The role of xylem class III peroxidases in lignification

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Abstract

Lignification is a cell wall fortifying process which occurs in xylem tissue in a scheduled manner during tissue differentiation. In this review, enzymes and the genes responsible for lignin biosynthesis have been studied with an emphasis on lignin polymerizing class III secretable plant peroxidases. Our aim is to understand the cell and molecular biology of the polymerization of lignin especially in tracheids and vessels of woody species but much of the experimental evidence comes from herbaceous plants. Class III peroxidases pose many problems for empirical work as their encoding genes are variable, their substrate specificities are wide and the half-life of many of the isozymes is very long. However, there is some evidence for the role of specific peroxidases in lignin polymerization through antisense mutants in tobacco and poplar and from tissue and cell culture lines of Picea abies and Zinnia elegans. Peroxidase enzyme action has been shown by substrate specificity studies and, for example, RT-PCR results have pointed out that many peroxidases have tissue-specific expression patterns. Tissue-level location of gene expression of some peroxidases has been studied by in situ hybridization and their cellular localization with antibodies and using EGFP-fusion genes. From these, it can be concluded that, although many of the xylem class III peroxidases have the potential for functioning in the synthesis of the lignin polymer, the combined information of catalytic properties, expression, and localization can reveal differences in the significance of different peroxidases in the lignification process.

Key words: Cell wall, lignin, peroxidase, polymerization.

Introduction

Lignin is a natural, branched plant biopolymer generated by radical coupling of hydroxycinnamyl subunits called monolignols, mainly coniferyl (CA), sinapyl (SA), and p-coumaryl alcohols (p-CA) and creates, together with hemicelluloses, a glueing matrix for cellulose microfibrils in tracheary elements (TEs) and fibres of higher plants (Boerjan et al., 2003). Lignification in TEs and fibres occurs along the secondary cell wall formation which is initiated in tree xylem cell corners and middle lamellae. Eventually, these cells die through programmed cell death. Lignin provides mechanical strength and resistance against pathogens, and makes the cell walls impermeable to water, thus enabling the transport of solutes via tracheids in the xylem tissue. In addition to lignification during growth, lignification also occurs as a stress response (Kim et al., 2008; Srivastava et al., 2007).

The biosynthesis of monolignols initiates from the general phenylpropanoid pathway where phenylalanine is converted to p-coumaryl CoA via a series of enzymatic reactions, catalysed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate coenzymeA: ligase (4CL) (reviewed by Boerjan et al., 2003; Fig. 1). Monolignols are synthesized from p-coumaryl CoA via aromatic ring hydroxylation, O-methylation and conversion of side chain carboxyl to an alcohol, after which they are transported to the cell wall (Boerjan et al., 2003; Fig. 1). The transport mechanisms of monolignols from the cytoplasm to the cell wall are still largely unknown. Monolignols are found in plants both as free and sparingly water-soluble monolignols and as water-soluble monolignol glucosides. The latter, namely coniferin, syringin, and p-coumaryl alcohol glucoside, have been proposed to be...
either transport forms, intermediates or storage forms of monolignols (Steeves et al., 2001; Tsuji and Fukushima, 2004). Coniferin accumulation has been found to correlate spatially and temporally with the beginning of secondary growth in conifers (Freudenberg and Harkin, 1963; Savidge, 1989). In Arabidopsis thaliana, coniferin and syringin accumulation has been observed in light-treated roots (Hemm et al., 2004). Monolignol deglucosylation is thought to occur in the cell wall, at the site of lignin polymerization by specific β-glucosidases. β-Glucosidases with the ability to catalyse the deglucosylation of monolignols have been identified in Norway spruce (Picea abies) (Marcinowsky and Grisebach, 1978), in some Pinus species (Leinhos et al., 1994; Dharmawardhana et al., 1995), and in A. thaliana (Escamilla-Treviño et al., 2006).

