Changes in Dehydrodiferulic Acids and Peroxidase Activity against Ferulic Acid Associated with Cell Walls during Growth of Pinus pinaster Hypocotyl

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Hydroxycinnamic acids associated with hypocotyl cell walls of dark-grown seedlings of Pinus pinaster Aiton were extracted with 1 N NaOH and identified by gas chromatography-mass spectrometry. The main hydroxycinnamic acid found was ferulic acid. Diferulic acid dehydrodimers were also found, with the 8,8-coupled isomer (compound 11) being the dehydrodiferulate present in the highest amount. However, the 5,5-coupled isomer, commonly referred to as diferulic acid, was not detected. Two truxillic acids, 4′-4′-dihydroxy-3′-3′-dimethoxy-α-truxillic acids I and II, were tentatively identified. The 8,8-coupled dehydrodiferulic acid (compound 11) was the phenolic acid that showed the most conspicuous changes with hypocotyl age as well as along the hypocotyl axis. Peroxidase activity against ferulic acid was found in the apoplastic fluid as well as being ionically and covalently bound to the cell walls. The peroxidase activity increased with hypocotyl age as well as from the subapical toward the basal region of the hypocotyls. A key role in the cell-wall stiffening of 8,8 but not 5,5 dimerization of ferulic acid catalyzed by cell-wall peroxidases is proposed.

The polysaccharides of plant cell walls contain hydroxycinnamic acids that are present as an ester-linked side chain. Ferulic acid has been identified as being ester linked to arabinoylans in monocotyledonous plants (Kato and Nevins, 1985; Hartley and Ford, 1989; Hartley et al., 1990b) and to pectic arabinans and galactans in dicotyledonous plants (Fry, 1982). Ferulic and p-coumaric acids have been identified as major hydroxycinnamic components bound to cellulosic secondary cell walls (Strack et al., 1987).

Ferulic acid, a major hydroxycinnamic acid, is able to undergo dimerization through peroxidase-mediated oxidative coupling to produce dehydrodiferulic acid, which cross-links matrix polysaccharides. The 5,5-coupled dehydrodiferulic acid, commonly referred to as diferulic acid, was the only dehydrodiferulate reported as a cross-link between cell-wall polysaccharides (Kato and Nevins, 1985; Wallace and Fry, 1994). Recently, however, Ralph et al. (1994) identified new dehydrodiferulates coupled through 8,8, 8,8-O,4, and 4-O-5 formed by peroxidases in grass walls. Cyclodimers formed photochemically from wall-bound p-coumaric acid and ferulic acid (truxillic and truxinic acid types) were also reported in monocotyledonous and dicotyledonous plants as a mechanism for cross-linking polysaccharide chains (Hartley et al., 1988, 1990a; Eraso and Hartley, 1990).

A key role of cell-wall peroxidases in the stiffening of the cell wall through the formation of biphenyl bridges between wall polymers and, consequently, in the cessation of cell elongation has been postulated (Fry, 1986). Peroxidases have been found to be soluble in the apoplastic fluid and linked by ionic or covalent bonds to Pinus pinaster wall (Sánchez et al., 1995).

The purpose of this work was to identify and quantify phenolic acids associated with pine hypocotyl walls at different stages of growth and to study peroxidase activity associated with pine cell walls in order to establish a relationship among hydroxycinnamic acids, cell-wall-associated peroxidases, and growth cessation.

MATERIALS AND METHODS

Plant Material

Seeds of Pinus pinaster Aiton were soaked in running tap water for 24 h and germinated and grown at 25°C in darkness (Sánchez et al., 1995). Whole hypocotyls were harvested at 7, 10, 13, and 16 d after soaking. Ten-day-old hypocotyls were divided into four 5-mm sections from the cotyledonary node toward the base and the sections were named I, II, III, and IV, as the distance from the cotyledonary node increased.

Cell-Wall Preparation and Hydroxycinnamic Acid Extraction

Whole hypocotyls or hypocotyl segments were immediately killed by boiling in methanol for 10 min and stored in methanol until further use. The rehydrated material was homogenized, treated with pronase and α-amylase from hog pancreas (Sigma), and washed with water, acetone, methanol:chloroform (1:1, v/v), and ethyl ether before air drying. The dry residue was considered to be the cell-wall preparation (Lorences and Zarra, 1986).

