Mol Gen Genet (1999) 262: 807–814

ORIGINAL PAPER

H.-S. Lee · K.-Y. Kim · S.-H. You
S.-Y. Kwon · S.-S. Kwak

Molecular characterization and expression of a cDNA encoding copper/zinc superoxide dismutase from cultured cells of cassava (*Manihot esculenta* Crantz)

Received: 22 April 1999 / Accepted: 10 August 1999

Abstract A cDNA, *mSOD1*, encoding cytosolic copper/zinc superoxide dismutase (CuZnSOD) was cloned and characterized from cell cultures of cassava (*Manihot esculenta* Crantz) which produce a high yield of SOD. *mSOD1* encodes a 152-amino acid polypeptide with a pI value of 5.84. Southern analysis using an *mSOD1*-specific probe indicated that a single copy of the *mSOD1* gene is present in the cassava genome. The *mSOD1* gene is highly expressed in cultured cells, as well as in intact stems and tuberous roots. It is expressed at a low level in leaves and petioles. Transcripts of *mSOD1* were not detected in nontuberous roots. Transcriptional level of *mSOD1* reaches a high level at stationary phase, and then sharply decreases during further culture. In excised cassava leaves, the *mSOD1* gene responded to various stresses in different ways. The stresses tested included changes in temperature and exposure to stress-inducing chemicals. Levels of *mSOD1* transcript increased dramatically a few hours after heat stress at 37° C and showed a synergistic effect with wounding stress. Levels decreased in response to chilling stress at 4° C and showed an antagonistic effect with wounding stress. The gene was induced by abscisic acid, ethephon, NaCl, sucrose, and methyl viologen. These results indicate that the *mSOD1* gene is involved in the response to oxidative stress induced by environmental change.

Key words Cassava (*Manihot esculenta*) · Chemical stress · Environmental stress · Oxidative stress · Superoxide dismutase

Communicated by H. Saedler

H.-S. Lee · K.-Y. Kim · S.-H. You
S.-Y. Kwon · S.-S. Kwak (✉)
Plant Biochemistry Research Unit,
Korea Research Institute of Bioscience and Biotechnology,
P.O. Box 115, Yusong, Taejon 305-606, Korea
e-mail: sskwak@mail.kribb.re.kr
Tel.: +82-42-860-4432; Fax: +82-42-860-4608

Introduction

Oxygen is essential for the existence of aerobic organisms, including plants, but toxic reactive oxygen species (ROS), such as superoxide radicals ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2) are generated in a number of different ways during normal metabolic processes. In particular, ROS are overproduced in plant cells under environmental stress. Injury caused by ROS is known as oxidative stress. Oxidative stress is the major cause of damage in plants exposed to environmental stress.

Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC1.15.1.1) constitutes the first line of cellular defense against oxidative stress. SOD is a metalloprotein that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The enzyme is ubiquitous in aerobic organisms, where it plays a major role in defense against ROS-mediated toxicity. Most plants contain a number of SOD isozymes that are located in various cellular compartments. Three classes of SOD have been identified based on the metals present at the active site. These are copper/zinc (CuZnSOD), iron (FeSOD), and manganese (MnSOD) SODs. CuZnSOD is generally found in the cytosol and the chloroplasts, MnSOD is located in mitochondria, and FeSOD is present within the chloroplasts of some plants (Bannister et al. 1987). Genes for CuZnSODs have been cloned and characterized from over 20 plants, including maize (Cannon and Scandalios 1989), rice (Sakamoto et al. 1992), sweet potato (Lin et al. 1993), and red pepper (Kim et al. 1997). Comparison of the amino acid sequences of SODs has shown a great degree of similarity among the enzymes from plant species.

In vitro growth of plant cells takes place under conditions of high oxidative stress. Thus, plant cell cultures are an excellent system for the production of antioxidants and the study of mechanisms of resistance to oxidative stress in plant cells. The physiological roles of antioxidant enzymes, including SOD, have not been

extensively studied in plant cell cultures, even though cell cultures are important in the field of plant biotechnology. We selected cassava (*Manihot esculenta*) and sweet potato (*Ipomoea batatas*) cell lines for their high yields of SOD and peroxidase (POD), respectively (Kim et al. 1994; You et al. 1996). In previous studies we isolated four POD cDNAs from suspension cultures of sweet potato and characterized these PODs in terms of their response to environmental stress. They were strongly expressed in cultured cells compared to intact tissues of sweet potato (Huh et al. 1997; Kim et al. 1999). In this study we report the molecular characterization of a cDNA encoding a CuZnSOD from cultured cells of cassava and its expression under temperature and chemical stress. Cassava is an important food source in Africa. Molecular cloning of cassava SOD has not previously been reported despite the importance of the plant as a food crop.

Materials and methods

Plant material

The cassava (*M. esculenta* Crantz) cell line was selected for its high yield of SOD. Calli were cultured on MS (Murashige and Skoog 1962) basal medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l) and sucrose (30 g/l) (MS1D). Two grams (fresh weight) of callus, subcultured at 4-week intervals, was inoculated onto MS1D solid medium and maintained at 25° C in the dark. Calli were collected at 5, 10, 25, 30, and 40 days after subculture for preparation of crude SOD and total RNA.

