Plant Mol Biol (2006) 61:719–732
DOI 10.1007/s11103-006-0043-6

Cloning, characterization and localization of three novel class III peroxidases in lignifying xylem of Norway spruce (*Picea abies*)

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Received: 27 January 2006 / Accepted: 9 March 2006

Abstract Plant class III peroxidases (POXs) take part in the formation of lignin and maturation of plant cell walls. However, only a few examples of such peroxidases from gymnosperm tree species with highly lignified xylem tracheids have been implicated so far. We report here cDNA cloning of three xylem-expressed class III peroxidase encoding genes from Norway spruce (*Picea abies*). The translated proteins, PX1, PX2 and PX3, contain the conserved amino acids required for heme-binding and peroxidase catalysis. They all begin with putative secretion signal propeptide sequences but diverge substantially at phylogenetic level, grouping to two subclusters when aligned with other class III plant peroxidases. In situ hybridization analysis on expression of the three POXs in Norway spruce seedlings showed that mRNA coding for PX1 and PX2 accumulated in the cytoplasm of young, developing tracheids within the current growth ring where lignification is occurring. Function of the putative N-terminal secretion signal peptides for PX1, PX2 and PX3 was confirmed by constructing chimeric fusions with EGFP (enhanced green fluorescent protein) and expressing them in tobacco protoplasts. Full-length coding region of *px1* was also heterologously expressed in *Catharanthus roseus* hairy root cultures. Thus, at least the spruce PX1 peroxidase is processed via the endoplasmic reticulum (ER) most likely for secretion to the cell wall. Thereby, PX1 displays correct spatiotemporal localization for participation in the maturation of the spruce tracheid secondary cell wall.

Keywords Lignin biosynthesis · Class III plant peroxidases · *Picea abies* · Gymnosperm · In situ hybridization · Secretion signal peptide · GFP · Endoplasmic reticulum

Abbreviations

AP	alkaline phosphatase
DEPC	diethylpyrocarbonate
DIG	digoxigenin
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FBPA	fructose-1,6-bisphosphate aldolase
IEF	isoelectric focusing
ORF	open reading frame
PBS	phosphate-buffered saline
POX	class III plant peroxidase
RT	room temperature
SS	signal sequence
UPM	Universal Primer mix

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Introduction

Class III plant heme peroxidases (POXs, EC 1.11.1.7) belong to a large family of plant secretory enzymes catalyzing oxidoreduction between a variety of phenolic sub-

strates and hydrogen peroxide (Welinder 1992). POXs are found from cell walls and vacuoles and they have been implicated in many physiological processes such as construction of the plant cell wall, stress responses and auxin metabolism (Hiraga et al. 2001). The catalytic mechanisms and structures are well characterized for several POX variants (Schuller et al. 1996; Gajhede et al. 1997; Smith and Veitch 1998; Østergaard et al. 2000) and recent molecular biology approaches have produced much information about structural diversity and expression patterns of POXs e.g. in thalegrass (*Arabidopsis thaliana*) and rice (*Oryza sativa*) (Welinder et al. 2002; Passardi et al. 2004). However, the precise functions for numerous POXs are only vaguely addressed or not known at all.

One of the earliest proposed functions of POX is oxidation of lignin monomers (monolignols) in lignin polymerization (Harkin and Obst 1973). Lignin is a branched, natural plant polymer generated by radical coupling of hydroxycinnamyl subunits, mainly coniferyl, sinapyl and *p*-coumaryl alcohols, and creates together with hemicelluloses a gluing matrix for cellulose microfibrils in the secondary cell walls of higher plants (Boerjan et al. 2003). Lignin provides mechanical strength and resistance against pathogens, and makes the cell walls impermeable to water, thus enabling transport of solutes via tracheids in the xylem tissue.

So far, it has been indicated that both anionic (Diaz-De-Leon et al. 1993; Christensen et al. 1998, 2001; Østergaard et al. 2000, Li et al. 2003) and cationic POXs (El Mansouri et al. 1999; Quiroga et al. 2000; Talas-Oğras et al. 2001; Blee et al. 2003; Koutaniemi et al. 2005; Gabaldón et al. 2005) play a role in xylem lignin formation. However, only a few POXs from gymnosperm tree species, such as spruces and pines, have been described (Tarkka et al. 2000; Fossdal et al. 2001; Koutaniemi et al. 2005), although they produce a large portion of the biomass on Earth in the form of highly lignified wood and are an important source of raw material both for construction and paper manufacture.

In gymnosperm trees, lignin consists mainly of guaiacyl (coniferyl) subunits linked by a series of ether and carbon–carbon bonds to form a heterogenous network (Higuchi 1997). It is likely that the amount and chemical properties of lignin in woody cell walls may be converted by affecting the expression, quantity and activities of specific plant POXs (see also Whetten et al. 2001). For this purpose, isolated xylem POXs and the respective genes must be studied to exclude the numerous related peroxidases and phenol oxidases possessing other specific roles and locations in plant cells.

Previously, we have studied the involvement of POXs to lignin biosynthesis in a gymnosperm tree species, Norway spruce (*Picea abies*) by following POX activities and isoform profiles in relation to developmental lignification, and by studying the monolignol substrate specifities of several spruce xylem POX fractions (Marjamaa et al. 2003, 2006). These studies have shown that there are several anionic and cationic coniferyl alcohol oxidising POX isoforms present in xylem extracts of Norway spruce during the period of xylem differentiation. The high number and similar catalytic properties of spruce xylem POXs emphasize the importance of gaining information at molecular level, in particular, on their spatiotemporal localization, to better determine their specific cellular functions.

In this study our aim was to find out which of the Norway spruce xylem POXs have potential for participation in the lignification of the tracheid cell wall, i.e. correct localization and substrate specificity. Here we report the cDNA cloning, characterization and localization studies of three new xylem-expressed Norway spruce POXs, PX1, PX2 and PX3. Tissue specific expression of the genes *px1, px2* and *px3* was studied by in situ hybridization in spruce seedlings and the function of the putative N-terminal secretion signal sequence (SS) propeptides was verified by transiently expressing PX1-, PX2- and PX3-SS-EGFP fusions in tobacco protoplasts. According to the localization results the most promising Norway spruce POX for lignin polymerization, PX1, was studied further. In order to specify the protein product of *px1* to the POX isoforms detected in the spruce xylem protein extracts, *Catharanthus roseus* hairy root cultures expressing *px1* were generated. The transgenic hairy root protein extracts contained an additional, highly cationic POX isoform. This putative *px1* protein product is similar to the monolignol oxidizing POX isoforms detected in Norway spruce xylem during lignification (Marjamaa et al. 2006).

Materials and methods

cDNA synthesis and amplification of *px1*, *px2* and *px3*

Norway spruce (*Picea abies*) wood samples were collected in August, which is at the time of lignification of young spruce xylem cells in the South of Finland (Marjamaa et al. 2003; Kukkola et al. 2003). An approximately 30-year-old tree was felled and the cortex and phloem were peeled off. Thin longitudinal sections were then cut from the differentiating xylem with a sharp knife and immediately frozen in liquid nitrogen. RNA was extracted from the differentiating xylem sections by guanidinium isothiocyanate/CsCl method (Nehls et al. 1998). Total RNA was reverse-transcribed using the Smart® RACE cDNA Amplification kit (Clontech). The first-strand synthesis was initiated with 1 μM of 3′RACE cDNA synthesis primer (3′CDS) or 1 μM of 5′RACE cDNA synthesis primer (5′CDS) and SMART

II oligonucleotides, according to the instructions of the manufacturer, and 200 U Superscript II reverse transcriptase (Invitrogen).

