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Three polyphenol oxidases from hybrid poplar are differentially expressed during development and after wounding and elicitor treatment

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The expression of three polyphenol oxidase (PPO; EC 1.10.3.1) genes was investigated in hybrid poplar (*Populus trichocarpa* × *P. deltoides*). *PtdPPO1* was previously isolated as a wound- and herbivore-inducible PPO (Constabel et al. Plant Physiol 124: 285–295, 2000), whereas *PtdPPO2* and *PtdPPO3* are two novel hybrid poplar PPO genes isolated from stem and root tissue, respectively. Sequence analysis revealed that the three PPOs were 60–66% identical at the amino acid level. Using gene-specific probes, the expression patterns of the three PPOs was investigated in various organs at different developmental stages. Under normal growing conditions, *PtdPPO1* mRNA was absent from all organs tested, while *PtdPPO2* was found to be expressed in mid-veins, petioles, stems and roots. *PtdPPO3* was expressed only in roots. *PtdPPO1* and *PtdPPO2* were induced by mechanical wounding and methyl jasmonate, but in different tissues. Overall, the expression patterns suggest that the three PPO genes may have specialized physiological functions in hybrid poplar.

Introduction

Polyphenol oxidase (PPO) is a copper-containing enzyme which uses molecular oxygen to oxidize *o*-diphenols to *o*-quinones (diphenolase activity; EC 1.10.3.1), and which in some plants can also hydroxylate monophenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1). The quinones are reactive compounds which are responsible for the damage-induced browning of many fruits and vegetables, and PPO activity is thus often associated with damaged and diseased plants (Mathew and Parpia 1971, Steffens et al. 1994). However, its physiological function has been debated. Due to its location in the thylakoid, PPO was once postulated to play a role related to photosynthesis or other primary metabolic functions (Vaughn et al. 1988); however, it is now becoming accepted that in some plants, PPO plays a role in plant defence against insects and pathogens (Steffens et al. 1994, Constabel et al. 1996, Li and Steffens 2002).

The antiherbivore role of PPO was first demonstrated in tomato by Felton et al. (1989), who showed that PPO-generated quinones could alkylate dietary proteins and reduce their nutritive value for insect pests. Further evidence for a function of PPO in defence against insects came from the discovery of PPO-induction by the defence hormones systemin and methyl jasmonate, which linked PPO expression to well-characterized defence signalling pathways in tomato (Constabel et al. 1995). PPO has been shown to be important in pathogen defence; transgenic tomato plants which overexpress PPO show enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Li and Steffens 2002). Nevertheless, PPO may perform different functions in other plants. For example, PPO-like enzymes have recently been shown to carry out hydroxylations in aurone and lignan biosynthesis in snapdragon and creosote bush, respectively (Nakayama et al. 2000, Cho et al. 2003).

Abbreviations – LPI, leaf plastochron index; MeJA, methyl jasmonate; PPO, polyphenol oxidase; TD, *P. trichocarpa* × *P. deltoides*.

PPOs are often encoded by gene families showing complex developmental patterns of expression. For example, in potato five PPO genes with expression in flowers, young leaves, trichomes, roots, and tubers were characterized (Thygesen et al. 1995). In tomato, there are at least seven PPO genes expressed in a similar range of tissues (Newman et al. 1993, Thipyapong et al. 1997). Four different PPO cDNAs were isolated from banana, and these show differential expression in vegetative tissues as well as fruits and flowers (Gooding et al. 2001). In some cases, individual PPO genes are up-regulated following stresses, in particular wounding and pathogen infection, which gives some hints as to functions. Interestingly, only one of the tomato PPO genes is wound- and stress-inducible, whereas the other six PPO family members are regulated developmentally (Thipyapong and Steffens 1997). These studies suggest that to fully understand the biology of PPOs, detailed expression of individual members of gene families need to be carried out.

We had previously isolated a wound- and herbivore-inducible leaf PPO (*PtdPPO1*) from hybrid poplar, *Populus trichocarpa* × *P. deltoides* (Constabel et al. 2000). This gene is a component of the inducible defence against leaf-eating herbivores, as it is also up-regulated by methyl jasmonate (MeJA) and co-ordinately expressed with other defence genes (Constabel et al. 2000). Furthermore, overexpression of *PtdPPO1* in transgenic *Populus* can result in enhanced resistance to forest tent caterpillar, *Malacosoma disstria* (Wang and Constabel 2004). Subsequent experiments revealed the presence of a second PPO protein (PPO-2) expressed primarily in stems and petioles, with distinct substrate specificity and other biochemical properties (Wang and Constabel 2003). Here we report the cloning of the corresponding stem PPO cDNA, as well as a third, root-specific, PPO cDNA. Our results demonstrate that the three hybrid poplar PPO genes show very distinct developmental and stress-induced expression patterns, which ultimately may provide clues as to their specific physiological roles.