For monolignol transport through the plasma membrane, either as free monolignols or as glucosides, four possible mechanisms can be envisaged: (i) passive diffusion through the plasma membrane based on hydrophobic interactions, (ii) Golgi-mediated vesicle transport, (iii) active transport

Fig. 1. Enzymatic pathway leading to the synthesis of monolignols CA, SA, and ρ-CA. See text for abbreviations. The transmission electron micrograph is of Norway spruce (Picea abies) mature tracheid cell wall which has been gold labelled with an antibody directed against dibenzodioxocin, a lignin substructure (for further details, see Kukkola et al., 2003).
via plasma membrane-located ATP-binding cassette (ABC) transporters, and (iv) facilitated diffusion through plasma membrane channels.

There is some evidence on the passive diffusion of monolignol-like compounds through membranes of artificial liposomes due to hydrophobic–hydrophilic interactions, but there is no evidence as yet that this transport possibility would be found in plant tissues (Boija and Johansson, 2006). An elaborate study by Kaneda et al. (2008) with radiolabelled $^{3}$H-phenylalanine fed to Pinus contorta seedlings supported a model where, as yet unknown, membrane transporters, rather than Golgi vesicles, would export the monolignols into the apoplastic space. Also, in global transcript profiling of A. thaliana stems, seven genes coding for ABC transporters have shown similar expression profiles to known monolignol biosynthetic genes (Ehlting et al., 2005). The cell wall macroarray analysis of maize (Zea mays) brown mid-rib (bm) mutants with altered lignin compositions has indicated that in one of the mutant lines, bm2, the decreased guaiacylglycerol lignin levels may be due to decreased transcription of one of the ABC-transporter genes (Guillaumie et al., 2007).

In the developing cell wall, lignin polymerization occurs by radical coupling reactions (Freudenberg, 1959). The secondary cell walls of xylem cells consist of three sublayers (S1, S2, and S3). Cell wall lignification initiates from cell corners and middle lamellae, proceeding through S1, S2, and S3 together with cell wall thickening. The deposition of specific lignin substructures has been demonstrated with immunolabelling techniques (Kukkola et al., 2003, 2004; see also Fig. 1). Monolignol oxidation/dehydrogenation is one of the earliest cellular functions proposed for peroxidases (PRXs) (Harkin and Obst, 1973). They are often found in lignifying tissues and they have the capability to oxidize a wide variety of small phenolic compounds, including monolignols. However, other enzymes with monolignol oxidizing activity, such as laccases and other phenol oxidizing PRXs. Interestingly, the sinapyl alcohol oxidizing PRX PalPrx08 (CWPO-C) from poplar cell culture is also able to oxidize larger substrates, synthetic lignin polymers, and ferrocyanochrome c, suggesting participation in radical formation in the lignin polymer (Sasaki et al., 2004). Structural modelling of PalPrx08 predicts that the substrate binding site of this enzyme is similar to those of the other plant PRXs suggesting that these large substrates do not interact with the haem, for which reason a second substrate oxidation site on the surface of the protein was expected

### PRX catalysis and lignification

In the regular PRX catalytic cycle, one molecule of hydrogen peroxide ($\text{H}_2\text{O}_2$) is reduced and two molecules of reducing substrates are oxidized (Fig. 2). Typically, class III plant PRXs are able to oxidize a wide variety of small phenolic compounds, including the lignin monomers, monolignols. However, differences between the abilities of different PRXs to oxidize different monolignols occur. While coniferyl alcohol is easily oxidized by most of the PRXs, sinapyl alcohol seems to be a poor substrate for many and it has been suggested that, even during lignin synthesis, sinapyl alcohol dehydrogenation is mediated by other phenolic radicals (Takahama and Oniki, 1994; Takahama, 1995). The reason for this kind of sinapyl compound discrimination of A. thaliana AtPrx53 (ATP A2) (Nielsen et al., 2001) has been studied by docking monolignols and ferulic acid to the substrate binding site AtPrx53 in the X-ray structure of A. thaliana PRX (Østergaard et al., 2000). Here, docking coniferyl alcohol and ferulic acid resulted in identical hydrophobic interactions with the enzyme, involving amino acid residues P69, I138, P139, S140, R175, and V178. Docking p-coumaryl alcohol gave fewer interactions, suggesting weaker binding of the substrate. Instead, docking sinapyl alcohol in the same orientation as the other substrates was not successful because of the overlapping of I138 and P139 with the second methoxyl group in sinapyl alcohol (Østergaard et al., 2000). Since P139 is conserved in the class III PRX superfamily, the authors proposed that this structural hindrance of sinapyl alcohol binding is occurring in all the family members.