Internal peroxidase cDNA fragments were amplified by degenerate primers designed corresponding to the conserved regions of POX sequences found in EMBL nucleotide data bank (sense 5′-CTI CA T/C TTT/C CAT/C GAG/A TGC-3′ and antisense 5′-GTG/A TGI G/CCI CCG/A/T/C GAT AGG GCG/A/T/C AC-3′). PCRs were carried out using 1 μl of cDNA template, 0.2 μM primers, 10 mM dNTP mixture, 1 × PCR amplification buffer, 2 mM $MgCl_2$ and 1 U Dynazyme II DNA-polymerase (Finnzymes). The following conditions were used in PCR: at 94°C for 2 min, 10 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 60 s, 10 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 60 s, 20 cycles of 94°C for 40 s, 56°C for 40 s and 72°C for 60 s, with final extension at 72°C for 5 min.

To amplify the 5′- and 3′-terminal cDNA ends of the transcripts, the Smart® RACE cDNA Amplification kit (Clontech) was used as instructed. The 5′-RACE and 3′-RACE products were amplified with gene-specific antisense and sense primers, respectively, and Universal Primer mix (UPM; Clontech) and nested universal primers. In RACE-PCR, the reagents of Smart® RACE cDNA Amplification kit (Clontech) were used. Advantage cDNA polymerase mix (Clontech) was used for amplification with following conditions: 20 cycles of 94°C for 40 s, 61°C for 40 s, and 72°C for 60 s. Amplified fragments were A-tailed with 1 U Dynazyme (Finnzymes) at 72°C for 15 min for T/A cloning.

Full-length open reading frame (ORF) cDNAs of the three peroxidase genes were amplified with gene-specific primers designed on 5′ and 3′ coding regions and untranslated regions. PCRs were carried out using 1 μl of cDNA template, 0.5 μM primers, 0.3 mM dNTP mixture, 1 × PCR amplification buffer, 1 × Enhancer solution (Invitrogen) and 1 U Platinum *pfx* DNA polymerase (Invitrogen). The following condition were used in PCR: 35 cycles of 94°C for 35 s, 50°C for 60 s ramping then 0.2°C/s up to 72°C for 120 s.

Cloning and sequencing

Amplified fragments were purified from 1% agarose gel using Concert Gel Extraction System (Invitrogen) and subcloned into pRT101 (Töpfer et al. 1987) or pCR2.1 vectors (Invitrogen). Sequencing of double-stranded plasmid DNA was automatically performed using ABI Prism 310 DNA Analyzer, and ABI BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The chromatograms were inspected using Chromas software (Technelysium Pty Ltd), nucleotide sequences were analysed and the ORFs translated using the software packages of EBI-EMBL server (http://www.ebi.ac.uk).

Protein sequence analysis

Database searches for similar amino acid sequences were performed with BLAST service of the National Center for Biotechnology Information (http://www.ncbi.nml.nih.gov). To predict the subcellular localization of the three translated peroxidase proteins SignalP (Nielsen et al. 1997) predictor (http://www.cbs.dtu.dk/services/SignalP), TargetP (Emanuelsson et al. 2000) (http://www.cbs.dtu.dk/services/TargetP/) and PSORT (http://psort.nibb.ac.jp) computer programs were used. Theoretical p*I*s and molecular masses of mature peroxidase proteins were estimated using Compute p*I*/Mw tool (http://expasy.cbr.nrc.ca/tools/) excluding the predicted N-terminal propeptides. Pairwise amino acid identities of full-length proteins were calculated from pairwise global alignments using the Needle program (CSC, Espoo, Finland), and multiple amino acid sequence alignments were performed with ClustalX using Gonnet substitution matrix of the CSC/CEDAR server bioinformatics package (CSC, Espoo, Finland). Phylogenetic tree construction using *A. thaliana* ascorbate peroxidase I (Q05431) as outgroup was performed with PHYLIP software package and Neighbour-joining, Fitch-Margoliash and Maximum-parsimony analyses at the CSC/CEDAR server.

In situ hybridization

cDNA coding regions of *px1, px2 and px3* and a 570-bp cDNA fragment of the transcript coding for Norway spruce fructose-1,6-bisphosphate aldolase (FBPA, EC 4.1.2.13, positive control) were digested from their cloning vectors as *Xba*I-*Sac*I fragments, ligated into pBluescript II SK+ vector (Stratagene) and linearized for use as a DNA templates for the synthesis of riboprobes. The positive in situ-control, referring to a constantly transcribed housekeeping gene, was obtained from the Norway spruce EST library (Koutaniemi, manuscript in preparation). Digoxigenin (DIG) labelled sense and antisense probes were synthesized with the (SP6/T7) Dig RNA labelling kit (Roche) using T7 and T3 RNA polymerases as described by Regan and Sundberg (2002). The resulting full-length DIG-labelled RNA-probes for the transcripts of *px1, px2 and px3* and the positive control gene were precipitated and redissolved in diethylpyrocarbonate (DEPC) treated water.

The two latest growth rings from 3-year-old Norway spruce seedlings growing in greenhouse conditions were used for in situ hybridization. Samples of 2 × 2 × 2 mm^2 were placed immediately in cold fixative (2% parafor-

maldehyde+1.5% glutaraldehyde in 0.05 M phosphate buffered saline (PBS), pH 7), fixed at +4°C overnight and embedded into Paraplast plus (Electron microscopy sciences, Fort Washington, USA) according to Leitch et al. (1994). Thin sections (10 μm) were cut from paraffin embedded samples using a microtome (Leica Instruments, Germany) and heat-fixed onto silanized glass slides (Electron microscopy sciences, Fort Washington, USA) for 3 days at +40°C. Paraffin was removed with Histoclear (National Diagnostics, Atlanta, Georgia, USA) treatment at room temperature (RT) for 20 min. Rehydration and pre-treatment of sections was made as described by Regan and Sundberg (2002) except sections were rinsed two times for 15 min with 0.1% DEPC in PBS at RT instead of acetylation. Before pre-hybridization, sections were dried using an ethanol series (25%, 50%, 75%, 100%) and air dried at RT for 15 min. Pre-hybridization was performed using hybridization buffer without Dextran at +58°C for 1 h. Hybridization was performed as described by Regan and Sundberg (2002) except that the RNA-probes were denatured at +80°C for 2 min. Hybridization was performed at +58°C for 20 h. Sections were hybridized with equal concentration (0.5–1 μg ml^{-1}) of either sense or antisense RNA probe.

Post-hybridization washes were carried out as described by Regan and Sundberg (2002). Sections were washed under high stringency conditions (0.3 M NaCl, 30 mM Na-citrate (2 × SSC), 30 min, 60°C) and then under low stringency conditions (0.1 × SSC, 30 min, RT). The hybridized DIG labelled RNA probes were detected using anti-DIG conjugated alkaline phosphatase (AP) and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) reaction as described by Leitch et al. (1994). Slides were mounted in glycerine gelatine and viewed with a light microscope (Zeiss Axioplan 2) and recorded using Evolution MP Digital Kit and Image Pro Plus software (Media Cybernetics).