Materials and methods

Plant materials and treatments

Hybrid poplar (*Populus trichocarpa* × *P. deltoides*; TD) clone H11-11 was propagated and grown in the University of Victoria's Forest Biology greenhouse facility under long day conditions and fertilized daily as described previously (Constabel et al. 2000). Plants were about 2 months old and typically had at least 25 leaves when used. Leaves were numbered from the apical meristem downward according to the leaf plastochron index (LPI) (Larson and Isebrands 1971), with the first developing leaf with a lamina of length ≥ 20 mm designated as the index leaf (LPI 0). Leaves were wounded by crushing at the margins with pliers, and stems by puncturing with a sterile needle. For MeJA treatment, whole trees were sprayed twice with 10 μM MeJA in 0.1% Triton X-100, 2 h apart. Induced tissues were harvested after 24 h unless stated otherwise. Young leaves and stems refers to tissues at LPI 2–4, and old leaves and stems refers to tissues at LPI 16. Young root samples were collected from root tips (5 cm length, approximately 1 mm diameter), while old root samples consisted of major roots (≥ 5 mm diameter) collected near the crown. All tissue samples were frozen in liquid N_2 and stored at $-80°C$. All experiments were repeated at least three times, with identical results.

Isolation of stem- and root-specific PPO cDNAs

Partial cDNA sequences corresponding to *PtdPPO2* and *PtdPPO3* were first amplified using PPO-specific degenerate primers (Constabel et al. 2000) with stem RNA as the RT-PCR template. The resulting fragments were gel-purified (Qiaquick Gel Extraction Kit; Qiagen, Mississauga, Canada) and cloned into the pGem-T Easy Vector (Promega, Nepean, Canada). Sequence analysis allowed these fragments to be clustered and used to design gene-specific primers. 3′-RACE was carried out using a Lock-Dock system (Borson et al. 1992) with gene-specific reverse primers (5′-AAGGAGATGC-GAATCCAG-3′ for *PtdPPO2* and 5′-ATATTCCA-CACGGTCCAG-3′ for *PtdPPO3*). To obtain the 5′-cDNA ends, unannotated sequence data of the *Populus* genome project were used to construct specific primers immediately upstream of the coding sequence. These primers (5′-GTATCCAAGAATCCTTCACCC-3′ for *PtdPPO2* and 5′-CCATGGCAAAAGGTGTTCAG-3′ for *PtdPPO3*) were then used to amplify the remainder of the PPO coding sequences from both stem and root total RNA. Sequence assembly, alignments and distances were performed using DNAStar software (DNAStar Inc., Madison, WI,). Based on the predicted sequence, additional PCR primers in both 3′ and 5′ untranslated regions were designed (5′-CCCCACACCAAAC-CAAACTA-3′, 5′-CAAGAAAAGGCGAAAGGAAA-3′ for *PtdPPO2*, and 5′-AACCATGGCAAAACCTGTTC-3′, 5′-ATTGCTCCTCGCACAAATCT-3′ for *PtdPPO3*) and used to amplify the entire cDNA, and the entire cDNA was cloned into pGem-T Easy and sequenced.

Gene-specific PPO probes and northern and Southern blot hybridization

Total RNA was isolated from plant tissues using the procedure described previously (Haruta et al. 2001). Aliquots containing 10 μg total RNA were electrophoresed on 1.2% agarose denaturing formaldehyde gels, and subsequently transferred onto Hybond N^+ membranes (Amersham, Baie d'Urfé, Canada). Three gene-specific probes were designed based on the 3′-cDNA sequences, which included the 3′-UTR of the PPO genes plus a short fragment of coding sequence. The PCR primers used to amplify the gene-specific probes were 5′-ATCTGGAAGCTGAAGGAG-3′ for *PtdPPO1*, 5′-GTTGAGGGTGATGACAGT-3′ for *PtdPPO2* and

5′-CCCAGGTCTAATAGTGTT-3′ for *PtdPPO3*, paired with the SP6 primer on the vector in each case. The sizes of the three gene-specific probes were 290 bp (PPO-1), 310 bp (PPO-2) and 350 bp (PPO3). The DNA fragments were ^{32}P-labelled using the RediPrime Kit (Amersham), and hybridizations were performed at 65°C and were washed at high stringency according to the method of Church and Gilbert (1984). Signals were detected and analysed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For Southern blots, genomic DNA was extracted from *P. trichocarpa* (clone 93–968) or TD hybrid H11-11 as previously described (Constabel et al. 2000). Aliquots containing 10 µg genomic DNA were digested with 60 Units *Eco*RI or *Hin*dIII (Amersham), separated on 0.8% agarose gels, and transferred onto Hybond N^+ membranes. Hybridizations were performed as for northern blots.

PPO activity assays, protein determination and immunoblotting

Poplar tissue was ground in 100 m*M* $NaPO_4$ buffer, pH 7.0, with 0.1% Triton X-100 and polyvinylpolypyrrolidone. After centrifugation at 12 000 *g*, the supernatant was used for protein determination (Bradford 1976), using BSA as a standard. PPO activity was determined by a spectrophotometric assay using dihydroxyphenylalanine (DOPA), as described previously (Constabel et al. 2000). For western blots, 10 µg of total soluble protein was separated on SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad, Mississauga, Canada). Detection was carried out using polyclonal antibodies raised against the recombinant *PtdPPO* protein (ME Christopher and CP Constabel, unpublished data). Immunocomplexes were detected using goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Bio-Rad) together with reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

Cell culture and elicitor treatment

Hybrid poplar suspension cells were obtained from Dr Brian Ellis (University of British Columbia, Vancouver, Canada) and maintained according to Haruta and Constabel (2003). Partially acid-hydrolysed chitosan was obtained from Dr Armand Seguin (Canadian Forest Service, Ste-Foye, Canada). For northern analysis, 3-day-old cultures were treated with the elicitors for a 3-h period before harvesting cells by centrifugation. Treatments consisted of final concentrations of 1 m*M* salicylic acid, 50 µ*M* MeJA (Bedoukian Research, Danbury, CT), 0.5 µg ml^{-1} chitosan, or 0.1% (v/v) *Phytophthora megasperma* (pmg) and *Venturia macularis* crude elicitor prepared as described (Lisker and Kuc 1977). All elicitors were diluted into sterile H_2O. Treated cells were harvested by centrifugation at 1600 *g* for 15 min, frozen in liquid nitrogen, and stored at −80°C until analysed.

