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THE MACROMOLECULAR AROMATIC DOMAIN IN SUBERIZED TISSUE: A CHANGING PARADIGM

MARK A. BERNARDS* and NORMAN G. LEWIS†

University of Northern British Columbia, 3333 University Way, Prince George BC, Canada, V2N 4Z9; †The Institute of Biological Chemistry, Washington State University, Pullman WA, 99164-6340, U.S.A.

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IN HONOUR OF PROFESSOR R. A. RYAN

Key Word Index—Biosynthesis; macromolecular assembly; cell wall; aromatic domain; suberin; hydroxycinnamate; lignin; review.

Abstract—As a structural feature of specialized cell walls, suberization remains an enigma, despite its obvious importance both during normal growth and development and as a stress response in plants. While it is clear that suberized tissues contain both polyaromatic and polyaliphatic domains, and that each of these has its own unique characteristics, whether there is a contiguous macromolecule that can be called suberin is an open question. From a structural perspective, the aromatic domain is unique and distinct from lignin, and is apparently comprised primarily of (poly)hydroxycinnamates, such as amides (e.g., feruloyltyramine). The aliphatic domain is also unique, being quite distinct from cutin in terms of both its chemical composition and cellular location. In the present paper, histochemical, structural and biochemical data, particularly regarding the polyaromatic domain of suberized tissues, are critically reviewed. A revised description of the polyaromatic domain of suberized tissues, based on the consensus that is emerging from the current data, is presented and especially includes a spatially distinct (poly)hydroxycinnamoyl-containing macromolecule.

THE SUBERIN ENIGMA

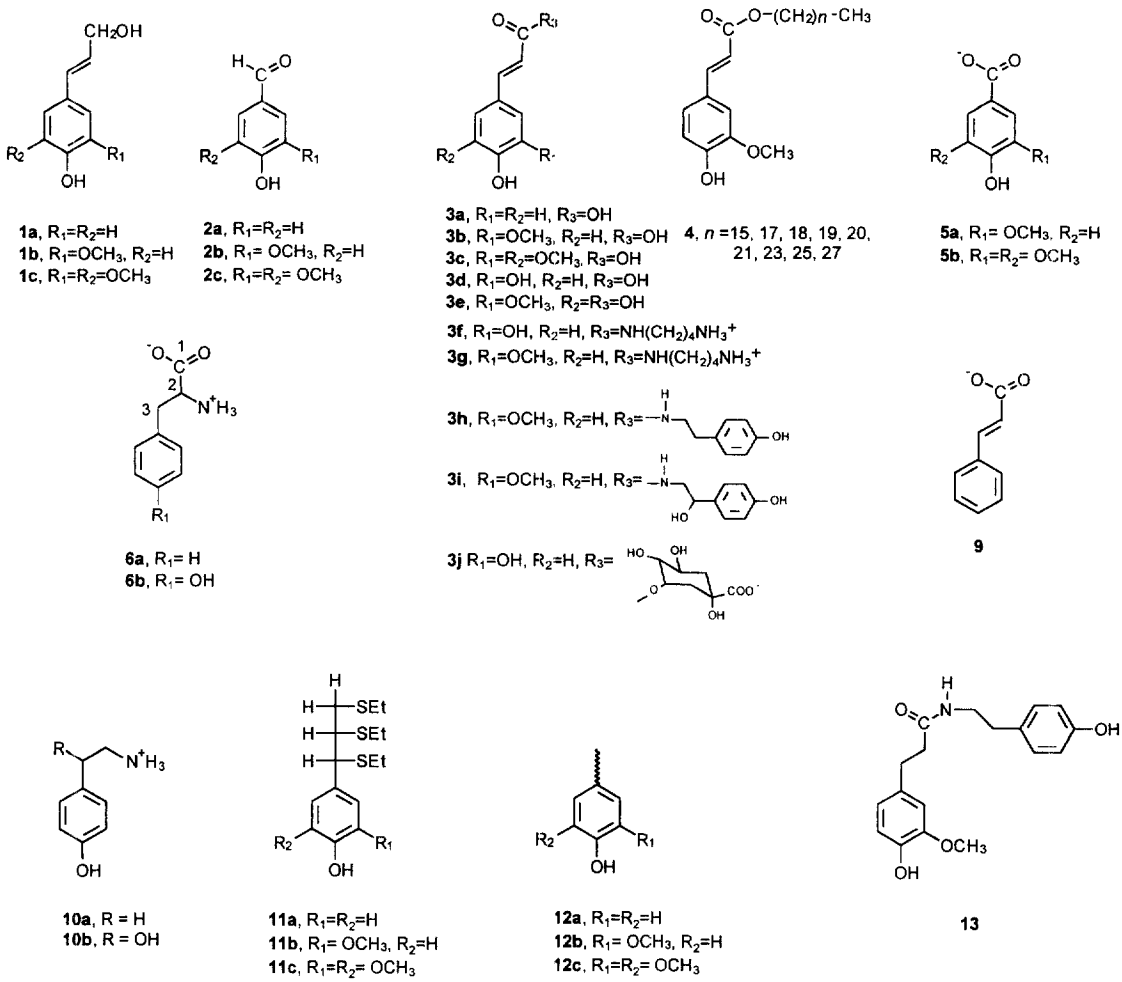
Over the course of evolution, plants evolved from a common single-celled aquatic ancestor into the myriad of aquatic and land-based species present today (e.g. [1, 2]). In making the terrestrial transition, the effects of a dry land habitat, such as desiccation, alterations in gravitational load perception and increased compressive forces had to be overcome [3]. This, in large part, was achieved through tissue-specific modifications of plant cell walls involving, for example, deposition of various (poly)aromatic and/or aliphatic matrices [3]. Interestingly, the former can range in complexity from simple phenolics to polymers such as those found in suberized periderm and lignified xylem vessels and phloem fibres [3, 4]. The aliphatics are also found in distinct forms, for example, as extracellular cutin or intracellular[1] suberin matrices [5, 6].

Amongst the various cell wall polymeric assemblies, suberization stands out as something of an anomaly, because it apparently involves the deposition of both polyaromatic and polyaliphatic domains [5, 6] within the same cell/tissue type. In terms of a function, it contributes to both cell wall strength and resistance to water loss during plant growth and development and may even predate lignification in land plant colonization [3]. It is also considered to be an important component of the wound- [5–15] and pathogen-induced [16–18] defence responses of many plant species. Under these stress conditions, its rapid, local deposition serves to prevent water loss through exposed and injured tissues, as well as to form a physical barrier to opportunistic pathogens and to the air [5–7].

But what of its identity in terms of biosynthetic pathway(s), building blocks and macromolecular

* Author to whom correspondence should be addressed. Fax (250) 960-5545, E-mail mark@unbc.ca.

[1] For the purpose of this review, intracellular is defined as including the primary cell wall.