However, class III PRXs with the ability to oxidize sinapyl alcohol have been purified from, for example, tomato (Lycopersicon esculentum) (Quiroga et al., 2000), poplar (Populus alba) cell culture (Ayoama et al., 2002), Zinnia elegans (Gabaldón et al., 2005), and silver birch (Betula pendula) (Marjamaa et al., 2006a), suggesting that an unmediated way to oxidize sinapyl alcohol by PRXs exists. Recently, Gomez Ros et al. (2007) identified common motifs in the primary structures of known sinapyl alcohol oxidizing PRXs. Interestingly, the sinapyl alcohol oxidizing PRX PalPrx08 (CWPO-C) from poplar cell culture is also able to oxidize larger substrates, synthetic lignin polymers, and ferrocyanochrome c, suggesting participation in radical formation in the lignin polymer (Sasaki et al., 2004). Structural modelling of PalPrx08 predicts that the substrate binding site of this enzyme is similar to those of the other plant PRXs suggesting that these large substrates do not interact with the haem, for which reason a second substrate oxidation site on the surface of the protein was expected...
PalPrx08 contains two specific Tyr residues, Tyr-74 and Tyr-177, which, according to the structural model, are both exposed on the protein surface and located near the haem, thus having potential for participation in electron transfer (Sasaki et al., 2008). Chemical modification of PalPrx08 Tyr residues to 3-nitrotyrosine with tetranitromethane (TNM) indeed resulted in decreased oxidation of syringaldazine, a dimeric sinapyl alcohol analogue, and prevented ferrocytochrome c oxidation, proposing the involvement of these residues in the oxidation of large substrates (Sasaki et al., 2008).

The peroxidative cycle of PRXs consumes hydrogen peroxide, which in the cell wall can be generated by, for example, NADPH oxidases (Ros Barceló et al., 2002), amine oxidase (Møller and McPherson, 1998), and oxalate oxidases (Caliskan and Cuming, 1998), and in certain conditions, for example, in the presence of NADH or during auxin oxidation even PRXs themselves (Liszkay et al., 2003; Kawano, 2003) are able to catalyse the formation of hydrogen peroxide. It can be argued that the availability of H₂O₂ has a role in lignification as one of the controlling factors in lignin polymerization. It has been found out that in the lignin-forming tissue-culture system of Norway spruce (Picea abies), inhibition of PRX activity through H₂O₂ scavenging caused a drastic reduction in lignin formation (Kärkönen et al., 2002). In addition, transgenic tobacco plants overexpressing the PRX gene IhPrx04 (swpa4) have shown increased H₂O₂ production which may have caused their increased lignin and phenolic content (Kim et al., 2008).

Induced lignification and increased expression of lignin biosynthetic genes has been found in transgenic hybrid aspen trees with elevated extracellular hydrogen peroxide concentrations due to the expression of antisense aspen lignin peroxidase dismutase (Srivastava et al., 2007). Accumulation of hydrogen peroxide in the cell walls is also controlled by reducing agents, most importantly ascorbate, the amount of which is apparently reduced to a minimum in lignifying cell walls in trees through ascorbate oxidase activity (Kärkönen and Fry, 2006; Ros Barceló et al., 2006).

The cell wall structural proteins, extensins, and ferulic acid residues in cell wall polysaccharides may also be the targets of PRX action causing cross-link formation, stiffening of the cell wall and cessation of cell elongation (Brownleader et al., 2000; Fry, 2004). However, in the presence of NADH and H₂O₂, PRXs may produce _OH which, in turn, causes cell wall loosening and promotes cell elongation (Liszkay et al., 2004).