Results

Hybrid poplar contains at least three distinct PPO genes

To isolate cDNAs encoding the stem-specific PPO-2 by RT-PCR, we used degenerate primers corresponding to the conserved copper-binding regions of PPO to amplify PPO fragments from cDNA synthesized from hybrid poplar stem RNA. Based on their nucleotide sequence, these fragments were clustered into three groups, representing the previously isolated *PtdPPO* (here named *PtdPPO1*) as well as two new PPO genes. We then used 3′-RACE, as well as additional PCR using primers designed from unannotated sequence data available at the JGI Poplar Genome Project to clone and sequence the entire coding sequences of two new poplar PPO cDNAs (see Materials and methods). Additional primers in the 5′- and 3′-untranslated regions were used to amplify and verify the entire coding sequences of the PPO cDNAs, which were named *PtdPPO2* and *PtdPPO3*. Conceptual translation of the *PtdPPO2* and *PtdPPO3* coding sequences predicted proteins of 581 and 590 amino acids with molecular weights of 64.9 and 66.3 kDa, respectively. PSORT analysis (http://psort.nibb.ac.jp/form.html) indicated that the predicted *PtdPPO2* and *PtdPPO3* proteins both included an N-terminal transit peptide of 81 amino acid residues, which target the protein to the plastids (Fig. 1). Removal of the predicted transit peptides would result in a predicted mass of 56.2 and 57.5 kDa for the mature peptides. Two conserved copper-binding domains, each with three highly conserved His residues that complex the catalytically active Cu^{2+} were also present (Fig. 1). The three cDNAs share 61–66% and 58–63% pairwise identity at the nucleic acid and amino acid levels respectively (Table 1).

The three poplar PPO cDNAs define small gene families

To perform genomic Southern analyses as well as to investigate differential expression of the three PPO genes in hybrid poplar, gene-specific probes for the three PtdPPOs were generated by PCR. These were composed of the 3′-UTRs plus a short fragment of coding sequence (65–150 bp) of the cDNAs (see Materials and methods). In this region, the three PPOs had the least similarity and cross-hybridization was negligible (Fig. 2A). We carried out Southern blots on genomic DNA restricted with *Eco*RI and *Hin*dIII, which do not cut within any of the probe sequences. In addition to the TD poplar hybrid, we also analysed genomic DNA of its *P. trichocarpa* female parent. In general, the banding pattern for the parental genotype was simpler than in the hybrid, reflecting the more divergent PPO alleles inherited from the *P. deltoides* parent. Hybridization with *PtdPPO1* confirmed that at least two *PtdPPO1*-type genes exist in hybrid poplar genome (Fig. 2B); (Constabel et al. 2000). When the same Southern blot was hybridized with the *PtdPPO2* and *PtdPPO3* probes, very different band patterns were observed (Fig. 2B,

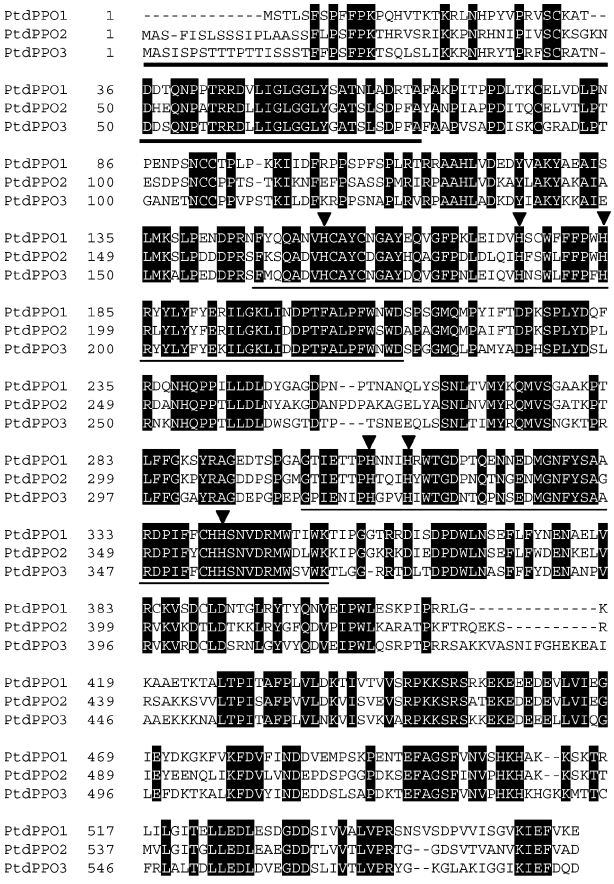

Fig. 1. Comparison of the deduced amino acid sequences of three hybrid poplar *PtdPPO* cDNAs. Triangles indicate the conserved His residues. The plastid transit peptides and copper-binding domains are indicated by heavy and light underscoring, respectively. The GenBank accession numbers are AF263611 (*PtdPPO1*), AY665681 (*PtdPPO2*) and AY665682 (*PtdPPO3*).

panels b and c), confirming that the probes each recognized distinct *PtdPPO* genes. Hybridization of restricted genomic DNA with the *PtdPPO2* probe detected five to seven bands in the *P. trichocarpa* parent, while hybridization with the *PtdPPO3* probe resulted in only one to two bands (Fig. 2). We conclude that both *PtdPPO1* and *PtdPPO2* belong to small gene families in the poplar genome, while *PtdPPO3* is represented by only one or two genes.

