGTG-3′) designed from the conserved regions of previously reported POD sequences (Huh et al. 1997; Kim et al. 1999) were used. PCR was performed with approximately 10–50 ng of cDNA, a PCR premix kit containing each dNTP at 250 µM, 1 U of *Taq* DNA polymerase, 40 mM KCl, 1.5 mM $MgCl_2$ in 10 mM TRIS-HCl (Bioneer, Daejeon, Korea), and 10 pmol of each primer. Amplification reactions consisted of 30 cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C. Five different PCR products, from 450 to 1000 bp in length, were cloned into pGEM-T easy vectors (Promega, Madison, Wis., USA) and used as probes to screen a cDNA library.

Construction and screening of the cDNA library

For the construction of a cDNA library, total RNA was isolated from suspension cultures of sweetpotato 20 days after subculture (DAS). Poly(A)$^+$-containing RNA was isolated by oligo(dT) cellulose chromatography (Stratagene, La Jolla, Calif., USA). The cDNA was synthesized from poly(A)$^+$ RNA with the ZAP-cDNA synthesis kit (Stratagene) according to the supplier's instructions. The ligated DNA was packaged with a lambda packaging kit, and the library was amplified in the *Escherichia coli* strain XL-1-Blue (Stratagene) before screening. The plaques were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden), UV cross-linked, and screened by hybridization with the radiolabeled probes. Plaques that gave positive signal were purified by at least three rounds of re-plating and screening. Phagemids were excised from the phages following the manufacturer's instructions (Stratagene).

Southern and Northern analysis

Genomic DNA of sweetpotato was extracted from calli according to Dellaporta et al. (1983), digested with *Eco* RI, *Hin* dII and *Hin* dIII (Roche Diagnostics, Mannheim, Germany), electrophoresed on 0.8% agarose gel and blotted onto Zeta-probe GT membrane (Bio-Rad, Hercules, Calif., USA). The blots were hybridized to a ^{32}P-labeled fragment that included the 3′-untranslated sequence (3′-UTR) specific to the APX cDNA (207 bp) or a fragment of the coding region of the APX cDNA (682-bp *Hin* dIII fragment). Hybridization was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 65°C. For Northern analysis of various intact tissues, suspension cultures, and infected leaves of sweetpotato, total RNA was extracted from plant materials by the LiCl method (Naqvi et al. 1998), denatured by heating at 70°C for 15 min in a denaturation buffer containing 50% formamide and 2.2 M formaldehyde, and blotted onto a Zeta-probe GT membrane. Hybridization was performed as above. All samples used in Northern experiments were subsequently subjected to RT-PCR analysis in order to confirm the results (data not shown).

RT-PCR analysis

To study APX expression in response to stress, the RT-PCR method was employed. An RT-PCR kit (Gibco BRL, Eggenstein, Germany) was used to identify the mRNA coding for *swAPX1* cDNA described in this study. Reverse transcription of 2 µg of total RNA was carried out as recommended by the kit manufacturer. As an internal control of reverse transcription, β-actin mRNA was used. The sense primer was 5′-TGGACTCTGGT GATGGTGTC-3′, and the antisense primer 5′-CCTCCAATC

CAAACACTGTA-3′. Conventional PCR was carried out on 0.2-μl samples (corresponding to ∼20–40 ng of RNA) of reverse transcribed mRNA by adding 1.5 μl of 5 mM MgCl$_2$, 5 μl of 10×PCR-buffer, 1 μl of 10 mM dNTP mix, 1 μl of sense primer (10 pmol), 1 μl of antisense primer (10 pmol), 1 μl of *Taq* DNA polymerase (1 U/μl), and water to 20 μl. PCR amplification reactions were initially incubated at 94°C for 5 min, followed by 25 cycles at 94°C for 30 s, and 58°C for 30 s, and 72°C for 30 s. Reaction products (20 μl) were analyzed by gel electrophoresis. Each RT-PCR experiment was performed at least three different times to ensure that the results were reproducible.

The gene-specific primers used for the PCR were 5′-AT GCCTTCTTTGCTGACTATCCA-3′ (sense) and 5′-TGATTTT CGCATACACTT-3′ (antisense); these primers amplified a 207-bp product from the *swAPX1* cDNA.

