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Gene structure and expression of the aspen cytosolic copper/zinc-superoxide dismutase (PtSodCc1)

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Abstract

Genomic and cDNA clones, corresponding to an ozone-induced cytosolic copper–zinc superoxide dismutase, were isolated from quaking aspen (*Populus tremuloides Michx*.). The cytosolic superoxide dismutase (SOD) appears to be part of a multi-gene family in aspen and is interrupted by five introns in the coding region. Northern blot analysis with a gene-specific probe revealed an increase in the expression of this gene in response to ozone in the leaves of an ozone-tolerant aspen clone, compared with an ozone-sensitive clone. Cytosolic SOD transcript expression levels in leaves were also found to increase significantly within 6 h of mechanical wounding, after which the level of the transcript decreases. Under normal growing conditions, immature male and female aspen floral bud tissues contained the highest levels of the cytosolic SOD gene transcript, whereas transcript levels were almost undetectable in older leaves. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Aspen; Oxidative stress; Ozone; Superoxide dismutase

1. Introduction

Oxidative stress is a negative consequence of life for all aerobic organisms resulting from the production of reactive oxygen species (ROS) [1]. Naturally occurring oxidative stress is occasionally enhanced by several air pollutants, of which ozone (O_3) is among the most common and also both a direct precursor of ROS in addition to being a ROS itself. Plants as well as other organisms have evolved various defense mechanisms that scavenge the ROS before they damage cells. Aerobic organisms contain superoxide dismutase (SOD) (superoxide:superoxide oxidoreductase, E.C. 1.15.1.1) as a means of removing the superoxide radical before it can react with hydrogen peroxide to give rise to the hydroxyl radical. Hydroxyl radicals are extremely reactive and hard to control and its formation is therefore best prevented. SOD is an essential enzyme, which protects cells from oxidative damage by catalyzing the reduction of the superoxide radical to hydrogen peroxide and molecular oxygen [2].

Numerous field studies as well as chamber fumigation experiments with a variety of plant species have shown that SOD can be activated in tolerant plants under oxidative stress [2–7]. Reports from a number of research groups suggest that the differential regulation of individual SOD genes at the transcriptional level is responsible for the differential expression of tolerance in response to environmental and chemical stimuli [8–10].

SODs are nuclear-encoded but are localized in different subcellular compartments [2]. The advantage of having SOD in different subcellular compartments is that it can scavenge the superoxide radical at the site of formation, minimizing dam-

Abbreviations: CSTR, continuously stirred tank reactors; RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated.

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age to the cellular components. Three types of SODs (Cu/Zn-SOD, Fe-SOD and Mn-SOD) have been identified and are classified according to the metal at the catalytic site and their sensitivity to cyanide and hydrogen peroxide, as well as their cellular location. Of the three types, the Cu/Zn-SODs are the most abundant in higher plants, and are localized to both the chloroplast and the cytosol, while the Mn-SOD is localized in the mitochondrial matrix and the Fe-SOD in chloroplasts [2]. Many cDNAs corresponding to all three types of SOD have been cloned and genomic clones for some of the SODs have been cloned from a few plant species [2,11–13]. We have previously cloned the cDNA corresponding to the chloroplast SOD from aspen [14]. Evidence from many investigations shows that the cytosol SOD protects plants from oxidative stresses such as ozone better than chloroplastic SOD [15–17], as was also observed in our preliminary studies [18]. Since the Cu/Zn type of SOD is the major isoform in the aspen plants, characterization of the Cu/Zn-SODs from aspen would provide a more complete picture as to the role of these SODs in protecting tissues from ozone-induced damage. We are working with two clones of aspen that differ in their ozone tolerance [19,20]. The existence of antioxidant defense systems in aerobic organisms is well documented but the underlying regulatory mechanisms have yet to be elucidated. Understanding the structure, regulation and expression of ozone-responsive genes will provide a better understanding of the defense mechanisms. In order to better explain the role of SODs in aspen, we have isolated the cDNA and genomic clones corresponding to an ozone-induced aspen cytosolic Cu/Zn-SOD.

2. Materials and methods

Aspen clone 216 has been classified as ozone-tolerant and clone 259 as ozone-sensitive [19,20]. Leaves from greenhouse-grown aspen plants were extracted to obtain genomic DNA, used in the construction of the genomic library, and also for RNA used in the reverse transcription-polymerase chain reaction (RT-PCR) experiments. RNA for northern blot analyses was obtained from trees exposed either to ozone or charcoal-filtered air in chambers known as continuously stirred tank reactors (CSTRs) [21]. These CSTRs allow for constant mixing of gases in the chamber and uniform distribution of concentration of the gases throughout the chamber. Standard molecular techniques were used for DNA and RNA analysis [22].

2.1. Assay for total SOD enzyme activity

Greenhouse-grown plants were fumigated in CSTRs with 200 ppb of ozone or exposed to charcoal-filtered air (control). Leaf samples were collected before fumigation (zero time), 3 and 6 h after fumigation. Samples were ground in liquid nitrogen and homogenized on ice in 100 mM potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA and 1% PVPP (polyvinylpolypirrolidone). The homogenate was centrifuged at $12\,000 \times g$ for 20 min at +4°C. The supernatant was filtered through two-layered Miracloth, fast-frozen in liquid nitrogen and stored at −80°C. Total protein content was assayed using the d.c. protein assay kit purchased from BioRad Laboratories with bovine serum albumen as the standard (Bio-Rad, CA). SOD activity was spectrophotometrically assayed as described by Beauchamp and Fridovich [23] and Dhindsa et al. [24]. The 1 ml reaction mixture contained 50 mM $KHPO_4$ buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium, 2 μM riboflavin and 300 μg total protein. Cyanide-resistant SOD activity was assayed as described, in the presence of 2 mM KCN [23].