The three *PtdPPO*s show differential expression in development and following stress

In healthy hybrid poplar saplings, *PtdPPO1* transcripts could not be detected in any vegetative organs tested, such as leaves, stems and roots (Fig. 3A). By contrast, *PtdPPO2* transcripts were abundant in young stems and old roots, but less so in old stems and young roots (Fig. 3A). Expression of *PtdPPO2* in young leaf tissue was barely detectable, and may be due to the presence of leaf veins in the sample (see below). *PtdPPO3* was

Table 1. Percentage identity among three hybrid poplar PPO sequences, calculated for both nucleotide and amino acid sequences using MEGALIGN (DNAStar). Protein identities are in bold.

	PtdPPO1	*PtdPPO2*	*PtdPPO3*
PtdPPO1	100	**62.9**	**59.5**
PtdPPO2	65.8	100	**57.5**
PtdPPO3	62.0	61.4	100

expressed only in young roots, and was not detectable in any other plant organs tested (Fig. 3A). Thus, the three *PtdPPO* genes have distinct constitutive expression patterns: *PtdPPO1* is not constitutively expressed in vegetative tissues, *PtdPPO2* is mostly expressed in conducting organs and *PtdPPO3* is root-specific.

To determine if *PtdPPO2* is preferentially expressed in younger tissues as was described for potato (Hunt et al. 1993), we investigated the developmental regulation of

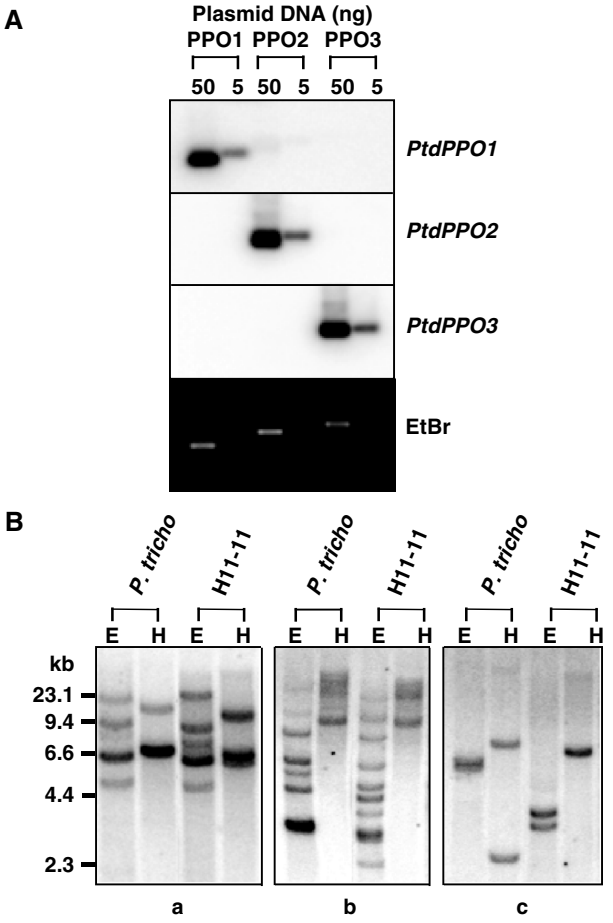

Fig. 2. Southern analysis of the *PtdPPO* genes using gene-specific PPO probes. (A) Plasmid DNA (50 and 5 ng) harbouring the three PPO cDNAs were hybridized with three PPO gene-specific probes. (B) Southern analysis of *P. trichocarpa* and TD hybrid H11-11. Ten micrograms of restricted genomic DNA was subjected to electrophoresis, blotted, and hybridized with labelled *PtdPPO1* (a), *PtdPPO2* (b) and *PtdPPO3* (c). E, *Eco*RI; H: *Hin*dIII.

