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ORIGINAL ARTICLE

A potential role for sinapyl *p*-coumarate as a radical transfer mechanism in grass lignin formation

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Abstract Grass lignins are differentiated from other lignin types by containing relatively large amounts of *p*-coumaric acid (*p*CA) acylating the C-9 position of lignin subunits. In the case of a mature corn (*Zea mays* L.) stems, *p*CA constitutes 15–18% of a dioxane soluble enzyme lignin. The major portion of the *p*CA is specifically attached to syringyl residues. Studies with isolated corn wall peroxidases show that *p*CA readily undergoes radical coupling in the presence of hydrogen peroxide, whereas sinapyl alcohol radical coupling proceeds more slowly. Analysis of corn wall peroxidases did not reveal specific enzymes that would lead to the preferred incorporation of sinapyl alcohol as seen in other plants. The addition of ethyl ferulate, methyl *p*-coumarate, or sinapyl *p*-coumarate conjugates to a reaction mixture containing peroxidase, sinapyl alcohol, and hydrogen peroxide stimulated the rate of sinapyl alcohol radical coupling by 10–20-fold. Based on spectral analysis it appears that *p*CA and ferulate radicals form rapidly, but the radical is readily transferred to sinapyl alcohol. The newly formed sinapyl alcohol radicals undergo coupling and cross-coupling reactions. However, sinapyl alcohol radicals do not cross-couple with *p*CA radicals. As long as hydrogen peroxide is limiting *p*CA remains uncoupled. Ferulates have similar reaction patterns in terms of radical transfer though they appear to cross-couple in the reaction mixture more readily then *p*CA. The role of *p*CA may be to internally provide a radical transfer mechanism for optimizing radical coupling of sinapyl alcohol into the growing lignin polymer. Attachment of some *p*CA to sinapyl alcohol ensures localization of the radical transfer mechanism in areas where sinapyl alcohol is being incorporated into lignin.

Keywords Lignin · Cell wall · Peroxidase · Hydroxycinnamic acid · *p*-Coumarate · Ferulate · Radical transfer mechanism

Abbreviations

FA	Ferulate
*p*CA	*p*-Coumarate
CoA	Coenzyme A
SA-pCA	Sinapyl *p*-coumarate
Et-FA	Ethyl ferulate
Me-*p*CA	Methyl *p*-coumarate
CA	Coniferyl alcohol
SA	Sinapyl alcohol

Introduction

Hydroxycinnamates are common in many land plants (Harris and Hartley 1980). Grass species contain significant amounts of ferulate (FA) and *p*-coumarate (*p*CA) esters acylating polysaccharides or, in the case of *p*CA, lignin. It has been speculated that the role of these hydroxycinnamates is to cross-link matrix components altering the

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structural integrity of the cell wall. In the case of ferulates this role has been clearly demonstrated by showing they cross-link arabinoxylans to each other via formation of dehydrodiferulates (Bunzel et al. 2004; Ishii 1991; Ralph et al. 2004) and cross-link arabinoxylans to lignin through radical coupling reactions (Grabber et al. 2000; Lam et al. 1992; Ralph et al. 1995). Ferulate dimers isolated from grass cell walls represent all possible free radical coupling reactions resulting in extensive cross coupling of arabinoxylans (Ralph et al. 1994b) and further coupling to lignin (Grabber et al. 2002; Ralph et al. 1995, 2004). The extensive cross-linking of wall polymers has a significant detrimental effect upon cell wall degradation, limiting nutrient availability to ruminants (Grabber et al. 1998a, b, 2008).

A role for *p*CA in plant development has not been so clearly demonstrated. It has been proposed that wall bound *p*CA and FA help limit cell wall degradation by *Sesamia nonagrioides* (stem borer) contributing to the resistance of specific corn (*Zea mays* L.) lines to this insect pest(Santiago et al. 2006). It is possible that *p*CA inhibits growth or general metabolism of insects such as corn borer leading to decreased cell wall damage. Although these studies are interesting, it is impossible to separate the individual contributions of *p*CA and FA (including FA dimers) to the observed insect resistance. It has also been shown that *p*CA has a strong inhibitory effect upon hypocotyl growth in mung bean (Demos et al. 1975). To our knowledge this hypothesis has not been rigorously tested. Similar growth inhibition studies have not been done in grasses like corn that accumulate large amounts of esterified *p*CA within cell wall matrices (Morrison et al. 1998; Ralph et al. 1994a). The accumulation of *p*CA in corn walls occurs in the lignin fraction and is shuttled out to the wall as sinapyl alcohol *p*CA esters (Grabber et al. 1996; Hatfield et al. 2008; Lu and Ralph 1999). Cyclodimers of *p*CA resulting from photo activation have been reported in grasses (Ford and Hartley 1990). These compounds are cyclobutane dimers formed by cross coupling involving the C1 and C2 of the propenonic acid side chain of two adjacent *p*CA or FA molecules producing t-2.c-4-diphenyl-r-1,t-3-cyclobutanedicarboxylic acid or 1-3,t-4-diphenyl-r-1,c-2-cyclobutanedicarboxylic acid. Substitutions of *p*CA on arabinoxylans have been reported, though frequency of attachment to arabinosyl side chains is lower than ferulate substitutions. In ryegrass, *p*CA substitution occurs at a frequency of about one *p*CA to every 15 FA molecules bound to arabinosyl residues (Mueller-Harvey et al. 1986). Recently Allerdings et.a., demonstrated *p*CA attached to arabinose side chains in corn bran (Allerdings et al. 2006). Although it is difficult to determine the exact concentrations *p*CA substitution on arabinose in corn bran it would appear to be in a range of approximately 1 mg per 100 g of bran. For corn stems we have been unable to consistently show attachment of *p*CA to arabinoxylans in rind tissue (Hatfield et al. 2008). The high amounts of *p*CA found in grasses overwhelmingly acylates lignin, not polysaccharides (Ralph et al. 1994a). The significance of *p*CA cyclodimers is difficult to assess due to generally low levels to begin with and, as such dimers were only identified after alkaline hydrolysis, it is not possible to determine where they were originally attached.

