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ORIGINAL ARTICLE

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Transcriptional and translational analyses of poplar anionic peroxidase isoenzymes

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Abstract Anionic peroxidases have been proposed to be a key enzyme for lignification in poplar. On the other hand, there are many genes encoding an anionic peroxidase in *Populus trichocarpa* genome, and their physiological functions are still unknown. Ampholine isoelectric focusing analysis revealed anionic peroxidases as dominant peroxidases in enzyme preparations from various organs. Using two-dimensional electrophoresis (2-DE) followed by peptide mass fingerprint (PMF) analysis, we surveyed the localization of anionic peroxidase isoenzymes in various organs of *Populus alba* L. Peroxidase isoenzymes were extracted from various organs and fractionated by a Concanavallin A Sepharose column. Each protein was separated by 2-DE gels and some anionic peroxidase isoenzymes in each organ were identified via PMF analysis. Transcript and protein of individual peroxidase indicate that the expression profile of each isoenzyme is quite different, for example, organ-specific gene, stress-response gene, and multifunction gene, even though they are in the same cluster. These results suggest that individual anionic isoenzymes in this small cluster were differently regulated at transcription, translation, or posttranslation.

Key words Anionic peroxidase · Lignification · Poplar · Real-time PCR · Two-dimensional gel electrophoresis

Introduction

One of the most studied functions of plant peroxidases is their role in lignification. Extensive studies, so far, have been carried out to identify whether some specific peroxidase isoenzymes are correlated with lignification in many plants, especially tobacco and poplar.[1–6] In these studies, anionic peroxidases have been discussed as being responsible for lignification because they have been often found as highly transcribed peroxidase genes in the developing xylem or as dominant peroxidase proteins in the enzyme preparation from the same organ.[3–6] One of anionic peroxidases, *Populus kitakamiensis prxA3a* gene, has been reported as a highly transcribed and translated gene in the developing xylem.[3,4] Li et al.[3] generated transgenic poplar trees (*Populus sieboldii* × *Populus gradidentata*) by an antisense strategy with a *prxA3a* gene and achieved a 20% reduction in lignin content of the transgenic plant at maximum when compared with control trees. These studies have proposed that anionic peroxidases are key enzymes in lignification.[3–6]

On the other hand, it has been known for a long time that plant peroxidases exist as a large family of isoenzymes in plants; for instance, 73 peroxidases and 138 peroxidase genes have been identified throughout the *Arabidopsis* genome and the *Oryza sativa* genome, respectively.[7,8] Very recently, the genomic sequence of *Populus trichocarpa* was completed by the United States Department of Energy (USDOE) Joint Genome Institute,[9] and the preliminary annotation data are available to the public (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). In our laboratory, at least 82 sequences encoding complete peroxidase protein were found with manual curation within the database (unpublished data). It was also found that 8 genes were highly similar to *prxA3a* among them. Therefore, it is of interest to investigate whether physiological functions of these isoenzymes are the same as *prxA3a*, because protein structure and substrate specificity of these isoenzymes are presumed to be highly similar.

Recently, some studies on *Arabidopsis thaliana* and *Oryza sativa* peroxidases were performed using the microarray and reverse-transcribed polymerase chain reaction (PCR) technique.[7,10,11] High density microarray analyses provide qualitative and semiquantitative data for large numbers of mRNAs;[12] however, the levels of gene expression do not always directly reflect active enzyme levels.[13] Enzyme activ-

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ity is often regulated through posttranslational modifications such as phosphorylation and acylation or through protein sorting, protein–protein interactions, and/or controlled proteolysis.[14] This is also true of plant peroxidases.[10] Therefore, the double expression analyses of proteins and transcripts will afford better understanding of the function of peroxidase gene expression.

In this study, we investigated protein and transcript level of the individual anionic peroxidase isoenzyme that is highly similar to *prxA3a* in various organs in the poplar tree to estimate the physiological function of these peroxidases.

Materials and methods

Plant materials

Poplar (*Populus alba* L.) callus was induced and developed on Murashige and Skoog basal medium supplemented with 3% sucrose, 1.0 ppm 2,4-dichlorophenoxyacetic acid, 0.5 ppm kinetin, and 0.8% agar. The callus was maintained on the medium at 25°C in darkness.[15] The poplar tree (*P. alba*) grown at Kyushu University was used for experiments of peroxidase gene cloning, transcription, and two-dimensional electrophoresis (2-DE) analyses.

Cloning of poplar peroxidases

Total RNAs from xylem, shoot, leaf, bark, petiole, and stress-treated leaves were prepared by Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized in a final volume of 20 µl that included 1 µg of total RNA, 1 µM oligo-dT 18-mer primer, 10 units of RNase inhibitor, and 10 units of AMV Reverse Transcriptase (Takara, Japan) according to the manufacturer's instructions. The PCR primers were selected in the sequence region specific to each peroxidase homolog of *Populus trichocarpa*. The following oligonucleotide primers were used in the PCR reaction: *PAPO1* and *PAPO2* sense primer (5′-GAAAAATG CATCTTTCTAAGGC-3′), *PAPO1* and *PAPO2* antisense primer (5′-TGTCAACAAGACTCCATTTGG-3′), *PAPO3* sense primer (5′-CTGGTTTGGAGGATCACTTCC-3′), *PAPO3* antisense primer (5′-TTTGATCTCAATCTTGTC TTCTC-3′), *PAPO4* sense primer (5′-GAGAAAATG AAGCTTTCAAAAC-3′), *PAPO4* antisense primer (5′-CAATTTTTTCTTGGCATAGTCTGGC-3′), *PAPO5* sense primer (5′-GTTCTGTTCAAGAAGAAAAACAAT GTC-3′), *PAPO5* antisense primer (5′-CTATCTAGTG ATCGCATAGC-3′), *PAPO6* sense primer (5′-CTCATAG CATAGAATTTCCGAAGACC-3′), and *PAPO6* antisense primer (5′-ATGTCTTCTGTTCTAGCTACAGT-3′). All PCR amplifications were carried out in 30 µl of reaction mixture containing primers (0.2 µM), dNTPs (0.2 mM each), and 1 unit of Takara *Ex-Taq* polymerase.