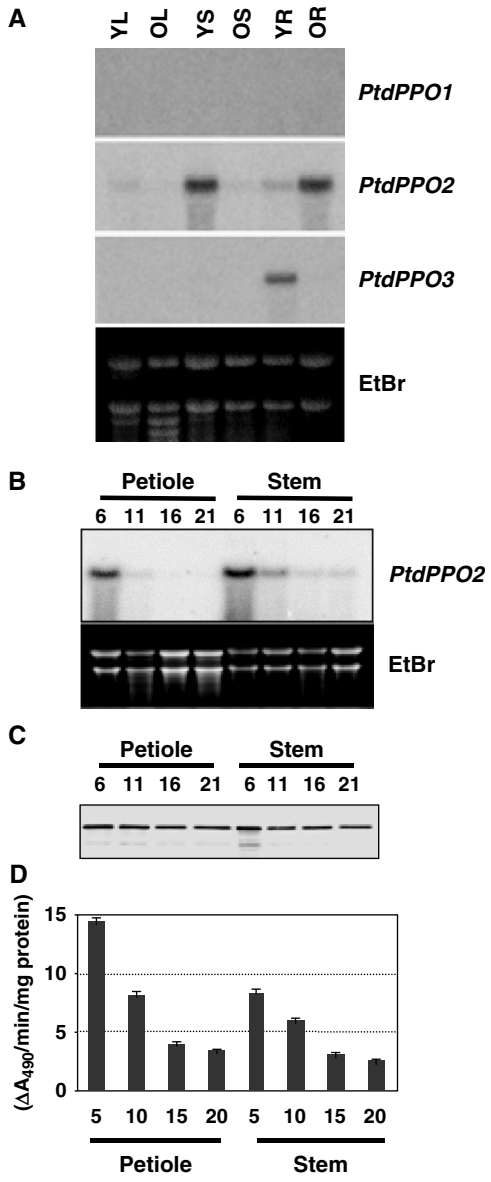

Fig. 3. Expression of three *PtdPPO* genes in various poplar tissues. (A) Northern analysis using the three gene-specific PPO probes. Total RNA was isolated from young and old leaves (YL, OL), stems (YS, OS) and roots (YR, OR), and analysed by northern blot. The EtBr-stained gel is included as a loading control. (B) Expression of *PtdPPO2* in petiole and stem at different developmental ages. Petiole and stem were collected from internode LPI positions as indicated by numbers. (C) Western blot analysis of the same tissue samples as in (B), using a polyclonal PPO antibody. Ten micrograms total protein was loaded per lane. (D) PPO activity of petioles and stems at different developmental ages. Error bars represent standard errors (n = 3).

PtdPPO2 expression in petioles and stems along the axis of the plant, which represents a tissue age gradient. Northern blots showed that *PtdPPO2* was strongly expressed only in the younger petioles and stems (LPI 6), with detectable but low expression at LPI 11, and very little expression at LPI 21 (Fig. 3B). However, when PPO protein levels in the same tissues were compared on western blots, PPO protein was present at all developmental stages including older tissues (LPI 21) (Fig. 3C). This distribution of PPO was confirmed using PPO activity assays, where younger tissues did show higher PPO activity than the older ones (Fig. 3D). Overall, our results would suggest that PPO-2 protein is primarily synthesized in young petioles and stems, but that it is very stable and thus remains present and active in older tissues. This pattern of early PPO protein synthesis in development coupled with very low protein turnover was also observed previously for potato PPO (Hunt et al. 1993).

As we had previously established that in leaves, *PtdPPO1* is inducible by wounding and MeJA, we used northern analysis to test for a response of *PtdPPO2* and *PtdPPO3* to these signals. Leaves and stems were wounded as described in Materials and methods, and tissues were harvested after 24 h. We also collected root samples from the wounded plants, but since only above-ground parts of the saplings were wounded, these roots are considered systemically wound-induced. As we had found previously, *PtdPPO1* transcripts accumulated in wounded leaves after mechanical wounding, and the induction was stronger in young leaves than in old leaves (Fig. 4A). *PtdPPO2* also responded to mechanical wounding by increasing transcript levels, and this inducibility was observed in both stems and roots, but not in leaves. The *PtdPPO2* gene also showed significant constitutive expression in young stems, consistent with our earlier observation (Fig. 3). In stems, the wound-induction was stronger in young tissues, whereas in roots, the induction was stronger in older tissues (Fig. 4). No wound induction of *PtdPPO3* was detected in root tissues, but after wounding, a very low induction of *PtdPPO3* could be seen in stems (Fig. 4).

Since jasmonates are key signals involved in plant defence, and we also tested the inducibility by MeJA of the three *PtdPPO* genes. As previously reported, *PtdPPO1* was highly inducible by MeJA in leaf tissues, although we also noted very faint expression in young stems and old roots (Fig. 4B). By contrast, *PtdPPO2* was dramatically induced by MeJA in stem and old root tissues. *PtdPPO3* expression in roots, however, did not respond to MeJA application (Fig. 4B). The induction pattern following MeJA treatment was very similar to that observed after wounding (compare Fig. 4, panels A and B). From both these experiments we conclude that (i) *PtdPPO1* is inducible and significantly induced only in leaves; (ii) *PtdPPO2* is constitutively expressed in stems and roots and is also inducible in these tissues; and (iii) *PtdPPO3* is essentially root-specific and not significantly induced by either MeJA or wounding.

PtdPPO genes are differentially regulated by various elicitors in poplar cell culture

Because of the inducible nature of PPO, we also wanted to test the responses of the *PtdPPO* genes to pathogen

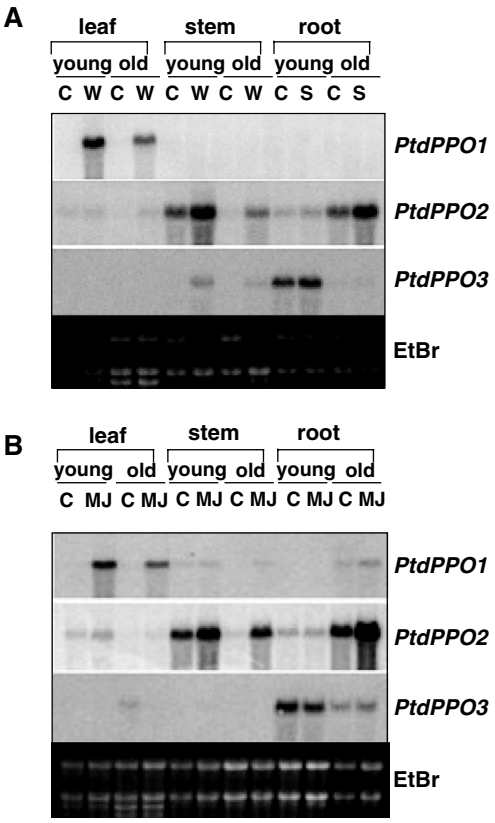

Fig. 4. Northern analysis of PPO gene expression in response to mechanical wounding and MeJA. Leaf and stem tissues were collected 24 h after wounding (A), or MeJA treatment (B). Root tissue was harvested from plants treated on leaves and was thus considered to be systemically induced. Young and old tissue refer to leaves at LPI 2–4 and LPI 16, respectively. C, control; W, wounded; S, systemically wounded; MJ, MeJA-treated.