EGFP fusion constructs and vectors

pEGFP (Clontech) was used to construct the enhanced green fluorescent protein (EGFP) expression cassettes. A fragment of the 35S promoter from cauliflower mosaic virus (CaMV) was digested from pRT101 and ligated into 5′ multiple cloning site of pEGFP. Endoplasmic reticulum (ER) retention motif HDEL (Munro and Pelham 1987; Pelham 1988) and nopaline synthase terminator (nos-ter) were amplified from pBIN m-gfp5-ER by PCR with sense (5′-ATG GAT GAA CTG TAC AAA CAT GA-3′) and 3′ anti-sense (5′-TGC GCG CAC TAG TTT GTT TTC-3′) primers using following PCR conditions: at 94°C 1 min 35 s, 35 cycles of 94°C for 35 s, 52°C for 60 s, ramping then 0.2/s up to 72°C for 120 s, with final extension 72°C for 10 min. The 250 bp fragment DNA product was ligated between the *Bsr*GI-site in the C-terminus of the EGFP encoding gene and *Spe*I-site of the 3′ multiple cloning site (Fig. 1). The 5′ signal sequences (SS) and part of the mature (N-terminal) region of the Norway spruce *px1, px2* and *px3* were amplified from full-length ORF *px1-3* cDNA clones by PCR using gene-specific sense primers and a degenerate antisense primer annealing to the site corresponding the conserved plant class III peroxidase distal active site pocket (5′-ACG TCG GTA CCT GAA CGA ACG AGT CG-3′).

As a positive control of fusion protein targetting, the DNA fragment encoding the N-terminal-SS of *A. thaliana* cationic vacuolar chitinase was amplified from pBIN m-gfp5-ER with sense (5′-GTG CGG ATC CAA TGA AGA CTA ATC TTT TTC TC-3′) and antisense (5′-GAC TCG GTA CCT GGA ATT CGG CCG AGG ATA ATG-3′) primers. The spruce *px1, px2* and *px3*, and *A. thaliana* chitinase 5′ SS-fragments were ligated in frame to the 5′ end of the *egfp* sequence in pEGFP (Fig. 1). A second set of fusion gene constructs containing the N-terminal-SS encoding 5′ sequences of the spruce peroxidases but lacking the 3′ ER-retention motif were prepared (Fig. 1). In addition, a plasmid construct without any 5′-SS insert fragment in front of *egfp* in the ER-tag fusion cassette was used as a negative control in transformations (Fig. 1).

Transformation and detection of tobacco protoplasts

For isolation of protoplasts, young leaves were harvested from greenhouse-grown tobacco plants (*Nicotiana tabacum* L.) and surface sterilized using 70% ethanol and 1.5–2% Na-hypochlorite. Protoplasts were prepared and transformed with electroporation using Gene Pulser™ (Bio-Rad) as described (Martens et al. 2002). Approximately 1.5×10^4 to 1.0×10^6 protoplasts and 3–10 μg of plasmid DNA (modified pEGFP) were used. Three to five individual transformations, with transformation efficiency of 3–10%, were done using each of the plasmid constructs.

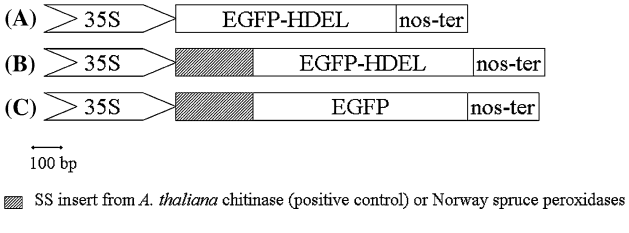


Fig. 1 35S-EGFP -constructs used in the transformation of tobacco protoplasts: EGFP lacking any N-terminal-SS (**A**); EGFP including N-terminal-SS of PX1-3 or *A. thaliana* chitinase and ER-retention motif (**B**), EGFP including N-terminal-SS but lacking ER retention motif (**C**). SS=(N-terminal) signal sequence, HDEL=ER-retention motif, nos-ter=nos-terminator.

After 24 h cultivation at 25°C in dim light, GFP fluorescence in the transformed tobacco protoplasts was detected by using confocal visualization system (Bio-Rad MRC1024) and krypton/argon laser light source mounted on a Zeiss Axiovert 135 M microscope. FITC filter block (excitation λ 488 nm, emission λ 520 nm) and 63 × magnifying oil objective were used. Confocal stack images were produced from optical sections (15–20 sections, 30–40 nm/section) using two-fold magnification and average of two images (Kalman filter). Of each transformation, at least ten GFP-expressing protoplasts were inspected and images of three of them were saved.

Expression of Norway spruce *px1* in *Catharanthus roseus* hairy roots

For expression in *Catharanthus roseus* hairy roots, the Norway spruce *px1* was introduced to the binary expression vector pK7WG2D (available from VIB-Ghent University) designed for *Agrobacterium*-mediated plant transformation (Karimi et al. 2002). Full-length coding cDNA-fragment *px1* was detached from pBluescript cloning vector as *Eco*RV-*Sac*I-fragment, blunt-ended with T4-DNA polymerase, inserted to Gateway™ pENTR 4 vector (Invitrogen) *Eco*RV-*Xmn*I-site and screened for a clone in which *px1* cDNA was in sense orientation to get an entry clone. The entry clone was used to introduce the *px1* into Gateway compatible pK7WG2D vector by site-specific recombination system (Gateway, Invitrogen). The resulting expression vector was introduced to *Agrobacterium rhizogenes* strain LBA 9402/12 by electroporation. The *A. rhizogenes* cells were grown in plates in the presence of 100 μg/ml rifampicillin, 20 μg/ml streptomycin and 100 μg/ml spectinomycin at +30 °C.

Young leaves from *C. roseus* grown in a phytotron with 16 h photoperiod at +25°C were surface sterilized by a short rinse with 70% ethanol followed by incubation in 0.6% hypoclorite for 10 min and 3 × 5 min rinses with sterile water. The sterilized leaves were infected with the relevant *A. rhizogenes* cells by gently scratching the adaxial side of the leaf with the tip of an injection needle dipped to a fresh microbial plate. The infected leaves were placed adaxial side up for co-cultivation on solid MS-medium without vitamins, 1% sucrose, 0.3% gelrite, pH 5.7 and incubated in growth chamber with 16 h photoperiod at +24°C for 16–24 h under dim light (under a sheet of facial tissue). After co-cultivation the leaves were transferred adaxial side up to fresh plates containing 500 μg/ml cefotaxime sodium and incubated further under the same conditions. The infected leaves were transferred to fresh cefotaxime plates every 2 weeks until the formation of hairy roots. Hairy roots were subcultured at 4-week intervals on half-strength Gamborg B5 medium with vitamins containing 2% sucrose and 0.2% gelrite, pH 5.8 at +24°C in darkness.

Total DNA was extracted from the hairy root cultures with Qiagen DNAeasy plant mini kit and subjected to PCR-analysis for the presence of *px1* with T-DNA boundary specific primers (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG C-3′) and (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG G-3′). The presence of RolB genes was verified with the primers (5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ and 5′-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′), whereas the absence of VirC genes was verified with the primers (5′-ATG TCG CAA GGA CGT AAG CCC A-3′ and 5′-GGT GTC TTT CAG CAT GGA GCA A-3′).

Transgenic hairy roots expressing *px1* and wild-type controls (0.5–2 g) were ground in liquid nitrogen with mortar and pestle. Root proteins were extracted with 0.1 M Na-acetate buffer pH 5 (6–12 ml) containing 10 mM $CaCl_2$, 1 Complete protease-inhibitor tablet (Roche)/50 ml buffer and 5% acid-washed PolyClarAT (Gap Corporation). Root proteins were also extracted with the same buffer solution containing 1 M NaCl in order to examine the cell-wall associated protein fraction. The root extracts were centrifuged for 30 min at 4°C at 6500 g to remove cell wall remnants and concentrated using Macro-Sep tubes (cut-off point 10 000 Da, Pall Filtron). Concentrated extracts were analyzed in isoelectric focusing gels (IEF) and peroxidases were visualized with guaiacol and hydrogen peroxide (Marjamaa et al. 2003).