The PCR procedure started with 5 min of denaturation at 94°C and was carried out over 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1.5 min of extension at 72°C. The PCR products were purified from the agarose gel with a GEL-M kit (Viogen, Japan) according to the manufacturer's instructions and were subcloned into pXcmkn12 vector. The resulting ligation product was transformed into *Escherichia coli* strain DH5α according to the manufacturer's protocols. The clones were sequenced by a dideoxy dye termination method (Thermo Sequence Cycle Sequence Kit, Amersham Bioscience, Piscataway, NJ, USA) with a LIC-4000 sequencer (Aloka, Japan). The region representing the signal peptide of the peroxidase homolog was predicted with a primary structure analysis program, SignalP (http://www.cbs.dtu.dk/services/SignalP/). The alignment was created with the GENETYX-Mac version 13. 0. 3. (Genetyx, Japan). The name of each sequence corresponds to the deduced protein of the following DNA accession numbers: PAPO1: AB206042, PAPO2: AB260988, PAPO3: AB260989, PAPO4: AB260990. PAPO5: AB260991, PAPO6: AB260992.

Stress treatment

Leaflets were cut off and subjected to wounding, cadmium, and H_2O_2 treatments. For wounding treatment, fully expanded leaves of healthy poplar plants were detached and immediately wounded with a needle-point holder. Wounded leaves were put on wet paper towel in petri dishes and incubated at 25°C in darkness.[16] For cadmium treatment, leaves were submerged in 1 l of aqueous 1 mM $CdCl_2$ solution at 25°C in darkness. For water treatment, leaves were submerged in 1 l of water at 25°C in darkness. For H_2O_2 treatment, leaves were submerged in 1 l of aqueous 200 mM H_2O_2 at 25°C in darkness.[17] All stress treatments were conducted for 1, 5, and 10 h, respectively, and the leaflets were then frozen in liquid nitrogen. For the control, leaflets, inner bark, petioles, shoots, and xylem were harvested, then immediately frozen in liquid nitrogen. In the preparation of xylem, bark was removed from the stem and thin flakes (thickness <1 mm) were whittled from the surface of the xylem using a feather knife.

Preparation of glycoproteins

Xylem, petiole, leaf, and inner bark organ were ground with liquid nitrogen and homogenized in 50 mM Tris-HCl buffer (pH 7.5). After centrifugation at 20000 *g* at 4°C for 30 min, the supernatant was salted out with ammonium sulfate with 80% (w/v) saturation. The protein precipitate was collected by centrifugation (20000 *g* for 30 min), then dissolved in 50 mM Tris-HCl buffer (pH 7.5). After centrifugation at 40000 *g* at 4°C for 30 min, the supernatant was desalted with a PD-10 column (Amersham Bioscience). The glycoproteins were separated by a Concanavallin A Sepharose column with the 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and 0.2 M α-methylglycopyranoside. Finally, purified glycoproteins were desalted by a PD-10 column with 50 mM Tris-HCl buffer (pH 7.5) and concentrated via

ultrafiltration (molecular weight cutoff <10000, Advantec, Japan).

The solution was heated at 80°C for 5 min and insoluble material was removed by centrifugation (15000 *g* for 30 min). Four volumes of cold acetone (–20°C) were added and the solution was incubated overnight (–20°C). After centrifugation (15000 *g* for 30 min), the precipitate was washed with cold acetone (–20°C), and the pellet was solubilized in 2-DE solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 0.5% IPG buffer (pH 4.0–7.0, pH 3.0–5.0; Amersham Bioscience), and a trace of bromophenol blue. The sample was then incubated for 2 h at room temperature, and insoluble material was removed by centrifugation (15000 *g* for 30 min).

Ampholine isoelectric focusing analysis

Isoelectric focusing (IEF) gel (pH 3.5–9.5) electrophoresis was performed according to the manufacturer's instructions. The gel was mounted on a water-cooled (10°C) electrophoresis unit (2117 Multiphor II; Amersham Bioscience). Protein samples were subjected to electrophoresis at 1500 V, 30 W, and 50 mA for 90 min. Then the gel was stained with 10 mM guaiacol and 1 mM hydrogen peroxide or coomassie brilliant blue R-250 reagent at room temperature. Peroxidases were visualized and the isoelectric points were estimated by reference to the mobility of marker proteins (Amersham Bioscience). Peroxidase activity was assayed using guaiacol as a substrate according to described methods.[18]

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as previously described.[19] Isoelectric focusing was carried out with an IPGphor system (Amersham Bioscience). Immobilized pH gradient strips (pH 4.0–7.0, pH 3.0–5.6, pH 3.5–4.5, 18 cm; Amersham Bioscience) were rehydrated for 12 h, and then 200 µg of protein in 2-DE solubilization buffer was focused in four steps at 500 V (1 h), 500–1000 V (1 h), 1000–8000 V (2 h), and 8000 V (8 h). After completion of focusing, strips were equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and a trace of bromophenol blue then with a buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 2% SDS, and a trace of bromophenol blue. The strips were loaded onto precast 10% or 12% homogenous polyacrylamide gels (20 × 20 cm). The lower running buffer contained 385 mM Tris, 50 mM glycine, 0.1% SDS, and 0.02% sodium azide, while the upper running buffer was identical except it lacked sodium azide. The system was run at 1000 V and 24 mA per gel. Gel slabs were stained in 7.5% acetic acid solution with 0.02% SYPRO Red (Takara) and incubated with gentle rocking at room temperature for 1 h. After removal of the staining solution, gels were washed in 7.5% acetic acid solution for 30 min.