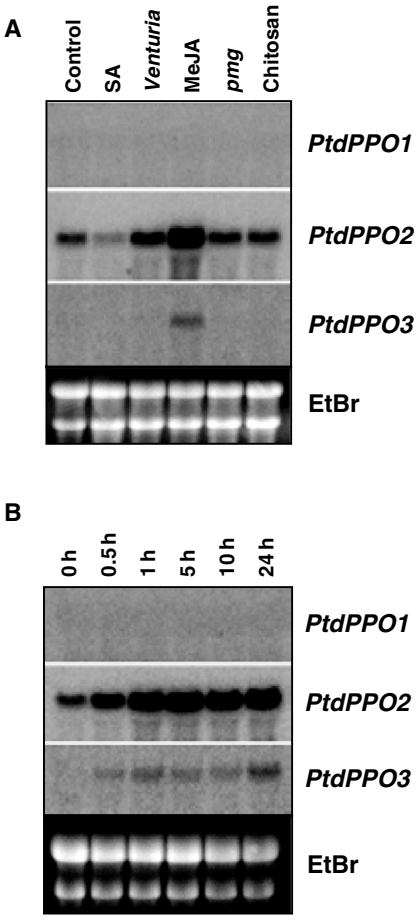

Fig. 5. Northern blot analysis of *PtdPPO* gene expression in hybrid poplar cell culture in response to different elicitors. (A) Cells were treated for 3 h with 1 m*M* salicylic acid (SA), 50 μ*M* MeJA, 0.5 μg ml^{-1} chitosan, 0.1% (v/v) *Phytophthora megasperma* (pmg) elicitor or 1% *Venturia macularis* extract. (B) Time course of PPO gene expression in cell culture following treatment with MeJA (50 μ*M*).

stress. For practical reasons, we chose to use a suspension culture, but one derived from the same TD poplar hybrid (de Sá et al. 1992). Cell cultures have been widely used to mimic defence gene induction (Sasabe et al. 2000, Andi et al. 2001, Haruta and Constabel 2003) and have the advantage of consisting of fewer cell types and being very sensitive to pathogen elicitors compared to whole plants. *PtdPPO2* was highly induced by MeJA in cell culture, as it was in the whole-tree experiments (Fig. 5A). *PtdPPO2* was also induced by a crude extract of the aspen pathogen *Venturia macularis*, but to a lesser extent than with MeJA. *PtdPPO2* was only slightly induced by the other pathogen-derived elicitors, a crude elicitor from *Phytophthora megasperma*, and chitosan (Fig. 5A). Interestingly, salicylic acid (SA), a well-known inducer of defence responses and systemic acquired resistance in many plants (Kunkel and Brooks 2002), repressed the expression of *PtdPPO2* after 3 h of treatment (Fig. 5A). SA-mediated repression of *PtdPPO2* transcripts was also observed when 1 m*M* SA was applied to wounded stem segments (data not shown). Compared to *PtdPPO2*, *PtdPPO3* was only slightly induced by MeJA in cell cultures (Fig. 5A). This was confirmed in time course experiments, which showed a strong maximal induction of *PtdPPO2* by 1 h after elicitation, with similar kinetics but a much weaker signal for *PtdPPO3* (Fig. 5B). We did not detect expression of *PtdPPO1* in cell cultures either before or after any of the elicitor treatments, despite the strong inducibility of this gene in leaves.

Discussion

The hybrid poplar PPO gene family

PPOs have been extensively studied in plants for their roles in browning reactions of fruit and vegetable during processing, and also for their functions in plant defence

against pests and pathogens (Duffey and Felton 1991, Constabel et al. 1995, Li and Steffens 2002). In hybrid poplar, we previously characterized the herbivore-inducible leaf *PtdPPO1* gene, which is implicated in defence against insect herbivores (Constabel et al. 2000). In this report, we describe the cloning and characterization of two novel PPO cDNAs, *PtdPPO2* and *PtdPPO3*, which are expressed constitutively in stems and roots. *PtdPPO2* was wound- and MeJA-inducible, while *PtdPPO3* expression was only slightly affected by wounding or MeJA treatment. In cell cultures, MeJA and a pathogen-derived elicitor induced *PtdPPO2* strongly and *PtdPPO3* very weakly, with no effect on *PtdPPO1*.

The proteins encoded by the newly described *PtdPPO2* and *PtdPPO3* have all the features of plant PPOs, including plastid targeting sequences. They give rise to predicted MWs of 56.2 and 57.5 kDa, respectively, for the processed peptides. However, on western blots, the PPO-2 protein we had previously isolated from stems migrated at approximately 66 kDa (Wang and Constabel 2003). Nevertheless, we believe that the PPO-2 protein is encoded by *PtdPPO2*, because this is the only PPO gene to be significantly expressed in petioles and stems (Figs 3–5). Furthermore, in MeJA-treated cell cultures, a single PPO protein band was detected at 66 kDa (data not shown), and *PtdPPO2* was also the major PPO gene expressed (Fig. 5). The discrepancy in MW for PPO-2 is probably due to migration artifacts, such as described in *Vicia faba*, where the deduced MW of mature PPO (58 kDa) is also lower than what is observed for isolated PPO protein (approximately 65 kDa). It is also possible that the size discrepancy is related to improper cleavage of the transit peptide, but we have not investigated this.