In corn all of the *p*CA acylates the C-9 position of lignin units (Ralph et al. 1994a) and is primarily on syringyl units (Grabber et al. 1996; Lu and Ralph 1999).The process of *p*-coumaroylation of grass lignin is dependent upon the phenylpropanoid pathway to produce the activated *p*-coumaroyl-CoA (*p*CA-CoA). Activated *p*CA is esterified by sinapyl or coniferyl alcohol before being shuttled into the wall free space (Hatfield et al. 2008; Ralph et al. 1994a, 2004). The formation of *p*CA-CoA is a key branch point in the phenylpropanoid pathway leading to three main groups of metabolites; monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols), polyphenols (flavonoids, isoflavonoids, anthocyanins) and in grasses *p*CA-sinapyl or coniferyl alcohol conjugates (Humphreys and Chapple 2002). Therefore the incorporation of *p*CA into cell wall lignin must be part of a coordinated flux of metabolites through the pathways to ensure acylation of the lignin at proper levels and correct timing.

Although sinapyl alcohol (SA) is incorporated into growing lignin polymer, the *p*CA portion of the sinapyl *p*-coumarate (SA-*p*CA) conjugate escapes radical coupling reactions. We have observed that SA undergoes only slow radical coupling in the presence of H_2O_2 and isolated corn wall peroxidases. Such low affinity of wall peroxidases for sinapyl alcohol has been noted in other plants (Church and Galston 1988; Serjiades et al. 1993) as well as plants that appear to have developed more specialized peroxidases to handle sinapyl alcohol (Takahama et al. 1996). Hydroxycinnamic acids have been demonstrated to enhance the oxidation of sinapyl alcohol (Takahama and Oniki 1997; Takahama et al. 1996). Recently, incorporation of SA-*p*CA conjugates into a model lignin polymer has been demonstrated (Grabber and Lu 2007), using a maize cell wall culture system (Grabber et al. 1995). As seen in native grass lignins, the *p*CA portion of SA-*p*CA does not undergo radical coupling, remaining as free-phenolic pendant groups on the growing model lignin polymer; only the SA portion becomes coupled into the lignin polymer. However, using this cell wall model system they noted a rapid decline in peroxidase activity as a result of adding the *p*CA-SA conjugate resulting in limited enhancement in the incorporation of SA into the lignin polymer (Grabber and Lu 2007). It is not clear why there would be decreased peroxidase activity as the work reported here clearly demonstrates that *p*CA is readily oxidized by corn peroxidases. Also structural studies clearly indicate corn lignin contains significant amounts of syringyl units in the lignin polymer isolated form mature stems (Lu and Ralph 1999; Marita et al. 2003; Morrison et al. 1998). This manuscript reports on the investigation into the potential role for SA-*p*CA conjugates to provide a radical transfer mechanism to enhance the incorporation of SA into grass lignins.

Materials and methods

Isolation of corn peroxidases

Peroxidases were isolated from cell walls of corn cell suspension cultures. Cells were harvested at the stationary growth phase (approximately 14–16 days after transfer). Initial work had shown that the cell culture medium contained high levels of peroxidase activity and that these peroxidases were similar to those extracted from the cell walls. This allowed us to utilize the culture media for peroxidase production while utilizing the cell walls for other experiments. Peroxidases were partially purified from cell culture media as briefly described.

Culture media (500 mL) was made 40% with ammonium sulfate and allowed to stir on ice for 1 h. Precipitated protein and carbohydrate were pelleted by centrifugation (10,000 × g for 30 min). The decanted supernatant was made 75% with ammonium sulfate and stirred on ice for another 2 h. Precipitated protein was pelleted as before, the pellet dissolved in 50 mM Tris buffer (pH 7.0), and dialyzed against Tris buffer (2 L 50 mM, pH 7.0) for 24 h (4°C) with three changes of buffer. The crude peroxidase sample was concentrated by utrafiltration (Amicon stirred cell 400 mL using a Amicon Polyethersulfone ultrafiltration membrane PBQK 10 kD cutoff) and loaded on a DEAE column (Spectra Gel, 2.5 × 15 cm) equilibrated with 50 mM Tris buffer (pH 7.0). Unbound proteins were eluted with equilibration buffer. Bound proteins were eluted with a 180 mL NaCl gradient (0–500 mM) in Tris buffer (pH 7.0). Two-mL fractions were collected starting with sample addition to the column and continuing until the end of the gradient. All even fractions were assayed for total protein (reading at 280 nm) and peroxidase activity using guaiacol/H_2O_2 as substrate. Fractions containing peroxidase activity were pooled giving four peroxidase fractions (D1, D2, D3 and D4). All four peroxidase fractions contained similar relative activities against ethyl ferulate (Et-FA), methyl *p*-coumarate (Me-*p*CA), coniferyl alcohol (CA), and sinapyl alcohol (SA) substrates. The order of reactivity always followed the pattern of Et-FA $\geq$ CA > Me-*p*CA $\gg$ SA. The peroxidase activity against SA was always 25–50-fold less than FA. Peroxidases D1 were used for these experiments.