2.2. Isolation of the coding sequence for the aspen cytosolic SOD genomic clone

The aspen genomic library was constructed by Clontech (Clontech, Palo Alto) in λDASHII (Stratagene, La Jolla), using genomic DNA from aspen leaves. The library was screened using a radiolabeled full-length 700 bp pea cytosolic SOD cDNA fragment kindly provided by B. Zilinskas [25]. Approximately 300 000 plaques were screened and positive clones were then isolated after several rounds of screening and purified to homogeneity. λDNA was isolated using standard procedures and digested by restriction enzymes. The restriction enzyme fragments were then separated by agarose gel electrophoresis, blotted onto nylon membranes and hybridized to the full-length pea cytosolic SOD cDNA fragment which was radiola-

beled with [α-^{32}P] dCTP using a Decaprime II labeling kit (Ambion, Austin). A 3.6 kb fragment hybridized very strongly and was subcloned into the *Eco*RI site of the pGEM 7Z vector (Promega, Madison). Nested deletions spanning the entire fragment were obtained by means of the Erase-a-Base system (Promega, Madison) and both strands of the entire fragment were sequenced using the Thermosequenase cycle sequencing kit (Amersham, Arlington Heights). The DNA was analyzed using the MACDNASIS program (Hitachi, CA) and homology searches from the Genbank database were performed using the BLAST program [26].

2.3. Isolation of the 5′ end of the aspen cytosolic SOD gene and determination of the transcription start point

The PCR was used to clone the upstream region corresponding to the cytosolic SOD gene. An antisense primer (5′-AAGAGACAAGAGT-TAGGACTGCAC-3′) was designed based on the 5′ non-coding intron region and was used in a PCR reaction together with the T3 primer using λDNA from a positive clone as the template. An approximately 5.5 kb fragment was amplified, which was then subcloned into the pT7-2 vector (Novagen, Madison). End sequencing was used to verify the identity of the DNA. About 1.5 kb of upstream sequence was obtained by primer walking and also by deletions from subclones generated using the Erase-a-Base system (Promega, Madison). Sequence analysis was performed using MACDNASIS software. The 5′-flanking region was analyzed using the TFSEARCH program [27]. Primer extension analysis was done using the AMV Reverse transcriptase primer extension system (Promega, Madison) in order to determine the transcription starting point (TSP) of the aspen cytosolic SOD gene. Briefly, 100 ng of a 5′-UTR antisense primer (5′-CTC AGA GCA CCC CTT AGA GAG AGA G-3′) and a 5′-ATG antisense primer (5′-TGC CAC TCA CAC CTT CAC TGC TAT T-3′) were end-labeled with [γ-^{32}P] dATP and hybridized with 20 μg of total RNA at 55°C for 2 h in a reaction mixture containing 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT (dithiothreitol) and 0.5 mM spermidine and 1mM dNTPs. The hybridized RNA/primer mixture was then extended by the addition of 40 U ribonuclease inhibitor RNasin and 1 U AMV reverse transcriptase at 42°C for 1 h. The reaction was terminated by the addition of loading dye mixture (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1%bromophenol blue). The reaction products were denatured at 90°C for 10 min and the entire reaction was loaded on an 8% denaturing polyacrylamide gel alongside sequencing ladders generated using the 5′-UTR antisense primer.

2.4. Isolation of the cytosolic SOD cDNA clone

Several gene-specific primers were designed spanning different exon sequences based on the sequence of the genomic clone. The full-length cDNA was obtained by RT-PCR using these primers. Briefly, 0.5 μg of poly A^+ was reverse transcribed in a 10 μl reaction containing 1 μM of oligo-dT primer, 40 U RNasin (Promega, Madison), 1.0 mM each dNTP, 50 mM Tris–HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 5 mM DTT and 100 U Superscript II (Life Technologies, Gaithesburg). The reaction was incubated at 42°C for 1 h and then heated to 70°C for 15 min to cancel the reaction. One microliter of the first-strand cDNA was then used in a 50 μl PCR reaction containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 μmol 5′ and 3′ PCR primers (5′-GAA-CAATGGTGAAGGCTGTAGCTG-3′) and an oligo-$(dT)_{20}$ primer, 5 U Taq DNA polymerase, 20 mM Tris–HCl (pH 8.4) and 50 mM KCl. The PCR reaction was then purified using the Qiagen PCR purification kit (Qiagen, Chatsworth, CA) and then ligated to the pT7-Blue vector (Novagen, Madison). The DNA was sequenced using the Thermosequenase kit (Amersham) and homology was ascertained using the BLAST program [26].

2.5. Analysis of cytosolic Cu/Zn-SOD gene expression in aspen

Cytosolic Cu/Zn-SOD expression was followed in aspen trees exposed to 165 nl l^{-1} of ozone in CSTRs. Leaves were harvested from plants before fumigation, as well as 3 and 6 h after fumigation. Plants exposed to charcoal-filtered air served as controls. RNA was extracted from the leaves using the LiCl-based method described by Parsons et al [28]. Total RNA was also extracted from aspen roots, xylem, immature male and female floral tissues to study patterns of tissue expression. For

wound-inducible expression of cytosolic SOD, a wounding experiment was also analyzed according to the method of Parsons et al. [28]. Leaves at the bottom of the plant were wounded using a one-hole leaf punch (three to six leaf discs were removed depending on leaf size) and non-wounded leaves at the top of the plant were collected at different time-points (3, 6 and 12 h) after wounding. Non-wounded plants were used as controls. The harvested leaves were frozen in liquid nitrogen and RNA was extracted from the leaves as already described [28]. Enzyme activity assays for SOD from wounded and control plants were also done in parallel to compare with RNA analysis. SOD enzyme assays were performed as described [24].