Results

cDNA cloning and sequencing of the spruce xylem peroxidases

Total RNA was isolated from differentiating xylem of Norway spruce (*Picea abies*) at the time of lignification. Reverse-transcription-PCR using degenerate primers corresponding to conserved, heme-binding distal and proximal regions of POXs, yielded three amplified cDNA fragments of correct size but varying at nucleotide sequence level. To obtain full ORF clones, we used RACE-PCR to amplify the lacking 5′ and 3′ end fragments. Nucleotide sequences of three different POX encoding gene cDNAs were then reconstructed and ORF clones were amplified by PCR from cDNA using gene 5′ and 3′ end sequence specific primer pairs. The full-length cDNA clones were sequenced from both ends to verify their identity, and the ORF sequences, between 1023 and 1056 nucleotides in length (Table 1), were deposited in the EMBL Nucleotide Sequence Database under accession numbers AJ566202 (*px1*), AJ566201 (*px2*), and AJ566203 (*px3*).

Characteristics of the translated spruce xylem peroxidases

The ORF of *px1* codes for a 340 amino acid polypeptide with a predicted cleavage site LDA-QS for N-terminal signal and propeptide, as deduced by the similarity with Norway spruce SPI2 (see below), between positions 33 and 34 (Fig. 2). The long sequence suggests a 21–22 amino acid signal peptide plus an additional 11–12 amino acid pro-peptide structure for the N-terminus with a cleavage site either between TA-VA or TAV-AL. As confirmation of the assumption of peroxidase function, all the critical amino acids required for heme-binding and peroxidase catalysis are found in the translated sequence of PX1 as well as in PX2 and PX3 (marked in red in Fig. 2). PX1 differs from the two other predicted spruce POXs (PX2 and PX3) in length being the shortest and lacking a C-terminal extension. Mature PX1 protein is the most cationic (Table 1) with estimated p*I* over 9 and contains five putative *N*-glycosylation sites (marked green in Fig. 2) following the general eukaryotic pattern NXS/T, where X is not D or N (Creighton 1993). In a BLAST search PX1 showed the highest identity at protein level (72%) with the Norway spruce pathogen-induced peroxidase SPI2 (Q9SC55).

The *px2* ORF codes for a 353 amino acid polypeptide (Table 1) beginning with a predicted 20 amino acid N-terminal signal peptide with ASR-EN cleavage site (Fig. 2), suggesting the length of 333 amino acids for the mature protein. Deduced PX2 is also cationic with estimated p*I* value over 8 but longer in length than PX1 containing C-terminal extension of 22 amino acids. A potential cleavage site for the predicted C-terminal propeptide may exist between SN-LAS, leaving asparagine at the end of the mature PX2 in accordance with the carboxyl terminus of PX1. All the conserved cysteines, and the amino acids required for heme peroxidase function, as with PX1, are found in the primary structure of PX2 with six potential *N*-glycosylation sites (Fig. 2). In BLAST search, PX2 showed the highest identity (78%) with tobacco peroxidase PER9-6 (Q94IQ1). Major differences between PX1 and PX2 are the absence of a potential *N*-glycosylation site and 5–6 amino acids within the peptide loop between helices F′ and F″ after the conserved tyrosine residue at position 220 in full-length PX2.

The ORF of *px3* encodes a polypeptide of 351 amino acids with a 22 amino acid predicted N-terminal signal peptide with cleavage site RA-VH (Fig. 2). The mature protein PX3 is calculated to be acidic (Table 1). Irrespective of the high difference of estimated p*I* values, that is over three pH units, the predicted PX3 and PX2 share 58% amino acid sequence identity within full-length alignment. The translated polypeptide contains all the eight conserved cysteines for disulfide bonds, all the indispensable amino acids required for heme-binding and peroxidase function and coordination of two Ca^{2+}-ions, and up to ten potential *N*-glycosylation sites (Fig. 2). In BLAST search, PX3 shows high level of identity (80%) to the deduced Scots pine (*Pinus sylvestris*) short-root peroxidase, PSYP1 (Q9FYS6), with almost identical C-terminal pro-peptides ending with –SYSM. The major difference between the predicted PX3 and PSYP1 is observed within the eight amino acids longer N-terminus in PSYP1, including a majority of repeated cationic amino acids (MRDRR——KKK-).

In situ hybridization

In situ hybridization experiments were conducted to reveal cell-specific gene expression patterns of *px1, px2* and *px3*. The *fbpa* gene encoding fructose-1,6-bisphosphate aldolase, which is common also in spruce xylem and participates in glycolysis, was selected as a positive housekeeping control in the experiments. Paraffin-embedded stem sections from seedlings of Norway spruce were treated with DIG-labelled sense and antisense RNA-probes corresponding to PX1, PX2, PX3 and FBPA encoding transcripts.

Table 1 Properties of the predicted peroxidases expressed in the xylem of Norway spruce (*Picea abies*)

Gene	ORF length, nt:s	Protein	Amino acids	N-terminal propeptide, aa:s	Molecular mass, mature protein (kDa)	p*I*, mature protein	C-terminal propeptide, aa:s	Potential glycosylation sites	Number of tyrosines	Amino acid sequence identity (%)		Potential localization
										PX2	PX3	
Pa-px1	1023	PX1	340	33	33.7	9.43	Not existing	5	9	39.7	40.8	Secreted
Pa-px2	1059	PX2	353	20	36.2	8.37	22	6	9	100	58.6	Secreted or vacuole
Pa-px3	1056	PX3	351	22	35.6	5.15	20	10	10	58.6	100	Secreted or vacuole

