Molecular cloning of a peroxidase gene from poplar and its expression in response to stress

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Summary  To elucidate the precise functions of peroxidase in poplar (Populus alba × P. tremula var. glandulosa), we cloned a peroxidase gene (PoPOD1) from poplar suspension culture cells and examined its expression pattern in response to various stresses. PoPOD1 showed the highest homology with a bacterial-induced peroxidase gene from cotton (Gossypium hirsutum L.). The PoPOD1 gene encodes a putative 316 amino acid protein with an N-terminal signal peptide of 23 residues. The DNA blot analysis indicated that PoPOD1 is a single copy gene in the poplar genome. The RNA blot analyses indicated that PoPOD1 shows cell-culture-specific expression. Expression of PoPOD1 is down-regulated by various treatments including treatment with some metals, NaCl, methyl viologen and polyethylene glycol, and by the plant growth regulators, jasmonic acid (JA) and gibberellic acid (GA3). The gene is significantly up-regulated by the bacterial-elicitor laminarin and by wounding. Thus, PoPOD1 gene expression is sensitively and specifically regulated at the transcription level. Because both JA and GA3 appear to be involved in the regulation of PoPOD1 expression in poplar cells, we postulate that the peroxidase encoded by PoPOD1 plays a pivotal role in defense against pathogen invasion, possibly through the formation of a cell wall barrier over the wound.

Keywords: bacterial-elicitor, oxidative stress, peroxidase, poplar suspension cell, wounding.

Introduction

Plant peroxidases are bifunctional enzymes involved in both reduction of hydrogen peroxide by transferring electrons to various donor molecules and production of reactive oxygen species (ROS) during oxidative burst and cell elongation (Chen and Schopfer 1999, Rodriguez et al. 2002, Passardi et al. 2004). Plant peroxidases, which are encoded by a large number of superfamily genes, are secreted from plant cells or transported into vacuoles via the endoplasmic reticulum (ER) (Tognolli et al. 2002, Welinder et al. 2002). Because peroxidases exist in numerous isoforms and have broad substrate specificity, they have been implicated in a wide range of physiological processes. These include lignification, suberization, auxin catabolism, cross-linking of cell wall proteins, defense against pathogen attack, salt tolerance, oxidative stress, senescence, somatic embryogenesis, ethylene biosynthesis, respiration, light-mediated processes and growth regulation (Ito et al. 1993, Hiraga et al. 2000, Rebmann et al. 1991, Reimers et al. 1992, Mohan et al. 1990, Ito et al. 2000, Kim et al. 2000, Yoshida et al. 2003, Valerio et al. 2004). Recent plant genome-wide projects make it possible to analyze simultaneously structural characteristics and expression patterns in relation to tissue-specificity and to environmental responses of the near-whole gene family of peroxidases, particularly in rice, Oryza sativa L. (Hiraga et al. 2000a, 2001, Passardi et al. 2004) and Arabidopsis thaliana L. (Tognolli et al. 2002, Welinder et al. 2002, Valerio et al. 2004).

Although massive efforts have been made to elucidate the precise roles of peroxidases, little is known about the specific function of each isof orm because of their high copy number, broad range of substrate specificity, complex expression profiles in response to temporal, spatial and environmental changes and the absence of simple correlations between sequence similarity and function (Kim et al. 2004, Wang et al. 2004). To elucidate the natural functions of plant peroxidases, it is necessary to obtain information on their precise physiological responses to a diverse range of cellular processes.

In this study, we isolated a peroxidase gene (PoPOD1) from poplar (Populus alba × P. tremula var. glandulosa) suspension culture cells. PoPOD1 showed cell-culture-specific expression. Expression of PoPOD1 is down-regulated in response to...
various environmental stresses, including some metals, NaCl, methyl viologen (MV) and polyethylene glycol (PEG), and to the plant growth regulators, jasmonic acid (JA) and gibberellic acid (GA₃). The gene is significantly up-regulated by a bacterial elicitor and by wounding. We investigated the expression pattern during normal cell growth. The expression patterns of PoPOD1 exhibited peculiar characteristics compared with known plant peroxidases, providing further insight into the diverse functions of the peroxidases.