Total RNA (20 μg) was denatured by heating to 65°C for 15 min in formaldehyde loading buffer. The RNA was then separated by electrophoresis on a 1% agarose gel containing formaldehyde and 1X MOPS (3-(*N*-Morpholino) propanesulfonic acid). The integrity and equal loading of RNA in each well is checked by SYBR Green (Molecular Bioprobes, Eugene, OR) staining after the electrophoresis. The RNA was then transferred onto a nylon membrane (Amersham) and prehybridized at 65°C in a buffer containing 7.5% sodium dodecyl sulfate (SDS), 0.5 M Na-phosphate (pH 7.2), 1mM EDTA and 100 μg ml^{-1} salmon sperm DNA. The coding region of the aspen cytosolic SOD cDNA fragment was labeled with [α-^{32}P] dCTP using the Decaprime random primed labeling kit (Ambion, Austin, TX). Hybridization was carried out overnight at 65°C and blot was washed at 65°C to provide high stringency (40 mM $NaHPO_4$ (pH 7.2), 1% SDS and 1 mM EDTA). Blots were subjected to autoradiography using Kodak X-AR film.

3. Results

3.1. The ozone-tolerant aspen clone can be induced to higher SOD enzyme activity than the ozone-sensitive aspen clone

Ozone-sensitive and -tolerant aspen clones were exposed to 200 ppb ozone for 3 and 6 h, and SOD enzyme activity was assayed in the total protein extracted from the leaf samples. SOD increased significantly only in the ozone-treated tolerant aspen clone 6 h after fumigation to about fourfold the control level (Fig. 1). The change in activity could not be detected as significant 3 h after ozone exposure in the tolerant clone. No SOD enzyme activity was detected in this assay in the presence of KCN, indicating the enzyme activity observed in these assays is derived from Cu/Zn-SOD. In-situ gel activity assays for SOD indicated that the increase in enzyme activity corresponds to a cytosol Cu/Zn-SOD isoform (data not shown).

3.2. Isolation and mapping of the PtSodCc1 gene

An aspen genomic library, constructed in λDASHII (Stratagene, La Jolla, CA), was screened for the cytosolic gene using a full-length pea cytosolic SOD cDNA [25]. Approximately 43 positive clones were obtained during the first round of screening. Four clones remained positive after three rounds of purification. Restriction enzyme and hybridization analysis resulted in the

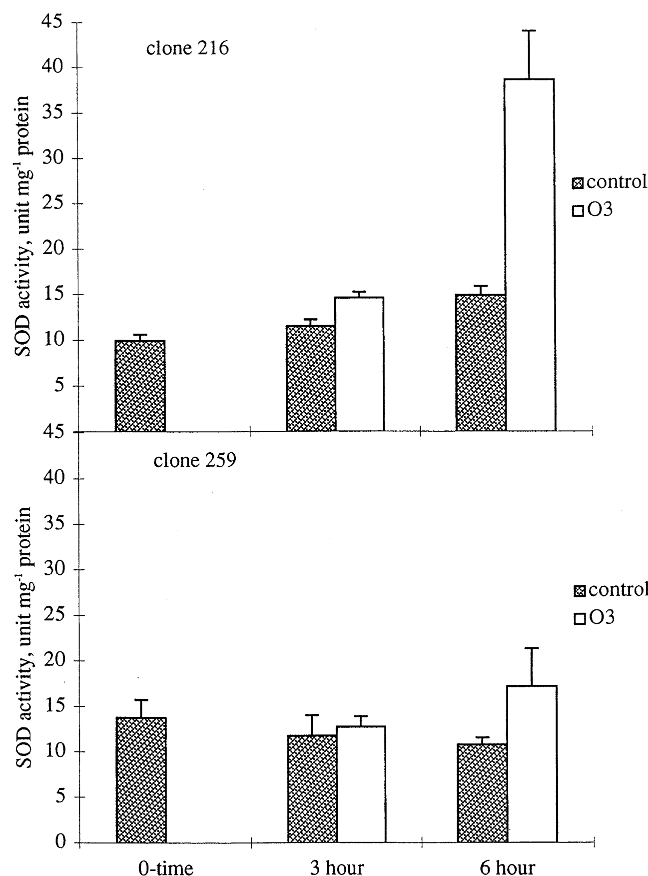

Fig. 1. Ozone inducibility of elevated levels of SOD activity in leaves of quaking aspen. Clone 216 (ozone-tolerant) and Clone 259 (ozone-sensitive) aspen. Data are averages of four different leaf samples; the standard deviations are shown only where there is a significant deviation from the solid line.