Scheme 1. Phenolic metabolites associated with lignified and suberized tissues, including derivatives isolated after chemical degradation. Side chain carbons of L-phenylalanine **6a** are numbered according to the 99% atom ^{13}C stable isotopes of **6a** used for labelling studies (e.g. [21]).

structure? Within the literature there are conflicting descriptions of suberin. These ranging from a "fatty substance in the cell wall of cork tissue and in the Casparian strip of the endodermis" [19] to a heteropolymer comprising both aromatic and aliphatic domains, found in the epidermal tissues of underground plant parts and periderm [5, 6]. This uncertainty has largely been due to the fact that it is not yet possible to obtain "pure" suberin in its native state, structurally intact and free from other contaminants [20, 21]. Accordingly, the question of suberin constitution, and hence even its definition, has not been resolved. Moreover, like almost every other plant polymeric cell wall constituent, the matter of its macromolecular assembly has *not* been established.

The intractable polyaromatic domain of suberin has often been likened to that of lignin [5, 6], which is primarily derived from the polymerization of monolignols **1a–c** (Scheme 1). The evidence for a putative lignin domain in suberized tissues (reviewed in [5, 6]) was largely based on the application of degradative techniques, originally developed for the analysis of lignins [reviewed in 22, 23] to suberized tissues. Thus, alkaline nitrobenzene oxidation of suberized potato tubers gave substituted benzaldehydes **2a–c** which, at that time, were viewed as markers for lignin [reviewed in 22]. Recently, non-destructive techniques (i.e., specific ^{13}C-labelling followed by solid state ^{13}C NMR spectroscopy *in situ* [21, 24–26]) as well as the more selective degradation method of thioacidolysis (e.g. [23, 27]) have been developed and applied to both lignifying (e.g. [24–27]) and suberizing (i.e., potato) [21, 28, 29] tissues. The results obtained for suberized potato tuber tissues gave a new perspective. They revealed the polyaromatic domain of suberin in this Solanaceous species as a hydroxycinnamate-derived polymer [21], primarily comprised of ferulic acid **3b** and *N*-feruloyltyramine **3h** [29], rather than a typical lignin.

Similarly, the aliphatic domain of suberin was first considered to resemble that of cutin [5], but again was later found to be quite distinct both morphologically

and chemically. For example, the aliphatics in suberized tissues are located between the primary cell wall and the plasmalemma,[2] whereas cutin is formed as a continuous layer on the surface of external tissues such as stems, fruits, flowers and leaves [5, 6, 19]. When revealed using histochemical staining coupled with electron microscopy, the aliphatics of suberized tissues typically display a characteristic multi-lamellar structure [5, 6, 19, 30]. They also generally contain longer chain fatty acids (i.e., >20 carbon units) than those that are cutinized and are also distinguished from the latter by the presence of α,ω-dioic acids [30, 31]. Indeed, the presence of C-16 and/or C-18 alkan-α,ω-dioic acids is considered diagnostic [31]. By contrast, cutins are largely comprised of shorter chain length (i.e., <20 carbon units), but more highly substituted (i.e., di- and tri-hydroxy, as well as epoxy) fatty acids, of which dihydroxyhexadecanoic acid is viewed as diagnostic [31–33]. Both aliphatic polymers are partially cross-linked via ester linkages as evidenced by their susceptibility to alkaline hydrolysis and they also contain associated waxes.

The role of suberization as a wound-healing matrix, and its distinctive monomeric building blocks establish suberin (or at least suberized tissues) as both a unique and distinct plant cell wall polymer relative to lignins and cutins. At the present time, however, the data on suberin and suberization is incomplete, often conflicting and limited to just a few plant species. The most comprehensive picture of "suberin" has been established from studies on a single plant organ, namely the potato tuber. This review critically evaluates our current understanding of the aromatic domain of suberized tissues by attempting to interpret the recent data regarding suberin and its macromolecular assembly.

SUBERIN AND THE SUBERIZATION PROCESS AS REVEALED BY HISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES

Histochemical and ultrastructural analyses of plant tissues often help clarify specific aspects of cellular processes and reveal distinct morphological features that are otherwise not readily observable in any other way. Staining and fluorescence visualization techniques can be used to reveal the presence of specific chemical functionalities in fresh or fixed tissue sections. In order to be effective, however, the dyes and/or probes used must be specific for the particular component(s) of interest. Since suberized tissue comprises both aromatic and aliphatic moieties, combinations of stains are required for selective visualization. On the other hand, transmission electron microscopy (TEM) of thin sections can reveal detailed morphological and molecular features of tissues, especially when used in combination with specific antibody probes. In general, thin sections are stained with electron dense OsO_4 to provide contrast between cell components.

Cell wall bound aromatics

Cell wall aromatics are easily detected in freshly prepared or fixed sections due to their autofluorescence under UV illumination. Aromatics bearing a phenolic hydroxyl (i.e., phenolics) can be stained with reagents that form characteristically coloured or fluorescent adducts. For example, phloroglucinol-HCl, and the Mäule reaction or fluorescent probes such as berberine/aniline blue or berberine/ruthenium red [8, 9, 34], are commonly used due to their selectivity for phenolics. Such approaches are limited by their inability to distinguish in any compelling manner between the type of phenolic substance (i.e., low molecular weight phenolics, lignin, suberin, etc.). In suberized potato tuber, for example, both autofluorescence and staining with berberine/aniline blue or berberin/ruthenium red clearly indicate the presence of phenolics in the walls of cells immediately below the exposed surface of the tissue (Fig. 1(a), (c)); that is, within the first 1–3 cell layers only.

Aliphatic components of cells

The aliphatic components of cells can be visualized using any of the Sudan family of dyes or fluorescent dyes such as neutral red [34]. The Sudan dyes only weakly stain the aliphatic domain of suberized tissues, and generally cannot distinguish between suberin associated aliphatics and other cell lipids [34]. Neutral red has the advantage that its fluorescence enhances its detection, but this is complicated by interference from autofluorescent species (i.e., cell wall bound phenolics). This problem can be overcome by using combinations of stains designed to allow the selective visualization of neutral red (i.e., aliphatic) fluorescence. For example, when neutral red is used in combination with toluidine blue O, the latter suppresses cell wall autofluorescence (under UV illumination) and allows a more distinct visualization of the neutral red (i.e., lipid) fluorescence [34]. When applied to suberized wound periderm in potato, the aliphatic domain is clearly revealed without any interfering background fluorescence (Fig. 1(b)).