In-gel tryptic digestion

In-gel tryptic digestion was performed as previously described[20] with a slight modification. The target spot was excised and cut into 2-mm cubes and the gel pieces were transferred into a 1.5-ml microcentrifuge tube and washed with 40% 1-propanol at room temperature for 15 min. After removal of 1-propanol solvent, 200 mM ammonium bicarbonate in 50% acetonitrile was added, and the sample was incubated at room temperature for 15 min. The gel pieces were then dried and covered with 20 ng/µl modified trypsin (Promega, Madison, WI, USA) in a minimal volume of 100 mM ammonium bicarbonate to rehydrate the pieces; they were then incubated at 37°C. After a 12-h incubation, the supernatant was collected, and the gel pieces were extracted once with 100 mM ammonium bicarbonate, followed by two extractions with 80% acetonitrile containing 0.05% trifluoroacetic acid. The supernatant and extracts were combined and concentrated to the required concentrations.

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The resulting peptide mixtures were desalted using ZipTips C18 (Millipore, Billerica, MA, USA) and eluted onto a 96-well MALDI target plate. Samples of 2 µl were mixed on the plate with 1 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid solution in 0.1% trifluoroacetic acid in H_2O/acetonitrile (1:1). Samples were then dried at room temperature. Mass spectral data were obtained using a Voyager DE mass spectrometer equipped with a 337 nm N_2 laser in the positive ion reflectron mode (Applied Biosystems, Foster City, CA, USA). Spectral data were obtained by averaging 64 spectra, each of which was the composite of 64 laser firings. Internal mass calibration was performed using bradykinin (904.45 Da) and adrenocorticotropic hormone (ACTH) (2465.75 Da).

Construction of putative in silico peroxidase protein library

The preliminary genomic annotations of *Populus trichocarpa* were opened to the scientific community by the USDOE Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html).[9] One hundred and eight peroxidase genes were listed as candidates by the JGI site. The lists of amino acid sequences of peroxidase genes and other genes were extracted as a fasta file, after which the data were converted into a MASCOT file.

Identification of proteins

Peptide mass fingerprint (PMF) was utilized for protein identification by analyzing the size of tryptic fragments via the MASCOT search engines (Matrix Science, Boston, MA, USA) using the entire NCBI protein database and the *Pop-*

ulus trichocarpa in silico protein library. For effective PMF analysis, it was assumed that peptides were monoisotopic, and the possibility that methionine residues were oxidized was considered. The fingerprinting method allowed for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted for matching the peptide mass values was 100 ppm. Scores greater than 65 were considered to be significant.

Real-time PCR analysis

Total RNA extraction and reverse-transcriptase reaction were performed as described in the cloning of poplar peroxidases. Real-time PCR was performed in a final volume of 10 µl with a Line Gene (Bio Flux, Japan). The SYBR *Premix Ex Taq* (Takara) was used according to the manufacturer's instructions with a final concentration of 0.2 µM for each primer. PCR amplification was performed as follows: (1) an initial denaturation at 95°C for 5 min, (2) 45 cycles, with 1 cycle consisting of denaturation 95°C for 15 s, annealing at 55°C for 15 s, and elongation at 72°C for 30 s. Amplicon specificity was verified by melting-curve analysis conducted at 65° to 95°C with stepwise fluorescence acquisition and by 2% agarose gel electrophoresis staining with ethidium bromide. No fluorescence was detected from real-time PCR amplification without a template. The PCR primers were selected in the sequence region specific to each peroxidase homolog and the PCR product was designed at a length of 240–258 bp. The following oligonucleotide primers were used for real-time PCR analysis: each primer of *PAPO1* and *PAPO2* was 5′-GTTGTTGATAGAATGAA GGCTTTG-3′ (forward) and 5′-GCTGACATTAGTGAA GCTCTC-3′ (reverse), respectively; each primer of *PAPO3* was 5′-GATAACATGAAGGCTGCAG (forward) and 5′-TACTGGTGTTGAGGCCCACGGCT-3′ (reverse), respectively; each primer of *PAPO4* was 5′-GTTGATATA ATGAAGGAAAGG-3′ (forward) and 5′-GTTGATATA ATGAAGGAAAGG-3′ (reverse), respectively; each primer of *PAPO5* was 5′-GTCGAACATTTAGCAACA GGC-3′ (forward) and 5′-TGACCAAAGTGACCGTGG CAGCG-3′ (reverse), respectively; each primer of *PAPO6* was 5′-CAAACCCCGATCCAACCTTAA-3′ (forward) and 5′-CTATCGAAGAAGGCGGCCTGG-3′ (reverse), respectively. *ACTIN* was used as a reference gene (accession number AB025795). Each primer sequence was 5′-GC CCAGAGGTCCTCTTCCAA-3′ (forward) and 5′-GGGG CTAGTGCTGAGATTTCCTTGC-3′ (reverse), respectively. Each reaction was repeated three times. The data were normalized by actin transcripts that were assumed to be constantly expressed in all organs, and the relative expression of the transcript in various stress treatments was calculated. Template DNA for a working curve was synthesized by the PCR technique. All PCR amplifications were carried out in 30 µl of reaction mixture consisting of each of the primers (0.2 µM), dNTPs (0.2 mM each), and 1 unit of Takara *Ex-Taq* polymerase. The synthesized PCR product was electrophoresed by 2% agarose gel staining with ethidium bromide. The PCR product was purified from the agarose gel with a GEL-M kit according to the manufacturer's instructions. The concentration of the PCR product was measured by spectrophotometer and used to establish a working curve.