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2701 tttttcccaaaatgcttaagcaaccaatgtaaaatgaaaaattaaataatcttctttgtt   2760
2761 ccacgacatcctttatgctcagttatttggagttctgtttgcaGGCCGCATTTTAATCCT   2820
61                                              G  P  H  F  N  P
2821 GTAGGCAAGGAGCATGGTGCCCCTGAGGATGAGAATCGTCATGCTGGTGATCTGGGAAAT   2880
67   V  G  K  E  H  G  A  P  E  D  E  N  R  H  A  G  D  L  G  N
2881 GTCACTGTTGGTGATGATGgtacgcatcactgtccatgtttctgtaaatagtttctaaag   2940
88   V  T  V  G  D  D
2941 caccaggattatacttttacaacataagctttcataacaattttattttgttgagtggtt   3000
3001 tcattttccatattctttgttcacattttaaaggagtctttttgaccaaattttggttgt   3060
3061 ggtagacaggtgccttttttagtaaatctggtttcacacttgttgggtttgtgacacttta  3120
3121 aatttttgtgtgatcagGCACTGCTGCTTTCACAATCATTGACTTTCAGATTCCTCTTAC   3180
94                    G T  A  A  F  T  I  I  D  F  Q  I  P  L  T
3181 TGGACCACATTCCATTATTGGAAGGGCTGTTGTTGTTCATGGAGATCCTGATGATCTTGG   3240
109   G  P  H  S  I  I  G  R  A  V  V  V  H  G  D  P  D  D  L
3241 CAAGGgtaatttatcaatatttcctttcaaggcttttgtactcactgatgacattgttgt   3300
130   K
3301 atgtgctgatgaatgatgagtaggtggcctgacacttaactagtattggatttctagctt   3360
3361 tatgagtctggattcctctaagcaaacaattttggagggtctggttgctctcaaaatgaa   3420
3421 agtttcaatttgttgaatataatctcaacataatttggttagcaaaaacatgcattggaa   3480
3481 cttttttttttaaggggtcagtgattcccacagcactgtcttattggtactcttgccta   3540
3541 tgatcattatcctttcccaagtggaccttcaattgtaattaagcagcatgatgtcttttc   3600
3601 gttgctgtagtgattggttgtttccttatgttttctgccttctgccaggctatttaattg   3660
3661 attacttttactagtttggaataggaaaggaagaagattcttcactcatttactgtttcc   3720
3721 aagggttggagtgtggatgtgaagattatgtttcttaatgacttttcccaggataggcaa   3780
3781 agcatgagctgtttacttcactaatttattgcatcattctaagcaagttatgttaataga   3840
3841 ttattgattctttatttccaggGAGGACATGAACTCAGCAAAACCACTGGTAATGCTGGC   3900
131                       G  G  H  E  L  S  K  T  T  G  N  A  G
3901 GGCAGAGTAGCATGCGgtaagtagcaagcttattgtctaacattaaacaaaataacatgt   3960
144   G  R  V  A  C
3961 catggttgtaaactatttatgacattgttttgagttagaagaattatcaagaaatggtgt   4020
4021 tcaaagtgtctatgaatcttcactctctcggccatgattcatggtttcatcaatctgtca   4080
4081 tattgtctcataattgtaaaggattagaatattgactccgtcattccttgtgtagGTATT   4140
149                                                       G  I
4141 ATTGGTCTGCAAGGTTGAACTGCTTCACTCTGCCACAGCACCGCCATAGTCTCCTGACAAT  4200
151  I  G  L  Q  G  *
4201 GCAGTCGCATGAAGAGAGTTCTGACGACCAGTGACTCAGTCTGAATAAAAGATCGAACCC   4260
4261 TAATATGCTTGTTTGCTGGATTGGTTTTGTAGCCGAAAATGGTGTGTTCTGAAAGATCTT   4320
4321 TATTTTCTACAGCTTGGTTCCTTGTTGGATGGTTGCAATTTTCCTTGAAATGATGTGTTT   4380
4381 ACTTCGGG                                                       4387
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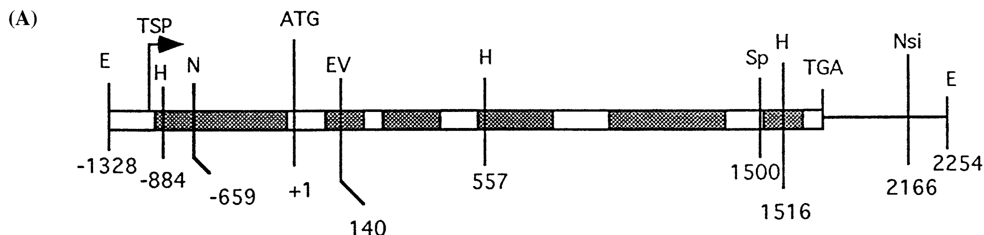
Fig. 2. (A) Restriction map and intron/exon organization of the cytosolic SOD genomic clone from ozone-tolerant aspen. The complete coding sequence of the gene is present on a 3.6 kb *Eco*RI fragment. The ATG translation initiation codon is designated +1. The 5′ non-coding region is designated by negative numbers. A bent arrow indicates the transcription start point. The position of the stop codon (TGA) is also indicated. Intron numbers in Roman numerals are designated below each intron. Some of the restriction enzymes that cut within the genomic clone are shown as: E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; EV, *Eco*RV; Sp, *Sph*I. (B) Alignment of the aspen cytosolic SOD genomic (PtSodCc1) and cDNA (PtSODCC1) clones. The coding sequence of the cytosolic SOD is spread over 3.2 kb in the genomic clone. Exons are represented in upper case with the predicted amino acid sequence below. Introns are designated in lower case. Oligonucleotides used for PCR amplification of the promoter region (lower case) and for primer extension analysis (upper case) are underlined. The transcription start point is in bold. The putative TATA box is underlined. Sequence corresponding to 1.5 kb of promoter region is shown here. Amino acids that are associated with the copper and zinc atoms are underlined. The stop codon is indicated by an asterisk. The putative polyadenylation sequence and the TGTGTTT sequence are also underlined. The accession numbers for the sequences are AFO16892 (PtSODCC1) and AFO16893 (PtSodCc1).

identification of a 3.6 kb *Eco*RI fragment containing the entire coding region of the cytosolic SOD gene (Fig. 2A). The coding sequence of the cytosolic SOD gene is spread over 3.2 kb. Like all other cytosolic SOD genes characterized to date, the aspen cytosolic SOD gene also contains an intron in the 5′ non-coding region, which is also the largest of all introns in the gene. A unique feature of the aspen gene versus the other cytosolic SOD genes is the number of introns. The aspen gene has one intron less than the other plant cytosolic SOD genes characterized to date. The position of all the other introns is the same as in rice, tobacco, tomato, sweet potato and maize [5,11,13,29–31]. The coding sequence of the gene is split into seven exons, starting in the second exon and ending in the seventh exon. The largest intron in the gene is 883 bp long and the smallest intron is 87 bp long. The absence of the intron between amino acids 91 and 92 distinguishes the aspen gene from its coun-

(A)



▒ = intron

☐ = exon

(B)

