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Update on Cell Walls

Covalent Cross-Links in the Cell Wall

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In current models of the organization of polymers in primary cell walls of plants it is proposed that cellulosic microfibrils are embedded in a matrix of interwoven noncellulosic polysaccharides and proteins (Talbott and Ray, 1992; Carpita and Gibeaut, 1993). There is good evidence that the microfibril surfaces are coated with noncellulosic polysaccharides such as xyloglucans and, possibly, arabinoxylans and glucomannans that are cellulose-like in their conformations. Further, it is envisaged that a proportion of these polysaccharides are hydrogen bonded to the surfaces of cellulose microfibrils and, by virtue of their length, are able to interact with surfaces of more than one microfibril and so act as an adhesive between them. Within the matrix there are other less well-defined hydrogen-bonding possibilities between component polysaccharides, as well as possibilities for ionic and salt interactions between polysaccharides, proteins, and one another.

In meristematic and differentiating cells, walls have to withstand osmotically generated turgor pressures that may reach values around 3 to 10 bar (0.3–1 MPa) (Carpita and Gibeaut, 1993; Cosgrove, 1993). Walls must be constructed so as not to fail under these conditions. The aggregate strength of noncovalent forces between wall polymers appears to make this possible. There is little evidence that covalent cross-linking between wall polymers is necessary to achieve this stability (see Talbott and Ray, 1992, for a discussion). The results with 2,6-dichlorobenzonitrile-adapted tomato and tobacco cells in suspension culture indicate that although the cellulose-xyloglucan network in these walls is greatly reduced, the cells remain viable under normal osmotic conditions (Shedletzky et al., 1992). In these adapted cells the integrity of the walls appears to be dependent on increased amounts of Ca^{2+}-bridged pectates. The altered walls have a lower tensile strength, but their porosity is the same as for walls of nonadapted cells. The situation is somewhat different in the case of barley, a graminaceous monocotyledon. Here, the adapted cells have elevated glucuronoarabinoxylan and (1→3,1→4)-β-glucan contents and a lowered cellulose content (Shedletzky et al., 1992). In addition, there is an increase in ester-linked phenolic acids, especially PCA, and an increase in polysaccharides released after esterase treatment. Covalent cross-linking could be involved and extension might involve transesterification.

Walls must not only resist turgor pressure, they must also allow wall expansion during cell growth. Cosgrove (1993) has discussed a mechanism by which stress relaxation in walls can lead to water uptake by the cell and allow wall expansion that accompanies cell growth. Stress relaxation in a wall composed of noncovalently associated polymers has been postulated to result from controlled relocation of adhesive associations between microfibrils, for example, by action of the wall-located xyloglucan transferase (see Cosgrove, 1993, for a discussion), but other possibilities are under consideration (Talbott and Ray, 1992; Carpita and Gibeaut, 1993).

At the completion of the expansion phase of cell growth, the mechanical properties of walls change and they are no longer capable of stress relaxation. These changes, like those during expansion, are under metabolic control. The end point of the expansion phase may involve creation of permanent (irreversible) associations between wall polymers by formation of covalent cross-links, the molecular equivalent of spot-welding a steel mesh frame. The nature of these covalent cross-links and their biosynthesis is the subject of this mini-review.

COVALENT CROSS-LINKS IN UNLIGNIFIED PRIMARY OR SECONDARY WALLS

Glycosidic linking of oligo- and polysaccharide chains to one another is possible through the hemiacetal hydroxyl at the terminal reducing end of one chain and a hydroxyl group on another chain. Branched homopolymers such as glycogen and amylopectin and substituted heteropolymers such as rhamnogalacturonans are constructed in this way. These associations are regarded as covalent substitutions or interlinks (Talbott and Ray, 1992) rather than covalent cross-links. Glycosidic cross-links would be possible only if a hemiacetal function was present on an intrachain monosaccharide unit. Painter (1983) reported on the isolation of such a molecule, the oxohexuronic acid D-*lyxo*-5-hexosulopyranuronic acid from walls of sphagnum moss and some grasses.