Hydroxycinnamic acid ester linked to polysaccharides was released from pine hypocotyl cell walls as described by

Abbreviations: FA-Fa, 4′-4′-dihydroxy-3′-3′-dimethoxy-α-truxillic acid; TMS, trimethylsilylated.
Eraso and Hartley (1990). Milled walls (100–50 mg) were shaken under N₂ with 1 N NaOH (5 mL) at 25°C for 24 h. Sinic acid (100 µg), not present in this material, was added as internal standard. The suspension was centrifuged and the residue was washed with H₂O (2 × 1 mL). The supernatant and washings were combined, acidified to pH 2.5 with 6 N HCl, and extracted with diethyl ether (4 × 4 mL). The combined ether extract was evaporated under vacuum and the residue was dried under a stream of N₂. The dry residue was silylated by the addition of 20 µL of N,O-bis(trimethylsilyl)trifluoroacetamide plus 20 µL of pyridine and shaken for 15 min.

All handling of phenolic solutions and their derivatives was carried out under white fluorescent light to avoid UV radiation, which causes cis-trans-isomerization of substituted cinnamic acid (Hartley and Jones, 1975).

Phenolic Acid Analysis

TMS derivatives (1 µL) were analyzed by GC-MS on a fused silica column (PTE-5, 30 m × 0.32 mm i.d., film thickness 0.25 µm, Supelco [Bellefonte, PA]). The column was held at 150°C for 2 min, programmed at 10°C min⁻¹ to 300°C, and held for 30 min. Samples were injected using the splitless mode. For quantitative determination of phenolic acids, TMS derivatives were separated by GC as described above and detected by flame ionization. The same column and chromatographic conditions were used, except that the column oven temperature was programmed at 5°C min⁻¹. The response factors for 8,8- and 8,5-dehydrodiferulates used for their quantification were those given by Ralph et al. (1994).

Protein Extraction

Apoplastic peroxidases from intact hypocotyls at all ages or from 10-d-old hypocotyl regions were collected from intercellular and wall spaces by vacuum infiltration and low-speed centrifugation (Fry, 1988; Sánchez et al., 1995). After that the hypocotyls (10 g) were homogenized in 50 mM NaCl (50 mL) at 4°C with a Polytron (Kinematica, Luzern, Switzerland) and filtered through Miracloth (Calbiochem), and the residue, after washing with acetone at −20°C and ice-cold distilled water, was considered the cell-wall preparation. Wall proteins were extracted with 1 M LiCl (50 mL) at 4°C for 24 h with magnetic stirring. The suspension was filtered (Whatman GF/A) and dialyzed against distilled water, and the filtrate was concentrated 4 mL). The combined ether extract was evaporated under vacuum and the residue was dried under a stream of N₂. The dry residue was silylated by the addition of 20 µL of N,O-bis(trimethylsilyl)trifluoroacetamide plus 20 µL of pyridine and shaken for 15 min.

Protein content of the different extracts was measured as described by Bradford (1976) using BSA as the standard.

Peroxidase Assay

Peroxidase activity was measured using ferulic acid as substrate (Takahama et al., 1992). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm (extinction coefficient = 11.3 mM⁻¹ cm⁻¹) in a reaction mixture (total volume 1 mL) containing 40 µM ferulic acid, 90 mM sodium-phosphate buffer, pH 4.0 to 5.5, 10 µg of protein extract, and 0.5 mM hydrogen peroxide. Apoplastic and covalently bound peroxidases showed maximum activity at pH 5.5 and 4.0, respectively. However, the ionically bound peroxidase showed two maxima at pH 5.5 and 6.5, and it was higher at pH 5.5.

RESULTS AND DISCUSSION

Phenolic Acids

The ester-linked phenolic acids were released from pine cell walls by treatment with 1 N NaOH and analyzed by GC-MS (Table I). Their release by NaOH hydrolysis at room temperature is in agreement with their association with cell walls through ester linkages probably linked to pectic polysaccharides, as has been shown for dicots (Fry, 1983). The retention time relative to both sinapic and 5,5-coupled dehydrodiferulic acid, as well as the mass fragmentation pattern, were used for qualitative analysis of phenolic acids. Trans- and cis-ferulic acid and trans-p-coumaric acid were identified as the major monomeric phenolic components in pine hypocotyl cell walls. These hydroxycinnamic acids are commonly found to be ester and/or ether linked to primary cell walls of higher plants (Bacic et al., 1988). Some hydroxybenzoic acids such as p-hydroxybenzoic, vanillic, protocatechuic acids, and aldehydes such as vanillin and syringaldehyde were also detected, although the amounts of these were very low (data not shown).