Peroxidase activity was also extracted from corn stems at a developmental stage when the tassel had emerged, but before being fully extended. Mature internodes 8, 9, and 10 and immature internodes 15,16, and 17 were excised from individual corn plants and pooled into a mature and immature groups. Three replicate plants were used in these studies. Mature internode sections were removed from the plant and divided into pith and rind tissues and cut into 5–10 mm sections before freezing in liquid nitrogen. Due to the small size of the immature internodes the stem tissues were not sub divided into rind and pith. These stem sections were cut into small sections 5–10 mm before freezing in liquid nitrogen and storing at −80°C prior to peroxidase extraction. Frozen corn stems sections were finely ground in a liquid nitrogen Freezer Mill (Spex Model 6750) using three cycles of 1 min at rate 15 followed by 0.5 min cool. All samples were pre-cooled for 2 min in liquid nitrogen before starting the grinding cycle. Immediately after grinding samples were quickly weighed into 50 mL Oakridge tube to obtain a recovered fresh weight before suspending in TRIS-acetate buffer (50 mM pH 7.0) at 2 mL g^{-1}. Samples were stirred for 2 h at 4°C before centrifuging (10,000×*g* 15 min) to pellet insoluble material and allow removal of the supernatant. Insoluble pellets were washed by suspending in 4°C TRIS-acetate buffer, stirring 10 min and the supernatant removed after pelleting the insoluble material (10,000×*g* 15 min). The wash cycle wash repeated a second time. All supernatants (original extract and washes) were combined frozen in liquid nitrogen and stored at −80°C until assayed for peroxidase activity. Insoluble materials (primarily cell walls) from the initial extraction were suspended in TRIS-acetate buffer containing $CaCl_2$ (2 mL g^{-1} fresh weight, 50 mM pH 7.0 + 200 mM $CaCl_2$) and stirred over night at 4°C. Calcium chloride extracts were recovered and the insoluble material washed as described for the original buffer extract. After the final wash after the $CaCl_2$ extraction the residue was suspended in TRIS-Acetate + LiCl (2 mL g^{-1} FW, 50 mM buffer pH 7.0 + 4 M LiCl) and stirred 24 h at 4°C. Extracted peroxidases were recovered from the cell wall residues as described above. For the $CaCl_2$ and LiCl extracts individual samples were dialyzed against cold TRIS-acetate buffer (10:1 dialysis ratio, fresh buffer after 8 h) for 24 h. All extracts after dialysis were frozen in liquid nitrogen and stored at −80°C until assayed for peroxidase activity.

Monitoring oxidation reactions

Ethyl ferulate (Et-FA), methyl *p*-coumarate (Me-*p*CA), coniferyl alcohol (CA), sinapyl alcohol (SA), sinapyl *p*-coumarate (SA-*p*CA) were prepared according to published methods (Lu and Ralph 1998a; Lu and Ralph 1998b) and used as phenolic substrates for peroxidase reactions using isolated corn peroxidases. All reactions were carried out in either 20 mM MES or 20 mM acetate buffer (pH 5.5). Single-substrate analysis reaction conditions used 140 nmoles of phenolic substrate, 450 nmoles H_2O_2, and 0.015 μg peroxidase in a total volume of 1 mL of buffer. Reaction rates were determined by monitoring the absorbance changes at the absorption maximum for each substrate (i.e., Et-FA 325 nm, Me-*p*CA 310 nm, SA 270 nm, SA-*p*CA 270 nm) over the course of the reaction (5–10 min depending upon the substrate). The phenolic substrate and peroxidase were added to the reaction buffer and the absorbance monitored for 1 min to obtain background readings before the reaction was initiated by the addition of H_2O_2. Rates of reactions were calculated from the linear portion of the decrease in absorbance curve.

For experiments determining the interaction of two phenolic substrates the primary substrate was sinapyl alcohol (SA) while the secondary substrate was Et-FA, Me-*p*CA or SA-*p*CA. Three different methods were used to monitor peroxidase-mediated oxidation reactions; spectrophotometric, HPLC, and NMR spectrometry. For the spectrophotometric analysis reaction conditions were as described above except the concentration of SA was increased to 266 nmoles while the secondary substrates were limited to 67 nmoles except for SA-*p*CA at 56 nmoles and 450 nmoles H_2O_2. Peroxidase was added to a final concentration of 2.4 μg mL^{-1} in a total final reaction volume of 1 mL. Reaction rates were monitored at 270 nm for 5 min. In some cases spectral scans (400–225 nm) were taken every 2-min to monitor changes in SA as well as the secondary substrate.

HPLC analysis of substrate utilization and product formation

To verify that primary substrates were being oxidized by the corn peroxidases, reactions were analyzed by HPLC to monitor the consumption of phenolic substrates. For HPLC experiments, reaction conditions were modified slightly to allow subsamples to be taken from the reaction mixture for HPLC analysis. The total reaction volume was 4 mL of buffer with SA at 1,064 nmoles, ET-FA or Me-*p*CA at 268 nmoles or SA-*p*CA at 236 nmoles, enzyme 0.452 μg, and 450 nmoles H_2O_2. Sinapyl alcohol oxidation in the total reaction mixture was monitored at 270 nm (spectrophotometric analysis as listed above) and samples of the reaction mixture (500 μL) removed, at times indicated, and added to 500 μL of ethanol to stop peroxidase activity. Samples were subjected to HPLC analysis on a C-18 column (Waters Spherisorb ODS2, 5 μm, 4.6 mm × 250 mm). Primary (sinapyl alcohol) and secondary (Et-FA, Me-*p*CA, SA-*p*CA) reactants were separated using an acetonitrile:H_2O gradient and dual wavelength monitoring (270 and 310 nm). The solvent gradient consisted of solvent A = H_2O with 1% acetic acid and solvent B = acetonitrile. Initial conditions consisted of 90% A and 10% B held for 2 min followed by a linear ramp to 65% B in 23 min, a second ramp to 100% B in three min and held for 6 min before returning to the start conditions in 3 min.