Materials and methods

Plant materials and growth conditions

Poplar leaf suspension cells were maintained by subculture every 2 weeks (Choi et al. 2001). At each subculture, 0.4 g (fresh mass) of cells was transferred to 100 ml of liquid MS medium (Murashige and Skoog 1962) containing 1 mg l⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg l⁻¹ NAA (1-naphthalene acetic acid) and 0.01 mg l⁻¹ BAP (6-benzylaminopurine). The shaker in a culture room at 22 ± 1 °C and 20 µmol m⁻² s⁻¹ provided ample light for the cell suspensions to maintain a cell density of 1406 BAE, LEE, LEE, NOH AND JO

Construction of cDNA library and isolation of peroxidase cDNA

Poplar suspension cells from an 8-day-old subculture were used for mRNA extraction and double-strand cDNA was synthesized by reverse transcriptase (Lee et al. 2005). The λZAPII vector (Stratagene, La Jolla, CA) was used to entrap the synthesized cDNA to construct the cDNA library. For expressed sequence tag (EST) analysis, the cDNA library was randomly excised in vivo with ExAssist helper phage and the plasmid DNAs were then isolated and 5'-single pass sequences were determined. Searches of the public databases were made with BLASTX to select clones homologous to peroxidase. The selected cDNA clones were sequenced and analyzed by GENETYX-MAC program (SDC software, Tokyo, Japan).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from the leaves of 1-year-old poplar plants growing in a nursery using a MagExtractor-Plant Genome kit (Toyobo, Osaka, Japan). Ten µg of the genomic DNA were digested with restriction enzyme BamHI, EcoRI or HindIII overnight. The DNA was fractionated on 1% agarose gel and transferred to Hybond-XL nylon membrane (Amersham-Phamacia, Piscataway, NJ) by the capillary transfer method (Southern 1975). The full-length PoPOD1 cDNA was used as the probe. Labeling of the probe, hybridization and membrane washing were done as described by Lee et al. (2005).

Stress treatments

Four-day-old cell suspensions were cultured in the presence of CdCl₂, AlCl₃·6H₂O, CuSO₄·5H₂O, ZnSO₄·7H₂O, Pb(NO₃)₂, KCl, MgSO₄·7H₂O, KNO₃, salicylic acid (SA), a-bispic acid (ABA), JA, GA₃, NaCl, MV, PEG or laminarin. All reagents were purchased from Sigma-Aldrich (St. Louis, MO). During the course of the plant–pathogen interaction, and within minutes of pathogen recognition, ion fluxes occur across the plasma membrane, leading to the alkalization of the extracellular medium (Dixon et al. 1994). Therefore, to determine the effect of laminarin, the pH of the culture medium was measured with a glass electrode every 5 min, starting 10 min before treatment with laminarin or water. As a physical stress, the cell suspension was subjected to agitation at 300 rpm on a gyratory shaker. For the wounding treatment, leaf discs (9 mm diameter) were floated on petri plates containing MS medium. To study the wounding response in intact attached leaves, the leaves of 1-year-old poplar plants were trimmed along the edge by about 1 cm with sterile scissors and the samples were collected. Amounts and duration of the treatments are indicated in the figure legends. All treated cells were immediately frozen in liquid nitrogen and stored at –70 °C until analyzed. All treatments were performed and analyzed twice in separate experiments.

Northern blot analysis

The steady-state mRNA levels of PoPOD1 were compared by northern blot analysis after the various stress treatments. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). Ten µg of the RNA was fractionated on 1.2% formaldehyde agarose gel and then transferred to Hybond-XL nylon membrane. Labeling of the probe, hybridization and membrane washing were done as described by Lee et al. (2005). As an internal control, elongation factor 1β was chosen because it shows little variation in expression (Brunner et al. 2004). The gene was PCR amplified with a pair of gene-specific primers 5'-AAGAGGACAAGAGGCAGCA-3' and 5'-CTAACCGCTTCATCCAC-3'. The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and confirmed by sequencing using T7 and SP6 primers (Promega).