Construction and screening of a cDNA library

A cDNA library was constructed with poly(A)$^+$ RNA from cultured cassava cells using a Uni-ZAP XR cloning kit (Stratagene) following the protocol supplied. Callus that had been subcultured for 25 days was used for cDNA synthesis. Four degenerate oligonucleotides were prepared based on the highly conserved region (two for the sense primer at the N-terminal end and two for the antisense primer at the C-terminal end) reported for SOD sequences from other plant species. These oligonucleotides were used as primers for PCR amplification of SOD cDNA. The PCR product was labeled with ^{32}P and was then used to screen the cDNA library. Hybridization was performed with a ^{32}P-labeled probe by random priming (Pharmacia) according to the manufacturer's instructions. After hybridization the membranes were rinsed once in 2 × SSC, 0.5% SDS at room temperature, then washed twice in 2 × SSC, 0.1% SDS

Sequencing and sequence analysis

The dideoxy method of DNA sequencing was performed according to the protocol supplied with T7 Sequenase v2.0 (Amersham). The sequence was analyzed with a DNA autosequencer (Applied Biosystems Model 476A).

Protein assay and enzyme activity

Cultured cells were homogenized on ice in 200 mM TRIS-HCl buffer (pH 7.8) using a Polytron homogenizer. SOD activity was assayed using xanthine/xanthine oxidase (XOD)/cytochrome *c* according to McCord and Fridovich (1969). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of XOD after 2 min at 25° C. Proteins were determined according to the method of Bradford (1976) using Bio-Rad protein assay reagents.

Southern and Northern blot analysis

Cassava genomic DNA was extracted from leaves according to Asemota (1995), digested with *Eco*RI, *Hin*dII and *Hin*dIII, electrophoresed on 0.8% agarose gel, blotted onto Zeta-Probe GT membranes (Bio-Rad), and hybridized to the 260-bp *mSOD1*-specific probe from the 3′ untranslated region (positions 509–768). Hybridization was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 60° C. After hybridization the blot was washed once with 40 mM sodium phosphate (pH 7.2), 1% SDS, and 1 mM EDTA at room temperature for 10 min, then twice with the same solution at 60° C. For Northern analysis, total RNA was extracted by the LiCl method (Naqvi et al. 1998), fractionated on a 1% agarose gel containing 0.67 M formaldehyde, and blotted onto a Zeta membrane (Bio-Rad). Hybridization was performed as above.

Stress treatments

Cassava plants were grown in a greenhouse. The third leaf from the top was removed from each plant for the purpose of investigating stress reactions and induction of *mSOD1*. For temperature stress the excised leaves were incubated under continuous light for 24 h in Falcon tubes containing 45 ml of sterile water and subjected to (1) heat stress at 37° C, (2) chilling stress at 4° C, or (3) kept at 25° C as a control. Chemical stresses included exposure to 100 μM ABA, 100 μM ethephon, 100 mM NaCl, 200 mM sucrose, or water as a control. Leaves were incubated in Falcon tubes containing 45 ml of sterile liquid at 25° C under continuous light for 24 h as a control. Plants sprayed with 10 mM methyl viologen were maintained in a greenhouse and leaf samples were taken at 1, 6, 30, 54, and 78 h after treatment. All treated plant materials were immediately frozen in liquid nitrogen and stored at −70° C until further use.

Results

Isolation and analysis of a cDNA clone encoding a cytosolic CuZnSOD

To isolate cDNAs encoding CuZnSOD, a PCR product that had been amplified using oligonucleotides corresponding to a conserved region of CuZnSOD sequences was used to screen a cDNA library constructed with mRNA from cultured cells of cassava. The clone that contained the largest insert was designated as *mSOD1* and subjected to further characterization. The *mSOD1* cDNA was 801 bp in length with an ORF of 152 amino acids and encodes a protein with a predicted molecular mass of 15,346 Da and a pI value of 5.84 (GenBank Accession No. AF170297). It contained the putative polyadenylation signal AATAAA, which was present 170 bp upstream from the poly(A) tail (Fig. 1).