Direct ester linkages between carboxyl groups of uronic acid residues in a polysaccharide, e.g. in glucuronoxylans or rhamnogalacturonans, and hydroxyls on a neighboring polysaccharide chain have been proposed (Fig. 1a). For example, the pectins in walls of maize coleoptiles are esterified to alcohols other than methanol, but the identity of these alcohols has not been established (Kim and Carpita, 1992). The existence of carboxylic esters in primary walls of spinach,

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Abbreviations: DDFA, dehydrodiferulic acid; DHP, dehydrogenative polymer of coniferyl alcohol; FA, ferulic acid; HRGP, hydroxyproline-rich protein; PCA, *p*-coumaric acid; PRP, proline-rich protein.

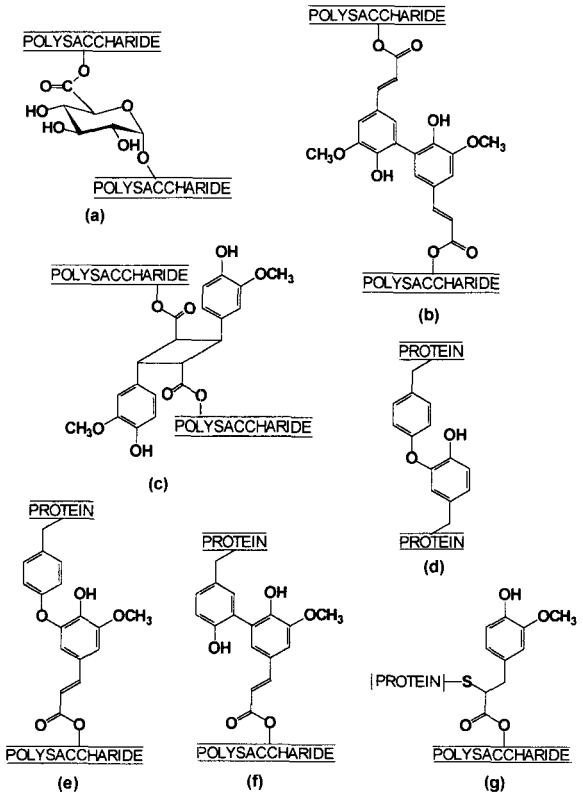

Figure 1. Structures of proposed covalent cross-links between polysaccharides and proteins in nonlignified walls.

carrot, rose, and fescue cells in culture that had been prelabeled with [6-^{14}C]glucuronate has been reported (Brown and Fry, 1993). After digestion of spinach walls with a mixture of polysaccharide hydrolases, many labeled glucuronate-containing oligomers were present in wall hydrolysates. One of these was a derivative of [^{14}C](1→4)-α-D-galacturonotriose arising from a pectic polysaccharide in which one carboxyl group was linked, via a very alkali-labile ester bond, to an as-yet unidentified nonpolar alcohol. These incomplete descriptions illustrate the methodological difficulties in identifying alcohols involved in ester links in walls. Whereas reduction of esters using tritiated borohydride reagents is a well-known and practical way of "marking" carboxylic acid groups involved in ester linkage, so far no comparable procedure is available to mark hydroxyl-bearing residues involved in ester cross-links.

The occurrence of the hydroxycinnamic acids FA and PCA in ester linkage to arabinoxylans in grasses, to pectic polysaccharides in spinach and sugar beet (see Bacic et al., 1988; Lam et al., 1990 for refs.), and to xyloglucans in bamboo (Ishii and Hiroi, 1990) is now well established. The possibility of covalent associations between esterified FA on wall polysaccharides was realized following the seminal experiment of Geissman and Neukom (1971), who showed that aqueous extracts of wheat flour could be made to gel in the presence of hydrogen peroxide and peroxidase and demonstrated that a dehydrogenative coupling between two esterified FA residues on arabinoxylans to form DDFA had occurred (Fig. 1b). This reaction captured the attention of wall biochemists as a possible covalent cross-linking mechanism, and since then many sightings of DDFA in walls of grasses have been made. In grasses the concentrations range from 0.01 to 0.05% of the dry wall. However, it was not until Ishii (1991) isolated and characterized the "linkage group" from bamboo arabinoxylans that definitive evidence for the existence of the DDFA ester cross-link was obtained. Sugar beet pectins that bear FA esterified to arabinose and galactose residues also gel following peroxidase-catalyzed, oxidative cross-linking (Rombouts et al., 1983).