Results and discussion

Isolation and structural characterization of the PoPOD1 gene

The poplar cDNA clone with the highest homology with a bacterial-induced peroxidase gene from cotton (Gossypium hirsutum L.) was selected following EST analysis. The selected clone, named PoPOD1, showed a high BLASTX score of > 300 to plant peroxidases. The PoPOD1 cDNA consists of...
1230 bp including a 951 bp open reading frame (ORF), a 69 bp 5′ untranslated region (UTR) and a 210 bp 3′ UTR (Figure 1). It has a putative polyadenylation signal (AATAAT) at 144 bp upstream (at 1059–1064 bp) of poly (A) + tail. The ORF of \( \text{PoPOD1} \) is predicted to encode a polypeptide of 316 amino acids. The protein encoded by \( \text{PoPOD1} \) contains an N-terminal signal peptide of 23 residues without a C-terminal extension, suggesting that it is targeted for secretion from plant cells via ER. The predicted isoelectric points of the preprotein (M, 33.4 kDa) and the mature protein (M, 31.0 kDa) of \( \text{PoPOD1} \) were 8.89 and 8.88, respectively, indicating that \( \text{PoPOD1} \) encodes a basic peroxidase.

Plant peroxidases are known to have highly conserved amino acids located in the putative catalytic domain (Welinder et al. 2002). The protein encoded by \( \text{PoPOD1} \) contains eight cysteine residues involved in the formation of disulfide bridges (Figure 1). It also has the catalytic distal sites Arg61 and His192 hydrogen-bonded to Asn93. The His192 is coordinated to heme Fe^{3+} and hydrogen bonded to Asp261 at the proximal site of the heme. The \( \text{PoPOD1} \) encoded protein has two putative glycosylation sites (Asn-Xxx-Thr/Ser) and the side chain ligands associated with two stabilizing Ca^{2+} ions are also conserved.

**Genomic organization of \( \text{PoPOD1} \) gene**

To identify the copy number of the \( \text{PoPOD1} \) gene in the poplar genome, Southern blot analysis was done with the full-length \( \text{PoPOD1} \) cDNA as a probe (Figure 2). Genomic DNA extracted from poplar leaves was digested with restriction enzyme BamHI, EcoRI or HindIII and hybridized with the probe under high stringency conditions. The probe hybridized strongly to single DNA fragments of about 23 and 2.5 kb in EcoRI- and HindIII-digested DNA, respectively, indicating that the \( \text{PoPOD1} \) gene exists as a single copy in the poplar genome. The two bands of about 9 and 7 kb in BamHI-digested DNA may reflect an internal BamHI site within the intronic region of the \( \text{PoPOD1} \) gene because there is no restriction site for the enzyme in the cDNA.

**Tissue-specific expression of \( \text{PoPOD1} \) gene**

The mRNA levels of \( \text{PoPOD1} \) were determined in various tissues including leaves, stems, roots, flowers and cell cultures. We found that \( \text{PoPOD1} \) is expressed only in suspension cultured cells (Figure 3). This finding is in agreement with previous studies showing that the expression of many peroxidase genes is tissue specific (Hiraga et al. 2000a, Tognolli et al. 2002, Welinder et al. 2002, Valerio et al. 2004). It has been suggested that peroxidase gene expression changes when the organ is subjected to stress such as during suspension culture. In sweet potato (\( \text{Ipomoea batatas} \)), expression of two peroxidase genes was high in calli and suspension cells, but lower in stem and undetectable in roots and leaves (Huh et al. 1997). Differential expression of peroxidase genes in cell suspension culture compared with the tissue from which the culture originated can be further explored in Arabidopsis (Welinder et al. 2002). Many of the peroxidase genes of Arabidopsis expressed in roots growing under standard conditions were down-regulated in cultured root cells. In our study, the \( \text{PoPOD1} \) gene was highly expressed in leaf cells grown in sus-
pension culture, whereas its expression was not detected in leaf tissue. Thus, PoPOD1 has a unique pattern of gene expression that has not been reported for other peroxidase genes. Our cells represent an unselected population derived from leaf tissue and thus differ from those of sweet potato selected for high yield of peroxidase (Kwak et al. 1994). Legendre et al. (1993) reported that suspension cultures could be stimulated to produce hydrogen peroxide simply by stirring the suspension vigorously. Therefore, we suggest that expression of the PoPOD1 gene in our cell suspension cultures is triggered by environmental factors such as mechanical shearing stress during suspension culture. This suggestion is supported by our findings that PoPOD1 expression is highly induced by high-speed agitation in suspension culture and by wounding of leaf tissues (see Figures 5 and 7).