Amino acid sequence comparisons indicated that mSOD1 showed a high degree of identity with the CuZnSOD sequences of several other plant species (Fig. 2). The overall comparison among the mature forms of the 17 sequenced SOD isoforms showed that mSOD1 shares the highest level of sequence identity with the enzymes from *Nicotiana plumbaginifolia*

```
TCTCGATCTTCTCTGTCTAAGCTCTAAAGGGGTGCTCTGAGATCACGTAAAACAATGGTG    60
                                                        M  V
AAGGCTGAAGCTGTTCTTACCAGTAGTGAGGGGGTTAGCGGAACAATCTTCTTTACCCAA   120
 K  A  E  A  V  L  T  S  S  E  G  V  S  G  T  I  F  F  T  Q
GAAGGAGATGGTCCTACCACTGTAACTGGAAACATTTCCGGCCTTAAGCCAGGGCTTCAT   180
 E  G  D  G  P  T  T  V  T  G  N  I  S  G  L  K  P  G  L  H
GGGTTCCACGTCCATGCCCTTGGAGACACAACAAACGGTTGCATGTCAACTGGGCCACAC   240
 G  F  H  V  H  A  L  G  D  T  T  N  G  C  M  S  T  G  P  H
TTTAACCCTTCTGGCAAAGATCATGGTGCCCCTGAGGATGAGATTCGTCATGCTGGTGAT   300
 F  N  P  S  G  K  D  H  G  A  P  E  D  E  I  R  H  A  G  D
CTGGGAAATGTCACTGCTGGTGATGATGGCACTGCTAGTTTCACAATTATTGACAAGCAT   360
 L  G  N  V  T  A  G  D  D  G  T  A  S  F  T  I  I  D  K  H
ATTCCTCTTTCTGGTCAAAATTCAATCATAGGAAGGGCAGTTGTTGTTCATGCAGATCCT   420
 I  P  L  S  G  Q  N  S  I  I  G  R  A  V  V  V  H  A  D  P
GATGATCTTGGCAGGGGAGGACATGAACTCAGTAAAACCACCGGAAATGCTGGTGGCAGA   480
 D  D  L  G  R  G  G  H  E  L  S  K  T  T  G  N  A  G  G  R
GTAGCATGCGGTATTATTGGTTTGCGAGGATAGAGTGCTTCTCCAGAGATCAATAACAAG   540
 V  A  C  G  I  I  G  L  R  G  *
ACAAAGACAGCTGAAACATGCACAGCCGGACAACCTTTAGAAGAACGTTAGGAGACCATT   600
AACTCATTTGAATAAAAGAAAGAATAATACTGTAGTTTTGGCTGGTTTGGTCTTGTGATC   660
TCAAGATGGTGTATGCTTTGTATGGTTTCGTGAAGTTTATTGAACTTTGAACTTTTTCGA   720
ATGGTAGGGCTTGCTCTTTGTCTGGTCCAAATTCAGGCCGTGGATGTTTTATACTGCTTT   780
AAAAAAAAAAAAAAAAAAAA                                           801
```

Fig. 1 Nucleotide and deduced amino acid sequences of a CuZnSOD cDNA, *mSOD1*, isolated from cultured cells of cassava (*Manihot esculenta*). The deduced amino acid sequence is shown in single-letter code *below* the nucleotide sequence. Numbers on the *right* refer to nucleotides. *Bold italics* represent putative poly(A) signal site. The *asterisk* denotes the stop signal

(86.8%) and sweet potato (86.1%). Putative copper and zinc binding sites, which are highly conserved in plant CuZnSOD isoforms, were found at His-45, His-47,His-62, His-70, His-79, Asp-82, and His-119 of the deduced amino acid sequence of mSOD1.

Genomic organization of *mSOD1*

In order to elucidate the genomic organization of the *mSOD1* gene, the genomic DNA was digested with *Eco*RI, *Hin*dII, and *Hin*dIII. Southern hybridization was performed using the ^{32}P-labeled 3′-untranslated region of *mSOD1* as a probe. Digestion with *Eco*RI, *Hin*dII, and *Hin*dIII generated single bands of 1.7 kb, 4.4 kb, and 1.6 kb, respectively (Fig. 3). This restriction pattern strongly suggests that cassava contains a single *mSOD1* gene.

Differential expression of *mSOD1* in cultured cells and intact cassava tissues

Total RNA isolated from callus, leaf, petiole, stem, nontuberous roots (nR), and tuberous roots (tR) was analyzed by Northern hybridization with an *mSOD1*-specific probe to examine the expression of the *mSOD1* gene in cultured cells and various tissues of whole plants (Fig. 4). The results demonstrated that there is considerable variation in the levels of *mSOD1* expression in different tissues of cassava. *mSOD1* transcripts were detected predominantly in callus, strongly in stem and tuberous root, and weakly in leaf and petiole. Transcripts were not detected in nontuberous roots. Tuberous roots contained a smaller transcript species (0.8 kb) than that found in other tissues (1.0 kb).

SOD activity and expression of the *mSOD1* gene during cell growth

Cell growth in callus cultures of cassava follows a typical sigmoidal growth curve (Fig. 5A). After an initial lag period of up to 10 days after subculture (DAS) cell mass increased exponentially, reaching a maximum at 30 DAS. SOD activity (units/g dry cell weight) remained low until the end of the exponential growth phase (25 DAS), then increased linearly up to 40 DAS during the stationary phase.

The expression profile of the *mSOD1* gene during cell culture was examined with an *mSOD1*-specific probe (Fig. 5B). The 1-kb *mSOD1* RNA was expressed as early as 5 DAS; however, expression decreased slightly on entering the early exponential growth stage (10 DAS). Expression levels of *mSOD1* were highest during the stationary phase (25–30 DAS), followed by a weak decrease during further culture (40 DAS).

Differential expression of the *mSOD1* gene under various stress conditions

Excised cassava leaves were subjected to high and low temperatures in order to examine the effect of temperature stress on the levels of *mSOD1* mRNA. The *mSOD1* gene in excised leaves responded differently to heat and chilling stress (Fig. 6). In excised (wounded) leaves treated at 25° C the *mSOD1* gene was induced at 2 h after treatment (HAT). Induction then gradually decreased to the basal level by 24 HAT. The *mSOD1* gene was strongly expressed at 2 and 5 HAT at 37° C, showing a synergistic effect with wounding stress. However, the *mSOD1* transcript level decreased dramatically at 10 HAT and was barely detectable at 24 HAT under heat stress. Under chilling stress at 4° C the *mSOD1* gene was not expressed at 2, 5, and 10 HAT, while at 25° C *mSOD1* transcripts were moderately expressed at the corresponding timepoints (Fig. 6B). Expression of the *mSOD1* gene was enhanced by chilling stress for 24 h, relative to the levels in leaves kept at 25° C.