Proton NMR

Proton NMR analysis was used to monitor radical coupling products formed from sinapyl alcohol in the presence of Me-*p*CA or Et-FA. Substrate disappearance and product formation was monitored directly in the NMR tube. Concentrations used for these experiments (SA = 2,666 nmoles, Me-*p*CA = 78 nmoles, Et-FA = 67 nmoles, H_2O_2 = 2,000 nmoles in 1 mL10 mM phosphate buffer, pH 5.5) allowed sampling times of 2.5 min over a 24 h period. Data presented for the first 4 h of reaction as most of the SA is completely oxidized when reaction mixtures contain additions of Me-*p*CA, or Et-FA. The peroxidase used was D1 4.8 μg, except for Et-FA where only 2.4 μg was used.

Results and discussion

Peroxidase preparations isolated from cell walls of both cell culture and internode tissues of corn were analyzed to determine their abilities to oxidize cell wall phenolic components (ferulic acid, *p*-coumaric acid, coniferyl alcohol, and sinapyl alcohol, as these are the most prominent phenolics associated with lignification of corn cell walls). In the case of peroxidases partially purified from corn culture cells the oxidative activity against Et-FA, Me-*p*CA, and CA was high compared to the activity with SA (Table 1). This would eliminate the possibility that peroxidases in primary corn culture walls lack the ability to readily oxidize *p*-coumarates so this would not have been a limitation in the in vitro lignification experiments described in the Introduction (Grabber and Lu 2007).

It is common that plant peroxidases do not oxidize the doubly methoxylated hydroxycinnamyl alcohol (SA) as read-

Table 1 Oxidation rates of various substrates via partially purified (D1) corn cell culture peroxidase. Each substrate was at an initial concentration of 150 nmoles, 400 nmoles H_2O_2, and peroxidase was added to a final concentration of 2.4 μg mL^{-1} of reaction buffer. Rates of reactions were monitored as the number of nmoles of substrate converted per mg of peroxidase per min of reaction time

Substrate	Reaction Rate (nmoles min^{-1} mg $protein^{-1}$)
Ethyl ferulate	226
Coniferyl alcohol	154
Methyl *p*-coumarate	122
Sinapyl alcohol	4

ily as the singly methoxylated hydroxycinnamoyl alcohol (CA) (Takahama et al. 1996). Some plants produce specific peroxidases or oxidases that readily oxidize sinapyl alcohol (Aoyama et al. 2002; Tsutsumi et al. 1998). Corn cell cultures readily incorporate peroxidases into their cell walls, but as the walls are not lignifying it is possible that the appropriate peroxidase is not being synthesized and exported to the wall space. To test this possibility we evaluated developing corn stems to determine if there was a stage of development when sinapyl alcohol-specific peroxidases were produced. If a specific peroxidase was being produced to oxidize SA, it's likely to be produced during the latter stages of secondary cell wall formation (mature stem internodes, 8, 9, & 10) when there is a shift to more syringyl-rich lignins (Terashima et al. 1993). At tassel extension corn stem development of the lower internodes (8–10) have reached near maturity and there is a shift to increased incorporation of SA into lignin (Morrison et al. 1998). Thakur identified several isozymes of peroxidases in corn (Thakur et al. 1981). However, the substrate specificity of these peroxidases has not been characterized and not all may be associated with the cell wall.

To test the possibility of a SA-specific peroxidase being synthesized during the stages of rapid cell wall lignification, peroxidases were isolated from two types of tissue; mature corn stem internodes (8, 9, 10) and immature internodes (15, 16, 17). Three peroxidase fractions were isolated; a buffer soluble, calcium chloride soluble, and a lithium chloride soluble fraction. The buffer soluble fraction would comprise peroxidases in the cytoplasm and free space of the cell wall, the $CaCl_2$ fraction would comprise weakly and ionic bound peroxidases, and LiCl would extract those peroxidases that are more tightly bound within the wall matrix. Although there was a difference in the total peroxidase activity extracted from mature internodes compared to immature internodes (Fig. 1a, b) the pattern of activity against different substrates was similar. The peroxidases solubilized in all three types of extractions oxidized Et-FA > CA > Me-*p*CA ≫ SA. In all cases SA was poorly oxidized in the presence of hydrogen peroxide (Fig. 1).

None of the corn extracts oxidized sinapyl alcohol as readily as the other substrates tested in these experiments. This would fit the observation that as the substitution by methoxy groups increases on the phenolic ring the ability of the native peroxidases to effectively oxidize these units decreases (Church and Galston 1988; Sato et al. 1995). However, it does not follow that activity would further increase with no methoxy substitutions on the ring. Methyl *p*-coumarate (an ester, not a hydroxycinnamyl alcohol, but with no methoxy substitutions on the ring), was not as readily oxidized by corn peroxidases as ferulate or coniferyl alcohol, both with one methoxyl substitution, at the three position, on the phenolic ring (Fig. 1). This also holds true for peroxidases partially purified from walls of corn cell cultures (Table 1). It is clear that the total amount of peroxidase activity varies with the development of the corn stem, but that the relative rates remain in the same order of reactivity, i.e., SA is always the most poorly oxidized phenolic.