Expression of PoPOD1 gene during cell growth

Expression of PoPOD1 during the normal growth of poplar leaf cells in suspension culture was identified by northern blot analysis. The growth kinetics of the culture was determined by monitoring cell mass at 2-day intervals following subculture. Cell growth reached stationary phase at 22 days after subculture (DAS) (Figure 4A). As shown in Figure 4B, PoPOD1 was highly expressed at 2 DAS, but expression was moderate during the remaining growth period. The temporal increase in the expression of PoPOD1 at 2 DAS is thought to be associated with fresh medium stress, i.e., a dilution effect (Huh et al. 1997).

Expression of PoPOD1 gene in response to plant growth regulators treatments

The mechanisms underlying the regulation of the peroxidase genes are complicated and individual genes of this family may have their own regulation (Agrawal et al. 2002). Transcript level of PoPOD1 was monitored in cells growing in suspension culture in the presence of growth regulators, heavy metals, other ions, or various osmotica. Figure 5A shows the expression patterns of PoPOD1 in response to exogenously applied physiological concentrations of various plant growth regulators. Abscisic acid (20 μM), which regulates many plant responses to water-limiting stresses, and SA (20 μM), which controls the systemic acquired plant pathogen response, did not affect the steady-state levels of PoPOD1 transcripts. However, JA (10 μM), which controls plant responses to wounding and pathogen attack, down-regulated the expression of PoPOD1, as did GA3 (20 μM). Down-regulation of peroxidase genes by addition of exogenous plant growth regulator has been reported previously. In rice, expression of a JA-inducible peroxidase gene, OsPOX, was strongly suppressed by ABA (Agrawal et al. 2002). Moreover, ABA treatment led to a strong reduction in expression of four peroxidase genes in sweet potato. However, unlike most wound-inducible genes that are induced by JA, PoPOD1 was down-regulated by JA. Similarly, in tobacco (Nicotiana tabacum L.), the peroxidase gene (pox) transcript accumulated rapidly and systemically after wounding, and expression of the pox gene was unaffected by SA, but suppressed by methyl jasmonate. Thus, these results suggest the presence of a novel signal transduction pathway for wound-induced expression of the peroxidase gene (Hiraga et al. 2000b, 2001).

Expression of PoPOD1 gene in response to heavy metals and abiotic stress

To determine the expression of PoPOD1 in response to metals, poplar leaf cells growing in suspension culture were treated with high concentrations of various metals (Figure 5B). PoPOD1 expression response varied greatly with the metal supplied. The steady-state levels of PoPOD1 transcripts were significantly decreased in aluminum- (AlCl3·6H2O), copper- (CuSO4·5H2O) and lead-treated (Pb(NO3)2) cells, but not in...
Peroxidase activities of dwarf bean (*Phaseolus vulgaris* L.) and *Arabidopsis* were increased by treatments with zinc and cadmium, respectively (Assche et al. 1988, Drazkiewicz et al. 2004); however, peroxidase activity of horseradish (*Armoracia rusticana* L.) hairy roots was inhibited by heavy metal treatments (Nepovim et al. 2004). In *Arabidopsis*, expression of the gene coding for peroxidase was up-regulated by iron deficiency (Thimm et al. 2001). Thus, although the evidence indicates that plant peroxidases are involved in heavy metal stress responses, the precise mechanisms remain unclear.