Transmission electron microscopy (TEM)

In thin sections prepared for transmission electron microscopy (TEM) by staining with OsO_4, suberized tissues are clearly distinguished by the presence of alternating light and dark bands, or lamellae (Fig. 2). These lamellae are initially located between the

[2] In mature periderm, secondary wall formation occurs after suberin deposition, resulting in the suberin lamellae being sandwiched between the primary and secondary cellulosic wall components (cf. [19, 30]).

primary cell wall and the plasma membrane, although they later become sandwiched between the primary and secondary cell walls in tissues that undergo cell wall thickening [19, 30]. The presence of these lamellae (as revealed by TEM) is considered diagnostic for tissues that are suberized. They are found in the periderm of a wide variety of plants, including both herbaceous and woody species [8, 9, 19, 30]. The origin of these lamellae has been attributed to alternating layers of aromatics and aliphatics (e.g., [5, 6]) but this has not been proven unequivocally (see below).

As discussed below, in spite of their limitations, histochemical and ultrastructural analyses of both natural and wound-induced suberins, from herbaceous and woody plants, have provided valuable insight into two important aspects of the suberization process:

(i) tissue specificity, and
(ii) spatial segregation of the aromatic and aliphatic domains.

Tissue specificity of suberization

Using Sudan dyes as a marker for suberin, the staining of a wide variety of plant tissues has demonstrated that during normal growth and development of both herbaceous and woody plants, suberized cells are primarily located in the epidermal tissues of underground plant parts (i.e., roots, stolons, tubers, etc.) as well as in the periderm (cork, bark) of aerial tissues that undergo secondary thickening [19]. Suberization also appears to be an integral component of Casparian band formation in root endodermal layers [19, 35, 36]. The presence of characteristic lamellae in TEM sections of these tissue types [19] supports this pattern of deposition.

When suberization is induced by either wounding [5–15] or pathogen attack [16–18], staining of freshly prepared sections with either Sudan dyes or neutral red reveals that it is restricted to cells immediately below (i.e., 2–3 cell layers) the exposed or attacked surface(s) [5–17]. The presence of lamellae in TEM sections is also restricted to these cells (e.g. [11]). Thus, in general, the suberization of cell walls is restricted to specific cells, both developmentally and in response to stress.

Is there a spatial segregation of the aromatic and aliphatic domains in suberized tissues?

Selective visualization of either polyphenolics (with the fluorescent probe berberine/aniline blue) or lipids (with neutral red/toluidine blue O) in serial sections obtained from natural and wound-induced suberized potato periderm, has revealed an important apparent spatial distinction between both domains [34]. The polyphenolics (visualized using berberine/aniline blue staining) appear to be predominantly *within* the primary cell wall, while the aliphatic component (visualized with neutral red/toluidine blue O) is observed to be restricted to the space between the plasmalemma and the primary wall [34]. In other words, careful examination of the staining patterns in differentially stained, suberized potato tubers, suggests that the deposition of aromatics and aliphatics are not coincident. For example, the presence of aliphatics apparently extends into deeper tissue layers than do phenolics (compare Fig. 1(a) and (b)). Similarly, when serial sections from the same tissues are stained with Sudan III/IV and viewed under long wave fluorescent light (365 nm), red staining lipid ribbons are clearly distinguished from the autofluorescent (i.e., phenolic containing) cell wall [34] (Fig. 1(c)). In some cells, these ribbons have pulled away from the primary cell wall, presumably due to plasmolysis. This again suggests a distinct spatial orientation for the aliphatic domain. Finally, fluorescent staining of lipids with neutral red in combination with toluidine blue O (to quench cell wall autofluorescence) shows a near absence of staining in the intercellular space (Fig. 1(d)). This is in stark contrast to the extensive berberine/aniline blue fluorescence (i.e., phenolics) within the same region (Fig. 1(e)).

The apparent spatial segregation of the polyaromatic and polyaliphatic domains in suberized potato tissues is also evident under conditions of wound-induced suberization in other plant species (e.g., woody angiosperms and gymnosperms [9] and herbaceous dicots [8, 10, 14, 15]). In these cases, a temporal pattern in the deposition of the individual domains of suberin is observed, where the phenolics are apparently detectable within the cell wall *in advance* of any aliphatics, often by as much as a day or two [8–10, 14, 15, 34]. When deposited later, the aliphatics are revealed as lamellae under the TEM. This is in marked contrast to the deposition of the polymer lignin, which is often viewed as the final cellular act prior to programmed cell death (e.g. [20]).

But what then of the lamellae apparent in TEM sections of suberized tissues? If the aromatic and aliphatic domains are as clearly delineated as the staining of suberized potato tubers suggests, then alternating layers of aliphatic and aromatic components cannot account for their presence. One hypothesis suggests that the aliphatic components themselves provide sufficient differences in polarity (i.e., some components are somewhat amphipathic) that they arrange themselves in a fashion analogous to membrane bilayers [30]. However, this hypothesis requires experimental verification.

In summary, the histochemical and ultrastructural data obtained thus far for a variety of suberized tissues (including both herbaceous and woody species) resulting from either normal growth and development or after stress-induction has provided new insight regarding *where* the two domains of suberin are located relative to each other. Based on these data, it can be *inferred* that the aromatic domain is incorporated *within* the primary cell wall matrix, while the aliphatic