The formation of DDFA ester bridges may play a role in termination of the expansion phase of cell growth in grasses. There is correlative evidence from Kamisaka's laboratory (Tan et al., 1992, and refs. therein) that the increase in FA and DDFA content of oat coleoptile walls 4 to 5 d after sowing is related to the decrease in wall extensibility and to the increase in minimum stress relaxation time and relaxation rate of the wall. In rice coleoptiles white light decreased wall extensibility, and this was correlated with an increase in the contents of FA and DDFA ester-linked to arabinoxylans. Since the ratio of DDFA:FA was constant, it was suggested that the feruloylation step is rate limiting. In maize coleoptiles a substantial proportion of aromatic material is incorporated into the wall after the cessation of elongation (Carpita, 1986). Most of this material is saponifiable FA, with small amounts of DDFA, but a significant fraction of unidentified aromatic material resists saponification and may be involved in cross-linking the primary wall during differentiation.

The DDFA cross-link has also been implicated in the aggregation of rice cells in suspension culture. Thus, Kato et al. (1994) have shown that the amounts of FA and DDFA esters in walls from rice suspension cultures depend on the growth medium. On an "AA" medium the phenolic ester content of walls was lower than on a "B5" medium, and more noncellulosic polysaccharides were found in the medium and the cell clumps were smaller. Carboxylesterase treatment of the walls facilitated solubilization of noncellulosic polysaccharides.

Yet other possibilities for dimerization of phenolic acids on polysaccharides exist. Thus UV-induced formation of a series of homo- and heterocyclodimers of the cyclobutane type by head-to-tail or head-to-head association of ester-linked PCA and FA (Fig. 1c) has been demonstrated (Morrison et al., 1992). These can be distinguished from other phenolic acid dimers in GC or HPLC separations. Their occurrence has not been unequivocally reported from unlignified primary walls, but they are regularly reported in walls of cells from plant tissues with both unlignified and lignified walls.

BIOSYNTHESIS OF HYDROXYCINNAMIC ACID ESTERS AND THEIR DIMERIZATION

Esterification of polysaccharides by hydroxycinnamic acids has been investigated at the whole-cell and enzymic levels. The kinetics of feruloylation of wall polysaccharides in cultured spinach cells was followed by administering [^{3}H]arabinose and tracing its incorporation into FA-(Ara)$_2$ units of its feruloyl-arabinan (Fry and Miller, 1989). The results indicated that arabinosylation and feruloylation occurred co-syntheti-

cally and intracellularly. However, Yamamoto et al. (1989) have suggested a wall location for feruloylation of matrix polysaccharides in barley coleoptiles based on incorporation of FA from [2-^{14}C]feruloyl-CoA into wall preparations and its release by an esterase-free polysaccharide-hydrolase mixture. With feruloyl-CoA as the donor, an intracellular location of feruloylation would be anticipated on the basis of cellular economics. This is supported by the demonstration of the enzymic feruloylation of an uncharacterized endogenous polymer by microsomal membranes from parsley cells using [2-^{14}C]feruloyl-CoA as a donor (Meyer et al., 1991). An alternative feruloyl donor could be a glycosidic ester of FA, e.g. 1-*O*-feruloyl-β-D-Glc. Thus, Mock and Strack (1993) have shown that the formation of 1-*O*-sinapoyl-β-D-Glc by UDP-Glc:hydroxycinnamate D-glucosyltransferase (EC 2.4.1.120) is a reversible reaction and calculated that the $\Delta G^{\circ\prime}$ for its hydrolysis is -35.7 kJ mol^{-1}. They conclude that such glycosidic esters are potential donors of acyl groups in *O*-esterification reactions.