Southern analysis indicated that PPO in poplar is encoded by at least three small gene families. Like most dicot PPOs, it appears that poplar PPOs contain no introns, as none were detected during our analysis of the *P. trichocarpa* genomic data. The Southern blots are therefore interpreted more easily, and suggest that both *PtdPPO1* and *PtdPPO2* belong to small gene families with two or three, and five to seven members, respectively. By contrast, there are only one or two *PtdPPO3*-type genes in the genome. These gene family estimates are consistent with two-dimensional gel electrophoresis and western blot analyses, where we could detect up to five PPO-1, six PPO-2, and two PPO-3 protein isoforms with the PPO antibody in wounded leaf, stem, and root extracts (data not shown). Tomato contains at least seven PPO genes, and five PPO cDNAs have been isolated from potato (Hunt et al. 1993, Thygesen et al. 1995). Interestingly, in a phylogenetic analysis the three *PtdPPO* genes did not cluster as a group. The inducible *PtdPPO1* and *PtdPPO2* grouped with an aspen wound-induced PPO, but *PtdPPO3* was most closely related to a cluster of apple PPO genes (Fig. 6). Unlike *PtdPPO3*, the apple PPO genes are wound-induced in leaves and fruit (Boss et al. 1995, Kim et al. 2001). Other PPO genes for which

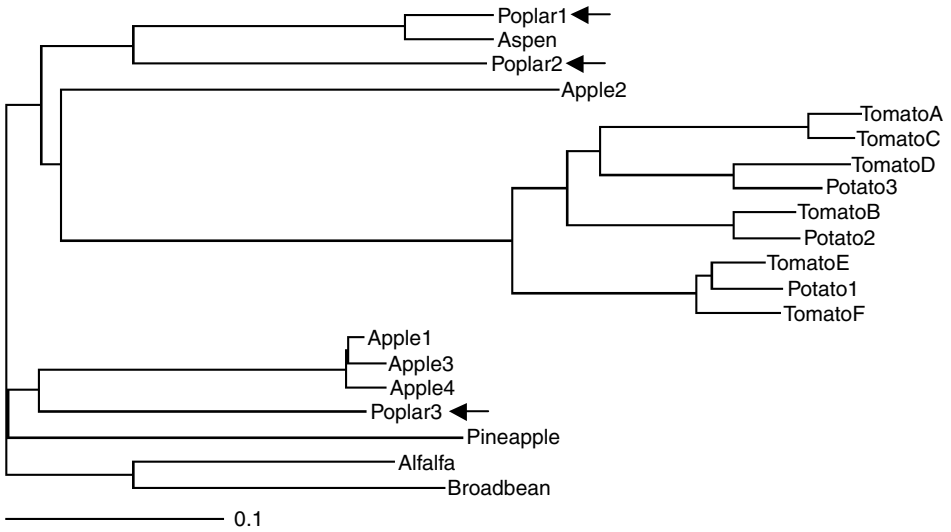

Fig. 6. Phylogenetic analysis of three hybrid poplar PPOs and other plant species-related PPO proteins. The predicted PPO amino acid sequences without the transit peptides or gaps were used for the alignment. The sequence similarity matrix was calculated with the CLUSTAL X package (Thompson et al. 1997) and submitted to a neighbour-joining analysis to generate a branching pattern. The consensus tree was drawn using TREEVIEW (version 0.3, Roderic D.M. Page, University of Glasgow, UK). Poplar1, Poplar2 and Poplar3 refer to *PtdPPO1*, *PtdPPO2* and *PtdPPO3*, respectively. Some other sequences are assigned arbitrary names for ease of presentation. GenBank accession numbers and species of origin for the sequences used are: Apple1 (*Malus domestica,* P43309), Apple2 (AAK56323), Apple3 (BAA21677), Apple4 (BAA21676), Aspen (*Populus tremuloides,* AAK53414), Alfalfa (*Medicago sativa* ssp. *sativa,* AAP33165), Broadbean (*Vicia faba*, S24758), Pineapple (*Ananas comosus*, AAK29782), TomatoA (*Lycopersicon esculentum*, Q08303), TomatoB (Q06355), TomatoC (Q08305), TomatoD (Q08306), TomatoE (Q08307), TomatoF (Q08296), Potato1 (*Solanum tuberosum,* Q06355), Potato2 (T07097), Potato 3 (T07096). The bar indicates 0.1 nucleotide substitutions per site.

wound-induction has been reported include tomato PPO-F and a pineapple PPO (Fig. 6). Thus there does not appear to be a correlation of wound-induced expression and primary sequence classification.