mSOD1 was also highly induced by various chemical stresses. Leaves excised from plants were incubated in the presence of NaCl (100 mM), ABA (100 μM), ethephon (100 μM), sucrose (200 mM), or water for 24 h (Fig. 7). All chemicals resulted in expression of much higher levels of *mSOD1* transcripts than did treatment with water. Sucrose had the most drastic effect on *mSOD1* transcripts. Methyl viologen (MV), a su-

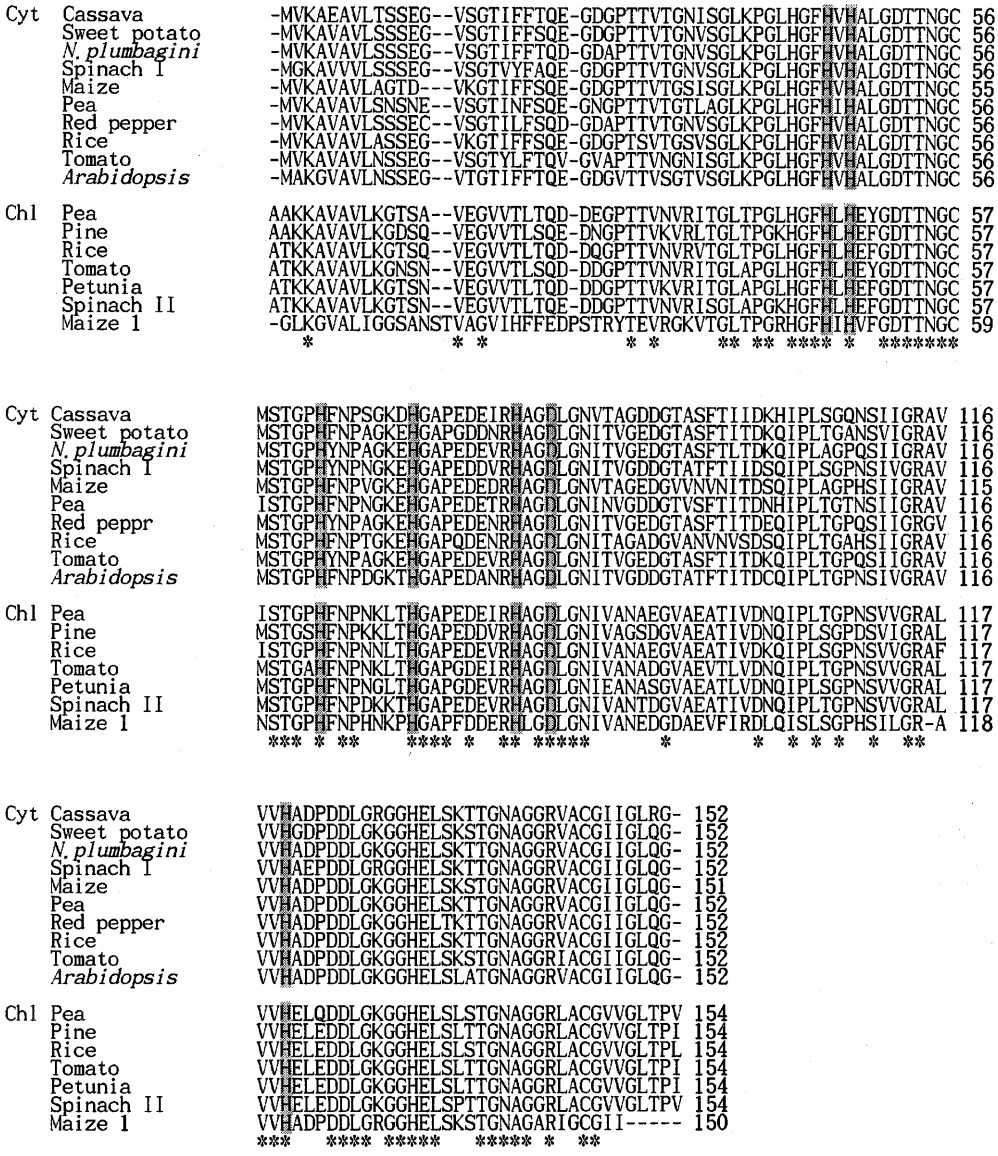

Fig. 2 Alignment of the amino acid sequence deduced from the CuZnSOD cDNA (mSOD1) with sequences of homologous SODs. Amino acid sequences of the cytosolic (Cyt) CuZnSODs from sweet potato (Lin et al. 1993), *Nicotiana plumbaginifolia* (Tsang et al. 1991), spinach I (Sakamoto et al. 1990), maize (Cannon and Scandalios 1989), pea (White and Zilinskas 1991), red pepper (Kim et al. 1997), rice (Sakamoto et al. 1992), tomato (Perl-Treves et al. 1988), *Arabidopsis* (Hindges and Slusarenko 1992), and the chloroplast (Chl) CuZnSODs of pea (Scioli and Zilinskas 1988), pine (Karpinski et al. 1992), rice (Kaminaka et al. 1997), tomato (Perl-Treves et al. 1988), petunia (Tepperman and Dunsmuir 1988), spinach II (Sakamoto et al. 1993), and maize I (Scandalios 1997) were deduced from cDNA sequences. *Asterisks* indicate 100% conservation. The seven metal binding sites in the protein are *shaded*

peroxide-generating herbicide, was applied at 10 mM to whole plants kept in the light. Leaf samples were taken at various times. *mSOD1* transcript levels were induced at 6 HAT and had increased markedly in response to MV at 30 HAT. Levels then decreased by 54 HAT and remained at these low levels thereafter (Fig. 8).