It has been suggested that other phenolics could improve the incorporation (oxidation) of sinapyl alcohol into lignins, particularly in plants that do not produce peroxidases that readily oxidize sinapyl alcohol (Takahama 1995; Takahama and Oniki 1994, 1997; Takahama et al. 1996). Takahama demonstrated that water-soluble esters of *p*-coumarates (apparently acylating a hexose) in epicotyls of *Vigna angularis* increased oxidation of sinapyl alcohol (Takahama and Oniki 1997). This raises the possibility that *p*-coumarates function in this fashion in corn and other grasses in general. It has already been shown that *p*CA is coupled to lignin, particularly to syringyl units, at the C-9 position of side chains (Grabber et al. 1996; Lu and Ralph 1999; Ralph et al.

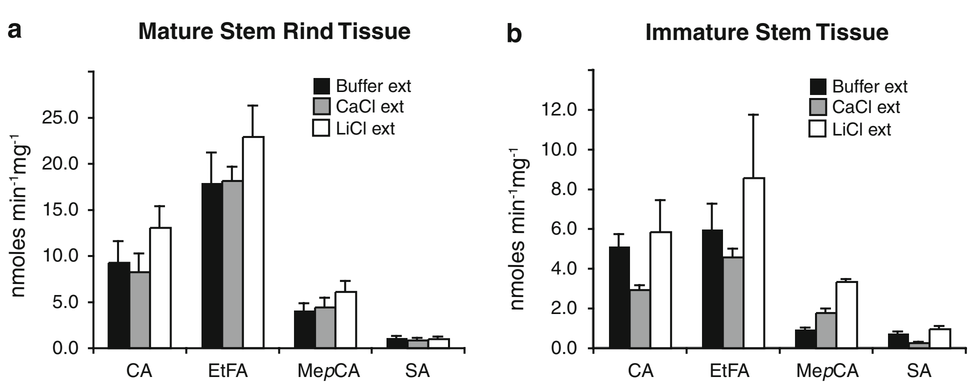


Fig. 1 Peroxidase activity extracted from corn stems tissues representing mature and immature portions of the stem at tassel emergence. **a** Stem rind tissues isolated form internodes 8, 9, and 10. **b** Total stem tissues from internodes 15, 16, and 17. Three different sets of plants were pooled and treated with sequential extractions to give buffer soluble, $CaCl_2$-soluble and LiCl-soluble protein fractions. All extracts were analyzed for peroxidase activity using coniferyl alcohol (*CA*), ethyl ferulate (*Et-FA*), methyl *p*-coumarate (*Me-pCA*), and sinapyl alcohol (*SA*). All reactions comprised 135 nmoles of phenolic substrate, 400 mmoles of H_2O_2, and protein varied from 4 to 9 μg per assay

1994a). This type of ester linkage formation can only occur through an enzyme-mediated process (Lu and Ralph 2002; Lu et al. 2004). The most logical route for *p*CA incorporation into lignin is therefore via lignification using *p*-coumaroylated monomers, i.e., conjugates with mainly SA. Such an arrangement would ensure the presence of an easily oxidized phenolic entity that could help to ensure the incorporation of SA into growing lignin polymers. A partially purified peroxidase from corn cell cultures was used to investigate this possibility in greater detail.

Corn peroxidase (D1) readily oxidized Et-FA, CA, and Me-*p*CA, but only slowly oxidized sinapyl alcohol (Table 1). Ethyl ferulate and methyl *p*-coumarate were tested as possible intermediates to aid in the oxidation of sinapyl alcohol. Analogs of both hydroxycinnamates are present in the cell wall at the time of lignification. Ferulates are anchored to arabinoxylans and are not free to diffuse throughout the wall free space. However, when lignification occurs, ferulates can become incorporated into the lignin polymer and may even act as nucleation sites for the onset of lignification (Ralph et al. 1995, 2004). It is possible that ferulates would aid in the oxidation of SA since they are readily oxidized by peroxidases and, by radical transfer, oxidized the less easily oxidized SA monomers (and syringyl units in the polymer). The real question is whether conjugates of *p*CA and sinapyl alcohol could also enhance SA oxidation for incorporation into lignin. To test these possibilities Et-FA, Me-*p*CA, and sinapyl *p*-coumarate (SA-*p*CA) were tested with the corn peroxidase.

When monitoring the oxidation/radical coupling reaction of sinapyl alcohol it is clear that the addition of FA or *p*CA enhances the rate of SA oxidation (Fig. 2). To closely monitor the peroxidase/H_2O_2 oxidation and radical coupling reactions, UV-vis spectral scans were taken of the reaction media. As illustrated in Fig. 3a little spectral change occurred when monitoring SA oxidation by