Expression and possible functions of hybrid poplar PPOs

The expression patterns of each of the three poplar PPOs were surprisingly different and organ-specific. We note that although we designed probes specific for each of the three cDNAs for this analysis, we cannot rule out that the probes are detecting expression of more than one very similar genes, which together contribute to the complex expression patterns. *PtdPPO1* was expressed only in stress-induced leaves, while *PtdPPO3* mRNA was almost exclusively expressed in roots. *PtdPPO2* expression was most widespread, being expressed in organs with conducting functions (root, petiole, mid-vein, and stem), as well as in cell culture. *PtdPPO3* was not wound- or MeJA-induced in roots and only very slightly induced by MeJA in cell cultures, and mostly expressed in roots. Surprisingly *PtdPPO1* did not respond to MeJA treatment in cell culture despite being MeJA inducible in leaves, unlike *PtdPPO2*. This specificity of the poplar PPO gene expression was in contrast to reports for other species such as tomato, where most of the PPO genes are expressed in a wide variety of tissues including leaf, root, stem, and reproductive tissues (Thipyapong et al. 1997). In potato, PPO expression is slightly more tissue-specific, but most genes are expressed in several different tissues (Thygesen et al. 1995). However, the highly root-specific expression of potato PPO gene Pot72 was similar to what we observed for *PtdPPO3*. Tight tissue-specific regulation could be indicative of some differentiation of function of the PPO isoforms (see below).

PtdPPO1 and *PtdPPO2* were both induced by wounding and MeJA in leaves and stems/roots, respectively (Figs 4 and 5). In previously reported western blots, we did not detect wound-induction of PPO-2 in stems (Wang and Constabel 2003); this discrepancy is probably due to the significant level of PPO constitutively present in stem tissues, and by the greater sensitivity of northern analysis relative to western blots. Wound-induction is a property of a variety of plant PPOs, and wound-induced PPOs have been described in tobacco, tomato, and apple (Boss et al. 1995, Constabel and Ryan 1998). Up-regulation of PPO genes following wounding and pathogen attack is often seen to be adaptive, and provides indirect evidence that PPO plays a role in defence against pests and pathogens. We therefore speculate that since *PtdPPO2* is induced in stems and roots, it might be effective against boring insects which attack these tissues. Defence of the stem to protect the conducting tissues is likely of great importance for the plant, and this could explain why plants maintain high constitutive PPO activity in petioles, stems and roots.

In addition to MeJA, *PtdPPO2* was up-regulated by an elicitor derived from the poplar pathogen *V. macularis*, as well as flg22 (data not shown), a bacterial pathogen-derived peptide elicitor (Felix et al. 1999). The induction of *PtdPPO2* in cell culture by pathogen elicitors suggests a potential role of this PPO in defence against pathogens. PPO induction in diseased tissues and during defence reactions has been observed in a variety of plants (reviewed in Constabel et al. 1996). In tomato, the importance of PPO-mediated phenolic oxidation in disease resistance was demonstrated using PPO-overexpressing transgenics; compared with the wild type, transformed leaves showed significantly fewer lesions and contained at least 100-fold fewer bacterial counts after inoculation with the pathogen *Pseudomonas syringae* (Li and Steffens 2002).

In contrast to the pathogen-derived elicitors, the defence gene inducer salicylic acid reduced *PtdPPO2* expression in cells (Fig. 5), as well as wound-induced *PtdPPO2* expression in stems (data not shown). The inhibitory effect of SA on wound- and MeJA-induced gene expression has been previously reported for the induction of proteinase inhibitors in the tomato as well as the *Arabidopsis* defence response (Doares et al. 1995, Kunkel and Brooks 2002). Such cross-talk may demonstrate the interaction of signalling pathways involved in wounding and pathogen defence in poplar, as has been suggested in other species. In contrast to the situation for *PtdPPO2*, the wound-induced tomato PPO-F gene was induced by SA as well as MeJA (Thipyapong and Steffens 1997). These data suggest that such patterns could be gene-specific, and confirm previous observations that PPO expression varies significantly between species (Constabel and Ryan 1998).

The tissue-specific differential expression of PPO genes observed here may reflect differences in function, especially if the three encoded enzymes show different substrate specificities and other biochemical properties. PPOs in general have broad substrate preferences and oxidize a variety of diphenolic compounds (Mayer and Harel 1979). Nevertheless, individual PPOs often have distinct preferred substrates. Our previous study on wound-induced leaf and stem PPO in poplar indicate that catechin and DOPA are better substrates for PPO-1 than PPO-2, and that PPO-1 in general appears to have broader specificity (Wang and Constabel 2003). Other differences include stability, pH optima, and differences in the requirement for activation by SDS. To extend these studies to root PPO, we carried out preliminary biochemical analysis of crude extracts from young roots where, based on our northern analyses, the predominant expressed PPO is expected to be encoded by *PtdPPO3*. These experiments indicated that root PPO had a higher preference towards chlorogenic acid and a lower preference for caffeic acid, compared to the leaf and stem PPOs (data not shown). In addition, the root PPO showed significant activity in the absence of SDS, unlike the other isoforms. Therefore, these biochemical differences between stem, root and leaf PPO may provide a rationale for the differential expression of their corresponding *PtdPPO* genes described here, as

conditions and substrate availability are likely to vary between these organs. Identification of the endogenous PPO substrates in different poplar tissues will be an important next step.

Additional insight into the specific roles of the hybrid poplar PPOs will come from investigations into cell-type-specific expression patterns. In preliminary experiments with tissue printing, we determined that in young stems, PPO activity and protein was primarily expressed in the epidermal or subepidermal cells in stems and petioles (data not shown), consistent with a role in defence. In older stems, PPO was additionally detected in phloem and xylem cells. Future work will continue to focus on cell-type localization of the hybrid poplar PPOs by in situ hybridization, from both defence and developmental perspectives.

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