Discussion

A cDNA clone encoding CuZnSOD (mSOD1) was isolated from a cDNA library constructed with RNA from cultured cassava cells which produced a high yield of SOD. Sequencing showed that mSOD1 was a full-length clone with a complete ORF coding for 152 amino acid residues and a polyA stretch at its C-terminal end (Fig. 1). No signal peptide was found in the mSOD1 sequence, suggesting that it encodes a cytosolic CuZnSOD. The translation start site (AACAATGG) matched the consensus sequence (AACAATGG) reported for plants (Lutcke et al. 1987).

Comparison of reported amino acid sequences for plant cytosolic and chloroplast CuZnSODs showed that mSOD1 shares a high degree of amino acid sequence identity (78–87%) with cytosolic forms and a lower level of identity (56–66%) with chloroplast forms (Fig. 2). The N-terminal regions of the SODs are generally divergent, showing no significant regions of homology. The highly conserved regions exist in the C-terminal end. It has been suggested that the functional roles of SOD in protecting cells against oxidative stress involve several residues in the C-terminal part of the protein. The data shown in Fig. 2 also clearly indicate that the metal-binding sites (His and Asp) are highly conserved.

Genomic Southern analysis indicated that there is a single *mSOD1* gene in the cassava genome. Cytosolic CuZnSOD genes exist in single copies within the genome of maize (Cannon and Scandalios 1989), *N. plumb-*

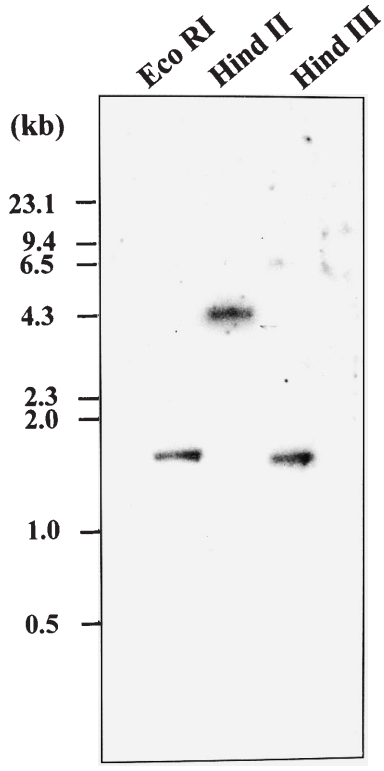

Fig. 3 Southern analysis of genomic DNA prepared from cassava leaves. Equal amounts (10 μg) of genomic DNA were digested with the enzymes indicated, electrophoresed in a 0.8% (w/v) agarose gel, and blotted onto a nylon membrane. The blot was hybridized with the 0.23-kb fragment specific to *mSOD1* as a probe. The positions of molecular weight markers are shown on the *left*

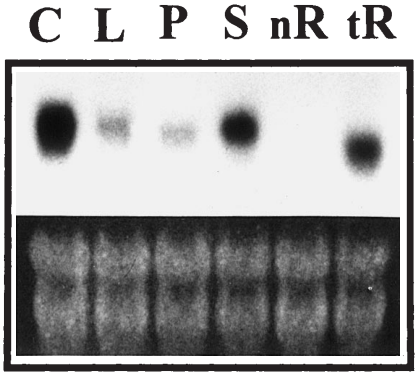

Fig. 4 Expression levels of *mSOD1* in cultured cells and various tissues of cassava. RNA was prepared from callus (C), leaf (L), petiole (P), stem (S), nontuberous root (nR), and tuberous root (tR). Equal amounts (40 μg) of each sample were loaded in each lane. An *mSOD1*-specific DNA fragment was used as a probe. Ethidium bromide-stained RNA served as a loading control

aginifolia SOD is also encoded by a single gene (Herouart et al. 1993), whereas multiple genes encoding SOD isoforms exist in most plants.

The *mSOD1* gene was expressed in callus, leaves, petioles, stems, and tuberous roots of cassava. However, the amount of *mSOD1* mRNA varied considerably between tissue types, with the highest levels in callus and moderate levels in stems and tuberous roots. The *mSOD1* gene was weakly expressed in petioles and leaves, and was not detected in nontuberous roots. Remarkably high levels of SOD transcripts have also been reported in cultured cells of *N. plumbaginifolia* (Tsang et al. 1991) and rubber (Miao and Gaynor 1993). Interestingly, we found that the transcript of the *mSOD1* transcript from tuberous root was smaller than that found in other tissues (Fig. 4). This smaller transcript may be produced by alternative splicing. It has been reported that synthesis of ascorbate peroxidases is controlled by alternative splicing in pumpkin and spinach (Mano et al. 1997; Yoshimura et al. 1999). A similar observation was also reported for CuZnSOD synthesis in *Caenorhabditis elegans* (Fujii et al. 1998).