Results and discussion

Peroxidase isoenzymes in enzyme preparations from various organs

When 2-DE followed by PMF analysis of the crude extract from poplar developing xylem was performed, over 20 proteins, such as enolase, triose-phosphate isomerase, and alcohol dehydrogenase were tentatively identified. However, no peroxidase protein was found among them (data not shown). In the Ampholine IEF followed by active staining, anionic peroxidases were found as dominant peroxidase proteins in the enzyme preparation from various organs (Fig. 1). In the inner bark organ, only anionic peroxidases were observed. Indeed, anionic peroxidases (pI 4.0–5.0) were also dominantly observed in other organs (Fig. 1). These results indicated that expression of peroxidase proteins was lower than that of major proteins (for instance, glycolytic enzymes and primary metabolism), and it appears that partial purification of peroxidase isoenzymes from crude protein preparation is needed in order to improve the detection limit for 2-DE analysis. It is known that plant peroxidases are glycoproteins. Therefore, peroxidase isoenzymes extracted from leaf, bark, xylem, and petiole were fractionated by a Concanavallin A Sepharose column. This

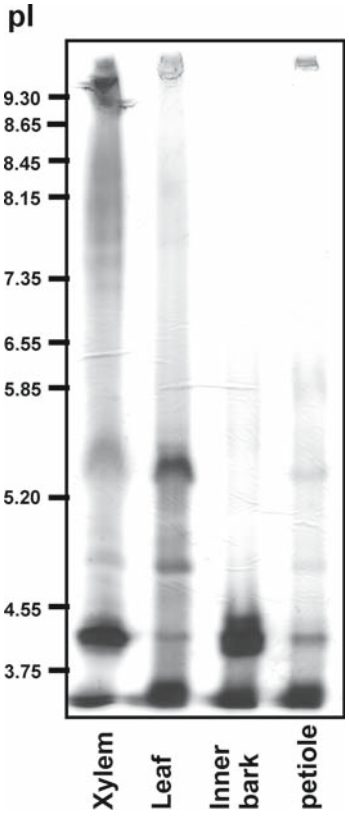


Fig. 1. Isoelectric focusing (IEF) analysis of crude proteins from various organs of *Populus alba*. Crude proteins extracted from various organs (guaiacol oxidation activity: 0.06 unit) were subjected to IEF analysis. After electrophoresis, the gel was stained with 10 mM guaiacol and 1 mM hydrogen peroxide at room temperature. Individual bands correspond to peroxidase isoenzymes

Table 1. Relative peroxidase activity of crude enzyme and ConA purified fraction (%)

Fraction	Leaf	Petiole	Bark	Xylem
Crude fraction	100	100	100	100
ConA purified fraction	90 ± 2	85 ± 5	91 ± 8	63 ± 5
ConA flow through fraction	ND	ND	ND	30 ± 3
Yield of protein	12.4	11.5	9.8	15.4

The peroxidase activity of each crude enzyme is defined as 100%
ND, not detected

Table 2. Peroxidases identified in various organs

Spot no.	Protein name	Accession number (*P. alba*)	JGI annotated gene (*P. trichocarpa*)	tpI	tMw	Coverage[a] (%)	Score
1	PAPO1	AB206042		4.03	33 944.36	38	71
2	PAPO2	AB260988		4.06	33 885.35	35	68
3	PAPO3	AB260989		4.51	33 572.05	23	90
4	Calreticulin		estExt_Genewise1_v1.C_LG_XIII0633	4.35	47 425.07	29	103

[a] Sequence coverage in peptide mass fingerprint analysis

separation step gave a concentration effect of 7.3 to 9.4 times and 90% recovery of peroxidase activity except that ca. 30% of activity in xylem did not adsorb onto the column (Table 1). However, it was confirmed that the main isoenzymes from xylem were recovered in the purified fraction in the Ampholine IEF analysis (data not shown). The fractionation by using a Concanavallin A Sepharose column appears to be appropriate for the concentration of peroxidases from crude enzyme preparations as a prefractionation procedure.

In Fig. 2a, high expression of spots nos. 1 and 2 were detected in callus. Individual spots were subjected to PMF analysis. These spots were most similar to EUGENE3.00010103 or GRAIL3.0116010201 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) of *Populus trichocarpa* peroxidases. However, the score of PMF was still low via a MASCOT search. Therefore, cDNAs of these peroxidase isoenzymes of *Populus alba* were cloned. The cluster containing EUGENE3.00010103 and GRAIL 3.0116010201 consisted of anionic peroxidase genes (EUGENE3.00010103, GRAIL3.0116010201, ESTEXT_GENEWISE1_V1.C_LG_III2714, ESTEXT_GENEWISE1_V1.C_LG_III2066, ESTEXT_GENEWISE1_V1.C_LG_XVI1838, EUGENE3.00031890, ESTEXT_FGENESH1_PG_V1.C_LG_I0098, and EUGENE 3.00010106). Based on the their DNA sequence we designed specific primers for each peroxidase homolog of *P. trichocarpa*, and six cDNAs encoding mature anionic peroxidases were cloned from *P. alba*.

Again, the PMF data of the protein spots were subjected to the MASCOT search of our peroxidase protein library including the cloned *P. alba* peroxidases. Spots nos. 1 and 2 were identified as the PAPO1 protein and PAPO2 protein, respectively, by PMF analysis (Table 2). Although PAPO1 and PAPO2 proteins were highly similar to each other (97.3% homology at amino acid level), MALDI-TOF-MS analysis was able to clearly detect the different molecular weights of the tryptic peptides (PAPO1: 150-DAANAFL***L***PPTLTLDQLR-167, M_w = 1969.25; and PAPO2: 150-DAANAFL***P***PPTLTLDQLR-167, M_w = 1953.20) from two peroxidases, respectively. In bark and petiole, the 2-DE profiles were nearly equivalent (Fig. 2b, c), and PAPO1 and PAPO2 proteins were identified (Table 2). In the leaf, the 2-DE profile was slightly different from the profiles of bark and petiole (Fig. 2b, c, d), and the PAPO1 protein was detected but not the PAPO2 protein. The 2-DE profile of xylem (Fig. 2e) was very different from those of other organs. The PAPO1 protein was also detected in the xylem, but its expression level was obviously lower than in the other organs (Fig. 2b, c, e). The PAPO1 protein was expressed in all organs. On the other hand, the PAPO2 protein was observed in the bark and petiole. *PAPO1* and *PAPO2* were highly similar to the *PXP3-4* in *P. trichocarpa* [accession number X97350, 94.6% homology at nucleotide level (*PAPO1* vs *PXP3-4*), 94.3% homology at nucleotide level (*PAPO2* vs *PXP3-4*)]. *PXP3-4* was reported as syringaldazine-oxidizing peroxidase among poplar xylem peroxidases.[5,6] The transcript of *PXP3-4* in xylem and bark is highest among other organs.[6] The high transcription in bark was consistent with the expression profile of PAPO2. *PAPO1* and *PAPO2* were also highly similar to the *PCY 2-6* gene in *Populus nigra* [accession number D83224, 97% homology at nucleotide level (*PAPO1* vs *PCY2-6*), 96.7% homology at nucleotide level (*PAPO2* vs *PCY2-6*)]. Kato et al.[21] investigated the transcriptional localization of *PCY2-6* in *P. nigra* xylem and phloem by in situ hybridization. The mRNA was observed in the phloem and inner bark, and the researchers speculated that *PCY2-6* might be involved in bark development.[21] In the 2-DE analysis, PAPO2 protein was observed as a highly expressed peroxidase in the bark and petiole compared with other organs. This observation is consistent with the study of Kato et al.[21] Therefore, *PAPO2* might be involved in suberization.