Abiotic stress treatments

For stress treatments, three different systems (intact plants, plants cultured in vitro, and excised leaves) were used, depending on the type of stress. For wounding and chemical treatments, sweetpotato plants grown in greenhouse for 1 month were used. The third leaves from the top were wounded by pressing with a needle puncher (Huh et al. 1997). Whole plants were exposed to methyl viologen (MV) by spraying them with a 50 μM solution in 0.125% Triton-X 100. For treatments with H$_2$O$_2$ (440 mM), NaCl (100 mM), abscisic acid (ABA, 100 μM), salicylic acid (SA, 100 μM) and methyl jasmonate (MeJA, 100 μM) treatments, the third leaves from the top were removed from each plant and incubated in Falcon tubes containing 30 ml of each chemical solution at 25°C for 48 h. Sterile water was used as a control for H$_2$O$_2$ and NaCl treatments, whereas 0.1% DMSO solution was used as a control for ABA, SA and MeJA treatments. For temperature treatment, plants cultured in vitro on solid agar containing MS basal medium at 25°C for 1 month were used. Plants were exposed to 4°C for 48 h or 37°C for 12 h. All treated plant materials were immediately frozen in liquid nitrogen and stored at –70°C until further use.

Inoculation with *Pectobacterium chrysanthemi*

The bacterial strain used in this study was *Pectobacterium chrysanthemi* (*Erwinia chrysanthemi*, KCTC 2569), which causes bacterial stem and root rot of sweetpotato. *P. chrysanthemi* cells were grown on nutrient agar medium at 30°C. Leaf discs (18 mm in diameter, nine per plate) were placed in a petri-dish (87×15 mm) and each was inoculated with *P. chrysanthemi* (1.3×10^4 cells/ml). Total RNA was extracted from the discs at various times, starting at 20 h after treatment.

Analysis of DNA and protein sequences

Homology searches were carried out using the BLAST program on the NCBI web-server, and multiple sequence alignments were constructed obtained with the programs ClustalX and Genedoc. To predict the isoelectric point (pI), molecular weight, signal peptide, and subcellular location of a deduced protein, we used TargetP (http://www.cbs.dtu.//services/TargetP/) and PSORT (http://psort.ims.u-tokyo.ac.jp) programs within ExPasy site (http://www.expasy.org/tools/).

Results and discussion

Isolation and sequencing of a cDNA clone encoding a cytosolic APX

A cDNA library prepared with RNA isolated from suspension-cultured cells of sweetpotato was screened with five probes produced by PCR using degenerate oligonucleotides designed from the conserved regions of POD sequences. 218 positive clones with inserts larger than 1 kb were isolated from the library. In all, 20 guaiacol-type POD genes, one APX and one peroxiredoxin gene were represented in the set of 218 positive clones (Park et al. 2003). In this paper, we describe the characterization of the APX gene.

The APX cDNA clone, named *swAPX1*, was 1046 bp in length. It contained an ORF coding for a predicted polypeptide of 250 amino acids (Fig. 1). The cDNA clone also contains a 198-bp 3′ UTR. The 3′ UTR includes a putative polyadenylation signal, AATAA(T), located 22 bp upstream from the start of the poly(A) tail. The G/C content of the coding region was 47.14%. The predicted molecular mass and pI of the *swAPX1* product are 27.5 kDa and 5.32, respectively. The lack of a transit peptide in the deduced amino acid sequence implies that *swAPX1* encodes a cytosolic isoform of ascorbate peroxidase.

The deduced amino acid sequence of the *swAPX1* product was compared with those of other plant cytosolic APXs available from GenBank. It displayed 80% homology to a cytosolic APX from *Arabidopsis thaliana* (gi 21554322) and 90% homology to a tobacco cytosolic APX (gi 559005), and very high homology to other plant APXs (Fig. 1). The APX active-site signature (the A region in Fig. 1) is present at positions 33–44 (AP-LMLPLAWHSA), together with the proximal heme-ligand motif (H region) between residues 155 and 163: DIVALSGGH (Bairoch 1991). Plants possess many isoforms of APX, including cytosolic, stromal and thylakoid membrane-bound isoforms (Chen and Asada 1989; Tanaka et al. 1991; Miyake and Asada 1992). The APX clone from sweetpotato is predicted to code for a cytosolic isoform; the clone and its corresponding gene were named *swAPX1*.

We suspected that *swAPX1* might be involved in antioxidative responses in cell cultures of sweetpotato, even though the level of ascorbate in cultured plant cell lines is much lower than those in intact plant tissues (Ahn et al. 1999).