Phenolic dimers were also found and identified by relative retention times and GC-MS to be some of the new dehydrodiferulic acids recently reported by Ralph et al. (1994) in grass cell walls. Comparison with the relative retention times of these dehydrodiferulates recently reported by Ralph et al. (1994) in grass cell walls, and to our knowledge this is the first report for gymnosperm cell walls. The identification by GC-MS of compound 5 corresponds to the presence in the cell walls of the ester-linked compound 5 formed via 8,5 dimerization. The detection by GC-MS of compounds 10, derived by the extraction procedure from compound 6 and/or 7, and 11 from 7 and/or 8, shows the presence in the cell wall of compounds 6 and/or 7, and 7 and/or 8, respectively. Although it is not possible to assess which of these three dehydrodiferulates (6, 7, or 8) are present in the cell walls, all of the three compounds are present.
formed through an 8,8-dimerization mechanism (Ralph et al., 1994). It seems clear that of the five possible types of dimerization, only two, the 8,5 and 8,8 coupling, are operative in pine hypocotyl cell walls.

However, the 5,5-coupled dehydrodiferulate commonly reported for monocot and dicot cell walls has not been found in our material. The absence of 5,5-coupled diferulic acid cannot be caused by its destruction during the extraction and derivatization procedure, because when it was exogenously added to wall preparations its TMS derivative was detected by GC-MS (data not shown).

Two stereodimers of ferulic acid were also tentatively identified. These compounds had similar mass spectra and showed the same major ions as the mass spectrum of the TMS derivative of trans-ferulic acid. The ratios of the relative quantities of m/z 338 to 308 and 249 to 219 of both isomers I and II were 3.1, 2.8 and 1.4, 1.34, respectively. These high ratios indicate that these compounds are FA-FA isomers I and II. This dehydrodiferulic acid increased with hypocotyl age (Fig. 2). Compound 11. This dehydrodiferulic acid increased with hypocotyl age (Fig. 2) as well as from region I through region IV (Fig. 3). Compound 10 (8,8-dehydrodiferulate) was detected only in the basal region of 10-d-old hypocotyls (Fig. 3). Its amount in the whole hypocotyl increased from d 10 to d 16 (Fig. 2). Compound 9 (8,5-dehydrodiferulate) was detected only in 13- and 16-d-old hypocotyls (Fig. 2). Although the diferulic content of cell walls appears to be somewhat low, Neukom (1976) suggested that even low levels would be enough to cause appreciable cross-linking of wall polysaccharides, modifying wall extensibility (Wallace and Fry, 1994). In fact, if the amount of dehydrodiferulic acid ester linked to pine cell walls (Figs. 2 and 3) is compared with the changes in growth capacity of pine hypocotyls with age (Lorences and Zarra, 1986) and along the hypocotyl axis (Lorences et al., 1990), an inverse relationship is found. Thus, it seems feasible that the formation of dehydrodimers between feruloyl residues ester linked to pectic polymers increases wall cross-linking, decreasing cell-wall capacity for extension. This mechanism has been proposed for dicots (Fry, 1986) and monocots (Kamisaka et al., 1990) through the formation of 5,5-dehydrodiferulic bridges. However, the absence of the 5,5-coupled dehydrodiferulate in pine hypocotyl cell walls and the presence of the other dehydrodiferulates, mainly 8,8-coupled, leads to the idea that the 8,8-coupled dehydrodimers must be the main agent responsible for such cross-linking. Furthermore, the presence of different dehydrodiferulates in greater

Table 1. Main phenolic acids associated with pine hypocotyl cell walls identified by full-scan GC-MS of their TMS derivatives