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   1 GGAGCAAAAAGTTAGTTGATCTGCAAACTTTTTTTGGTTTTCTTGTTTTGCTGGACCAAC   60
  61 ATGTGATGTTCAGATCTCTGAAATGGTAGCATTTGGATAGTCGGAGATAGTAGAGACAGA  120
 121 GAAGACATGTCTCTGTATACCTAAGGACAAATTTGCACCAAAACTATTTCAGATTTCTCT  180
 181 GTCTCTCCAACCATCCTTTTGTATTTGTCTCGTTTGTCATGAGATACTAACCGAATGCAA  240
 241 TCTAATGATCTAGAGACAATCTAATAAACGATAATACTAACATAATTCTGCTGCCAAACA  300
 301 TGTGAAATGAATGTAATCCCTCATCAAATTCATACTTCCATTGTATGTTCTTTACGGTTC  360
 361 ACCCTTCTGATTCGGTTTAGGGAAGAACTTGTGTTCATGAGTCCGTTCTCAAATGCTCAT  420
 421 TCGGTATCCAACACACAGGCCTGGCTTTCATATTTCTGGAGAAGAGCCAAAAACCATGGT  480
 481 CTGGAACCCGACATAGCAGAAGAACGTTTGCAATTCTGGATCAATCACAGTAGTCGGTCT  540
 541 TCCTCGTCACACGATGCAGTCGATGGTAATCTCCCTCCCTATCCTCTTATCCGACACACA  600
 601 CAGGCACACGCACACGCACGTGTTGAACACAATCATCTTCCTTCTCCTCATTCTCGCAAT  660
 661 CTTTCTTTTAATTTACTTGGTTATGATTCACGCCAATGCGCAGTTGAGCGCGGACTCATG  720
 721 GAGTTCAGGAGATTAGGCATTGAGAACCAGCTATGGCAAGCATCTCGAAGAGGGCTCGAG  780
 781 GTTGACTCCTCTCCAAGGCCAATCTTGAGTCCGATTTCTGATGTAACGTTCAAGCTGTTG  840
 841 TCTGACACAATACTCATTCGAATTGGGAAATCTCCTCTACGTGTTCATATCAGCTAGCTG  900
 901 ATAATTTTCTTTAATGCTTCATATTGGGAGCTATTCATCTCCAACTACCTTATTCCTGGC  960
 961 TCCATACTTCCACTCATGTAAATTTTCTATGGCAACGAGACATAACTGTAACCAAATACA 1020
1021 TTTAATTTGATACGTACAACGTATTCTATGGCAGCCACACGAATTCAATTTCCCTGAACA 1080
1081 GATTTTGTCATAATTCCAACGATGATTCGGACCTGTGTCCGGTTCGATTATTATAATCAT 1140
1141 GCTAATAAAAAGAGTAATGATTTATTTTGTTGGTGTACAAAATCAACAAATAGAAAAGGA 1200
1201 AGCCATTACGCTAGGATGCCCAATGGACTGACTAAAACCCAGTAGCCCCATCCTATCCCA 1260
1261 GGTGTCACCTTACTGTAAATAAATCCATTAATTTTACTGTCAGCAATGCATTCATTTTCA 1320
1321 CCACACTTGAAAATTACCTAGAGTGGGTGGTTGGGGTAAAAACTAAAAGAAAGGAAGTCT 1380
1381 TGCTTACCAGACTCTCATTTGTCCAGTATTGAACCATTCCCTTCTATAAAAAAAGGTTAA 1440
1441 AAAACACCTATCTCCTTCACTCTCAAACTCAACAAACAAACATACTGTCTCTCTCTCCCT 1500
1501 CTCTCTCTAAGGGGTGCTCTGAGgtaaagcttcaacctttccactctcttctttgatcac 1560
1561 attcaagggtcttagttatcatctcgtgatcatttgatctttgatctttgatcagaatct 1620
1621 tgtgtttttgttttggctttgttatgctttgactttttaaagatcagccttttatgattt 1680
1681 gtttgatgtgattaactagtagtagcttgatgatcatactagtgttatctcgttttttt 1740
1741 ttttttttatcatatgtgctctctttattcacaaagtgttgagtcggtacttagatttg 1800
1801 gtgtttttttgttgttactgctggtgggttgtatgtttttttttttttttcactgatctc 1860
1861 tctcaagttttgttcttttaataaaggtaagctgggtattgttagacctcatttttagtt 1920
1921 ttttttttttttcatatataaaactatatgtaccttgtttgggttttgtagatagaat 1980
1981 agacaaagggcaggagagaaaattaaagtttttttaaattaggaattgggtcgataaat 2040
2041 gggtattttgtataaattagaactacttaatgatactgaagatgtgctaatttgttttgg 2100
2101 aatcatcaacttgacgtgggtacttgtagctttgttttgagtagttttgctgggttcttt 2160
2161 agcttcgattagagtgatttagtaggtactgcattgacgtgtactcttgcgcatagaagt 2220
2221 ttaaattttaatttggctgttttgagctgcgaatcttttggcgggccgttcatgtgcaat 2280
2281 gtgccttttatttttttgttcccttgttgtattcggtcaggatgtggtgtttgtgtggtt 2340
2341 tttttaaagaatttaggtgattgtagatgtgcagtcctaactcttgtctctttagATC 2400
2401 ACATAGAACAATGGTGAAGGCTGTAGCTGTTCTTAATAGCAGTGAAGGTGTGAGTGGCAC 2460
   1             M  V  K  A  V  A  V  L  N  S  S  E  G  V  S  G  T
2461 CATCTTCTTTACCCAAGAAGGAGATGGtaagtgaaagctccaatcaattggtcttgcaat 2520
  18 I  F  F  T  Q  E  G  D  G
2521 ttttgttgctcatttttctggatagttaatgatatctgaaccatttttttcttcagCCAAC 2580
  27                                                      P  T
2581 TACTGTAACTGGAAACCTTTCTGGTCTTAAGCCAGGCCTTCATGGCTTCCACGTCATGCC 2640
  29 T  V  T  G  N  L  S  G  L  K  P  G  L  H  G  F  H  V  H  A
2641 CTTGGAGACACCACAAATGGCTGCATGTCAACTGgtattgctgaatttaactggacttc 2700
  50 L  G  D  T  T  N  G  C  M  S  T
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Fig. 2. (*Continued*)

terparts from other species, all of which have an intron splitting this region into two exons. Structural alignment between the genomic and the cDNA clone showed that there is complete agreement between the cDNA sequence and the putative exons in the genomic clone (Fig. 2B). Sequences at the exon/intron junctions are in agreement with the donor–acceptor consensus sequences. The polyadenylation signal sequence was found 86 bp downstream of the stop codon and it matches the animal consensus sequence AATAAA. For the proper formation of mRNA 3′ termini, a consensus sequence YGTGTTYY is thought to be important [32], and a sequence matching this consensus sequence was found 124 bp downstream of the polyadenylation sequence.