Genomic organization of the *swAPX1* gene

To elucidate the genomic organization of the *swAPX1* gene, the 3′-UTR segment of the cDNA was amplified by PCR, cloned in the pGEM-T easy vector, sequenced and used as probe on Southern blots of genomic DNA (Fig. 2A). The coding region of the APX cDNA, obtained by restriction digestion with *Hin* dIII (682 bp), was also used as a hybridization probe (Fig. 2B). Southern analysis of *Hin* dIII-, *Eco* RI-, and *Hin* dII-digested genomic DNAs is shown in Fig. 2. The *swAPX1* cDNA has two internal recognition sites for *Hin* dIII. The results therefore indicate there are at least two genes corresponding to *swAPX1* in sweetpotato.

Fig. 1 Alignment of the deduced amino acid sequence of the product of *swAPX1* from sweetpotato (*Ipomoea batatas*) with those of APX genes from other plants. Conserved residues are indicated in *white on a black background*. The *horizontal lines* indicate the conserved APX active site (A) and the proximal heme-ligand motif (H). Sequence data were obtained from dbEST and GenBank, and aligned using ClustalX and GeneDoc. (GenBank Accession Nos. of APXs are as follows: gi 559005 for tobacco, gi 7488966 for pepper, gi 2738949:1–250 for strawberry, gi 7484752 for cucumber, gi 7488687 for soybean, gi 12082341 for rice, gi 7484672 for spinach, and gi 21554322 for *Arabidopsis*). The GenBank Accession No. for *swAPX1* is AY206407
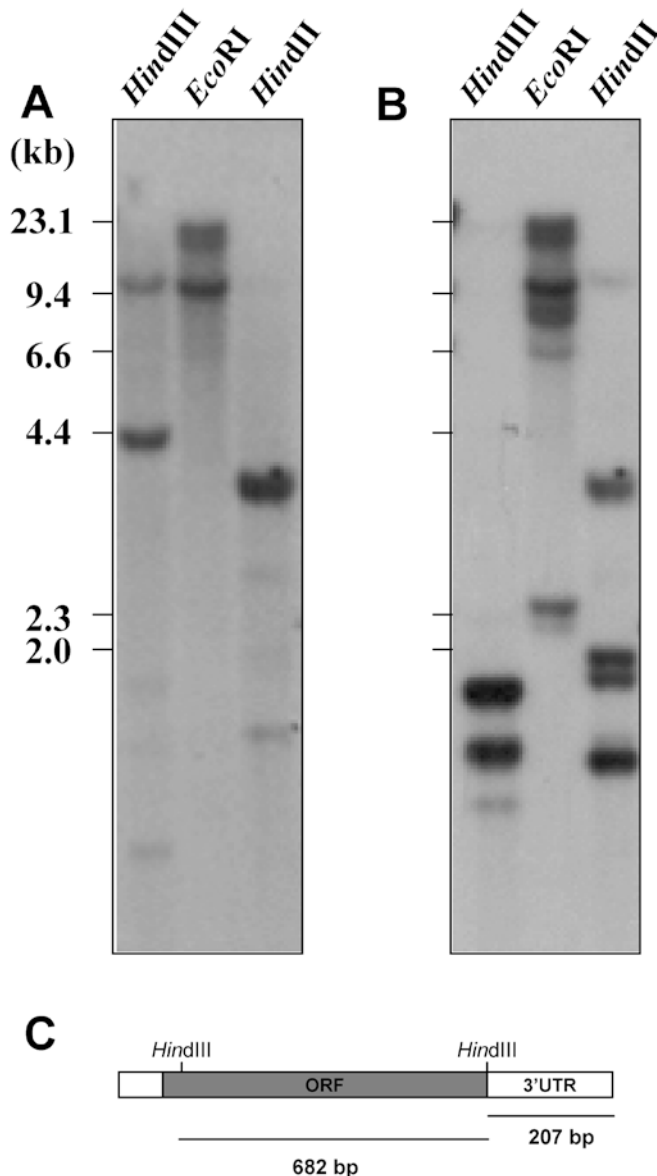
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                                      A
swAPX1      MG-KCYPTVSEEYKKAIEKLRRKLRGFIADKNCAPLMLPLAWHSAGTYDVSSKTGGPFGTMRL   62
Tobacco     MG-KCYPTVSEEYLKAVDKCKRKLRGLIAEKNCAPLMLRLAWHSAGTYDVCSKTGGPFGTMRL   62
Pepper      MG-KCYPTVSEEYLKAVDKCKRKLRGLIAEKNCAPLMLRLAWHSAGTYDVCSKTGGPFGTMRF   62
Strawberry  MG-KCYPTVSEKAIDKAKRKLRGLIAEKNCAPLMLRLAWHSAGTYDVKTKTGGPFGTMKQ   62
Cucumber    MG-KCYPTVSEEYLKAVDKCKRKLRGLIAEKNCAPLMLRLAWHSAGTYDVCSKTGGPFGTMRF   62
Soybean     MG-KSYPTVSADYQKAVEKAKKKLRGFIAEKRCAPLMLRLAWHSAGTYDVSSKTGGPFGTIKH   62
Rice        MGSKSYPTVSDEYLAAVGKAKRKLRGLIAEKNCAPLMLRLAWHSAGTFDVSSRTGGPFGTMKN   63
Spinach     MG-KSYPTVSENYQKSIEKARRKLRGLIAEKQCAPLMLRLAWHSAGTFDCTSKTGGPFGTMKH   62
A.thaliana  MT-KNYPTVSEDYKKAVEKCRRKLRGLIAEKNCAPIMVRLAWHSAGTFDCQSRTGGPFGTMRF   62