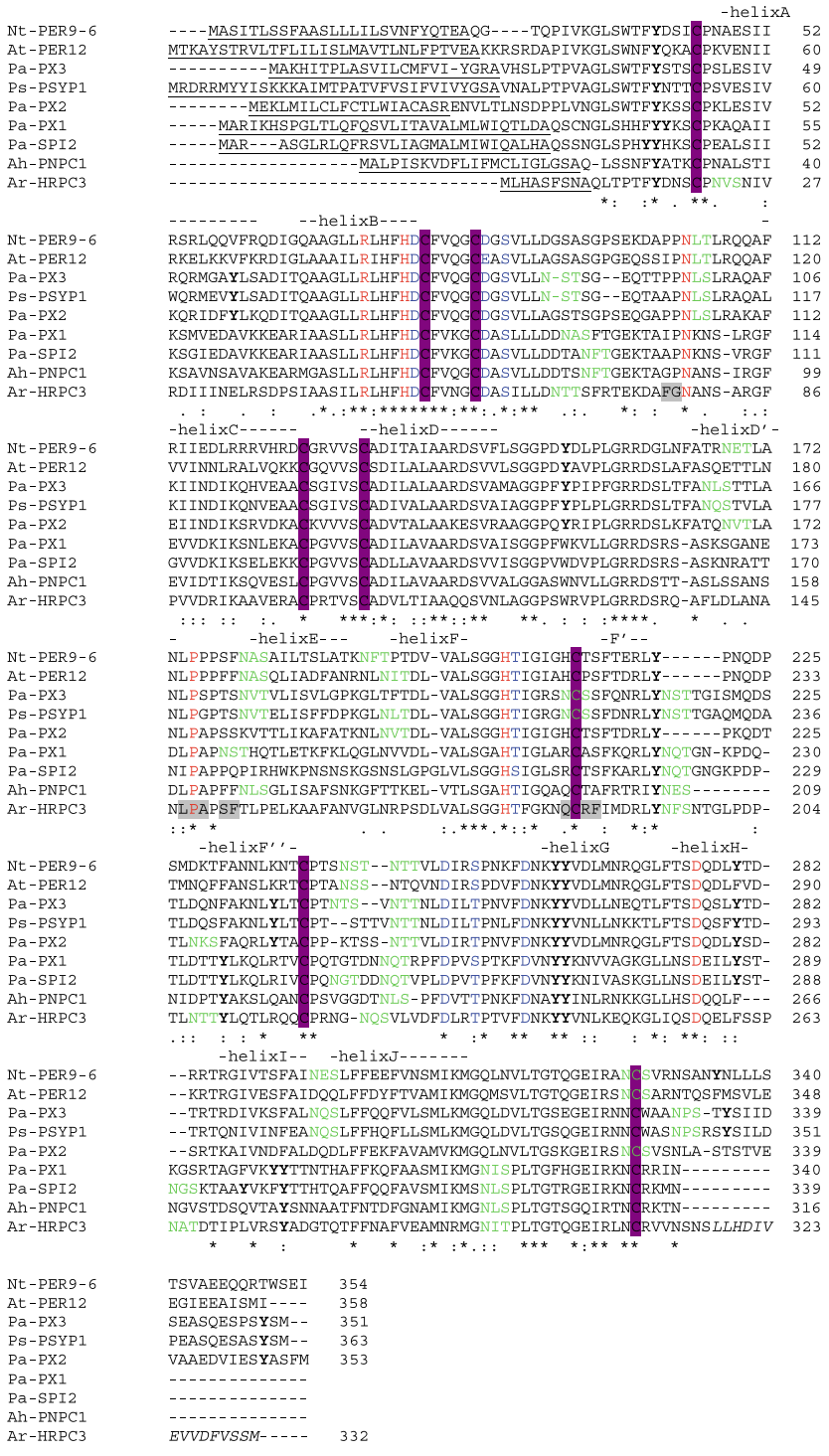
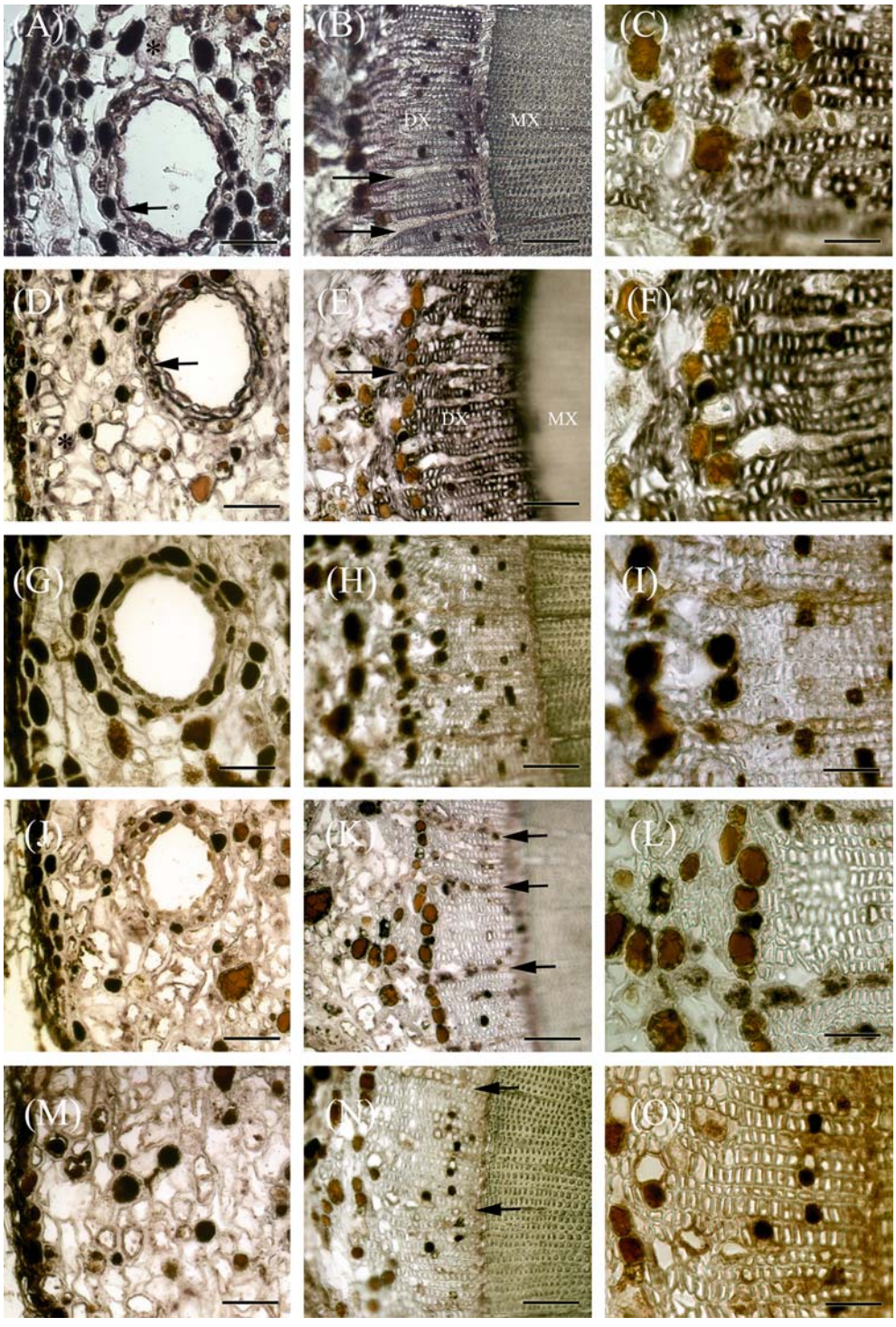


Fig. 2 Clustal W (1.82) multiple amino acid sequence alignment of Norway spruce PX1-3 (Pa-PX1, Pa-PX2 and Pa-PX3) with POXs retrieved from EMBL-Uniprot database according to accession numbers: Nt-PER9-6 (*Nicotiana tabacum*; Q94IQ1), At-PER12 (*Arabidopsis thaliana*; Q96520), Ps-PSYP1 (*Pinus sylvestris*; Q9FYS6), Pa-SPI2 (*Picea abies*; Q9SC55), Ah-PNPC1 (*Arachis hypogaea*; P22195) and Ar-HRPC3 (*Armoracia rusticana*; P15233). Predicted N-terminal-SSs are underlined, tyrosine residues are in bold, the heme-binding sites are marked in red, the calcium-binding sites in blue, S-S-bridge forming cysteines in purple, putative *N*-glycosylation sites in green and the C-terminal propeptides are indicated in italics

Antisense RNA-probes for the transcripts of *px1* and *px2* were detected inside the differentiating tracheids within the zone of developing xylem, but not in the mature tracheids, ray cells or cambium (Fig. 3B–C, E–F). Weak hybridization signal was also detected in cortex, especially in the resin duct epithelial cells (Fig. 3A, D). In sections treated with the antisense probe for *px3* transcripts, no hybridization signal was detected (data not shown). With the *fdpa* antisense probe, hybridization signal pattern was distinctly different from those observed for *px1* and *px2*, as the most intense DIG-AP staining was seen in ray cells within the zone of differentiating xylem (Fig. 3J–L). Sections incubated with the sense probes showed only very weak staining indi-



cating minimal unspecific binding of the probes (Fig. 3G–I and M–O).