In the 2-DE analysis, the PAPO3 protein was detected only in the xylem (Fig. 2e, Table 2), suggesting that *PAPO3* might be one of the lignification-specific peroxidase isoenzymes. The result of NCBI blast search showed that *PAPO3*

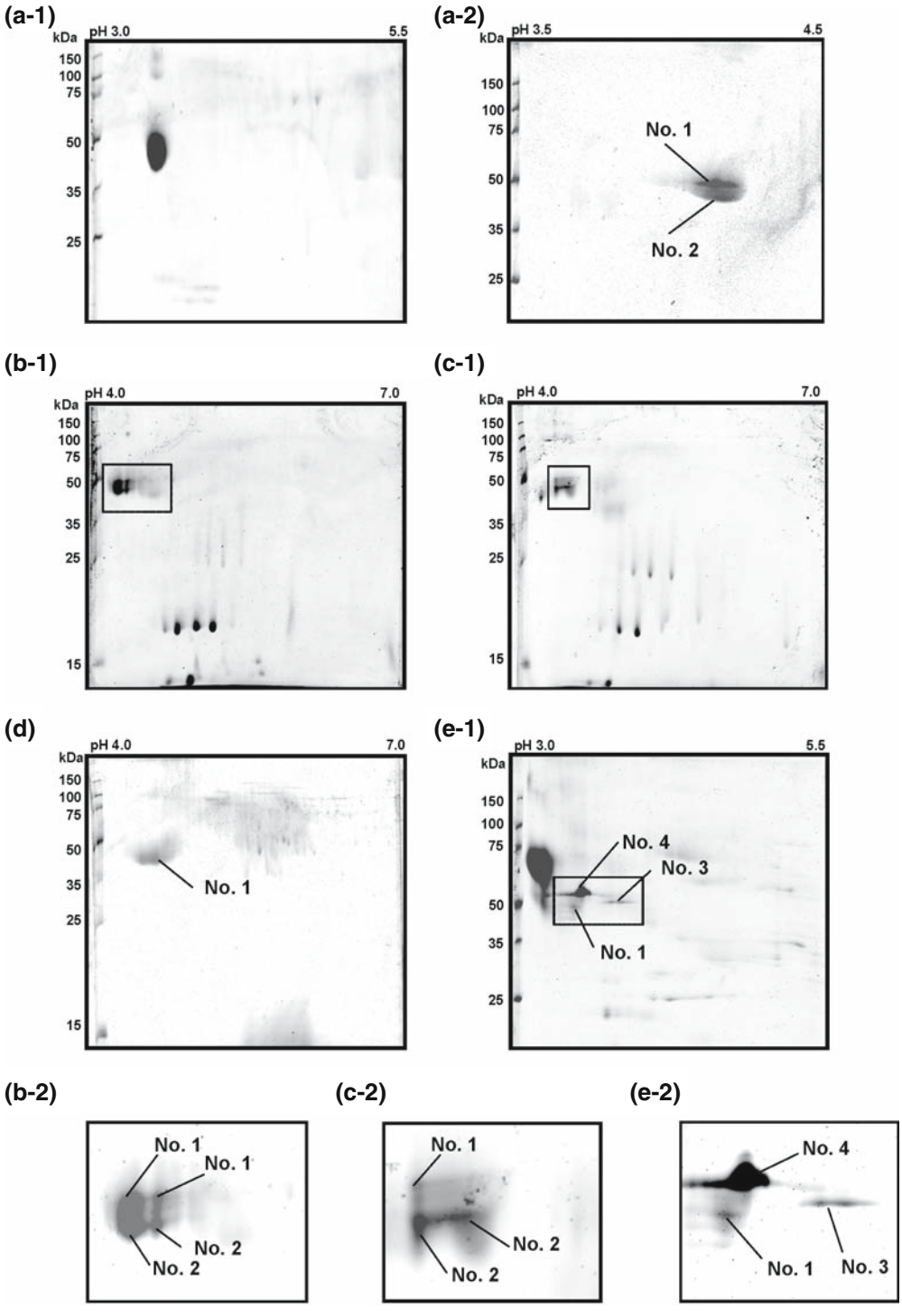


Fig. 2.a–e. Two-dimensional electrophoresis of glycoproteins of various organs of *Populus alba*. **a** Glycoproteins of callus (*a-1*, 200 µg; *a-2*, 50 µg). **b** Glycoproteins of petiole (*b-1*, 200 µg; *b-2*, inset from b-1). **c** Glycoproteins of inner bark (*c-1*, 200 µg; *c-2*, inset from c-1). **d** Glycoproteins of leaf (200 µg). **e** Glycoproteins of xylem (*e-1*, 200 µg; *e-2*, inset from e-1). Proteins (*Nos. 1–4*) were identified by peptide mass fingerprint analyses (see Table 2)