The TSP for the gene was determined using conventional primer extension analysis (data not shown). The 5′-UTR antisense primer was used in both the primer extension analysis and sequencing in order to determine the TSP. The ATG-antisense primer was essentially used as a control primer to confirm the validity of the primer extension reaction. The presence of the intron in the 5′ non-coding region prevented the use of the ATG-antisense primer in sequencing reactions as a reference for the primer extension reaction. The TSP was mapped to an 'A' residue, 78 bases upstream of the translational start codon. A putative TATA box motif was present at −28 bp upstream of the determined TSP (Fig. 2B).

3.3. Isolation of the cytosolic SOD cDNA (PtSODCC1)

The cytosolic SOD cDNA was isolated using a RT-PCR method. Gene-specific primers were designed spanning different exons based on the sequence of the genomic clone. The 5′ primer included six bases upstream of the ATG start codon, and the full-length cDNA minus the 5′-untranslated region was obtained by performing reverse transcription on poly-A^+ RNA with an oligo-dT primer. The cDNA obtained using the afore-mentioned primers is 752 bp long, of which 464 bp is the coding sequence and the 3′-untranslated region is 288 bp long. The polyadenylation signal sequence AATAAA is present 86 bp downstream of the stop codon. Another sequence, matching the consensus YGTGTTYY, which is thought to be important for the efficient formation of the 3′ end, was found 124 bp downstream of the stop codon (Fig. 2B).

3.4. Cytosolic SOD gene expression in response to ozone

Short-term ozone exposure experiments were carried out to assay the expression of cytosolic SOD in the initial stages of oxidative stress in aspen. The two aspen clones were exposed to ozone in CSTRs and leaves were collected 3 and 6 h after ozone treatment for subsequent analysis. Control plants were treated with charcoal-filtered air and leaves were collected at the same time intervals as for ozone exposure. There appears to be an approximately twofold increase in the amount of cytosolic SOD transcript after 3 h of ozone exposure in the ozone-tolerant clone (Fig. 3A). The transcript for the cytosolic SOD decreased after 6 h relative to the 3 h and control leaf samples. The ozone-sensitive clone, 259, does not show any significant changes in the transcript for the cytosolic SOD within 6 h of ozone treatment (Fig. 3A). The same results were obtained when we reprobed these blots with 3-UTR specific to the PtSODCC1 cDNA.

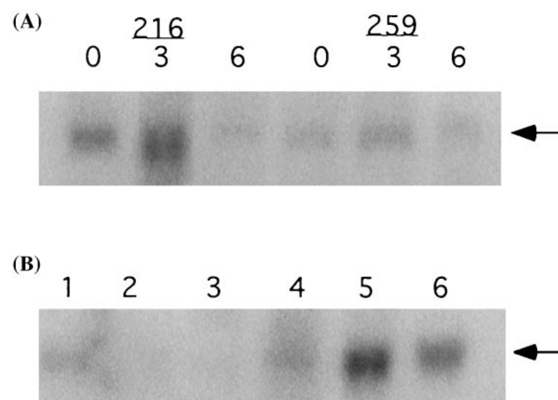

Fig. 3. (A) Ozone-induced increased transcript level of the cytosolic Cu/Zn-SOD in aspen. The probe used for this blot was the full-length aspen cytosolic SOD cDNA described in this paper. Twenty micrograms of RNA isolated from the indicated aspen clones was loaded in the following order. Clone 216: lane 0, control (0 h); lane 3, 3 h O_3; lane 6, 6 h O_3. Clone 259: lane 0, control (0 h); lane 3, 3 h O_3; lane 6, 6 h O_3. (B) Tissue-specific expression of Cu/Zn-SOD in ozone-tolerant aspen clone 216. Lane 1, young leaf; lane 2, mature leaf; lane 3, xylem; lane 4, root; lane 5, male flower; lane 6, female flower. Clone 216 is the ozone-tolerant aspen clone and clone 259 is ozone sensitive.

3.5. Tissue-specific expression of PtSODCC1 in aspen

Northern analysis of cytosolic SOD was performed on RNA extracted from different tissues of aspen clone 216. The corresponding mRNA was most abundant in immature male and female flower tissues of aspen, approximately half as abundant in young leaves, about a quarter of it in roots, and barely detectable in xylem tissue (Fig. 3B). It has been proposed that free radicals play an important role in the initiation of senescence and this correlates well with the decreased antioxidant potential of older leaves [24]. The SOD transcripts were virtually undetectable in mature aspen leaves, which is consistent with the proposed decrease in a leaf's antioxidant potential (Fig. 3B). Similar results are found in the SOD enzyme levels of mature aspen leaves (data not shown).

3.6. Expression of PtSODCC1 in response to mechanical wounding

Northern analysis of RNA isolated from wounded leaves after 0, 3 and 6 and 12 h showed that the expression of PtSODCC1 mRNA was highly increased 3–6 h after wounding and decreased 12 h after wounding (Fig. 4A). This observation is in accordance with the oxidative burst observed during wounding or hypersensitive response to increase the levels of H_2O_2, which is postulated to be involved in acting as a signal molecule for activating transcriptional modulators, which, in turn, serve to regulate expression of genes with protective function [33]. Analysis of SOD enzyme activity from these leaf samples also showed that the enzyme levels increased slightly 3 h after wounding and significantly after 6 h (Fig. 4B). The enzyme levels 12 h post-wounding were below that of control non-wounded plants (Fig. 4B).