The dimerization of feruloyl ester groups to form cross-links between polysaccharide chains must occur in muro. To permit this dimerization the feruloyl groups on the same or a different polysaccharide chain must be juxtaposed. We can envisage that in a gel-like primary wall, matrix polysaccharides will have a mobility that could bring feruloyl residues together so that, on occasion, dimerization could occur either enzymically, or in the case of cyclodimers, opportunistically, by photoinduction (Hanley et al., 1993; Turner et al., 1993).

Peroxidases, found in multiple forms in walls, are candidates for the catalysis of the dehydrogenative dimerization of FA esterified to arabinoxylans. These peroxidases could not only generate the reactive (free radical) intermediates of ester-linked FA, but could also generate the hydrogen peroxide needed to achieve this from various hydrogen donors. Several mechanisms have been proposed for hydrogen donor generation (see Delmer and Stone, 1988, for a summary), but no definitive proof for any of them has been forthcoming.

INVOLVEMENT OF PROTEINS IN COVALENT CROSS-LINKING IN UNLIGNIFIED WALLS

Proteins are ubiquitous wall components. They are functionally involved in preabsorptive hydrolysis of nutrient molecules (e.g. glycosidases, etc.), in wall metabolism (e.g. xyloglucan transferases, peroxidases, laccases, etc.), as inhibitory molecules (e.g. thionins, etc.), as transport proteins (e.g. lipid transfer protein), or as structural proteins (e.g. Gly-rich proteins, PRP, and HRGP) (Showalter, 1993).

Structural proteins become insoluble on secretion into the wall, possibly as a result of cross-linking. The notional possibilities for direct covalent cross-linking within and between structural wall proteins are quite extensive (Fry, 1986). However, few of these possibilities have been conclusively demonstrated. The isodityrosine (Fig. 1d), intramolecular cross-link between HRGPs is well established (Fry, 1986), but its putative intermolecular counterpart remains elusive. The isodityrosine ether linkage is believed to be created by a peroxidase-catalyzed reaction in muro. Many wall structural proteins contain Tyr-rich, repeated sequences that could be involved in isodityrosine cross-links (Showalter, 1993). PRPs that are rich in Tyr are rapidly insolubilized into walls of soybean cell cultures in response to stress or elicitor treatment (Bradley et al., 1992), and this appears to be mediated by an oxidative cross-linking. Similarly, in the walls of vegetative cells of the green alga *Chlamydomanas reinhardtii*, HRGPs related to those in walls of higher plants are made insoluble with a burst of hydrogen peroxide production and extracellular peroxidase activity. Both isodityrosine and dityrosine were found in hydrolysates of the insoluble vegetative wall layer (Waffenschmidt et al., 1993). Protein-protein cross-linking, even if restricted to intramolecular associations, could lead to insolubilization due to changes in protein conformation.

Various speculative proposals for covalent cross-links between hydroxycinnamic acids esterified to wall polysaccharides and Tyr's or Cys's on wall proteins through dehydrogenative dimerization have been made (Fig. 1, e–g; see Bacic et al., 1988), but no direct evidence for their occurrence has been adduced so far.

COVALENT CROSS-LINKS IN LIGNIFIED WALLS

Plant tissues contain cells whose walls are functionally involved in mechanical support or in water conduction. This group includes sclerenchyma, fiber cells, and xylem tracheary elements. The mature cells usually lack protoplasts and the wall is the only significant cellular structure present. Typically, the walls of these cells consist of a thin primary layer, a thicker, multilamellate secondary layer, and sometimes a tertiary layer. The secondary wall layer is rich in cellulose and the noncellulosic polysaccharides are qualitatively different from those of the primary wall. Many but not all secondarily thickened walls are also lignified. The lignin is formed by polymerization of monolignol precursors in muro after polysaccharide deposition to form the secondary wall layers has begun. Lignin deposition is initiated first at cell corners, then in the middle lamella, and proceeds through the primary wall into the secondary wall. The three monolignol precursors in angiosperms and the two of gymnosperms arise from the phenylpropanoid amino acids Phe and Tyr and may be delivered to the wall as their soluble β-glucosides (see Delmer and Stone, 1988; Iiyama et al., 1993, for refs.).