swAPX1      KAEQAHGANNGLDIAVRLLEPFKEQFPIVSYADFYQLAGVVAVEITGGPDVPFHPGREDKTEP   125
Tobacco     KAEQGHGANNGIDIAIRLLEPIKEQFPILSYGDFYQLAGVVAVEVTGGPDVPFHPGREDKTEP   125
Pepper      KTEQSHGANNGIDIALRLLEPLGEQFPIVSYADFYQLAGVVAVEVTGGPDVPFHPGREDKPEP   125
Strawberry  SAELAHGANNGLDIAVRLLEPIKEQFPILSYADFYQLAGVVAVEVTGGPDVPFHPGREDKPEP   125
Cucumber    KTEQSHGANNGIDIALRLLEPLGEQFPILSYADFYQLAGVVAVEVTGGPDVPFHPGREDKPEP   125
Soybean     PSELAHGANNGLDIAVRLLEPLKAEFPILTYADFYQLAGVVAVEVTGGPEVPFHPGREDKPEP   125
Rice        PGEQSHAANACLDIAVRLLDPIKDQLPILSYADFYQLAGVVAVEVTGGPEVPFHPGRQDKPEP   126
Spinach     QAELAHGANNGLVIAVRLLEPIKEQFPEITYADFYQLAEFVAVEVTGGPEVPFHPGREDKPEP   125
A.thaliana  DAEQAHGANSGIHIALRLLDPIREQFPTISFADFHQLAGVVAVEVTGGPDIPFHPGREDKPQF   125

                                          H
swAPX1      PVEGRLPDATQGNDHLRDVFVKQMGLSDKDIVALSGGHTLGRCHKERSGFEGPWTTNPLIFDN   188
Tobacco     PVEGRLPDATKGSDHLRDVFVKQMGLSDKDIVALSGGHTLGRCHKERSGFEGPWTTNANPLIFDN   188
Pepper      PVEGRLPDATKGSDHLRDVFVKQMGLSDQDIVALSGGHTLGRCHKERSGFEGPWTANPLIFDN   188
Strawberry  PPEGRLPDAGKGSDHLREVFGKTMGLSDQDIVALSGGHTLGRAHKERSGFEGPWTPNPLIFDN   188
Cucumber    PVEGRLPDATKGSDHLRDVFVKQMGLSDQDIVALSGGHTLGRCHKERSGFEGPWTANPLIFDN   188
Soybean     PPEGRLPDATKGSDHLRDVFGKAMGLSDRDIVALSGGHTIGAAHKERSGFEGPWTSNPLIFDN   188
Rice        PPEGRLPDATQGSDHLRQVFSAQMGLSDKDIVALSGGHTLGRCHKERSGFEGANTSNPLIFDN   189
Spinach     PQEGRLPDATKGCDHLRDVFIKQMGLTDQDIVALSGGHTLGRCHKDRSGFEGANTTNPLVFDN   188
A.thaliana  PPEGRLPDATKGCDHLRDVFAKQMGLSDKDIVALSGGAHTLGRCHKDRSGFEGAWTSNPLIFDN   188