Transient expression of EGFP fusion constructs in tobacco protoplasts

Subcellular localization of the PX1, PX2 and PX3 was studied by expressing chimeric fusions of EGFP and the N-terminal-SS-fragments of PX1, PX2 and PX3 in tobacco protoplasts (Fig. 1). In protoplasts expressing PX1-3 N-terminal–SS–peptide–EGFP fusions containing the ER-retention motif HDEL, GFP fluorescence was seen inside the cells in network-like structures (Fig. 4C–E). Distribution of the GFP signal was similar in the protoplasts expressing EGFP fusions of N-terminal–SS–peptide fragment of the *A. thaliana* secreted chitinase and EGFP

◀ **Fig. 3** Localization of the transcripts coding for Norway spruce PX1, PX2 and positive control, fructose-1,6-bisphosphate aldolase (FBPA) in Norway spruce stem xylem with in situ hybridization using DIG-labelled RNA-probes and anti-DIG antibody conjugated to AP. Treatment with antisense RNA-probes for *px1* (**A**–**C**) and *px2* (**D**–**F**), sense RNA-probe for *px1* (**G**–**I**), and with the antisense (**J**–**L**) and sense (**M**–**O**) RNA-probes for mRNA encoding FBPA. Bar length is 100 μm in all figures, except in **C**, **F**, **I**, **L** and **O**, where it is 50 μm. (**A** and **D**) Low hybridization signal can be seen in the cytoplasm of cortical parenchymal cells (asterisks) and resin duct epithelial cells (arrows). (**B** and **E**) The signal is seen in the differentiating xylem (DX), but not in the mature xylem (MX) in the growth ring of the previous year, which consists mainly of dead tracheids. Note the absence of hybridization signal within the living parenchymal ray cells (arrows). (**C** and **F**) Close-up photomicrographs of the differentiating tracheids where the hybridization signal is seen in the cell cytoplasm. (**G**) In the sense control section of the cortex no signal is detected. (**H**) Hybridization signal is absent in the sense control section of the differentiating xylem and the growth ring of the previous year. (**I**) No signal is seen in a close-up photomicrograph of the sense control section of differentiating tracheids. (**J**) There is no hybridization signal in the cortical parenchymal cells and resin duct epithelial cells. (**K**) The positive control signal is clearly seen in parenchymal ray cells (arrows). (**L**) Close-up photomicrograph of the differentiating xylem. The positive control antisense signal is seen in the parenchymal ray cells. (**M**–**O**) No signal is seen in the cortical cells nor in the differentiating tracheids treated with the sense probe for positive control. Rays are marked with arrows

(Fig. 4B). In the absence of any N-terminal-SS-fragments, GFP fluorescence was observed evenly in the cytosol and in the nucleus of the tobacco cells (Fig. 4A). Removal of the ER-retention motif from the 3′ end of the expression cassette (Fig. 1) only made the GFP signal weaker and thereby, more difficult to detect (Fig. 4F).

Heterologous expression of Norway spruce *px1* in hairy root culture of *C. roseus*

C. roseus hairy root cultures heterologously expressing full-length cDNA of *px1* were generated in order to correlate the protein product of *px1* to the multiple POXs detected in the xylem protein extracts of Norway spruce in our recent studies (Marjamaa et al. 2006). Protein extracts of the transgenic hairy roots expressing *px1* contained one highly cationic (basic) peroxidase isoform, which was not seen in the wild-type hairy roots (Fig. 5). Isoelectric point of the additional cationic POX was approximately 10 being ca. 0.6 units higher than the calculated p*I* of PX1 protein. The relative amount of the additional cationic POX increased when proteins were extracted with a buffer containing 1 M NaCl, indicating that the enzyme is attached to cell wall with ionic linkages (data not shown). The PX1-corresponding highly cationic POX isoform was also detected in IEF gels prepared from Norway spruce xylem protein extracts (Fig. 5).

Discussion

We report here cDNA cloning and primary structure characterization of three new class III peroxidases (POX) of a gymnosperm tree species, Norway spruce (*Picea abies*). So far, the only POX representatives cloned and described from Norway spruce are *spi2*, isolated from a cDNA library of pathogen-infected roots of Norway spruce trees (Fossdal et al. 2001), and *px4-7* from a lignin-forming spruce tissue culture (Simola et al. 1992; Koutaniemi et al. 2005). The cDNA clones of the new spruce genes (*px1, px2* and *px3*) were generated from Norway spruce xylem RNA-extracts upon the time of active lignification, during which several guaiacol and coniferyl alcohol oxidising peroxidase isoforms have been observed in spruce xylem protein extracts (Kukkola et al. 2003; Marjamaa et al. 2003, 2006).

In the present work, we have used the in situ hybridization technique successfully to study cell-specific POX encoding gene expression in spruce xylem sections. Only a few earlier studies exist where this technique has been applied for mature wood stems with extensive secondary growth (Hawkins et al. 1997, 2003; Regan et al. 1999; Wu et al. 2000; Groover et al. 2004; Kalluri and Joshi 2004). To our knowledge, this is the first time when in situ hybridization is exploited to follow gene expression in a gymnosperm tree species.

According to the multiple amino acid sequence alignment with HRPC and various other POXs (Fig. 2), the spruce peroxidases PX1, PX2 and PX3 all contain the conserved amino acids involved in heme-binding and peroxidase catalysis (Fig. 2). PX1-3 also contains the eight conserved cysteine residues, the conserved Ca^{2+}-ion binding sites, and recognizable eukaryotic N-terminal signal peptides (Nielsen 1997) for possible ER-targetting and further processing. The eight conserved cysteines, together with other similarities at protein sequence level, imply similar folding of the deduced mature PX1-3 with four disulphide bridges holding the α-helixes in compact globular structure, correspondingly as in the 3-D crystal structures of HRPC and other structurally described POXs (Schuller et al. 1996; Gajhede et al. 1997; Østergaard et al. 2000).

The primary structure of PX1-3 shows a high number of tyrosine residues (Fig. 2, marked in bold), which have been associated with peroxidase-lignin cross-linking, assumingly occurring in the plant cell wall (McDougal et al. 2001). The theoretical molecular weights and isoelectric points of the mature PX1, PX2 and PX3 range from 33.7 to 36.2 kDa and from p*I* 5.15–9.43, respectively. The differences in polypeptide length, in particular at the C-terminus, and other diversities within the deduced amino acid