In the lignification process the hydrophobic lignin replaces the water in the wall and encrusts the cellulosic and noncellulosic polysaccharides and protein components. The net result is that the already thickened walls of high tensile strength, composed largely of highly organized, cellulosic microfibrils, are infiltrated with a nonwettable, phenolic copolymer that effectively makes the wall impermeable as well as imparting extra strength to the mechanical load-bearing cells. The hydrophobic surfaces of the lignin deposits are intimately associated with and overlie the surfaces of wall polysaccharides and proteins. At this interface there is an opportunity for covalent cross-linking.

DIRECT COVALENT CROSS-LINKS IN LIGNIFIED CELL WALLS

Three types of cross-links proposed between lignin and polysaccharides are shown in Figure 2. Two are direct cova-

lent linkages. The first is a direct ester link between uronic acids on, for example, glucuronoxylans or rhamnogalacturonans, and hydroxyl groups on lignin surfaces to give α- or γ-esters on monolignol side chains (Fig. 2a). There is reasonable evidence for the existence of such esters based on their alkali lability and the results of borohydride reduction and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone oxidation studies (see Lam et al., 1990, for a summary). The α-ester linkage could be formed by reaction of quinone methide intermediates of monomeric lignin units, induced by peroxidase, with uronic acid carboxyls.

The second type proposed is the direct ether linkage between polysaccharides and lignins (Fig. 2b). 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone oxidation studies with gymnosperm and angiosperm woods suggest that Glc or Man residues are involved (see Lam et al., 1990). If the ether linkages involve primary hydroxyls on terminal monolignol units, they would be quite labile to alkali, but if nonterminal units are involved they would be stable to alkali. Again, these linkages could be formed by reaction of quinone methide intermediates on the lignin surfaces with monosaccharide residues on polysaccharides.

Carbohydrates in glycosidic linkage to terminal phenolic or side chain hydroxyls on lignin have also been reported (Bacic et al., 1988; Lam et al., 1990). They may involve single monosaccharides or short oligosaccharides and are not regarded as true cross-links between polymers. Their biosynthetic route is uncertain.

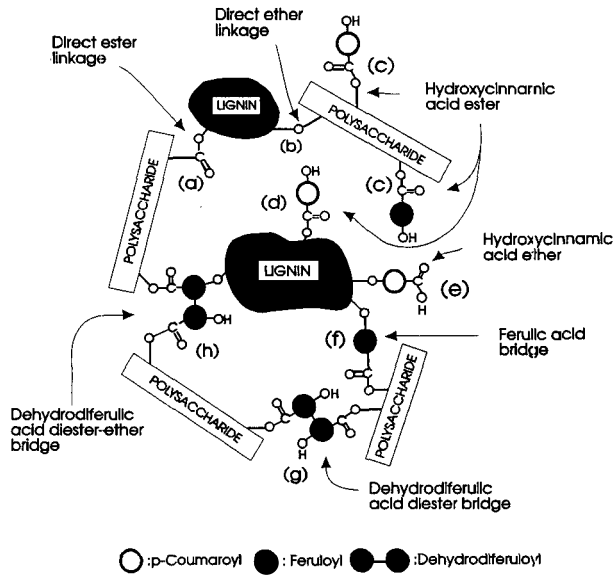

Figure 2. Schematic diagram showing possible covalent cross-links between polysaccharides and lignin in walls. O, PCA; ●, FA; ●—●, dehydrodiferulic acid. a, Direct ester-linkage; b, direct ether-linkage; c, hydroxycinnamic acid esterified to polysaccharides; d, hydroxycinnamic acid esterified to lignin; e, hydroxycinnamic acid etherified to lignin; f, FA ester-ether bridge; g, dehydrodiferulic acid diester bridge; h, dehydrodiferulic acid diester-ether bridge.

HYDROXYCINNAMIC ACID ESTER-ETHER BRIDGES BETWEEN POLYSACCHARIDES AND LIGNIN

Hydroxycinnamoyl esters of polysaccharides are also encountered in lignified walls from internode and leaf cells of grasses (Fig. 2c). In addition, hydroxycinnamic acids are also known to be directly esterified (Fig. 2d) or etherified (Fig. 2e) to lignin surfaces (Bacic et al., 1988; Lam et al., 1990).