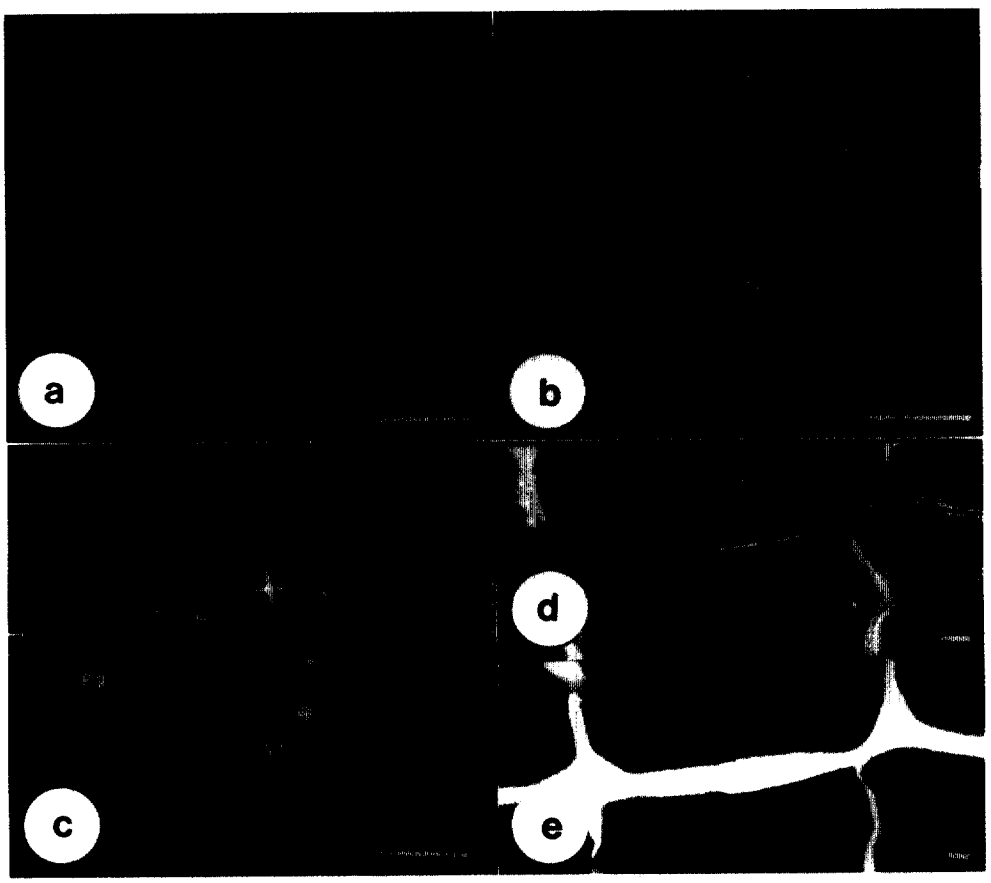


Fig. 1. Histochemical anatomy of suberized potato tuber. Serial sections (5 μm) of wound-healed potato tubers as revealed by staining with (a) berberine/ruthenium red, (b, d) neutral red/toluidine blue O, (c) Sudan III/IV (viewed under long wave UV light) and (e) berberine/aniline blue. The absence of lipid in cell walls that stain for phenolics is evident in (c), and when (a) is compared with (b), or (d) is compared with (e). Of particular importance is the clear delineation of the intercellular space when the tissue is stained with neutral red/toluidine blue O (d). Sections are oriented with the cut surface at the top. Scale bars are 100 μm (a, b, c) and 10 μm (d, e). A = autofluorescence, IS = intercellular space, ITW = inner tangential wall, R = ribbon-like lamella. (Reproduced from Ref. [34] with permission.)

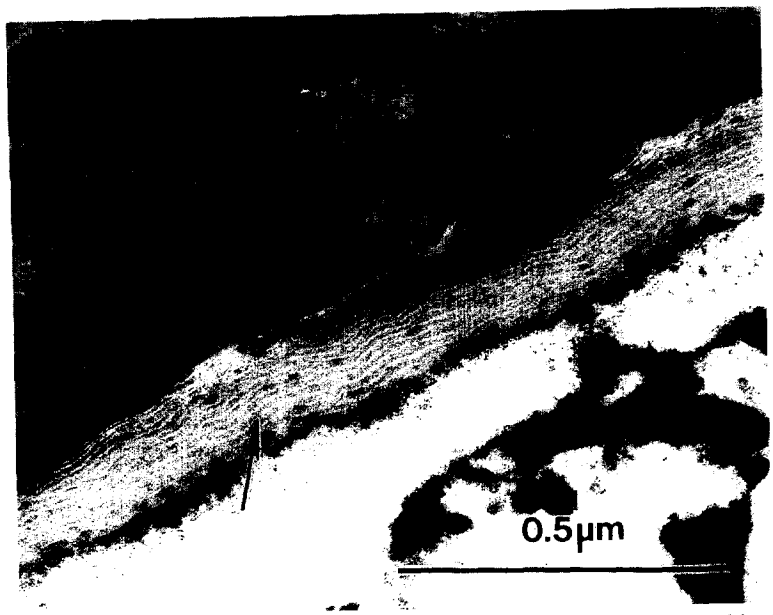


Fig. 2. Transmission electron microscopy of suberized potato periderm. Thin section cut from wound-healed potato periderm, five days post wounding. Arrow indicates lamella characteristic of suberized tissues.

domain exists as a discrete component *between* the plasmalemma and the primary cell wall matrix. Consequently, even though cells and tissues can be suberized, the notion that suberin exists as a single heteropolymer is misleading. However, both domains may well be covalently linked at their interface.

THE CONSTITUTION OF THE AROMATIC DOMAIN OF SUBERIZED TISSUES: STRUCTURAL ANALYSIS

The aromatic domain of suberin is described as intractable since it has not yet been isolated in its native state free from other constituents such as carbohydrates [21, 22]. Based largely on chemical degradation studies of suberized potato tubers, it was initially imperfectly characterized as an alkyl-aryl ether linked lignin-like polymer with esterified hydroxycinnamic acids [5, 6]. The turning point in our understanding of its constitution came with the development [24–26] and application [21] of specific carbon-13 labelling techniques, which allowed it to be "viewed" in its native state within the plant cell wall matrix, using the non-destructive technique of solid state ^{13}C NMR spectroscopy. Additional and supporting evidence next came from thioacidolysis studies, which confirmed and extended these observations [29]. Numerous other analytical techniques were also applied over the last 10–20 years to the analysis of suberized tissues, including solvent extraction of suberin-associated phenolics [4, 37–47], analysis of putative polymeric materials presumed to represent suberin [48–51] and identification of fragments revealed by chemical degradation of suberin-enriched tissues [13, 28, 29, 47]. These data are reviewed below in an attempt to clarify the reasons for previous difficulties in defining the polyaromatic domain in suberized tissues.

Analysis of soluble phenolics that accumulate during suberin formation

Analysis of organic solvent extractable components of natural and wound-induced potato periderm [37, 38], stem bark of Doublas fir (*Pseudotsuga menziesii*) [37, 39], and other species [40, 41], resulted in the identification of a series of hydroxycinnamate esters (principally ferulic acid **3b** derived) linked to long chain (i.e., C-16 to C-32) alcohols (e.g., **4**). Similarly, extraction of suberized green cotton (*Gossypium hirsutum* c.v. green lint) fibres yielded a diester of glycerol comprising caffeic **3d** and ω-hydroxydocasanoic acids [42, 43], the latter of which is common to many suberized tissues [31]. Because of their accumulation in suberizing tissues and the assumption that they contain linkages found in suberin, these metabolites were suggested as potential suberin precursors [37, 38]. On the other hand, it must be cautioned that when wounding or other stresses induce suberization, various phenolics can also accumulate in the tissues and not all are necessarily involved. For example, elicitation of suberization by wounding of potato tubers results in the accumulation of chlorogenic acid **3j** [38, 44, 45], *N*-(hydroxycinnamoyl) amides **3f–i** [4, 38, 46, 47] and a variety of other low molecular weight phenolic acids and aldehydes [13, 47]. Of these, only *N*-feruloyltyramine **3h** has thus far been shown to be incorporated into the polyaromatic domain of potato suberin [29]. Consequently, the accumulation of soluble phenolics in suberized tissues cannot be taken as proof for their role as precursors. Instead, they may serve other func-

tions such as helping disinfect the wound zone during "healing", or to act as plasticizers for the polymeric matrices.