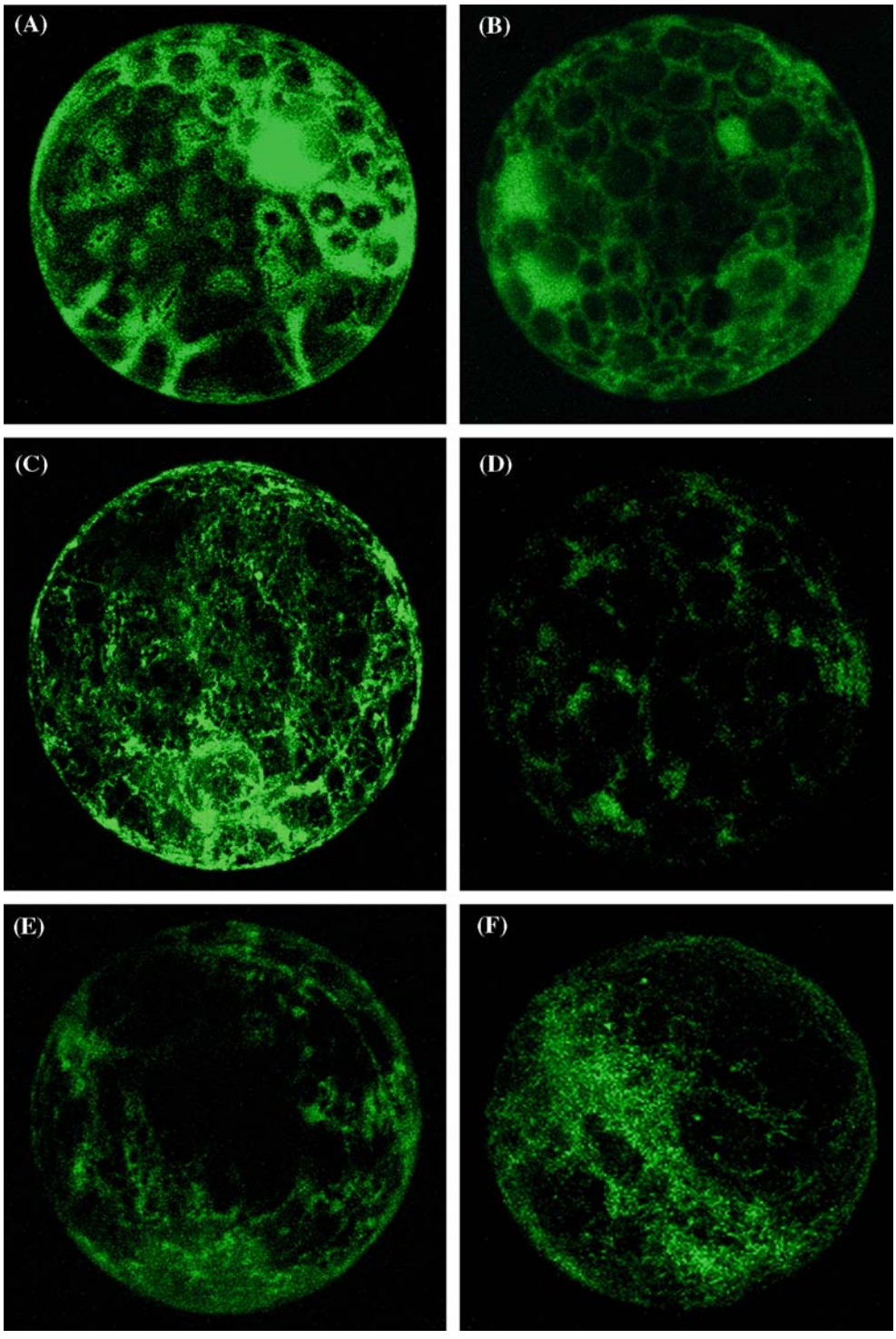


Fig. 4 Confocal images of tobacco protoplasts transiently expressing chimeric fusions of the N-terminal signal sequence (SS) peptide part of Norway spruce PX1-3 or *A. thaliana* secreted chitinase, fused to EGFP–ER-tag construct (see also Fig. 1). Protoplasts showing GFP signal were transformed with EGFP-constructs, lacking any N-terminal SS-peptide (**A**), including *A. thaliana* N-terminal SS-peptide (**B**), including Norway spruce PX1 N-terminal-SS-peptide (**C**), including PX2 N-terminal-SS-peptide (**D**), including PX3 N-terminal-SS-peptide (**E**) and including Norway spruce PX3-N-terminal-SS-peptide but without HDEL-ER-tag (**F**)

sequences lead to only 40–59% pairwise identity between PX1, PX2 and PX3 (Table 1).

As seen in Fig. 6, PX1, PX2 and PX3 fall into distinct phylogenetic groups with other POXs found as complete translated ORF sequences in the nucleotide sequence databases. According to the phylogram, PX1 is paralogous to the pathogen-induced spruce peroxidase SPI2, which is expressed in the developing spruce roots at the initiation of lignification, and is suggested to affect lignin structures in transgenic plants (Fossdal et al. 2001; Elfstrand et al. 2001, 2002). PX1 and SPI2 group with POXs isolated from lignin-forming Norway spruce tissue culture (PX4-7) and with POXs from leguminous plants such as the structurally well described peanut cationic peroxidase (Schuller et al.

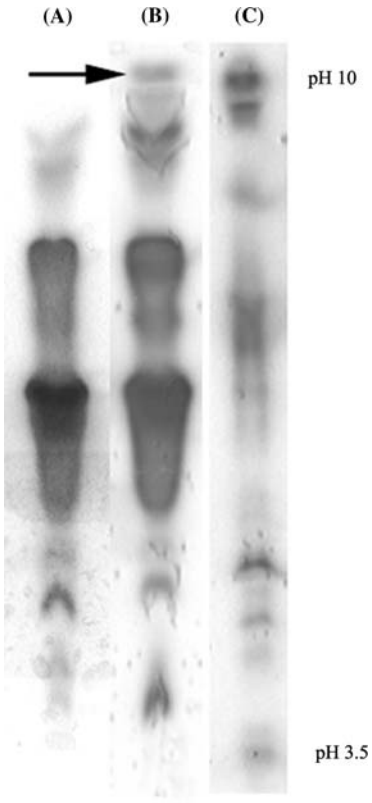


Fig. 5 Peroxidase isoforms in IEF gels recognized with guaiacol–H_2O_2 reaction: *Catharanthus roseus* hairy root extracts (**A**) wild-type hairy roots and (**B**) hairy root culture expressing *px1* (**B**); Norway spruce xylem proteins (**C**) extracted with buffer containing 1 M NaCl as described in Marjamaa et al. (2006). The putative *px1* protein product is marked with an arrow

1996) and a lignin-related peroxidase SHPRX6 from *Stylosanthes humilis* (Talas-Oğras et al. 2001). However, the two other Norway spruce xylem-peroxidases PX2 and PX3 are more related to POXs from spinach, flax, parsley and barley (Fig. 6).

Spatio-temporal localization is an important factor in determining the function of different POXs, since they form a large family of oxidoreductases with similar substrate specificities (Welinder 1992). Localization of PX1, PX2 and PX3 was studied here by determining cellular and tissue-specific sites of *px1, px2* and *px3* expression using in situ hybridization, and by studying the subcellular GFP-localization directed by N-terminal SS-peptides of PX1, PX2 and PX3 in tobacco protoplasts.

In situ experiments showed that the transcripts of spruce peroxidases *px1* and *px2* are found in differentiating tracheids, which indicates that respective proteins are translated and produced in the lignifying xylem cells. However, transcripts coding for PX3 were not detected in any of the spruce xylem sections. This indicates that, in opposition to the genes *px1* and *px2*, *px3* is not constitutively expressed in Norway spruce xylem cells, or that the level of transcription of *px3* in Norway spruce seedlings is very low.

Chimeric fusion proteins of the N-terminus of HRPC and some other plant enzymes have been previously transiently expressed in tobacco protoplasts in order to study protein localization (Di Sansebastiano et al. 1998; Matsui et al. 2003). For all three spruce xylem POXs (PX1-3), it was observed that the N-terminal-SS-part directed transgenic EGFP into ER. Due to the fact that PX1 obviously contains no additional localization signals, it is therefore predicted to be secreted *via* ER to the cell wall.

However, in addition to predicted N-terminal SS-peptides, PX2 and PX3 contain putative C-terminal pro-peptides, which may indicate possible vacuolar localization of the mature proteins (Theilade et al. 1993; Matsui et al. 2003). To solve the final localization of mature PX2 and PX3 enzymes, further transgenic expression studies of reporter fusion proteins are required.