To determine the abiotic stress responses of *PoPOD1*, poplar cells were treated with NaCl, MV, PEG and high-speed agitation (Figure 5C). *PoPOD1* expression was down-regulated by NaCl, MV and PEG, which induce the generation of ROS in plant cells (Donahue et al. 1997, Cazale et al. 1998). Vigorous stirring of the cell suspension cultures also causes ROS production possibly by increased contact of cells in the culture (Legendre et al. 1993, Yahraus et al. 1995, Cazale et al. 1998). In our cell suspension cultures subjected to 300 rpm of mechanical stress, the *PoPOD1* transcript level increased within 30 min of the start of treatment, but returned to a moderate level at the end of the 6-h treatment.

Expression of *PoPOD1* gene in response to a bacterial-elicitor and wounding treatments

Poplar *PoPOD1* has the highest homology to a bacterial-induced peroxidase gene from cotton (GenBank Accession No. AF155124), and JA, which controls plant responses to wounding and pathogen attack, down-regulated expression of *PoPOD1*. To examine the pathogenic bacterial-related response of *PoPOD1*, poplar cells were treated with the bacterial-elicitor laminarin. Laminarin, a linear β-1,3 glucan purified from the brown alga *Laminaria digitata*, triggers defense responses in plants (Klarzynski et al. 2000). During the course of the plant–pathogen interaction, and within minutes of pathogen recognition, ion fluxes occur across the plasma membrane leading to the alkalinization of the extracellular medium (Dixon et al. 1994). As shown in Figure 6A, poplar cell suspension cultures responded to laminarin with a rapid and transient alkalinization of the incubation medium. The maximum pH shift was reached after 30 min and was followed by a gradual re-acidification of the culture medium to its original pH value by 150 min after addition of the elicitor. To determine whether laminarin treatment resulted in changes in *PoPOD1* transcript levels, RNA isolated from cultured cells was analyzed by northern blot analysis. A slightly elevated *PoPOD1* transcript level was detected after 30 min of laminarin treatment (Figure 6B). The amount of the transcripts...
peaked 5–10 h after the onset of the treatment and gradually decreased after 24 h. The increased accumulation of PoPOD1 transcripts 5–10 h after the start of the laminarin treatment suggests that the gene product may play a critical role in plant defense system against microbial infection. It is well known that, within a few hours after pathogen recognition, a broad spectrum of metabolic modifications, such as accumulation of antimicrobial activities including pathogenesis-related proteins and phytoalexins, are followed by a variety of early events such as alkalization (Kombrink and Somssich 1995).

To analyze the response of PoPOD1 to wounding, leaf discs (9 mm diameter) were floated in petri plates containing MS medium (Figure 7A). No PoPOD1 transcript was detected within 3 h of the wounding treatment, in agreement with previous findings that healthy leaves contain no PoPOD1 (Figure 3). Expression of PoPOD1 was induced within 6 h of the wounding treatment. Expression gradually increased up to 48 h and then decreased. Wounding intact attached leaves induced transcript accumulation comparable with that in the leaf discs (Figure 7B).

In conclusion, we isolated a peroxidase gene, named PoPOD1, from poplar cells grown in suspension culture. PoPOD1 is present as a single copy in Populus alba × P. tremula var. glandulosa and showed cell-culture-specific expression. An unusual characteristic of PoPOD1 expression is that it is down-regulated by various treatments including some metals, NaCl, MV and PEG, and the plant growth regulators, JA and GA3. However, expression of the gene is significantly up-regulated by the bacterial-elicitor laminarin and by wounding. Thus, PoPOD1 gene expression is sensitively and specifically regulated at the transcription level, and JA or GA3, or both, may be involved in regulation of PoPOD1 expression in poplar cells.

Peroxidases are involved in plant cell wall cross-linking reactions via control of hydrogen peroxide availability (Passardi et al. 2004). These reactions are triggered by external factors such as wounding and pathogen interactions, indicating that rigidification of the cell wall mediated by peroxidases facilitates formation of a barrier to an invading pathogen. The involvement of PoPOD1 in plant defense against pathogen attack is supported by the observations that its expression is highly induced by the bacterial-elicitor laminarin and by wounding, and is also regulated by JA.

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References