swAPX1      SYFTELLSGEKEGLLQLPTDKALLNDSVFRPLVEKYAADEDAFFADYPEAHLKLSELGYAEA   250
Tobacco     SYFTELLSGEKEGLLQLPSDKALLSDPAFRPLVEKYAADEDAFFADYAEAHLKLSELGFAEA   250
Pepper      SYFKELLGGEKEGLLQLPSDKALLSDPAFRPLVEKYAADEDAFFADYAEAHLKLSELGFAEA   250
Strawberry  SYFTVLLSGEKEGLLQLPTDKALLSDPVFRPLVEKYAADEDAFFSDYALAHQRLSELGFAEA   250
Cucumber    SYFKELLGGEKEGLLQLPSDKALLSDPAFRPLVEKYAADEDAFFADYAEAHLKLSELGFAEA   250
Soybean     SYFKELLSGEKEGLLQLPSDKALLSDPVFRPLVEKYASDEDAFFADYAEAHQKLSELGFAEA   250
Rice        SYFTELVSGEKEGLLQLPSDKAIMADPAFRPLVEKYAADEDAFFADYAEAHLKLSELGFAEE   251
Spinach     TYFKELLSGEKEGLLQLPSDKALLSDPVFRPLVEKYAADEDAFFADYAEAHLKLSELGFADA   250
A.thaliana  SYFKELLSGEKEGLLQLVSDKAILDDPVFRPLVEKYAADEDAFFADYAEAHMKLSELGFADA   250
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Expression of the *swAPX1* gene in various tissues and during growth in cell culture

The expression patterns of the *swAPX1* gene in various tissues [leaf, (L), stem (S), non-storage root (R) and storage root (SR)] of whole plants and in cultured cells (Su, suspension cells) were examined by Northern analysis (Fig. 3). Transcripts of *swAPX1* were clearly detected in leaves and in cultured cells, weakly in stems, and not detected at all in non-storage and storage roots (Fig. 3A). The expression level of *swAPX1* was higher in mature leaves than in immature leaves (Fig. 3B), indicating that the expression of this gene is differentially regulated by growth stage in leaves.

As expected, the expression level of *swAPX1* was high in cell cultures from which the cDNA had been derived. The *swAPX1* gene was strongly expressed in suspension-culture cells, implying that this gene is involved in antioxidative defense responses in relation to culture stress.

Northern analysis indicated that the level of the *swAPX1* transcript increased during the cultivation of suspension cells (Fig. 3C). *swAPX1* was highly expressed just after subculture (0.5 days after subculture, DAS), but present at quite a low level at 5 DAS (during the early exponential growth phase). Then, the level of expression increased from the exponential growth phase to the end of the culture period (20 DAS). Growth in suspension cultures of sweetpotato cells follows a typical sigmoidal curve (Kwak et al. 1995; Huh et al. 1997). Cell growth is maximal at 11–14 DAS, which is equivalent to the early stationary phase. Cells cultured in vitro are subjected to greater oxidative stress than are whole plants, as indicated by the evaluation of antioxidant activity (Kwak et al. 1995; You et al. 1996). The highest levels of *swAPX1* expression were found just after subculture and after the onset of the stationary phase. It seemed likely that *swAPX1* might be associated with oxidative stresses in relation to subculture and nutrient depletion (including carbon sources). Indeed, the results strongly indicate
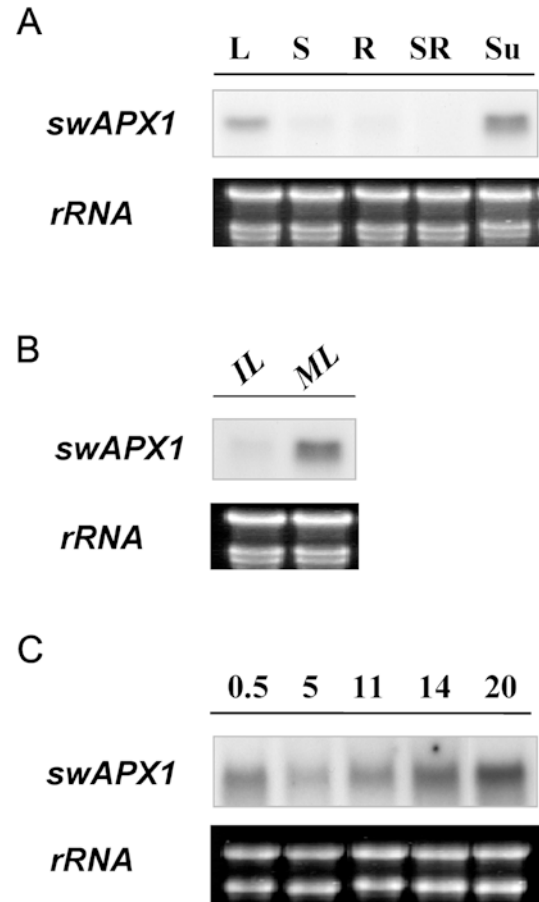
Fig. 3A–C Northern analysis of the expression of the *swAPX1* gene in various intact tissues and cultured cells of sweetpotato. **A** Transcription levels of *swAPX1* in leaves (L), stems (S), non-storage roots (R), storage roots (SR) and suspension-cultured cells (Su). **B** mRNA levels in immature leaves (IL) and mature leaves (ML). **C** Changes in the levels of *swAPX1* transcripts in suspension cultures of sweetpotato during cell growth. RNA was extracted 0.5, 5, 11, 14 and 20 days after subculture (DAS). Equal aliquots (20 µg) of total RNA were electrophoresed in each lane, transferred onto Zeta-probe GT membrane and probed with ^{32}P-labeled gene-specific DNA fragments