Recently, we have extracted and partially purified POX isoforms from the xylem of Norway spruce (Marjamaa et al. 2006). In order to correlate the predicted protein product of the *px1* to the numerous Norway spruce xylem POX isoforms, *C. roseus* hairy root cultures expressing *px1* cDNA were generated. Transgenic *C. roseus* hairy roots introduced an additional cationic guaiacol-oxidising cell-wall-associated POX isoform into root protein extracts (Fig. 5). The p*I* value of this POX isoform (p*I* 10) was ca. 0.6 pH units higher than the value predicted for the mature PX1 protein, which may be explained to be a consequence of binding the two Ca^{2+} ions and heme into the properly folded POX proteins (Welinder et al. 2002). This additional cationic POX isoform, the putative protein product of *px1* cDNA, shows similar p*I* value with guaiacol-oxidising POX isoforms that were detected in Norway spruce xylem protein extracts during developmental lignification (Marjamaa et al. 2003).

Involvement of cationic POXs to the biosynthesis of lignin has been proposed in several plant species (El Mansouri et al. 1999; Quiroga et al. 2000; Talas-Oğras et al. 2001; Blee et al. 2003; Koutaniemi et al. 2005; Gabaldón et al. 2005). In Norway spruce, the partially purified cationic POX isoforms (p*I* over 9), efficiently oxidized the monolignol coniferyl alcohol (Marjamaa et al. 2006). The cationic POXs isolated from lignin-forming Norway spruce tissue culture also showed high affinity for monolignol substrates (Koutaniemi et al. 2005). Here we have shown that the *px1* gene coding for the highly cationic Norway spruce PX1 is expressed in lignifying tracheids and contains a localization signal sequence for secretion to the tracheid cell wall. Therefore, this specific spruce POX has suitable properties for participating in the lignification of secondary cell walls during xylem differentiation.

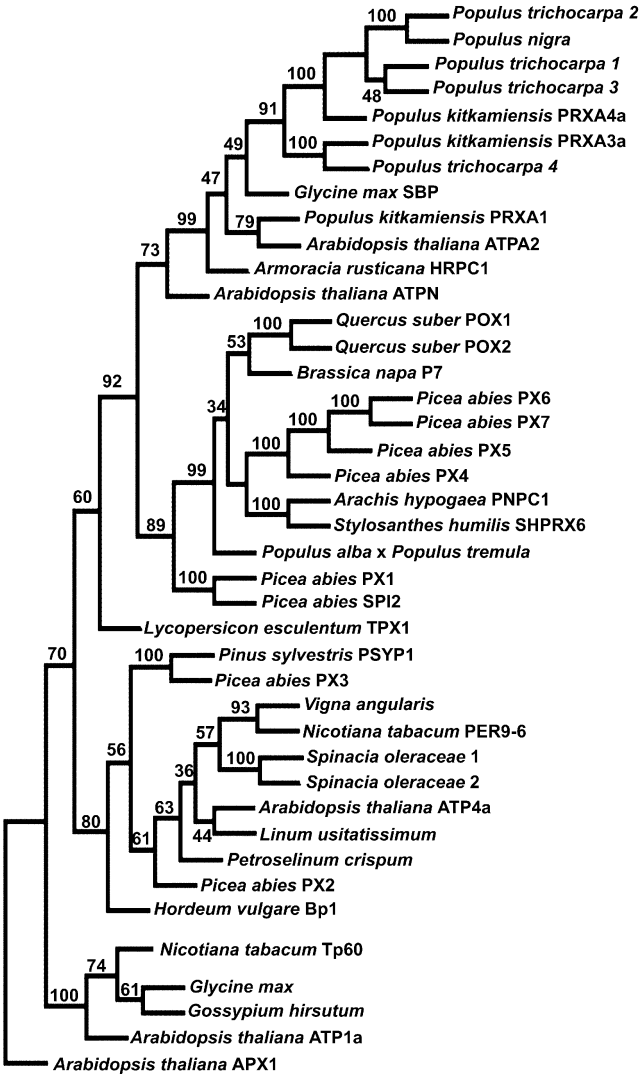


Fig. 6 Neighbour-joining tree visualizing the similarities in primary structures of various POXs including the Norway spruce PX1, PX2 and PX3 presented here. Maximum parsimony and Fitch-Margoliash methods produced Phylip trees with similar branching patterns. Bootstrap analysis with 100 replicates was performed to estimate the accuracy of the tree structure. Deduced amino acid sequences for following POXs were used in the analysis: *Arabidopsis thaliana* ATP1a (thalegrass, Q9SB81), *Arabidopsis thaliana* ATPA2 (Q42578), *Arabidopsis thaliana* ATP4a (Q96520), *Arabidopsis thaliana* ATPN (Q39034), *Arachis hypogaea* PNPC1 (peanut, P22195), *Armoracia rusticana* HRPC1 (horseradish, P00433), *Brassica napa* P7 (turnip, P00434), *Glycine max* (soybean, O64970), *Glycine max* SBP (O22443), *Gossypium hirsutum* (cotton, Q08671), *Hordeum vulgare* BP1 (barley, Q40069), *Linum usitatissimum* (flax, Q43782), *Lycopersicon esculentum* TPX1 (tomato, Q07446), *Nicotiana tabacum* PER9-6 (tobacco, Q94IQ1), *Nicotiana tabacum* TP60 (Q9XFL2), *Petroselinum crispum* (parsley, Q43032), *Picea abies* PX1 (Norway spruce, Q5W5I3), *Picea abies* PX2 (Q5W5I4), *Picea abies* PX3 (Q5W5I2), *Picea abies* PX4 (Q4W2V5), *Picea abies* PX5 (Q4W2V4), *Picea abies* PX6 (Q4W2V3), *Picea abies* PX7 (Q4W2V2), *Picea abies* SPI2 (Norway spruce, Q9SC55), *Pinus sylvestris* (Scots pine, Q9FYS6), *Populus alba* × *Populus tremula* var. glandulosa (Q58GF4), *Populus kitkamiensis* PRXA1 (aspen, Q43055), *Populus kitkamiensis* PRXA3A (Q43049) , *Populus kitkamiensis* PRXA4A (Q43050), *Populus nigra* (lombardy poplar, Q40949), *Populus trichocarpa* 1 (western balsam poplar, (Q43099), *Populus trichocarpa* 2 (Q43100), *Populus trichocarpa* 3 (Q43101), *Populus trichocarpa* 4 (Q43102), *Quercus suber* POX1 (cork oak, Q6T1C8), *Quercus suber* POX2 (Q6T1D0), *Spinacia oleraceae* 1 (spinach, P93545), *Spinacia oleraceae* 2 (P93547), *Stylosanthes humilis* SHPRX6 (Townsville stylo, L36110) and *Vigna angularis* (Azuki bean, Q43854). *Arabidopsis thaliana* ascorbate peroxidase I (Q05431) was used as outgroup

Acknowledgements This investigation was funded by the Academy of Finland (grant no. 43091) and the National Technology Agency TEKES under The Finnish Forest Cluster Research Program WOOD WISDOM (1998–2002) and University of Helsinki (grant no. 2108015). The project is a part of the Academy of Finland Center of Excellence in Plant Biology and Forest Biotechnology (project no. 164346). The confocal laser-scanning microscope of the Institute of Biotechnology of Helsinki University was used in this study. Kaisa Marjamaa is a member of the Viikki Graduate School of Biosciences (VGSB), which is acknowledged for financial support.

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