### Class III plant PRX proteins and gene expression in lignifying tissues

Participation of class III plant PRXs in lignin synthesis has been studied in many plant species and tissue culture systems, most importantly in tobacco (Nicotiana tabacum) (Lagrimini et al., 1987, 1997; Blee et al., 2003), Arabidopsis (Sasaki et al., 2008). PalPrx08 contains two specific Tyr residues, Tyr-74 and Tyr-177, which, according to the structural model, are both exposed on the protein surface and located near the haem, thus having potential for participation in electron transfer (Sasaki et al., 2008). Chemical modification of PalPrx08 Tyr residues to 3-nitrotyrosine with tetranitromethane (TNM) indeed resulted in decreased oxidation of syringaldazine, a dimeric sinapyl alcohol analogue, and prevented ferrocytochrome c oxidation, proposing the involvement of these residues in the oxidation of large substrates (Sasaki et al., 2008).

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Induced lignification and increased expression of lignin
Peroxidases in lignification

PRXs in lignifying xylem of trees

During secondary growth, trees produce large amounts of secondary xylem thus providing researchers with a good source of tissue material undergoing lignification. In addition, lignin in trees has commercial significance since it affects the utilization of wood material for construction, for pulp and paper manufacture, and even for cellulose hydrolysis for bioethanol production.

Genes and enzymes involved in monolignol biosynthesis and also lignin polymerization have been studied extensively (Boerjan et al., 2003; Marjamaa et al., 2007). PRX activity arizing from several PRX isoforms has been found often in protein extracts of lignifying secondary xylem of trees (Polle et al., 1997; Fagerstedt et al., 1998; Tsutsumi et al., 1998; Christensen et al., 1998, 2001; McDougal, 2001; Marjamaa et al., 2003, 2006a, b; Koutaniemi et al., 2005, 2006). In these studies, inhibition of PRX activity through H$_2$O$_2$ scavenging caused a drastic reduction in lignin formation (Kärkönen et al., 2002).
sequence of *Populus trichocarpa* is now available and provides valuable material for studying lignification related genes in trees (Tuskan et al., 2006). In this poplar genome, 34 phenylpropanoid and lignin biosynthetic genes have been identified and in the PeroxiBase (http://peroxidase.isb-sib.ch/), the PRX specific database (Bakalovic et al., 2006), sequence information of 105 class III PRXs can be found at the moment. Determination of the role of these PRXs in lignification is a future challenge.

Several PRX isoforms have been found in protein extracts of Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and silver birch (*Betula pendula*) stems during secondary growth (Marjamaa et al., 2003). In Norway spruce, the cationic forms are dominant whereas, in silver birch, most of the activity seems to arise from the anionic isoforms (Marjamaa et al., 2003). When partially purified, the PRX fractions from Norway spruce, in general, preferred coniferyl alcohol as a substrate, while in silver birch at least one of the anionic isoforms showed the highest oxidation rate with sinapyl alcohol (Marjamaa et al., 2006a). In an RT-PCR study of lignin biosynthesis-related genes of Norway spruce by Koutaniemi et al. (2007), it was shown that at least 11 of the 17 PRX genes tested were expressed in lignifying xylem of adult trees, but the highest expression in all the lignin-forming materials (lignifying tissue-culture line, mature xylem, young vertical xylem, and young compression wood) was related to PabPrx02, PabPrx03, PabPrx08, PabPrx13, and PabPrx14 genes. However, based on up-regulation under both pathogen attack and in compression wood, PabPrx02, and PabPrx03 appeared to have a general stress-induced function (Koutaniemi et al., 2007). When tissue and cell-specific expression of three Norway spruce PRXs was studied using *in situ* hybridization, it was found that at least PabPrx01 and PabPrx02 transcripts coding for cationic PRXs were found in developing tracheids but not in ray cells of Norway spruce xylem (Marjamaa et al., 2006b).