Fig. 2A–C Genomic Southern analysis of the *swAPX1* gene from sweetpotato (*I. batatas*). **A, B** Equal amounts (10 µg) of DNA were digested with the indicated restriction enzymes *Hin* dIII, *Eco* RI and *Hin* dII, fractionated by electrophoresis, transferred onto a Zeta-probe GT membrane and probed with ^{32}P-labeled gene-specific DNA fragments: a 207-bp 3′-UTR fragment (**A**), and the 682-bp coding region of the APX cDNA (**B**). **C** Partial map of the *swAPX1* gene showing the positions of the *Hin* dIII sites in the coding region

that *swAPX1* is involved in antioxidative responses in cultured cells.

Differential expression of the *swAPX1* gene in response to abiotic stresses

The expression profile of the *swAPX1* gene upon exposure to various abiotic stresses, such as wounding, chilling, heating and stress-related chemicals, was studied by

RT-PCR, using gene-specific primers. In Fig. 4, the results for each type of plant material are displayed in separate panels: data for (1) whole plants grown in pots in the greenhouse were used for wounding and MV treatments (Fig. 4A), (2) whole plants cultured in vitro were subjected to chilling and heat (Fig. 4B), and (3) leaf tissues from whole plants grown in the greenhouse were exposed to hydrogen peroxide, NaCl, ABA, SA and MeJA (Fig. 4C).
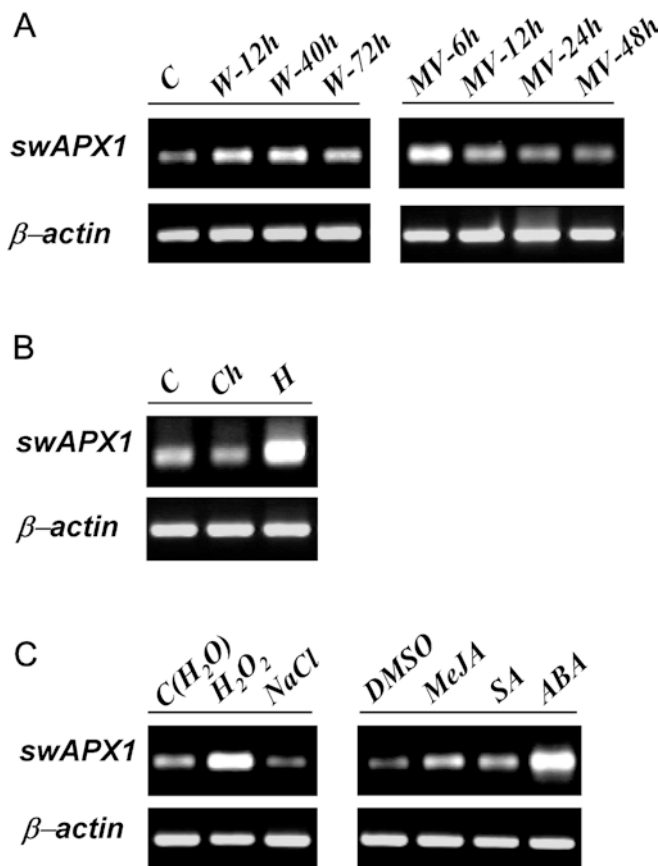
The expression of the *swAPX1* gene in leaves was investigated 0, 12, 40 and 72 h after wounding (W; Fig. 4A). The expression level of *swAPX1* gene increased only slightly in response to wounding, attaining its highest level at 40 h after treatments (HAT, W-40 h), and decreasing slightly by 72 h. The expression of *swAPX1* in the "untreated" control (C) is due to unavoidable wounding stress caused by the detachment of the leaves. It is known that wounding stress inhibits

normal growth and reproduction of plants, and enables pathogens to penetrate easily into plant tissues.