Characterization of putative polyaromatic suberin derivatives

Two methods originally developed for the analysis of lignins have been used in an attempt to characterize soluble and presumed suberin-enriched polymeric derivatives, i.e., the extraction of a so-called Björkman preparation [52], and thioglycolic acid derivatization (e.g. [53]). In the Björkman method, a finely milled, extractive-free cell wall residue is extracted with aqueous dioxane (i.e., dioxane:H_2O, 9:1) with the soluble cell wall derivatives subsequently precipitated by addition of organic solvents such as diethyl ether [52]. When applied to suberized (i.e., cork) tissues of *Rubus idaeus* [48], *Quercus suber* [48–50], potato [48] and *Fagus sylvatica* [51], the Björkman protocol yielded high molecular weight preparations (>5000 MW, based on gel permeation chromatography on Sephadex LH 20 [48]) in the dioxane:H_2O solubles, in amounts approximately 5-fold lower than that typical for lignified woody tissue [48, 49]. Analysis of the precipitates gave conflicting results, however, when different suberized tissues were examined. For example, cork layers of *R. idaeus*, *Q. suber* and *S. tuberosum* extracted by the Björkman protocol and analysed by both ^1H and ^{13}C NMR spectroscopy [48], did not contain signals typical of milled wood lignins, thus emphasizing that this method can also solubilize components other than lignins. Instead, the spectra contained both aromatic and aliphatic signals and the authors concluded that while suberized tissues had an aromatic component, it was not a typical lignin. By contrast, both IR and ^{13}C NMR spectroscopic analyses of the diethyl ether precipitate obtained from the dioxane:H_2O extract of *F. sylvatica* bark [51] suggested the presence of a lignin, based on similarities between the spectra obtained and those of authentic milled wood lignins prepared in the same manner [51]. Additionally, methanol precipitation of the diethyl ether solubles remaining in the *F. sylvatica* extract, followed by treatment with BF_3/MeOH, gave various fatty acids (ranging in chain length from C-12 to C-24) as well as vanillin **2b**, vanillic acid **5a**, ferulic acid **3b** and syringic acid **5b**. The benzoates and benzaldehydes were assumed to be artefacts of the BF_3/MeOH depolymerization, and were presumed to be derived from **3b** and **3c** in the preparation. Unfortunately, no spectroscopic (e.g., IR and ^{13}C NMR), chemical (e.g., to analyze for characteristic suberin aliphatics such as alkane-α,ω-dioic acids, ω-hydroxyalkanoic acids) or degradative (e.g., thioacidolysis) analyses were carried out on this so-called suberin fraction, and its true composition remains uncertain. It must also be emphasized that bark tissues are still poorly characterized and many of the proposed suberin constituents may reflect species-dependent differences in the overall chemical composition of the bark itself.

A second approach to obtain soluble aromatic suberin-derivatives from extractive free cell wall preparations involves treatment with thioglycolic acid/HCl, followed by extraction with aqueous NaOH to release phenolic fragments [53]. Thus, when suberization was monitored in potato tubers, in a time course fashion by preparing thioglycolic acid derivatives at specific times after wounding, the yield of soluble phenolic derivatives increased with time (as measured by an increase in absorbance at 280 nm in the solubles obtained [54]). It is important to note, however, that thioglycolic acid derivatization of cell wall bound phenolics is a non-selective process, in the sense that it can result in the release of soluble phenolic derivatives regardless of their biosynthetic origin [23]. Since no further characterization of the solubles obtained was carried out (such as UV spectroscopy, which would at least help delineate between monolignol-derived and hydroxycinnamate-derived moieties), no indication of the origin or identity of the phenolics was obtained.

Thus, although both the Björkman protocol and thioglycolysis confirmed the presence of (poly) aromatic cell wall material in suberized tissues, neither was definitive in determining its constitution. Furthermore, these studies again underscored the difficulties in obtaining cell wall polyaromatics in pure form and the need for the use of either more selective extraction techniques or ones in which the polyaromatic constituents of plant cell walls could be examined *in situ*.

Nuclear magnetic resonance spectroscopic analysis of suberized tissues in situ

The use of non-destructive solid state carbon-13 CP-MAS NMR spectroscopy [21, 55–58] to study the cell walls of suberized potato tissues provided the first real insight into the constitution of its aromatic domain. While natural abundance spectra are dominated by carbohydrate signals [55–58], the selective enrichment of specific carbons in the aromatic component of suberized plant cell walls, through the judicious use of specifically enriched carbon-13 metabolic precursors permitted a more incisive evaluation. While this technique was initially developed for lignins [24–26], it was adapted for suberized tissues through the administration of L-[1-^{13}C]-, [2-^{13}C]- and [3-^{13}C] phenylalanines **6a**[3] to wound healing potato tubers [21]. These precursors were used since (a) they were readily metabolized by the suberizing potato tissue and (b) many of their potential metabolic products (e.g., hydroxycinnamates, monolignols) could easily be distinguished by their characteristic NMR spectro-

[3] Side chain carbons are labelled 1 through 3, with 1 being the carboxylic acid. See Scheme 1.

scopic signals. Thus, sterile solutions of either natural abundance, L-[1-^{13}C]-, [2-^{13}C]- or [3-^{13}C] phenylalanines **6a** were administered to wound healing potato tubers (immediately following wounding), and the tissues allowed to metabolize for 7 d. Following extraction with buffer, EtOH, EtOH:$CHCl_3$, 90% DMSO and oxalic acid, the extractive free tissue was subjected to solid state ^{13}C CP-MAS NMR spectroscopy [21].

Of particular significance in the delineation of the constitution of the aromatic domain of suberized potato tissue was an examination of the apparent metabolic fate of L-[1-^{13}C]-phenylalanine. For example, its metabolism into the hydroxymethyl group of monolignols, which have chemical shifts (resonances) at approx. δ 63 ppm, could readily be distinguished from its incorporation into protein (approx. δ 175 ppm) or its metabolism being limited to the formation of hydroxycinnamates (approx. δ 170–175 ppm). Accordingly, the difference spectra of the extractive free, suberized tissue obtained following metabolism of L-[1-^{13}C]-phenylalanine **6a** and subtraction of natural abundance resonances, revealed a single enhanced resonance at δ 171.0, indicative of carboxyl function (i.e., either an amide, an ester or both) (Fig. 3(a)). While a small signal at δ 63.1 ppm revealed a trace of monolignol in the preparations (Fig. 3(a)), these data provided the first direct evidence for the constitution of the aromatic domain in suberized tissues, and that it was predominantly a hydroxycinnamic acid-derived polymer.