3.7. Cytosolic SOD is encoded by a multi-gene family in aspen

In order to determine the copy number of the cytosolic SOD in aspen, genomic DNA (20 µg) of aspen was digested with *Eco*RI and *Pst*I, and hybridized under stringent conditions with a radioactively labeled aspen cytosolic SOD cDNA fragment containing only the coding region. The

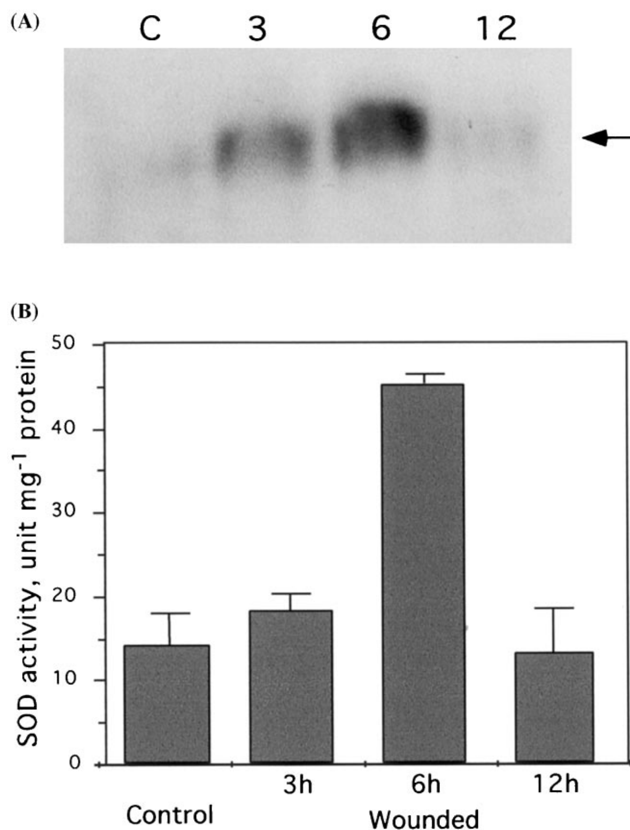

Fig. 4. (A) Wound-induced expression of cytosolic Cu/Zn-SOD RNA in ozone-tolerant aspen clone 216. Aspen cytosolic Cu/Zn-SOD coding sequence is used as a probe for hybridization with the leaf RNA samples. Total RNA (20 µg) was used for each lane. Lane C, RNA from control non-wounded plant; lane 3, RNA sample 3 h after wounding; lane 6, RNA sample 6 h after wounding; lane12, RNA sample 12 h after wounding. (B) Wound-induced expression of cytosolic Cu/Zn-SOD enzyme in ozone-tolerant aspen clone 216. Enzyme activity was measured from leaves collected after 0, 3, 6 and 12 h post-wounding. Data represents mean values from four replicates for each time point. Enzyme activity was measured as described in Fig. 1. Error bars indicate standard deviation.

restriction enzymes used for southern analysis do not digest the genomic or cDNA clones internally. A fragment in the 3.5–4 kb range hybridizes very strongly to the probe in the lane corresponding to the *Eco*RI digested DNA. In addition, an approximately 2 kb *Eco*RI fragment hybridizes strongly to the probe in addition to a number of bands that react with the probe less intensely. In the *Pst*I digested genomic DNA, two bands hybridize to the cDNA: one is 6 kb and the other approximately 3 kb in size (Fig. 5). In addition, there was also a faint signal from the approximately 15 kb range. There are no internal *Pst*1 sites within the genomic fragment containing the coding sequence and more than 2 kb promoter, which may explain the hybridization signal at 15 kb range. Since there

are several faint bands that are smaller in size than the known size of the PtSodCc1 that hybridize to the cDNA probe in the *Eco*RI digested genomic DNA (Fig. 5), it appears that the cytosolic SOD is encoded by at least two genes and is part of a multi-gene family in aspen. Some of the minor bands maybe a result of cross-hybridization of the probe with the chloroplastic SOD despite the high stringency hybridization and washing conditions. Since there is 54% homology between the aspen cytosolic SOD and chloroplastic SOD, this result is not unexpected. Reprobing of these blots with a 3′-UTR probe yielded hybridization to only the two bands in the 5–2 kb range (data not shown).

4. Discussion

Resistance to oxidative stress has traditionally been associated with higher expression and/ or activity of antioxidant enzymes [2,34–36]. However, the higher expression of oxidative stress-related genes is not constitutive but regulated [8,34]. SODs have been shown to be differentially regulated during environmental stress in a number of species including tomato, tobacco and *Arabidopsis* [2,8,9]. Ozone caused an increase in the cytosolic SOD transcript in *Arabidopsis* after about 12 h of ozone exposure [7]. Gupta et al. [4] have found that *Populus* responded to 180 ppb O_3 treatment with increased levels of SOD in 90 min. The work by Tsang et al. [10] with transgenic *Nicotiana tabacum* cv. PBD6 showed that the activity of already functional SOD (both cytosolic and chloroplastic) increases as a result of O_3 fumigation. However, the mRNA levels of corresponding enzymes did not rise until visible injury occurred.

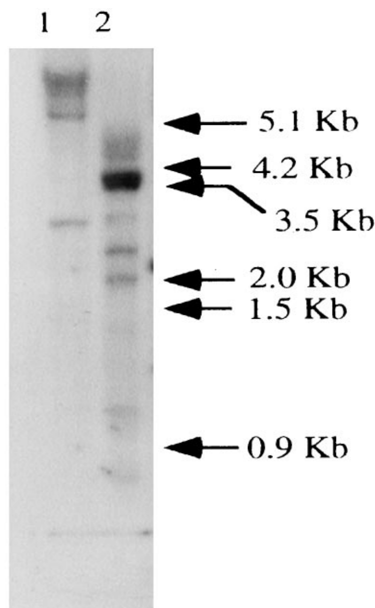

Fig. 5. Genomic Southern blot. Aspen genomic DNA (10 μg) was digested with *Eco*RI and *Pst*I, and probed with the coding region of the aspen cytosolic Cu/Zn-SOD cDNA. Lane 1 represents *Pst*I digested DNA and lane 2 contains *Eco*RI digested genomic DNA. The migration of the standard DNA markers (λDNA digested with *Eco*RI and *Hin*dIII) is indicated by arrows and corresponding sizes.