Whole plants were exposed to methyl viologen (MV, paraquat), a ROS-generating herbicide (Babbs et al. 1989). The expression of the *swAPX1* gene was evaluated at 6, 12, 24, 48 h after spraying of MV (50 µM) (Fig. 4A). The *swAPX1* transcript was strongly expressed as early as 6 HAT, compared to untreated control (C), and then decreased with time. MV is a well known non-selective herbicide which causes massive, light-mediated accumulation of superoxide radicals in photosynthetic tissues (Babbs et al. 1989). Among the several known types of APX enzymes, such as cytosolic APX (*c*APX), microbody APX (*m*APX), chloroplastic APX (*clh*APX), only *c*APX showed increased transcription in MV-treated spinach (Yoshimura et al. 2000). It seems likely that the induction of *c*APX expression during an early stage of oxidative stress plays an important role in removing H_2O_2 and minimizing photooxidative damage. Overproduction of a cytosolic APX from pea in tobacco plants provides increased protection to the photo-oxidative stress associated with exposure to MV (Allen et al. 1997; Kwon et al. 2002).

Sweetpotato plants cultured in vitro were subjected to chilling at 4°C, or kept at 37°C, for 48 h, in order to examine the effects of temperature stress on the level of *swAPX1* mRNA (Fig. 4B). The level of expression in the control plants (C) that had been cultured in vitro for 1 month was higher than in plants grown in the greenhouse (the untreated control in Fig. 4A), indicating that this enhanced expression is induced by the stress of long-term in-vitro culture. The *swAPX1* gene showed different expression levels in response to chilling and heating treatments. The expression level of *swAPX1* was slightly decreased by chilling at 4°C, but was increased by heating stress at 37°C. Temperature stress raises ROS levels in plants and would induce various kinds of antioxidant enzymes to overcome oxidative stress (Murata et al. 1992). It has been shown that *c*APX genes in pea and *A. thaliana* are also very strongly induced by heat treatment (Mittler and Zilinskas 1994; Storozhenko et al. 1998). Recently Panchuk et al. (2002) reported that *Apx2* is a novel heat shock gene in Arabidopsis, which serves to compensate for the heat stress-dependent decline in APX1 activity in the cytosol, indicating that each of these APX enzymes has a unique physiological function.

Leaves excised (with a petiole) from sweetpotato plants grown in the greenhouse were incubated in the presence of H_2O_2 (440 mM), NaCl (100 mM), ABA (100 µM), SA (100 µM), or MeJA (100 µM) for 48 h (Fig. 4C). As a control, the leaves were kept in distilled water. In the control, the gene was slightly induced, probably reflecting the effects of wounding during leaf detachment (Fig. 4A). H_2O_2 as a substrate of the APX reaction had dramatic effects on levels of APX transcripts. H_2O_2 treatment strongly induced the expression of *swAPX1*. Some interesting observations regarding the regulation of APX gene expression by H_2O_2 have been

Fig. 4A–C RT-PCR analysis of *swAPX1* expression in response to various abiotic stresses. **A** Effects of wounding (W) and methyl viologen (MV) treatments on the level of transcription of the *swAPX1* gene in leaves of sweetpotato plants. RNA was extracted from leaves 0, 12, 40 and 72 h after wounding, whereas RNA was extracted from leaves 0, 6, 12, 24 and 48 h after treatment with MV. **B** Induction of the *swAPX1* gene in leaves of plants cultured in vitro and subjected to chilling at 4°C for 48 h (Ch) or to heat stress (37°C for 12 h; H). Control plants (C) were kept at 25°C. **C** Expression of the *swAPX1* gene in detached leaves of sweetpotato plants exposed to various stress-related chemicals. Excised leaves with petioles were incubated in the presence of H_2O_2 (440 mM), NaCl (100 mM), ABA (100 µM), or MeJA (100 µM) for 48 h. Control plants were exposed to distilled water (H_2O) instead of H_2O_2 or NaCl treatment, and 0.1% DMSO (DMSO) instead of ABA and MeJA. Total RNA was extracted from plant materials by the LiCl method (Naqvi et al. 1998). Reverse transcription of 1–2-µg samples of total RNA was carried out, followed by conventional PCR amplification using gene-specific primers. *β*-Actin was used as a internal control. Reaction products (20 µl) were analyzed by gel electrophoresis

reported recently. Thus, treatment of cultured soybean cells with exogenous H_2O_2 resulted in the alteration of cytosolic APX transcription levels (Lee et al. 1999b). High salt concentrations are also known to induce oxidative stress in plant cells (Bueno et al. 1998). Interestingly, treatment of leaves for 48 h with 100 mM NaCl reduced the expression of the *swAPX1* gene.