The carbon-13 NMR spectra obtained for suberized cell wall preparations following the metabolism of [2-^{13}C]- and [3-^{13}C]-phenylalanines by wound healing potato tubers provided further evidence for this hydroxycinnamate-derived aromatic domain (Fig. 3(b), (c)). Of particular note is the absence of any significant resonance at approx. δ 38 ppm in the difference spectrum generated from [3-^{13}C]-enriched tissues (Fig. 3(c)), which ruled out the possibility of any meaningful level of phenylalanine **6a** being incorporated into cell wall bound proteins. In addition, enhanced resonances at δ 120.5 (C-2) and 142.1 (C-3) revealed the presence of hydroxycinnamates (and their conjugates). More importantly, enhanced resonances at δ 84.5 and 55.2 ([2-^{13}C]-enriched tissues) and δ 86.2 and 74.5 ([3-^{13}C]-enriched tissues) suggested the appearance of covalently bound side chain carbons in cross-links between aromatic monomers other than ester linkages. While the exact nature of these linkages have not yet been established, the enhanced ^{13}C signals are considered to be consistent with those derived, for example, from β-O-4′ **7** and phenylcoumarin (β-5′) **8** type linkages [25, 26] (Scheme 2). These will be identified in future work.

Re-evaluation of the natural abundance spectra presented for suberized potato tubers [55–58] supports these data, since the presence of esters/amide carbons in the suberized tissue was clearly evident by a large resonance at ca. δ 170 ppm [58], as well as aromatic

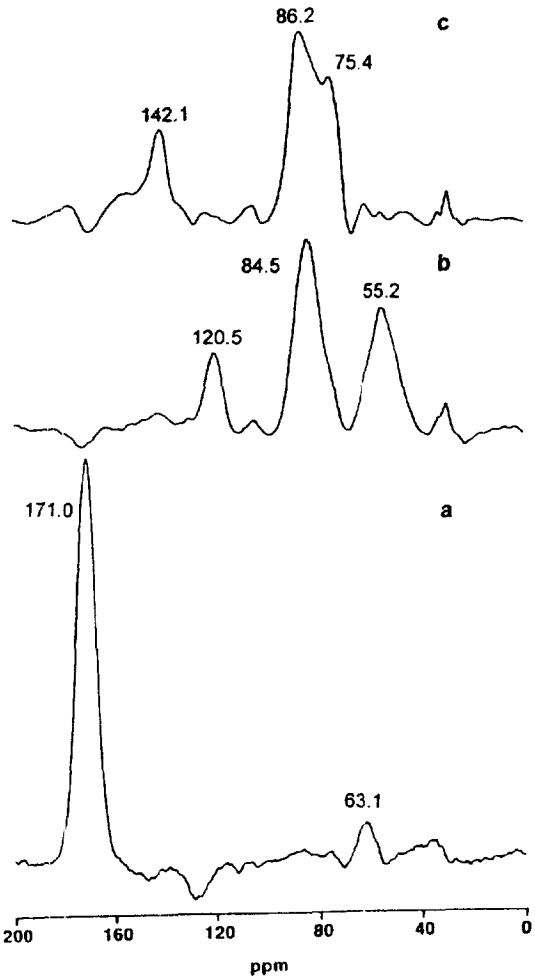
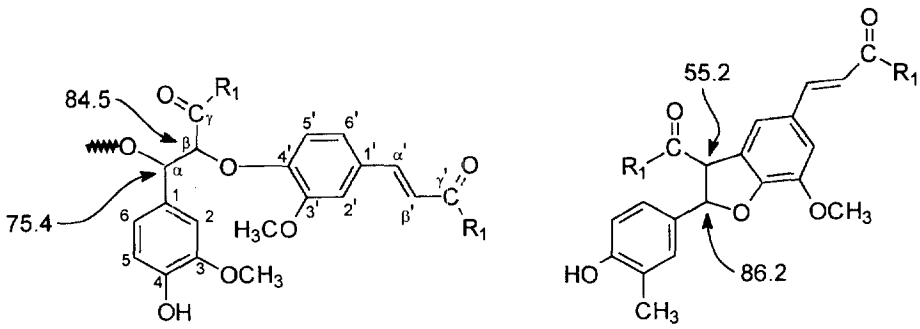


Fig. 3. Solid state ^{13}C CP-MAS NMR difference spectra of ^{13}C-enriched potato suberins. Samples were prepared from wound-healed potato tuber periderm, 7d after administration and metabolism of (a) L-[1-^{13}C]-phenylalanine, (b) L-[2-^{13}C]-phenylalanine or (c) L-[3-^{13}C]-phenylalanine. The major enhanced resonances are labelled in ppm. (Adapted from Ref. [21].)

signals consistent with hydroxycinnamates [56, 58]. Moreover, analysis of proton rotating-frame relaxation times, $T_{1\rho}$(H), carbon rotating-frame relaxation times, $T_{1\rho}$(C), and carbon spin-lattice relaxation times, T_1(C), gave direct evidence for distinct polymeric domains within suberized tissues (i.e., one aromatic and one aliphatic) as well as for the attachment of the aromatic moieties to both the carbohydrate cell wall and the aliphatic components [57]. That is, the motional freedom of carbohydrate, aromatic and aliphatic carbons and protons is more restricted within suberized tissue than when measured in non-suberized tissue, suggesting that they are covalently linked together. Thus, in apparent contrast to the histochemical evidence reviewed earlier, solid state ^{13}C CP-MAS NMR spectroscopy predicts a covalent cross-linking between the two distinct polymeric



7 β-O-4' **8** β-5' (phenylcoumarin)

Scheme 2. Possible initial coupling products of ferulic acid and/or *N*-feruloyl amides after treatment with peroxidase/H_2O_2. The pattern of coupling is analogous to that predicted for monolignols treated with peroxidase/H_2O_2. R_1 = OH, amide (e.g., tyramine) or ester. (Adapted from Refs [21, 26].)

domains found in suberized tissues, as well as between the aromatic and carbohydrate moieties within the cell wall. It may turn out, however, that the attachment occurs at the interface between the polyaromatic and polyaliphatic domains.

With the establishment of this new insight into the constitution of the polyaromatic domain of potato suberin, it is useful to re-evaluate the data generated by the myriad of other analytical techniques that have been applied to suberized tissues.

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy of the "milled cork lignin" (MCL) prepared from the cork of *Q. suber* by the Björkman procedure [49] gave spectra dominated by absorption bands characteristic of carboxylic acid derivatives, predominantly esters (ca 1740 cm^{-1}) with less intense absorption bands consistent with amides (ca 1650 cm^{-1}), and side chains of hydroxycinnamic acids. While the authors concluded that the polyaromatic material in their preparation could at best be called a "lignin-like polymer", they may in fact have presented the first real evidence for the hydroxycinnamic acid nature of the polymer. When the cork was first refluxed with sodium methoxide prior to extraction by the Björkman procedure, and the resulting dioxane:H_2O solubles fractionated by sequential precipitation (diethyl ether followed by methanol), the FTIR spectra were no more conclusive. The precipitate obtained with diethyl ether revealed a material more characteristic of a typical spruce milled wood lignin, in small amounts, whereas the spectra of the diethyl ether solubles had many absorption bands characteristic of esters, carboxylic acids and to a lesser extent amides, while maintaining some aromatic character. The question remains as to which fraction, if any, was truly representative of the polyaromatic domain of suberized tissues.