We subjected the aspen plants to chamber fumigations to demonstrate the ozone-inducibility of SOD genes (Fig. 1). The absence of detectable SOD enzyme activity in assay containing KCN indicates that most of the SOD in leaves constitutes Cu/Zn-SOD. In the ozone-tolerant aspen clone, the cytosolic SOD transcript increased rapidly within 3 h of ozone exposure, to which the peak in protein activity followed at 6 h (Fig. 1). Since the level of the transcript decreased by about 6 h, it suggests that the cytosolic SOD play a defensive role in the initial stages of oxidative stress and may be transcriptionally regulated. The ozone-tolerant aspen clone showed an almost fourfold increase in SOD enzyme activity relative to the control 6 h after ozone treatment. The ozone-sensitive aspen clone, on the other hand, showed an approximately 1.5-fold increase in SOD enzyme activity after 6 h of ozone fumigation. The ozone-sensitive aspen clone did not show any significant increase in the cytosolic SOD transcript levels within 6 h of ozone exposure.

Long-term ozone exposure studies have provided us with data about the expression patterns of different antioxidant transcripts in the two aspen clones, showing that the cytosolic SOD transcript increases in both, but especially in the tolerant clone 216 [18]. Recently, Van Camp et al. [37] isolated the promoters for two tobacco Mn-SOD genes and demonstrated, by reporter gene analysis, that for one of the two MnSOD genes, GUS staining was strongest at the base of an emerging bud. We examined the expression of the cytosolic SOD gene in different tissues of aspen and found that the highest levels of cytosolic SOD transcript are in the developing male and female flower tissues with a slightly higher amount in the male tissues. A possible explanation for the high expression levels in the developing floral tissues could be the high metabolic rate of actively developing tissues. Van Camp et al. [37] proposed that

the high respiratory activity in the pollen would result in the generation of superoxide radicals with a consequent increase in the SOD expression. It appears from the level of expression of the Cu/Zn-SOD in leaf tissue that, under normal growth conditions, there is more superoxide generation in the chloroplasts as a result of photosynthesis, and therefore the cytosolic SOD is expressed at relatively low levels. However, the ozone-tolerant clone seem to have constitutively higher levels of cytosolic Cu/Zn-SOD transcript, compared with ozone-sensitive clone. In addition, the transcription of cytosolic Cu/Zn-SOD also induced, in response, ozone in the ozone-tolerant clone.

Based on the Southern analysis, it appears that the cytosolic SOD gene is represented in the aspen genome as at least two separate entities (Fig. 5). Investigations by other groups in different species of plants have revealed that the cytosolic SOD is generally present as a multi-gene family. In maize, two highly homologous but distinct SOD genes have been identified and the two genes appear to exhibit differential tissue-specific response to stressors [5]. Similar instances have been reported from other species such as rice for the cytosolic SOD and tomato for the chloroplastic-SOD genes [31,38].

The genomic structure of all the plant cytosolic SODs is similar with respect to number and position of introns; however, the sizes of the introns vary. The AT content in introns ranges from 65 to 75%, which is a common feature of plant introns. All plant cytosolic SOD genes characterized to date contain an intron in the 5′ untranslated but transcribed region [5,11,12,29,30], whereas it is absent in all other eukaryotic SOD genes. This is also the largest intron in every plant SOD gene. In rice, the presence of this intron supposedly increases the transcription of the GUS reporter gene, indicating that the intron may play a regulatory function in the transcription of this gene [13]. In tobacco, the intron does not affect transcription of the GUS gene [29]. Currently, we are in the process of characterizing the promoter of the aspen cytosolic SOD, especially with regard to its regulation by ozone. The aspen cytosolic SOD (PtSodCc1) differs from other plant cytosolic SOD genes cloned to date in that its coding region is split by five introns as opposed to six in rice, maize, tomato and tobacco. The significance of this difference is not known. It would prove interesting to see whether the other aspen cytosolic SOD genes (if any) would also be lacking this intron and whether the lack of this intron is a feature common to SODs from tree species. Since no SOD genomic counterparts have been cloned from any other tree species, this would be mere speculation at this point.

Oxygen radicals have been shown to be produced in response to a variety of adverse environmental conditions such as wounding, cold stress, pathogen infections, heat stress and drought [36]. In response to wounding and pathogen infection, it has been shown that there is an increase in hydrogen peroxide which is believed to cause localized cell death, thereby preventing spread of the infection [39]. The plant also becomes more tolerant of the same stress during subsequent attacks—a condition known as systemic acquired resistance (SAR), which is immunity throughout the plant to the stressor. This increase in H_2O_2 was shown to be due to a decrease in catalase activity during SAR as a response to increased salicylic acid concentration in tobacco during pathogen infection [39]. Aspen plants, when mechanically wounded, showed an increase in the level of the cytosolic SOD transcript within 3 h of wounding. Since SOD converts superoxide radicals to hydrogen peroxide, an increase in the expression of SOD would result in an increase in the amount of hydrogen peroxide. Sequence analysis of the promoter for the PtSodCc1 gene revealed the presence of sequence elements similar to binding sites for transcription factors such as NF-κB and AP-1, which are known to be redox regulated. Thus, it may be speculated that the regions of interest produced during ozone fumigation may act as signaling molecules, which in turn regulate the expression of the cytosolic SOD gene through mediation of similar factors. Preliminary analysis of the promoter region indicates that it is ozone inducible.

In both ozone-induced stress as well as mechanical wounding, there seem to be similar temporal changes in the transcription and enzyme activity of cytosolic Cu/Zn-SOD in ozone-tolerant aspen clone. The only difference is in mechanically wounded tissue; the increase in transcripts was seen over a period of 6 h post-wounding, whereas an ozone-induced increase in transcription peaked by 3 h. Thus, it may be likely that a common regulatory mechanism exists for the expression of

cytosolic Cu/Zn-SOD in ozone-tolerant aspen. Studies are underway in our laboratory to characterize the promoter by promoter-GUS fusions and we would ultimately like to identify the signal molecule(s) and factors that initiate the transcription of the SOD gene in response to ozone stress.

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