As expected, MeJA and SA also enhanced the expression of *swAPX1* mRNA. ABA treatment strongly increased transcription of *swAPX1*. ABA mediates many physiological and developmental processes in plants in

response to various environmental stresses (Moore 1989). Thus ABA enhanced total POD activity by approximately 50% in suspension cultures of sweetpotato, without a significant effect on cell growth, when added to the growth medium at the late logarithmic phase of growth (Kwak et al. 1996).

Response of the *swAPX1* gene to inoculation with a bacterial pathogen

The expression of APX is rapidly induced in response to stresses that result in the accumulation of ROS. Pathogen attack also leads to the production of ROS in plants, and we therefore examined the expression of the *swAPX1* gene after inoculation with the bacterial pathogen *P. chrysanthemii*.

The *swAPX1* transcript accumulated following inoculation of sweetpotato with *P. chrysanthemii* (Fig. 5). The typical symptoms of disease, rot in the leaf disc, first appeared 24 h after treatment. Several studies have demonstrated that APX enzymes are involved in plant defense responses against viral and bacterial pathogens (You et al. 2002; Mittler et al. 1998, 1999a). Thus transgenic tobacco plants expressing antisense RNA directed against APX, which have a reduced capability to detoxify ROS, were reported to be hypersensitive to pathogen infection (Mittler et al. 1999b). Overexpression of a gene encoding a peroxisomal APX showed resulted in increased protection against oxidative stress damage in *Arabidopsis* (Wang et al. 1999).
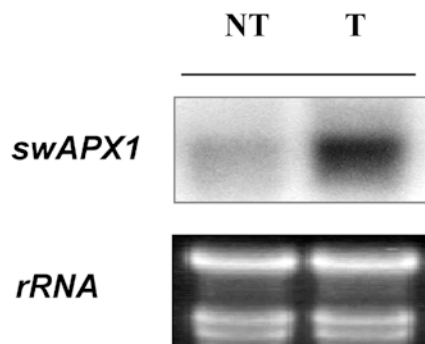
Photosynthetic organisms, including higher plants and eukaryotic algae, have developed ROS-scavenging systems, including APX isoenzymes. APX isoenzymes are induced by distinct regulatory mechanisms in response to various environmental stresses or cellular conditions, and play cooperative roles in protecting each organelle and thus minimizing tissue injury. Cloning of

cDNAs and genes encoding APX isoenzymes has facilitated a diverse range of molecular and physiological studies on these enzymes. In the present report, we have measured the level of transcription of the *swAPX1* gene in plant tissues exposed to biotic and abiotic stress conditions. The *swAPX1* gene responded to all stresses except NaCl. These results are similar to those for other cytosolic APXs, which respond to high light levels, wounding, pathogen infection, fruit ripening, and MV (Mittler and Zilinskas 1992; Mittler et al. 1999b; Morita et al. 1999; Schantz et al. 1995; Yoshimura et al. 2000). *c*APX genes respond to environmental changes, while the transcription levels of *chl*APX and *m*APX genes in spinach did not change in response to any of the stress treatments tested (Yoshimura et al. 2000). Our results suggest that *swAPX1* might be involved in the antioxidative mechanisms that are activated in cell cultures and whole plants of sweetpotato in response to various environmental stresses. Increased H_2O_2-scavenging capacity provided by the anionic (*swpa1*) POD (a-guaiacol-type POD) contributes to increased protection against MV-mediated oxidative stress (Yun et al. 2000). To understand the biological roles and regulation of APX gene expression in sweetpotato, it will be necessary to isolate more APX cDNAs and characterize them using transgenic technology.

Fig. 5 The effects of the pathogen *Pectobacterium chrysanthemi*, the causative agent of stem and root rot, on levels of the *swAPX1* transcript in sweetpotato leaves. NT and T indicate no treatment and inoculation with the pathogen, respectively. *P. chrysanthemi* was inoculated into leaf discs as amount of 1.3×10^4 cell/ml. RNA was extracted from the discs 20 h after inoculation. Equal aliquots (20 µg) of total RNA were electrophoresed in each lane, transferred onto a Zeta-probe GT membrane and probed with ^{32}P-labeled gene-specific DNA fragments

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