Cells cultured in vitro are grown under high oxidative stress, allowing comparison of the antioxidative defense mechanisms expressed by cultured cells with the response in whole plant cells. As shown in Fig. 5, changes in total SOD activity and levels of the *mSOD1* transcript occur during cell growth. *mSOD1* transcription levels were not coordinated with total SOD activity during cell

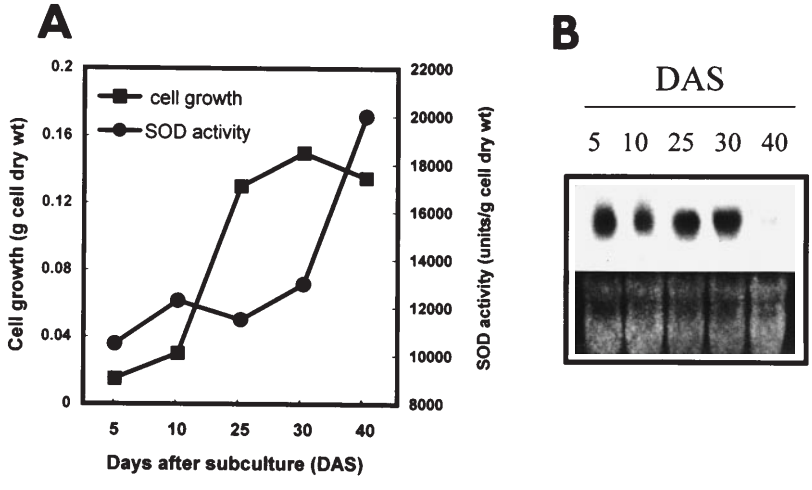

Fig. 5A, B Changes in SOD activity (**A**) and *mSOD1* transcript accumulation (**B**) during growth of cassava callus cultures. SOD activity was determined by the method of McCord and Fridovich (1969). RNA was extracted at 5, 10, 25, 30, and 40 days after subculture. Equal amounts (40 μg) of each sample were loaded in each lane. An *mSOD1*-specific DNA fragment was used as a probe. Ethidium bromide-stained RNA served as a loading control

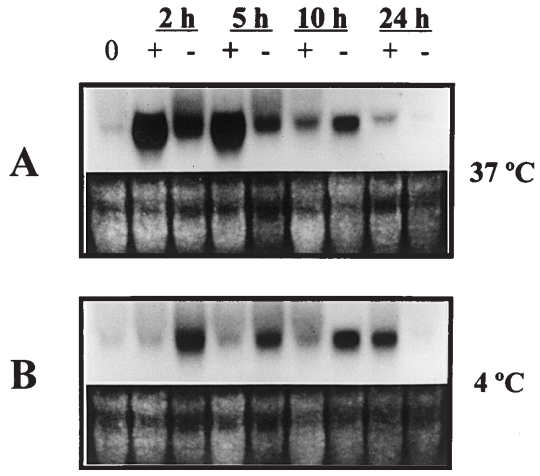

Fig. 6A, B Changes in *mSOD1* transcript levels in response to heat stress (**A**) and chilling stress (**B**) in excised leaves. Total RNA was extracted from leaves at 0, 2, 5, 10, and 24 h after stress treatment (+). Control samples were incubated at 25° C (-). Equal amounts (40 μg) of each sample were loaded in each lane. An *mSOD1*-specific DNA fragment was used as a probe. Ethidium bromide-stained RNA served as a loading control

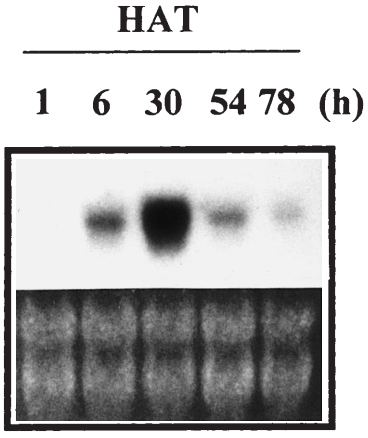

Fig. 8 Northern analysis of total RNAs isolated from methyl viologen-treated cassava leaves. Samples were taken at the indicated times (in hours) after treatment (HAT). Equal amounts (40 μg) of each sample were loaded in each lane. An *mSOD1*-specific DNA fragment was used as a probe. Ethidium bromide-stained RNA served as a loading control

growth. The high levels of *mSOD1* expression found during both the early initial lag phase and the stationary period seemed to be associated with oxidative stresses associated with subculture and nutrient depletion (carbon sources). At least seven SOD isozymes have been identified in cell cultures of cassava (You 1998). In suspension cultures of sweet potato, four POD genes exhibit different expression patterns during cell culture (Huh et al. 1997; Kim et al. 1999). These results indicate that each isozyme plays a different role in the antioxidative mechanism during cell culture. The key factor that induces MnSOD in cultured cells of *N. plumbaginifolia* and rubber is sugar (Bowler et al. 1989; Miao and Gaynor 1993). In cassava, sucrose also caused a dramatic induction of *mSOD1* mRNA. It is however, not clear whether induction by sucrose is due to metabolic activities of cells related to the carbon source or to osmotic effects (Fig. 7), although *Sod3* (MnSOD) transcripts in maize were induced by a high concentration of sucrose, but not by high osmolarity as such (Zhu and Scandalios 1994).