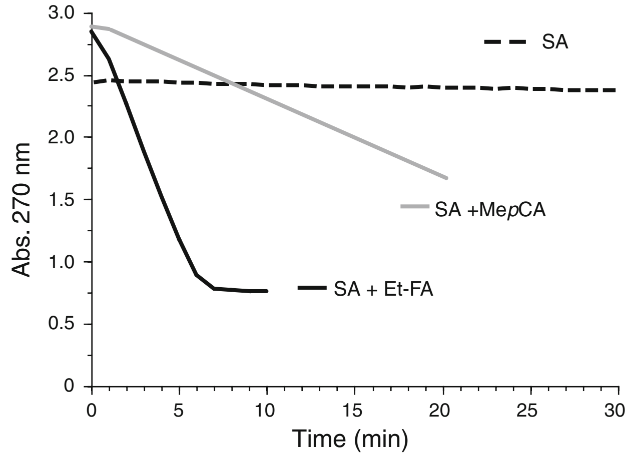
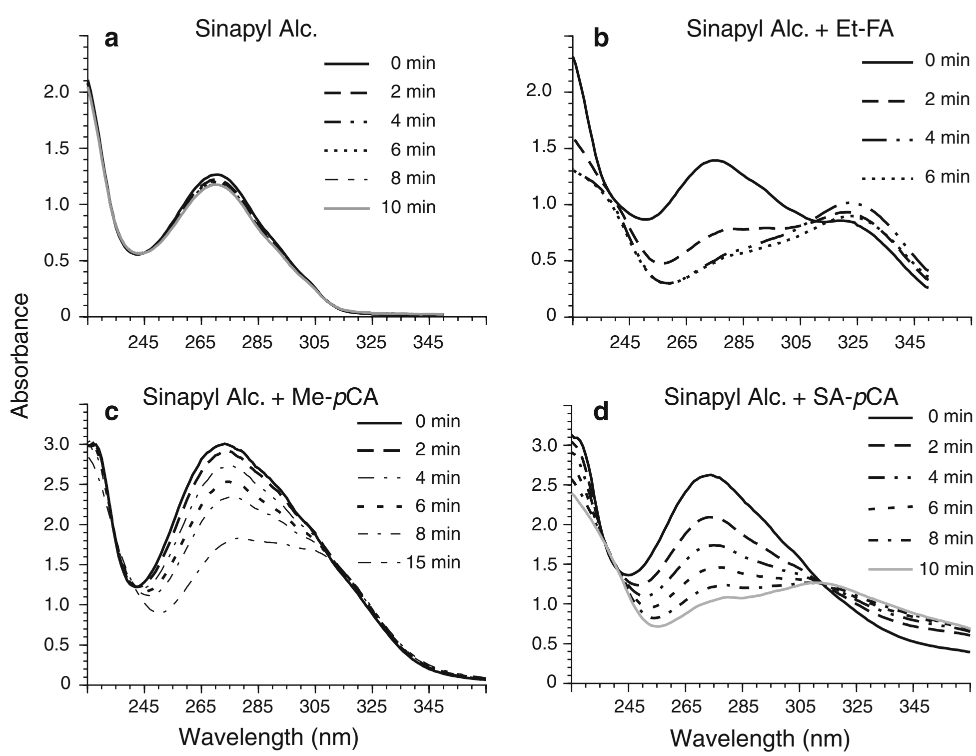


Fig. 2 Monitoring the oxidation of sinapyl alcohol (*SA*) by corn peroxidase T8D1 alone or with the addition of limiting amounts of methyl *p*-coumarate (*Me-pCA*) or ethyl ferulate (*Et-FA*). Reaction conditions were: SA = 1,064 nmoles, Me-*p*CA = 268 nmoles, Et-FA = 268 nmoles, H_2O_2 = 1,600 nmoles in 4 mL of 20 mM acetate buffer (pH 5.5). Separate experiments were run for SA only, SA + Me-*p*CA and SA + ET-FA. Reactions were monitored at 270 nm to determine rates of sinapyl alcohol oxidation

Fig. 3 Monitoring sinapyl alcohol (*SA*) oxidation as a function of time using scanning spectral analysis from 225 to 345 or 365 nm. SA was oxidized alone (**a**) or in the presence of ethyl ferulate (*Et-FA*, **b**), methyl *p*-coumarate (*Me-pCA*, **c**) or sinapyl *p*-coumarate (*SA-pCA*, **d**). Reaction conditions were as follows; SA = 266 nmoles, Et-FA = 67 nmoles, Me-*p*CA = 67 nmoles, SA-*p*CA = 56 nmoles, H_2O_2 = 450 nmoles in 1 mL of MES buffer (10 mM pH 5.5)



itself. However, the spectrum shifts quickly and significantly with the addition of Et-FA (Fig. 3b). A similar spectral pattern shift occurs when Me-pCA or SA-pCA is added (Fig. 3c, d). Although each added component (Et-FA, Me-pCA, SA-pCA) has spectral characteristics that contribute to the wavelengths of interest (225–400 nm) the limited amount added to each reaction mixture is a negligible contribution to the total composite spectrum that is largely due to SA. It is clear that adding FA or pCA to the reaction increases the rate of SA oxidation and is more closely aligned with the oxidation rate of the phenolic adduct.