is highly similar to the *prxA3a* gene from *Populus kinakaminesis* (accession number D38050, 93.9% homology at nucleotide level). Li et al.[3] generated transgenic poplar trees (*Populus sieboldii* × *Populus gradidentata*) by an antisense strategy with a *prxA3a* gene under the control of the original promoter. The peroxidase activities were lower in transgenic lines than in the control plants. In addition, lower lignin content and modified lignin composition was associated with the reduction of peroxidase activity.[3] Li et al.[3] also discussed that *prxA3a* would be involved in the lignification of xylem. Takeuchi et al.[22] investigated the localization of the PrxA3a protein in differentiating poplar xylem, and found that the protein localizes in the cells in the early stage of secondary wall formation. Thus, the peroxidase encoded by *prxA3a* was proposed to be involved in the early stage of lignification.[22] NCBI blast search showed that *PAPO3* is highly similar to *PXP1* in *P. trichocarpa* (accession number X97348, 92.6% homology at nucleotide level).[6] PXP1 protein was found as the dominant protein in enzyme preparations from xylem,[5] which is consistent with our study (Fig.

2e). The physiological function of *PXP1* was not discussed in the previous reports;[5,6] however, these three highly similar genes, *prxA3a*, *PXP1*, and *PAPO3*, appear to be involved in lignification.

In the xylem, spot no. 4 protein was identified as calreticulin. The plant calreticulin is often *N*-glycosylated.[23] Calreticulin participates in the folding newly synthesized proteins and glycoproteins. It is an important component of the calreticulin/calnexin cycle and the quality control pathways in the endoplasmic reticula.

Transcript profile of identified peroxidase genes

Three peroxidase isoenzymes were identified by 2-DE followed by PMF analysis and they belong to a small cluster, although the cluster contains at least six peroxidase genes. It is, therefore, of interest that the transcriptional regulation of the peroxidase genes in this cluster and transcription of these peroxidases in various organs and under the several stress conditions were investigated by real-time PCR analysis. Because *PAPO1* and *PAPO2* are very similar, specific primers were not able to be designed to amplify these transcripts separately. Thus, consensus primers for *PAPO1* and *PAPO2* were designed simultaneously. Figure 3 shows the transcription profiles of six peroxidases in this cluster.

For *PAPO1* and *PAPO2*, real-time PCR analysis indicated that the transcript in the bark and petiole was the highest among various organs. The PAPO1 and PAPO2 proteins were also detected as the major peroxidases in the bark and petiole (Fig. 2b, c). The transcript and translation of these peroxidase genes are highly consistent concerning their organ specificity. The transcript level of *PAPO3* was constant in various organs and lower than *PAPO1* and *PAPO2* (Fig. 3); however, the PAPO3 protein was a major peroxidase detected only in the xylem. The transcription profile of *PAPO4* was quite similar to that of *PAPO3* (Fig. 3), but PAPO4 protein was not detected in xylem (Fig. 2). These results suggested that the expression of *PAPO3* and *PAPO4* appears to be controlled in the translation level or posttranslation level rather than in the transcript level.

Besides lignification, many reports have suggested that plant peroxidases play a role in resistance to injury, pathogens, and oxidative stress.[24–31] Therefore, the transcription of these peroxidase genes was determined in the stress-treated leaf in order to identify which peroxidase genes are involved in the plant defense mechanism. The transcripts of *PAPO3* and *PAPO4* were decreased or unchanged by all stress treatments (Fig. 4b, c). Under the nonstressed condition, the PAPO5 and PAPO6 proteins were not detected by 2-DE analyses (Fig. 2) and their transcripts were quite low in real-time PCR analysis (Fig. 3). On the contrary, *PAPO5* and *PAPO6* were upregulated by wound stress or water stress (Fig. 4d, e). *PAPO5* was specifically upregulated by wounding, suggesting that this peroxidase is involved in the plant defense mechanism against injury. The transcripts of *PAPO1* and *PAPO2* were increased by hydrogen peroxide- and water-stress treatments (Fig. 4a). Figure 3 and 2-DE analysis show that *PAPO1* and *PAPO2* were highly expressed in inner bark (Figs. 2 and 3). Therefore, *PAPO1* and *PAPO2* seem to be involved in suberization and the plant defense mechanism.

The transcriptional and translational profiles of paralogous genes encoding anionic peroxidases are summarized in Fig. 5, which indicates that the expression of each peroxidase in this cluster is individually regulated. Double expression analyses identified organ-specific expression genes