SOD activity in plants is increased by a number of environmental stresses (Bowler et al. 1989). Temperature stress and wounding raise ROS levels, resulting in oxidative stress. In maize seedlings the levels of catalase transcripts and POD activity were elevated during cold-induced oxidative stress, whereas SOD activity was not affected (Prasad et al. 1994). During heat shock stress at 37° C the gene that encodes the cytosolic CuZnSOD of *N. plumbaginifolia* appeared to be the most highly expressed SOD gene (Tsang et al. 1991). In excised leaves of cassava at 25° C, the *mSOD1* gene was induced at 2, 5, and 10 HAT by wounding stress (Fig. 6). The level of *mSOD1* transcripts was increased by heat stress at 37° C, showing a synergistic effect with wounding stress. The level was decreased by chilling stress at 4° C, showing an antagonistic effect with wounding stress. These results indicate that the *mSOD1* gene may be more sensitive to heat stress than to chilling stress.

Biosynthesis of the phytohormone ethylene increases in plants under oxidative stress (Scandalios 1997). Ethephon is metabolized by plants to ethylene and phosphoric acid. Uptake of ethephon by cassava leaves induced *mSOD1* expression (Fig. 7). *Sod4* in maize (Scandalios 1992), *SodCp* in tomato (Perl-Treves and Galun 1991), and MnSOD in rubber tree (Miao and Gaynor 1993) were induced by ethephon in a similar

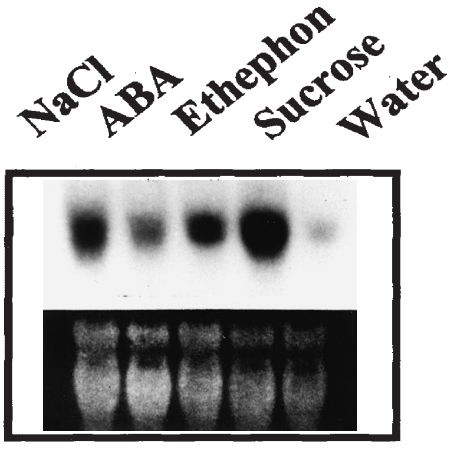

Fig. 7 Changes in levels of *mSOD1* transcripts in response to various chemical stresses. The third leaf from the top was excised and treated for 24 h with 100 mM NaCl, 100 μM ABA, 100 μM ethephon, or 200 mM sucrose. Equal amounts (40 μg) of each sample were loaded in each lane. An *mSOD1*-specific DNA fragment was used as a probe. Ethidium bromide-stained RNA served as a loading control

manner. We found that the cassava *mSOD1* gene was induced by exogenous ABA. A similar observation was also reported for maize SOD: the two closely related maize CuZnSOD genes *Sod4* and *Sod4A* are induced by ABA (Guan and Scandalios 1998). As expected, NaCl also highly induced the *mSOD1* gene since high salt concentrations induce oxidative stress in plant cells (Bueno et al. 1998). After treatment with MV, *mSOD1* mRNA was highly induced at 30 HAT (Fig. 8). In *N. plumbaginifolia*, exposure to MV drastically increases levels of mRNAs for FeSOD, CuZnSOD, and MnSOD under light conditions (Tsang et al. 1991). *Sod4* and *Sod4A* responded differently to MV in the dark and the response to MV was tissue specific (Kernodle and Scandalios 1996).

The *mSOD1* gene isolated from cultured cells of cassava responds to environmental stresses, including wounding, high temperature, and stress-inducing chemicals. Using the mSOD1 cDNA we are currently trying to develop transgenic plants that overexpress or downregulate SOD in order to understand the physiological role of the gene in whole plants subjected to environmental stress.

Acknowledgements This work was supported by Grants-in-Aid (AG650 M and NB0840). We thank Dr. C. Harn for his critical reading of this manuscript.

References

Asemota HN (1995) A fast, simple, and efficient miniscale method for the preparation of DNA from tissues of yam (*Dioscorea* spp.). Plant Mol Biol Report 13:214–218

Bannister JV, Bannister WH, Rotilio G (1987) Aspects of the structure, function and applications of superoxide dismutase. CRC Crit Rev Biochem 22:111–180

Bowler C, Alliotte T, De Loose M, Van Montagu, Inze D (1989) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. EMBO J 8:31–38

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bueno P, Piqueras A, Kurepa J, Samoure A, Verbruggen N, Van Montagu M, Inze D (1998) Expression of antioxidant enzyme in response to abscisic acid and high osmoticum in tobacco BY-2 cell cultures. Plant Sci 138:27–34

Cannon RE, Scandalios JG (1989) Two cDNAs encode two nearly identical Cu/Zn superoxide dismutase proteins in maize. Mol Gen Genet 219:1–8

Fujii M, Ishii N, Joguchi A, Yasuda K, Ayusawa D (1998) A novel superoxide dismutase gene encoding membrane-bound and extracellular isoforms by alternative splicing in *Caenorhabditis elegans*. DNA Res 5:25–30

Guan L, Scandalios JG (1998) Two structurally similar maize cytosolic superoxide dismutase genes, *Sod4* and *Sod4A*, respond differentially to abscisic acid and high osmoticum. Plant Physiol 117:217–224

Herouart D, Van Montagu M, Inze D (1993) Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana*. Proc Natl Acad Sci USA 90:3108–3112

Hindges R, Slusarenko A (1992) cDNA and derived amino acid sequence of a cytosolic Cu, Zn superoxide dismutase from *Arabidopsis thaliana* (L.) Heyhn. Plant Mol Biol 18:123–125

Huh GH, Lee SJ, Bae YS, Liu JR, Kwak SS (1997) Molecular cloning, and characterization of cDNAs for anionic and neutral peroxidases from suspension cultured cells of sweet potato and their differential expression in response to stress. Mol Gen Genet 255:382–391