Degradative analysis of the cell walls of suberized tissues

Tissues containing intractable cell wall polymers such as lignin and suberin are frequently subjected to harsh chemical treatments, at elevated temperatures in an attempt to isolate representative fragments. For example, alkaline hydrolysis, alkaline nitrobenzene oxidation, cupric oxide oxidation, pyrolysis-MS and thioacidolysis are routinely used to characterize lignified and suberized tissues [13, 28, 29, 44, 47, 54, 59–61]. Of these approaches, only alkaline hydrolysis and thioacidolysis release phenylpropanoid moieties (from primary esters and alkyl–aryl ethers, respectively) with their side chains intact [23, 27], thereby allowing for a more exact assessment. In the context of the changing paradigm regarding the aromatic domain of suberin, a re-evaluation of previous findings using these approaches is necessary.

The presumption of a lignin-like polymer comprising the polyaromatic domain of suberin was essentially based on the recovery of [^{14}C]-labelled alkaline nitrobenzene oxidation products (i.e., benzaldehydes **2a–c**) released following administration and metabolism of either [^{14}C]-phenylalanine **6a** or [^{14}C]-cinnamic acid **9** to wound healing potato tuber disks [44]. During nitrobenzene oxidation the 3-carbon sidechain of phenylpropanoids is cleaved between the α and β carbons, resulting in the release of a series of substituted benzaldehyde fragments (e.g., **2a–c**). Thus, the recovery of [^{14}C]-labelled *p*-hydroxybenzaldehyde **2a**, vanillin **2b** and syringaldehyde **2c** was taken as evidence for [^{14}C]-phenylalanine **6a** or [^{14}C]-cinnamic acid **9** having been metabolized into monolignols and subsequently incorporated into a lignin-like polymer [44]. A similar analysis of potato suberin phenolics resulting from cupric oxide oxidation [54], afforded the same benzaldehyde products (i.e., **2a–c**). From a quantitative perspective, the alkaline nitrobenzene oxidation procedure released a significant amount of

2a (48–66 μmol g^{-1} extractive-free residue), **2b** (40–146 μmol g^{-1} extractive-free residue) and **2c** (10–23 μmol g^{-1} extractive-free residue) [13, 44, 47], from wound-healed and naturally suberized potato tubers, respectively. It is important to recognize that both alkaline nitrobenzene oxidation and cupric oxide oxidation result in side chain cleavage of phenylpropanoid-derived moieties [23]. Consequently, the identity(ies) of the parent phenylpropanoids which yielded the substituted benzaldehydes **2a–c** could not be assigned unambiguously, and the narrow interpretation that the substituted benzaldehyde products **2a–c** obtained by treatment of suberized potato tuber could only have come from the corresponding monolignols **1a–c** did not constitute a proof. In fact, many other cell wall bound phenylpropanoids could account for the same products. For example, the same benzaldehyde derivatives **2a–c** expected of a monolignol **1a–c**-based lignin are also recovered after alkaline nitrobenzene oxidation of artificial "lignins" made from hydroxycinnamic acids [62]. Taken together, these data cannot exclude the possibility that the benzaldehyde fragments **2a–c** released from suberized tissue predominantly arose from hydroxycinnamic acids or amides.

Alkaline hydrolysis (i.e., 1 N NaOH, 1 h reflux) of extractive-free potato wound periderm tissue [47] released cell wall bound *p*-hydroxybenzaldehyde **2a** (2.5 μmol g^{-1}), vanillin **2b** (8.9 μmol g^{-1}), vanillic acid **5a** (2.4 μmol g^{-1}) *p*-coumaric acid **3a** (0.3 μmol g^{-1}), ferulic acid **3b** (16.7 μmol g^{-1}), tyrosine **6b** (11 μmol g^{-1}) and tyramine **10a** (3.3 μmol g^{-1}). Since alkaline hydrolysis under these conditions results mainly in the cleavage of primary ester linkages (and presumably amide linkages to a limited extent), these analyses indicate that there is a substantial amount of ester (and/or amide) linked phenolics in the cell walls of suberized potato tissue. The presence of **2a** and **2b** in the alkaline hydrolysate remain unexplained, though they could be linked via ester bonds through their phenolic hydroxyl groups. (Note that under the conditions employed, the possibility of artefacts from side chain cleavage of **3a** and **3b** was ruled out.) More rigorous alkaline hydrolysis (2 N NaOH, 3 h, 160°C) yielded substantially more tyramine **10a** (27 μmol g^{-1} extractive-free residue), presumably indicative of the slow cleavage of amide linkages. Importantly, the identification of a number of *p*-hydroxy-substituted aromatics (i.e., **2a**, **3a**, **6b**, **10a**) in a yield totalling approx. 40.8 μmol g^{-1} extractive-free residue provides an explanation for the large amounts of *p*-hydroxybenzaldehyde (e.g., 48 μmol g^{-1} extractive-free residue) recovered after alkaline nitrobenzene oxidation treatment. However, since the total yield of products from alkaline hydrolysis did not equal that predicted by the more chemically harsh alkaline nitrobenzene oxidation treatment (see above) there remained an uncharacterized phenolic-rich residue after hydrolysis [47].

Thioacidolysis, a degradative technique based on the treatment of polyaromatics with BF_3/CH_3CH_2SH, results in selective cleavage of alkyl-aryl ether bonds and yields characteristic diastereomeric mixtures of 1,2,3-trithioethane derivatives **11a–c** of the three common monolignols, as well as dimeric and trimeric products (e.g., 23, 27, 28). In lignified tissues, yields are generally modest (i.e., 10–30% of the original lignin content), but they nevertheless can provide a useful assessment of the monomeric composition (i.e., *p*-hydroxyphenyl **12a**, guaiacyl **12b** and syringyl **12c** moieties) in the lignins examined. When applied to suberized potato wound periderm, thioacidolysis released only minute amounts of 1,2,3-trithioethane derivatives **11b** and **11c** in roughly 2:1 ratio and approximately one-fifth the yield of that predicted by alkaline nitrobenzene oxidation. Significantly, no *p*-hydroxyphenyl **11a** derivatives were recovered [13, 28, 29, 47]. In addition mixtures of dimeric compounds [28, 29] were also obtained, which after reductive de-sulfuration with Raney Ni, yielded *N*-(dihydro)feruloyltyramine **13** (a derivative of *N*-feruloyltyramine **3h** [29]) as the major compound. Being recovered in yields > 16 μmol g^{-1} extractive-free residue, **13** provides an explanation for the presence of hydroxycinnamic amides in both the soluble and alkali-labile components of suberized potato tissue. It does not, however, account for all of the vanillin **2b** liberated by alkaline nitrobenzene oxidation. But the latter technique is more chemically harsh and results in a greater degree of polymer degradation than either alkaline hydrolysis or thioacidolysis.