On the basis of the properties of model compounds, Scalbert et al. (1986) proposed that the bifunctional FA could form covalent ester-ether bridges between polysaccharides and lignins (Fig. 2f). Circumstantial evidence that such linkages are present in wheat straw has been provided by Scalbert et al. (1985) and Iiyama et al. (1990). It has now been demonstrated by direct chemical analysis of dioxane-water-soluble, lignin-polysaccharide complexes from extract-free wheat and phalaris internodes that all etherified FA present is also esterified, presumably to polysaccharide, although lignin-lignin FA bridges are not excluded (Lam et al., 1992a). PCA is not involved in similar bridges. DDFA in diester linkage between polysaccharides (Fig. 2g) may also be etherified to lignin (Fig. 2h) (Lam et al., 1992b).

Hydroxycinnamoyl residues on polysaccharides can add to quinone methides to give benzylaryl ethers (α-ethers), and this was proposed as a mechanism of formation of the ester-ether bridges to lignin (Scalbert et al., 1986). Another route has been demonstrated by Ralph et al. (1992), who showed the incorporation of a feruloyl-arabinose ester into a synthetic lignin (DHP) through a peroxidase-catalyzed co-polymerization. In vivo, a competition is envisaged between α-etherification through quinone methide intermediates and incorporation into β-ethers and other products through radical co-polymerization with lignin monomers. Ralph et al. (1992) point out that the β-etherified FA may not be easily identified following its incorporation into the lignin structure.

COVALENT PROTEIN-LIGNIN ASSOCIATIONS

There is evidence that both HRGPs and Gly-rich proteins are associated with lignin and possibly act as foci for lignin polymerization (see Bacic et al., 1988; Iiyama et al., 1993). However, no information as to the nature of possible covalent linkages or their biosynthetic route is available.

None of the polysaccharide-lignin or protein-lignin cross-links have been confirmed by isolation of the "linkage group," i.e. the chemical entity (fragment) bearing the elements of the polysaccharide and lignin (protein) structure joined by the covalent linkage in question.

SIGNIFICANCE OF COVALENT CROSS-LINKS IN LIGNIFIED WALLS

Lignin in walls is important for the mechanical support of aerial parts of plants and also in resisting gravitational compressive forces. Additionally, in walls of conducting tissues, lignin may have a role in resisting tensile forces generated during transpiration (Raven, 1977). Covalent cross-links between lignins and wall polysaccharides and possibly proteins may provide additional associative forces that are important in these functions. Some indication of this possibility is given by the properties of tissues affected by the "rubbery wood" disease of applies, where in addition to a lowered lignin

content and altered lignin monomer constitution, there are reportedly also changes in the association of lignins with wall polysaccharides (see Raven, 1977, for refs.). The infiltration of lignin into walls to form a hydrophobic matrix phase creates a barrier to access of water and enzymes to the underlying polysaccharide and protein. The effectiveness of this barrier would be enhanced by the presence of covalent cross-links between lignin and other wall polymers. The physical and chemical modifications to the wall brought about by lignification are significant in resistance to attack by pathogens. Lignification of walls in cells in the vicinity of infection foci is a well-documented plant response to attack by pathogenic microorganisms. Oxidative processes in walls are stimulated by wounding, and elicitors leading to insolubilization of PRP into walls (Bradley et al., 1992) and similar oxidative processes would also be involved in the lignification response. Cross-linking of wall polymers would reduce accessibility of the pathogen's hydrolytic enzymes to their substrates (polysaccharides, hydroxycinnamoyl esters, and other wall components).

Covalent cross-linking between wall polymers is a physiologically significant strategy contributing to the termination of wall extensibility, wall strengthening, and the blocking of the ingress of pathogens. In practical terms, the cross-links are significant in relation to the selection or engineering of plants with enhanced availability of wall carbohydrates as substrates for microbial growth, e.g. in the rumen of herbivores or in industrial fermentations (Iiyama et al., 1993). Our understanding of the chemical nature of the cross-links, the extent of their occurrence, and the steps in their formation is still incomplete. Innovative approaches and new techniques are needed to obtain this information.

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