<table>
<thead>
<tr>
<th>Phenolic Acid</th>
<th>RRT* (SA)</th>
<th>RRT (DFA)</th>
<th>Major Ions (relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Ferulic acid</td>
<td>0.74</td>
<td>0.35</td>
<td>73 (100), 219 (36), 249 (53), 293 (25), 308 (36), 323 (37), 338 (M, 68)</td>
</tr>
<tr>
<td>trans-p-Coumaric acid</td>
<td>0.75</td>
<td>0.36</td>
<td>73 (100), 219 (94), 249 (41), 293 (54), 308 (M, 51)</td>
</tr>
<tr>
<td>trans-Ferulic acid (1)b</td>
<td>0.88</td>
<td>0.43</td>
<td>73 (100), 219 (32), 249 (53), 293 (32), 308 (49), 323 (53), 338 (M, 97)</td>
</tr>
<tr>
<td>8,8-Diferulic acid (11)b</td>
<td>1.5</td>
<td>0.76</td>
<td>73 (100), 147 (60), 273 (18), 467 (42), 556 (36), 659 (4), 674 (M, 2), 675 (1)</td>
</tr>
<tr>
<td>8,8-Diferulic acid (10)b</td>
<td>1.6</td>
<td>0.77</td>
<td>73 (100), 147 (23), 209 (24), 389 (2), 467 (7), 556 (1), 659 (1), 674 (M, 2)</td>
</tr>
<tr>
<td>8,5-Diferulic acid (9)b</td>
<td>1.73</td>
<td>0.84</td>
<td>73 (100), 147 (80), 393 (4), 496 (7), 674 (M, 7), 675 (4)</td>
</tr>
<tr>
<td>FA-FA I</td>
<td>1.61</td>
<td>0.78</td>
<td>73 (100), 219 (31), 249 (43), 293 (23), 308 (18), 323 (13), 338 (M-338, 56)</td>
</tr>
<tr>
<td>FA-FA II</td>
<td>1.74</td>
<td>0.81</td>
<td>73 (100), 219 (29), 249 (39), 293 (15), 308 (16), 323 (22), 338 (M-338, 45)</td>
</tr>
</tbody>
</table>

* RRT, Relative retention time to sinapic acid (SA) and 5,5-diferulic acid (DFA).  
  b Compound number as referred to in Figure 1.
Figure 1. General chemistry of formation and saponification of dehydrodiferulates detected in pine hypocotyl cell walls. Dimerization of ferulate esters via phenoxy radical 2 gives intermediates 3 and 4, which react in the cell wall to form dehydroferulate esters 5 to 8. During chemical analysis involving saponification and HCl acidification, esters 5 to 8 are converted to dehydrodiferulic acids 9 to 11. Only 8,5- and 8,8-dimerization involved in the formation of intermediates 3 and 4 are shown. Reagents and conditions are as follows: i, peroxidase; ii, NaOH; iii, HCl. (Adapted from Ralph et al., 1994.)
amounts than the 5,5-coupled ones in grass cell walls (Ralph, 1994) is in agreement with that hypothesis.

**Peroxidase Activity**

Biochemical interest in hydroxycinnamic acids ester linked to wall polysaccharides is due to the fact that they apparently undergo oxidative coupling in vivo to yield cross-linked polysaccharides. Such cross-linking is thought to decrease extensibility of cell walls, leading to the cessation of cell growth. The oxidative coupling was carried out by peroxidases associated with plant cell walls (Wallace and Fry, 1994). The peroxidase activity associated with pine hypocotyl cell wall as a possible agent responsible for ferulic acid dimerization was also studied. Peroxidase activity against ferulic acid has been found to be associated with pine cell walls to different extents, freely soluble in the apoplastic fluid, as well as ionically and covalently bound to cell walls (Figs. 4 and 5). Most of the peroxidase activity was present in the covalent fraction, whereas the lowest activity was found in the apoplast fluid. The peroxidase activity in the three fractions, apoplastic and ionically and covalently bound, increased with hypocotyl age (Fig. 4) and as hypocotyl growth decreased (Lorences and Zarra, 1986; Sánchez et al., 1995). When the peroxidase activity was studied along the hypocotyl axis, its activity in the three fractions increased from the subapical (II) through the basal region (Fig. 5). Because the growth capacity decreased from the apical to the basal region of the hypocotyl (Lorences et al., 1990), it is possible to assume a negative relationship of peroxidase activity toward ferulic acid and growth capacity. A similar relationship between peroxidase and growth along the plant axis (Goldberg et al., 1987), as well as with plant age (Valero et al., 1991), has already been proposed for dicot axes, suggesting a role for peroxidases in the cell-wall stiffening. Furthermore, the ability of pine peroxidases to catalyze wall cross-linking through ferulic acid esterified to polysaccharides has already been shown (Whitmore, 1976).

**CONCLUSIONS**

We have presented evidence that dimerization of ferulic acid in pine hypocotyl cell walls takes place primarily...
through 8,8- and 8,5-coupling mechanisms and not through 5,5, as has been proposed for other higher plants. The capacity of peroxidases associated with pine cell walls to oxidize ferulic acid and the inverse relationship found dimerization of ester-linked feruloyl as an important factor in the wall stiffening, causing growth cessation.

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LITERATURE CITED


Whitmore FW (1976) Binding of ferulic acid to cell walls by peroxidases of Pinus elliottii. Phytochemistry 15: 375–379