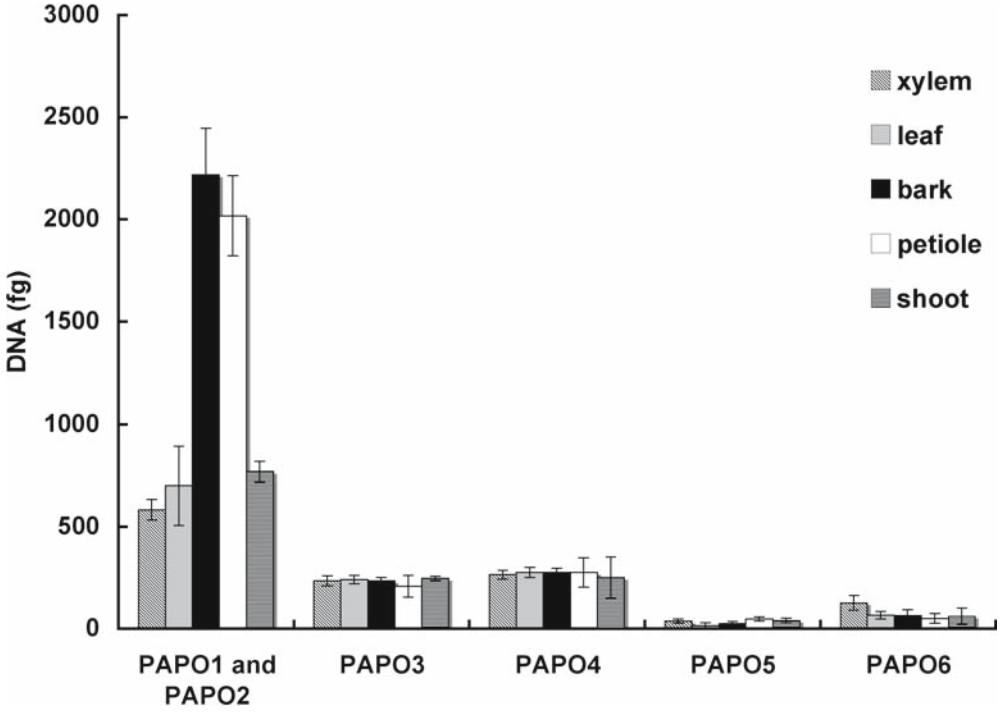
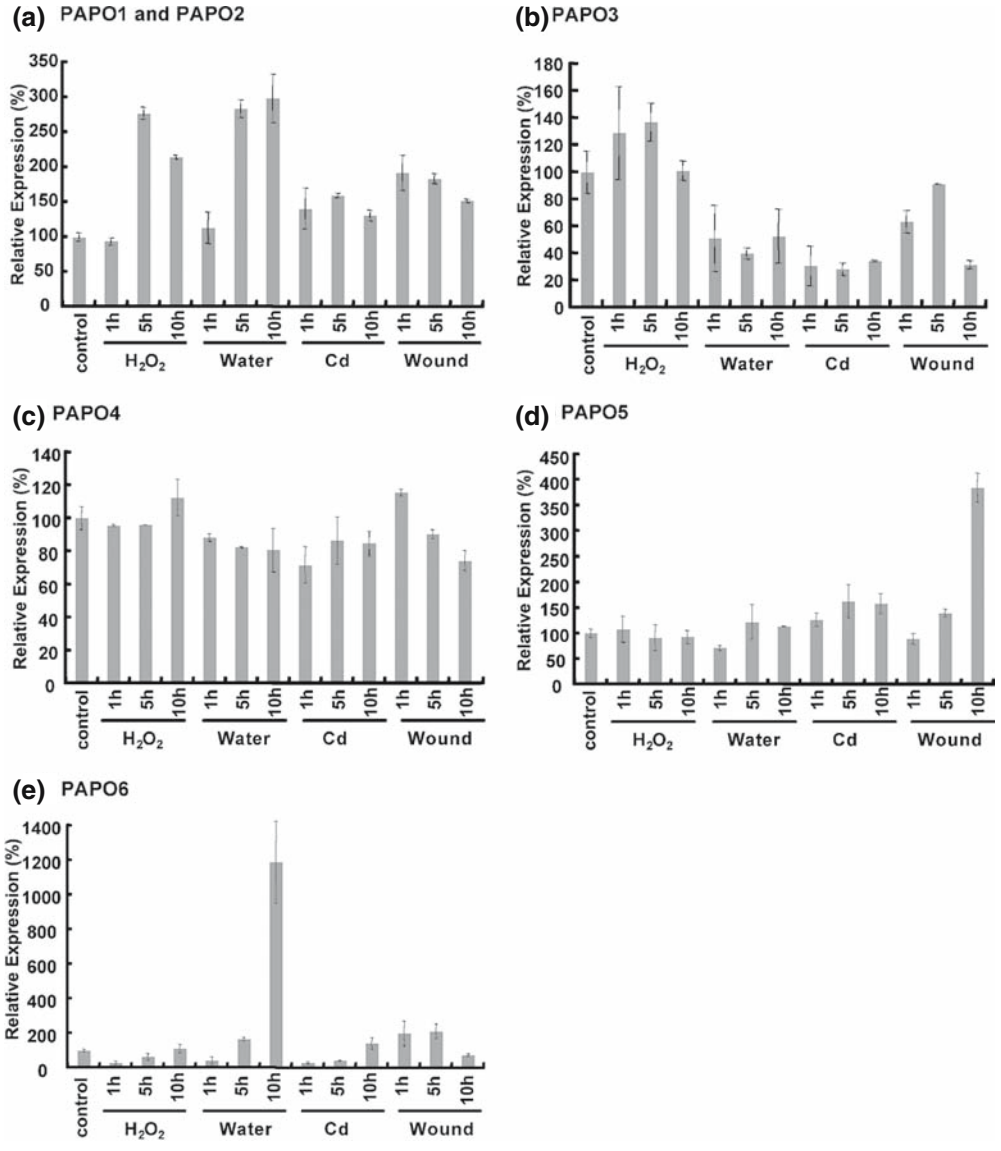


Fig. 3. The transcription profile of anionic peroxidase genes in various organs of *Populus alba.* The transcripts of the anionic peroxidase genes were quantitatively determined by real-time polymerase chain reaction (PCR). Data are given as mean ±SD ($n = 3$). The concentrations of individual DNA were calculated from a working curve



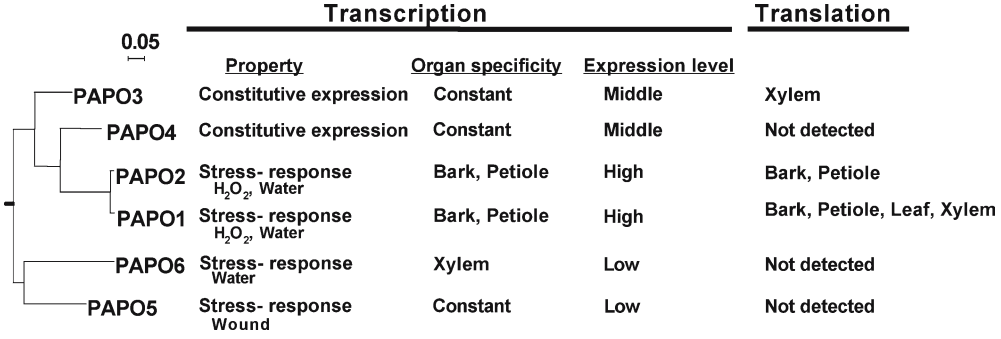


Fig. 4a–e. The transcription profiles of anionic peroxidase genes in stress-treated leaves of *Populus alba.* **a** *PAPO1* and *PAPO2*, **b** *PAPO3*, **c** *PAPO4*, **d** *PAPO5*, **e** *PAPO6*. The transcripts of the anionic peroxidase genes were quantitatively determined by real-time PCR. The data were normalized by actin transcript, which was assumed to be constantly expressed in this experiment, and the relative expression was calculated for each gene in stress-treated leaves. Data are given as mean ±SD ($n = 3$)