Kaminaka H, Morita S, Yokoi H, Masumura T, Tanaka K (1997) Molecular cloning and characterization of a cDNA for plastidic copper/zinc-superoxide dismutase in rice (*Oryza sativa* L.). Plant Cell Physiol 38:65–69

Karpinski S, Wingsle G, Olsson O, Hallgren JE (1992) Characterization of cDNAs encoding CuZn-superoxide dismutases in Scots pine. Plant Mol Biol 18:545–555

Kernodle SP, Scandalios JG (1996) A comparison of the structure and function of the highly homologous maize antioxidant Cu/Zn superoxide dismutase genes, *sod4* and *sod4A*. Genetics 144:317–328

Kim KY, Huh GH, Lee HS, Kwon SY, Hur Y, Kwak SS (1999) Molecular characterization of two anionic peroxidase cDNAs isolated from suspension cultures of sweet potato. Mol Gen Genet 261:941–947

Kim SK, Kwak SS, Jung KH, Min SR, Park IH, Liu JR (1994) Selection of plant cell lines for high yields of peroxidase. J Biochem Mol Biol 27:132–137

Kim YK, Kwon SI, An CS (1997) Isolation and characterization of cytosolic copper/zinc superoxide dismutase from *Capsicum annuum* L. Mol Cells 7:668–673

Lin CT, Yeh KW, Kao MC, Shaw JF (1993) Cloning and characterization of a cDNA encoding the cytosolic copper/zinc-superoxide dismutase from sweet potato tuberous root. Plant Mol Biol 23:911–913

Lutcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J 6:43–48

Mano S, Yamaguchi K, Hayashi M, Nishimura M (1997) Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin. FEBS Lett 413:21–26

McCord JM, Fridovich I (1969) Superoxide dismutase, an enzymatic function for erythrocuprein. J Biol Chem 244:6049–6055

Miao Z, Gaynor JJ (1993) Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (*Hevea brasiliensis*). Plant Mol Biol 23:267–277

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Naqvi SM, Park KS, Yi SY, Lee HW, Bok SH, Choi D (1998) A glycine-rich RNA-binding protein gene is differentially expressed during acute hypersensitive response following tobacco mosaic virus infection in tobacco. Plant Mol Biol 37:571–576

Perl-Treves R, Galun E (1991) The tomato Cu-Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. Plant Mol Biol 17:745–760

Perl-Treves R, Nacmias B, Aviv D, Zeelon EP, Galun E (1988) Isolation of two cDNA clones from tomato containing two different superoxide dismutase sequences. Plant Mol Biol 11:609–623

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and regulatory role for hydrogen peroxide. Plant Cell 6:65–74

Sakamoto A, Ohsuga H, Wakaura M, Mitsukawa N, Hibino T, Masumura T, Sasaki Y, Tanaka K (1990) Nucleotide sequences of cDNA for the cytosolic Cu/Zn-superoxide dismutase from spinach (*Spinacia oleracea* L.). Nucleic Acids Res 18:4923

Sakamoto A, Ohsuga H, Tanaka K (1992) Nucleotide sequences of two cDNA clones encoding different Cu/Zn-superoxide dismutases expressed in developing rice deed. Plant Mol Biol 19:323–327

Sakamoto A, Ohsuga H, Wakaura M, Mitsukawa N, Hibino T, Masumura T, Sasaki Y, Tanaka K (1993) cDNA cloning and expression of the plastidic copper/zinc-superoxide dismutase from spinach (*Spinacia oleracea* L.) leaves. Plant Cell Physiol 34:965–968

Scandalios JG (1992) Molecular biology of free radical scavenging systems. Current communications in cell and molecular biology,

vol. 5. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Scandalios JG (1997) Molecular genetics of superoxide dismutase in plants. In: Scandalios JG (ed) Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp 527–568

Scioli JR, Zilinskas BA (1988) Cloning and characterization of cDNA encoding the chloroplastic copper/zinc-superoxide dismutase from pea. Proc Natl Acad Sci USA 85:7661–7665

Tepperman J, Dunsmuir P (1988) Cloning and nucleotide sequence of a petunia gene encoding a chloroplast-localized superoxide dismutase. Plant Mol Biol 11:871–872

Tsang Ed WT, Bowler C, Herouart D, Van Camp W, Villarroel R, Genetello C, Van Montagu M, Inze D (1991) Different regulation of superoxide dismutases in plants exposed to environmental stress. Plant Cell 3:783–792

White DA, Zilinskas BA (1991) Nucleotide sequence of a complementary DNA encoding pea cytosolic copper/zinc superoxide dismutase. Plant Physiol 96:1391–1392

Yoshimura K, Yabuta Y, Tamoi M, Ishikawa T, Shigeoka S (1999) Alternatively spliced mRNA variants of chloroplast ascorbate peroxidase isoenzymes in spinach leaves. Biochem J 338:41–48

You SH (1998) Antioxidant enzyme activity and isoenzyme pattern in plant cultured cell lines with a high SOD activity. MS thesis, Chungnam National University, Taejon, Korea

You SH, Kim SW, Kim SH, Liu JR, Kwak SS (1996) Selection and isoenzyme analysis of plant cell lines for high yields of superoxide dismutase. Korean J Plant Tissue Culture 23:103–106

Zhu D, Scandalios JG (1994) Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid high osmoticum. Plant Physiol 106:173–178