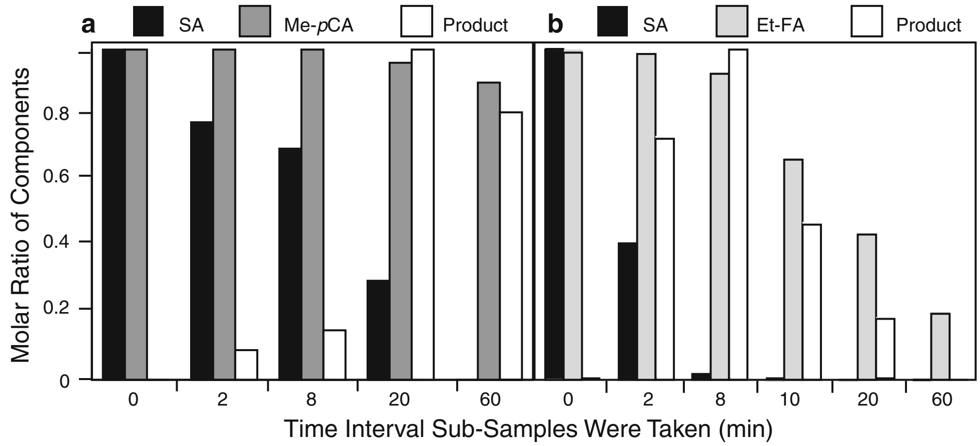


Fig. 4 Monitoring the loss of substrate from peroxidase reactions using HPLC to separate and identify substrates. Monitoring the oxidation of sinapyl alcohol (*SA*) by corn peroxidase D1 alone or with the addition of small amounts of methyl *p*-coumarate (*Me-pCA*) or ethyl ferulate (*Et-FA*). Reaction conditions were: SA = 1,064 nmoles, Me-pCA = 268 nmoles, Et-FA = 268 nmoles, H_2O_2 = 1,600 nmoles in 4 mL of 20 mM acetate buffer (pH 5.5). Separate experiments were run for SA only, SA + Me-pCA and SA + ET-FA. At given time points 500 µL of total reaction mixture was removed and mixed with 500 µL of ethanol to stop the reaction and sub-samples were subjected to HPLC analysis on a C-18 column (Waters Spherisorb ODS2, 5 µm, 4.6 mm × 250 mm)

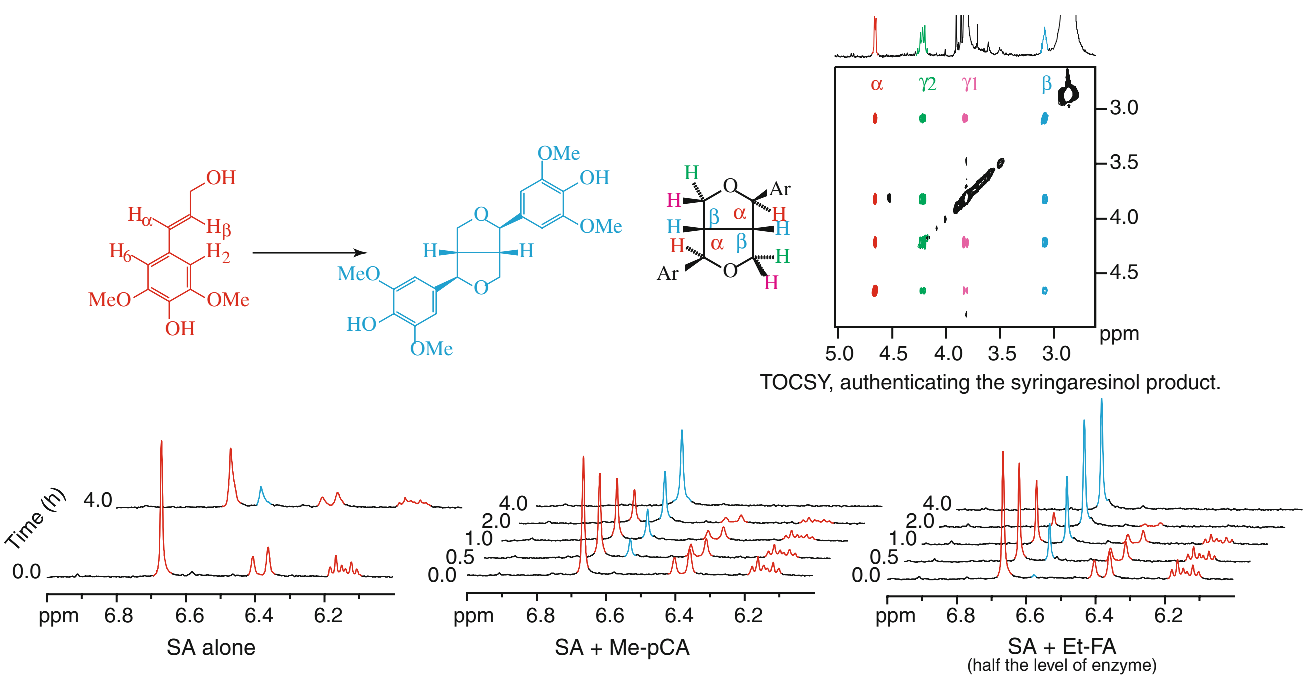


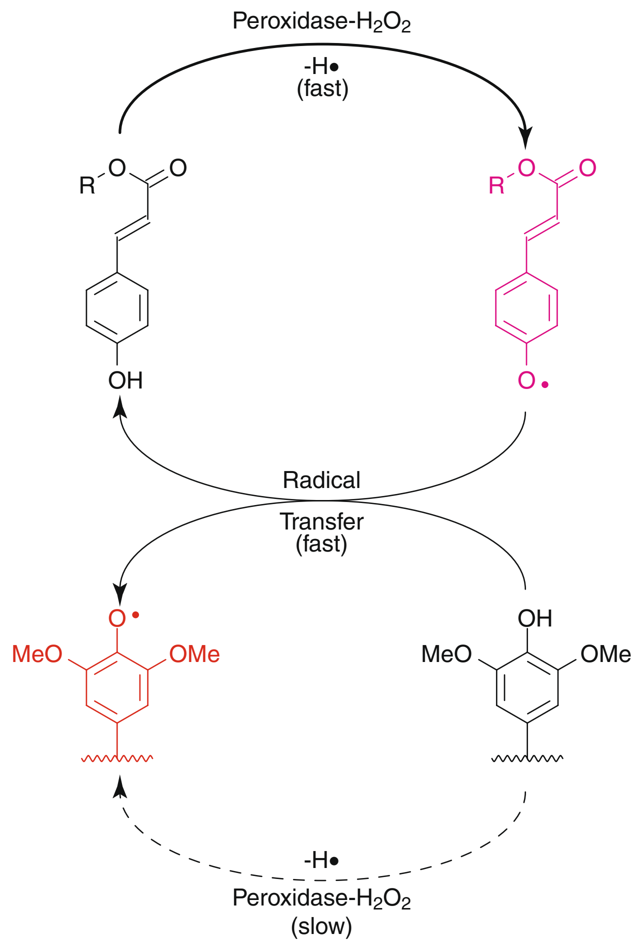
Fig. 5 Proton NMR analysis of radical coupling products formed from sinapyl alcohol (*SA*) in the presence of *Me-pCA* or *Et-FA*. Substrate loss (SA *red*) and product (syringaresinol *blue*) were monitored every 2.5 min for 4 h (only 0, 0.5, 1, 2 and 4 h proton spectra are shown). Initial reaction conditions were SA = 2,666 nmoles, Me-pCA = 78 nmoles, Et-FA = 67 nmoles, H_2O_2 = 2,000 nmoles in 1 mL phosphate buffer (10 mM pH 5.5). Peroxidase was corn cell culture D1 and 4.8 µg was added to each reaction except for Et-FA when only 2.4 µg was added. The 2D TOCSY NMR spectrum on the right authenticates the production of the sinapyl alcohol dehydrodimer, syringaresinol (after 4 h in the presence of limited pCA)