Fig. 5. Characteristics of the expression of six anionic peroxidases in *Populus alba*. PAPO1, PAPO2, and PAPO3 proteins were identified by PMF analyses in *P. alba. PAPO3* and *PAPO4* were constitutively expressed, and the other genes were stress-enhanced. *PAPO1* and *PAPO2* genes were highly transcribed and upregulated by hydrogen peroxide stress and water stress. The *scale bar* represents 0.05 substitutions per amino acid residues

(*PAPO3*), stress-response genes (*PAPO5*, *PAPO6*), and multifunctional genes (*PAPO1*, *PAPO2*) (Fig. 5). In this study, double expression analysis showed that positive correlations between transcript and their protein of peroxidases were not necessary. It is interesting that anionic peroxidases may have physiological diversity by means of acquiring different regulation of gene expression rather than modifying their protein structure and substrate specificity.

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References

1. Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc Natl Acad Sci USA 84:7542–7546
2. Blee KA, Choi JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP (2003) A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. Phytochemistry 64:163–176
3. Li Y, Kajita S, Kawai S, Katayama Y, Morohoshi N (2003) Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin characteristics. J Plant Res 116:175–182
4. Osakabe K, Koyama H, Kawai S, Katayama Y, Morohoshi N (1995) Molecular cloning of two tandemly arranged peroxidase genes from *Populus kitakamiensis* and their differential regulation in the stem. Plant Mol Biol 28:677–689
5. Christensen JH, Bauw G, Welinder KG, Van Montagu M, Boerjan W (1998) Purification and characterization of peroxidases correlated with lignification in poplar xylem. Plant Physiol 118:125–135
6. Christensen JH, Overney S, Rohde A, Diaz WA, Bauw G, Simon P, Van Montagu M, Boerjan W (2001) The syringaldazine-oxidizing peroxidase PXP 3-4 from poplar xylem: cDNA isolation, characterization and expression. Plant Mol Biol 47:581–593
7. Tognolli M, Penel C, Greppin H, Simon P (2002) Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene 288:129–138
8. Passardi F, Longet D, Penel C, Dunand C (2004) The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 65:1879–1893
9. Tuskan GA, and 109 authors (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313:1596–1604
10. Valerio L, De Meyer M, Penel C, Dunand C (2004) Expression analysis of the *Arabidopsis* peroxidase multigenic family. Phytochemistry 65:1331–1342
11. Welinder KG, Justesen AF, Kjaersgard IV, Jensen RB, Rasmussen SK, Jespersen HM, Duroux L (2002) Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. Eur J Biochem 269:6063–6081
12. Aharoni A, Vorst O (2001) DNA microarrays for functional plant genomics. Plant Mol Biol 48:99–118
13. Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19:1720–1730
14. Serino G, Deng XW (2003) The COP9 signalosome: regulating plant development through the control of proteolysis. Annu Rev Plant Biol 54:165–182
15. Sasaki S, Nishida T, Tsutsumi Y, Kondo R (2004) Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β-*O*-4 linkage. FEBS Lett 562:197–201
16. Sasaki K, Hiraga S, Ito H, Seo S, Matsui H, Ohashi Y (2002) Wound-inducible tobacco peroxidase gene expresses preferentially in the vascular system. Plant Cell Physiol 43:108–117
17. Nanjo T, Futamura N, Nishiguchi M, Igasaki T, Shinozaki K, Shinohara K (2004) Characterization of full-length enriched expressed sequence tags of stress-treated poplar leaves. Plant Cell Physiol 45:1738–1748
18. Tsutsumi Y, Sakai K (1994) Lignin biosynthesis in woody angiosperm tissues II. Peroxidase related to syringyl and guaiacyl lignin biosynthesis in *Populus* callus cultures. Mokuzai Gakkaishi 40: 744–750
19. Shimizu M, Yuda N, Nakamura T, Tanaka H, Wariishi H (2005) Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. Proteomics 5:3919–3931
20. Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P (1992) In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem 203:173–179
21. Kato Y, Sakuma Y, Azuma T, Ando A, Miura K, Takabe K, Tamai Y (2001) Isolation and characterization of a novel anionic peroxidase cDNA found in poplar (*Populus nigra*) suspension cultured cells. J Wood Sci 47:165–170
22. Takeuchi M, Takabe K, Fujita M (2005) Immunolocalization of an anionic peroxidase in differentiating poplar xylem. J Wood Sci 51:317–322
23. Navazio L, Miuzzo M, Royle L, Baldan B, Varotto S, Merry AH, Harvey DJ, Dwek RA, Rudd PM, Mariani P (2002) Monitoring endoplasmic reticulum-to-Golgi traffic of a plant calreticulin by protein glycosylation analysis. Biochemistry 41:14141–14149
24. Bestwick CS, Brown IR, Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 118:1067–1078
25. Bolwell GP, Butt VS, Davies DR, Zimmerlin A (1995) The origin of the oxidative burst in plants. Free Radical Res 23:517–532
26. Bradley DJ, Kjellbon P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70:21–30
27. Kawaoka A, Matsunaga E, Endo S, Kondo S, Yoshida K, Shinmyo A, Ebinuma H (2003) Ectopic expression of a horseradish peroxidase enhances growth rate and increases oxidative stress resistance in hybrid aspen. Plant Physiol 132:1177–1185
28. Kristensen BK, Bloch H, Rasmussen SK (1999) Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens. Plant Physiol 120:501–512
29. Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5:9–23
30. Stoessl A (1967) The antifungal factors in barley. IV. Isolation, structure, and synthesis of the hordatines. Can J Chem 45: 1745–1760
31. Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322:681–692