The final localization of the PRX proteins must be considered when attempting to reveal their functions. The amino acid sequences of class III plant PRXs typically begin with an N-terminal secretion signal peptide for ER targeting, which can usually be easily recognized. In some of the PRXs, the signal peptide is followed by an N-terminal extension with unknown function (Welinder et al., 2002). Many PRXs are also found in vacuoles of plants (Carter et al., 2004), and the vacuolar localization is often thought to be related to the presence of a C-terminal extension peptide (Matsui et al., 2003), which however, are often variant in structure. In addition, PRXs with binding ability to pectin (Carpin et al., 2001), lignin (Koutaniemi et al., 2008), and the plasma membrane (Mika and Lüthje, 2003) have been identified. It is well known that the microenvironment in the cell walls, including, for example, structural carbohydrates, affects the lignin structures. In addition, the local concentrations of monolignols or polymerizing enzymes and pH affect the radical and lignin polymer formation and ratios of specific linkages in the polymer, suggesting that spatial distribution of PRXs or other oxidative enzymes may have an impact on lignin polymer formation (Brunow et al., 1998; Sipilä et al., 2004; Mechlin et al., 2007).

**Transgenic plants and mutant lines with altered PRX expression and lignification**

Despite intensive research of many years, there are only a few examples where modification of PRX gene expression has been reported to have an impact on xylem lignification in transgenic plants. This may be due to functional redundancy of various PRXs located at the lignifying cell wall. Down-regulation of the gene coding for a cationic PRX *NiPrx60* (*TP60*) in tobacco resulted in up to a 50% reduction in the amount of lignin measured by the acetyl bromide method (Blee et al., 2003), while overproduction of a tobacco anionic PRX showed no impact on the lignin content of roots, leaves or stems of the transgenic tobacco plants (Lagrimini et al., 1997). However, the plants suffered from decreased root biomass and root branching which caused wilting. In further experiments with tobacco anionic PRX, Lagrimini et al. (1997) showed that this PRX gene (under its own promoter) is developmentally regulated and tissue-specific and is expressed in the epidermis and trichomes and in parenchymal tissues associated with vasculature but not in vascular tissue. Hence, this particular PRX does not seem to take part in lignin polymerization in TE. In transgenic aspen lines, lignin content determined by the modified acetyl bromide method decreased up to 20% resulting from the down-regulation of an anionic PRX *PkPrx03* (*PrxA3a*) (Li et al., 2003). *Arabidopsis thaliana* T-DNA mutant lines with defective genes encoding homologues of *PaPrx08*, with Tyr-177 or Tyr-74 showed 12.6% and 15.9% reduction in lignin, respectively (Tsutsumi et al., 2008). Over-expression of the gene coding for a cationic PRX caused ectopic lignification in transgenic tomato (*Lycopersicon esculentum*) plants (El Mansouri et al., 1999).

It has been reported that altered PRX expression may cause structural changes in lignin. The low lignin transgenic aspen lines described by Li et al. (2003) showed an increased proportion of syringyl units. Over-expression of a cationic PRX *PabPrx08* (*SP12*) caused reduced flexibility, deeper red phloroglucinol staining (a lignin stain with the highest reactivity to coniferaldehyde), and slightly increased frequency of aldehyde end groups in some transgenic tobacco lines (Elfrstrand et al., 2002). Taken together, and depending on the species in question, it may be necessary to modify the expression of multiple genes coding for cell wall located PRXs in order to achieve a controlled reduction in lignin polymerization.

**Conclusions**

The large number of class III secreteable plant PRXs poses many problems in the identification of the true lignin polymerizing PRXs in lignifying tissues. As seen through substrate specificity, RT-PCR, and *in situ* hybridization studies, many PRXs are expressed in the right location and
have the ability to oxidize monolignols resulting in lignin polymer formation. It is only through detailed work on the various PRXs and their signal sequences and substrate specificities and tissue-specific modification of expression levels of possibly multiple PRX genes that our knowledge on the last steps in the lignification process can be increased.

As there is some detailed information on the effects of the microenvironment (pH, amount of substrates and inhibitors, antioxidants, effects of other cell wall components) in the cell walls on the specific linkages formed in the lignin polymer by PRXs or laccases, we cannot expect to get natural lignin formed in test tube experiments. In addition, the formation of particular lignin structures in plants in situ has to be studied further, as well as their relations to other cell wall components.

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**References**


and larger products, and possible role in inter-polymeric cross-linking. *Phytochemistry Reviews* 3, 97–111.


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