Monitoring this same type of enhanced oxidation using HPLC to separate reactants, SA is consumed in the combined oxidation reaction and is consumed before the corresponding adduct (FA or *p*CA) (Fig. 4a, b). This is clearly demonstrated in the *p*-coumarates/sinapyl alcohol reaction mixtures (Fig. 4a). It appears the preferred steps in the peroxidase/H_2O_2 mediated oxidation of the *p*CA/SA combination is for *pCA* to be oxidized followed by the transfer of its oxidation state to sinapyl alcohol. In the process the *p*CA radical is returned to its ground state allowing the whole process of oxidation-transfer-ground state to be repeated (Scheme 1). From the HPLC experiments *p*CA does not enter into coupling reactions until SA is exhausted and even then appears to prefer to transfer its oxidation state to the products formed from SA coupling most likely the *β*–*β*-dehydrodimers (syringaresinol) of sinapyl alcohol. The preference of *p*CA to transfer its oxidation state to another phenolic such as SA rather than undergo radical coupling fits the observation that the majority of wall bound *p*-coumarates remain as free-phenolic pendant groups, unattached beyond their initial ester linkages (Lu and Ralph 1999; Ralph et al. 1994a).

In a solution state, as in these buffer-based reaction systems, the predicted product for sinapyl alcohol radicals would be syringaresinol. To test that indeed sinapyl alcohol residues were being oxidized and followed by subsequent coupling reactions the peroxidase mediated oxidation reactions were monitored by ^{1}H-NMR (Fig. 5). When SA is the only phenolic in the reaction mixture the formation of oxidative radical coupling products is quite slow and only a small amount of the syringaresinol product is formed after 4 h. Over the next 20 h SA oxidation continues slowly with nearly complete oxidation by the end of 24 h. However, the addition of the ferulate or *p*-coumaroyl esters enhanced the rate of sinapyl alcohol oxidation leading to the rapid formation of the sinapyl alcohol *β*–*β*-coupling product syringaresinol. This demonstrates that even though sinapyl alcohol itself oxidizes poorly with the corn peroxidase over extended periods of time, addition of ferulates or *p*-coumarates (at limited concentrations) to the reaction mixture enhances the rate of product sinapyl alcohol dehydrodimer formation. Sinapyl alcohol therefore becomes oxidized under these conditions, logically via radical transfer from the more readily oxidized ferulate or *p*-coumarate esters.

Conclusions

Grass lignins are unique, containing substantial acylation derived from lignification using monolignol *p*-coumarate ester conjugates. Based on the experimental data presented here, a possible role for *p*-coumarates is as an oxidation shuttle (Scheme 1). A redox shuttle involving manganese oxalate has been shown to produce monolignol radicals without direct contact with an oxidase (Önnerud et al. 2002). The *p*CA component of a SA-*p*CA conjugate can be readily oxidized and will subsequently transfer its oxidation state to a suitable acceptor molecule such as sinapyl alcohol. The work presented here clearly demonstrates that such an oxidation shuttle operates in an in vitro system made up of corn peroxidases, H_2O_2, and the phenolic substrates, and presumably could also take place within the lignifying cell wall matrix. Although oxidized ferulate could also exchange its oxidative state with sinapyl alcohol, such a role may be limited due to the nature of ferulate attachment to cell wall arabinoxylans. Ferulates in grass cell walls acylate the C-5 hydroxyl of arabinosyl side chains of arabinoxylans; their mobility in the wall would be limited. However, if FA were oxidized by a peroxidase in the presence of a sinapyl alcohol unit, FA radical could transfer its unpaired electron to the sinapyl alcohol to form a sinapyl alcohol radical. In the case of SA-*p*CA conjugates there would be greater possibilities for diffusion into the wall free



Scheme 1 A diagram illustrating the possible mechanism by which *p*-coumarates act as an oxidative shuttle to enhance the oxidation of sinapyl alcohol during lignification of grass cell walls

space along with sinapyl alcohol residues. The oxidized *p*-coumarate units do not radically couple with oxidized sinapyl alcohol (or a syringyl units in lignin) and largely remain uncoupled (Scheme 1). Formation of *p*CA dimers does occur under conditions of excess H_2O_2/POD and if there are no other components to which radical transfer can occur. However, in the cell wall, once the sinapyl alcohol end is incorporated into lignin, mobility is limited and radical coupling between two immobilized *p*CA units would presumably be a rare event even if the system was not oxidant-limited.

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