The recovery of **13** in the thioacidolysis products obtained from suberized potato tuber supports the prediction that its polyaromatic domain is largely comprised of hydroxycinnamic acids and their derivatives [21]. In addition, since thioacidolysis selectively cleaves ether linkages, it has been proposed that this dimer may have been ether-linked in the original aromatic polymer, consistent with the solid state ^{13}C NMR spectra data presented earlier.

Recently, a comparative analysis between suberized and lignified tissues of *Clivia miniata* using cupric oxide oxidation, thioacidolysis and BF_3/MeOH treatments was undertaken [60, 61] and serves to emphasize the difference between lignified (i.e., xylem vessels) and suberized (i.e., hypodermal and endodermal) tissues. For example, thioacidolysis of xylem vessel tissue of *C. miniata* released typical trithioethane monomers (e.g., **11b, c**), as well as dimeric derivatives (e.g. approx. 210 μmol g^{-1} extractive-free residue), with little evidence of hydroxycinnamic acids. In contrast, the suberized hypodermal cell walls contained relatively large amounts of hydroxycinnamic acid moieties (e.g., approx. 175 μmol g^{-1} extractive-free residue), principally *p*-coumaric acid **3a**, accounting for greater than 70% of the recovered phenolics. The same distribution pattern of monolignol- and hydroxycinnamic acid-derived phenolics was found with alkaline cupric oxide oxidation and BF_3/MeOH transesterification analysis. Thus, as with potato periderm,

the suberized *C. miniata* tissues (e.g., hypodermis) contained predominantly hydroxycinnamic acids and/or their derivatives. Interestingly, alkane-α,ω-dioic acids (i.e., "chemical" markers for the aliphatic domain of suberized tissue) were only present in the putative suberized tissues, coincident with hydroxycinnamic acids. Unfortunately, the authors did not analyze for hydroxycinnamoyl amides in their various extracts.

Lastly, pyrolysis-GC/MS has been directly applied to extractive-free suberin-enriched preparations of *Q. suber* cork [59]. As with alkaline nitrobenzene oxidation, the pyrolysis of phenylpropanoid polymers can result in cleavage of side-chain carbons to yield characteristic substituted aromatic derivatives (e.g., substituted benzaldehydes **2a–c** and benzoates **5a–b**) as well as cell wall polysaccharide breakdown products. When applied to suberized tissues (e.g., *Q. suber* cork), only 29% of the peaks in the pyrogram could be identified. Nearly two thirds of these were of phenolic origin and of the phenolics, over 85% were of the guaiacyl **12b** type. Thus in agreement with alkaline nitrobenzene oxidation, alkaline hydrolysis and thioacidolysis, this treatment predicts a polyaromatic domain rich in 3-methoxy-4-hydroxy substituted phenolics.

In summary, while the structural analysis of the aromatic domain of suberized plant materials using a variety of degradative techniques have provided somewhat conflicting results, a clearer picture is beginning to emerge. The use of improved analytical tools and a re-evaluation of previous interpretations of analytical data has led us to the following conclusions:

(i) alkaline hydrolysis, alkaline nitrobenzene oxidation, cupric oxide oxidation, BF_3/MeOH depolymerization and pyrolysis-MS analyses demonstrate that a significant amount of the cell wall-bound phenolics present in suberized tissues have a 3-hydroxy-4-methoxy-substitution pattern, including significant amounts of hydroxycinnamic acids,

(ii) thioacidolysis establishes that in addition to only a small amount of alkyl-aryl ether-linked monolignols **1a–c** being present, suberized potato tuber tissue contains significant amounts of covalently (i.e., ether)-linked *N*-feruloyl amides (e.g., **3h**), and

(iii) solid state ^{13}C NMR spectroscopic analyses of suberized potato tissue preparations (both natural abundance and specifically labelled with ^{13}C) have clearly demonstrated that the majority of the aromatic moieties in suberized potato tissues are hydroxycinnamates, and that they are covalently attached to each other. It would also appear that these are linked to other components of the primary cell wall and the components of the aliphatic domain.

It should be cautioned once again, however, that the majority of these data have been obtained from a single plant species (and organ), namely the potato tuber. Additional studies, using other species/tissues, are required for both confirmation of these conclusions and the establishment of chemical markers to be used as criteria in verifying the presence of the phenolic domain of suberin in plant tissues.

BIOSYNTHESIS OF AROMATIC POLYMERS IN SUBERIZED CELL WALLS[4]

The *de novo* biosynthesis of suberization-specific aromatic monomers and their subsequent macromolecular assembly at the cell wall/plasmalemma interface is poorly understood. Nevertheless, there continues to be considerable interest in the biosynthesis and metabolic fate of the phenylpropanoid derivatives associated with suberizing tissue [18, 65–73] and these are discussed below. As with the structural studies reviewed above, however, the majority of the enzymology (i.e., biochemistry) regarding suberization has come from studies using wound healing potato tubers as a model system. Note that several general aspects of hydroxycinnamic acid biosynthesis have recently been extensively reviewed [e.g. 20] elsewhere and only those salient to suberization are considered below.

Based on the experiments using ^{14}C- and ^{13}C-phenylalanine as substrate, it is established that the phenylpropane skeleton(s) of the aromatic moieties in suberized tissues are derived from phenylalanine **6a**. This process begins with the elimination of ammonia to yield *trans*-cinnamic acid **9** (Fig. 4) in a reaction catalyzed by the enzyme phenylalanine ammonia-lyase (PAL) [20, 74, 75]. Although tyrosine **6b** is another potential precursor, especially to the tyramine **10a** component of **3h**, its role in the suberization process has as not yet been directly investigated. The ammonia liberated during PAL activity in both lignifying *Pinus taeda* and wound healing potato tubers is recycled in a compartmentalized fashion via glutamic acid and the shikimic acid pathway [76, 77], thereby enabling more **6a** to be formed without increasing the demand for nitrogen. Presumably some of the arogenate formed during this cycle is channelled into tyrosine biosynthesis and ultimately ends up as tyramine. Given the high demand for phenylpropanoids during suberization, it is not surprising that wounding

[4] This review focusses on the aromatic domain of suberized tissues and the biosynthetic aspects of the aliphatic domain are not considered. The reader is directed to the few recent reports [63, 64] describing ω-hydroxylation of fatty acids (i.e., the first step toward the biosynthesis of the characteristic ω-hydroxyalkanoic and alkane-α,ω-dioic acids found in suberized tissue) in *Vicea sativa*, which are representative of the limited experimental work that has been reported regarding the biosynthesis of the aliphatic components of suberin since the reviews by Kolattukudy [5–7].

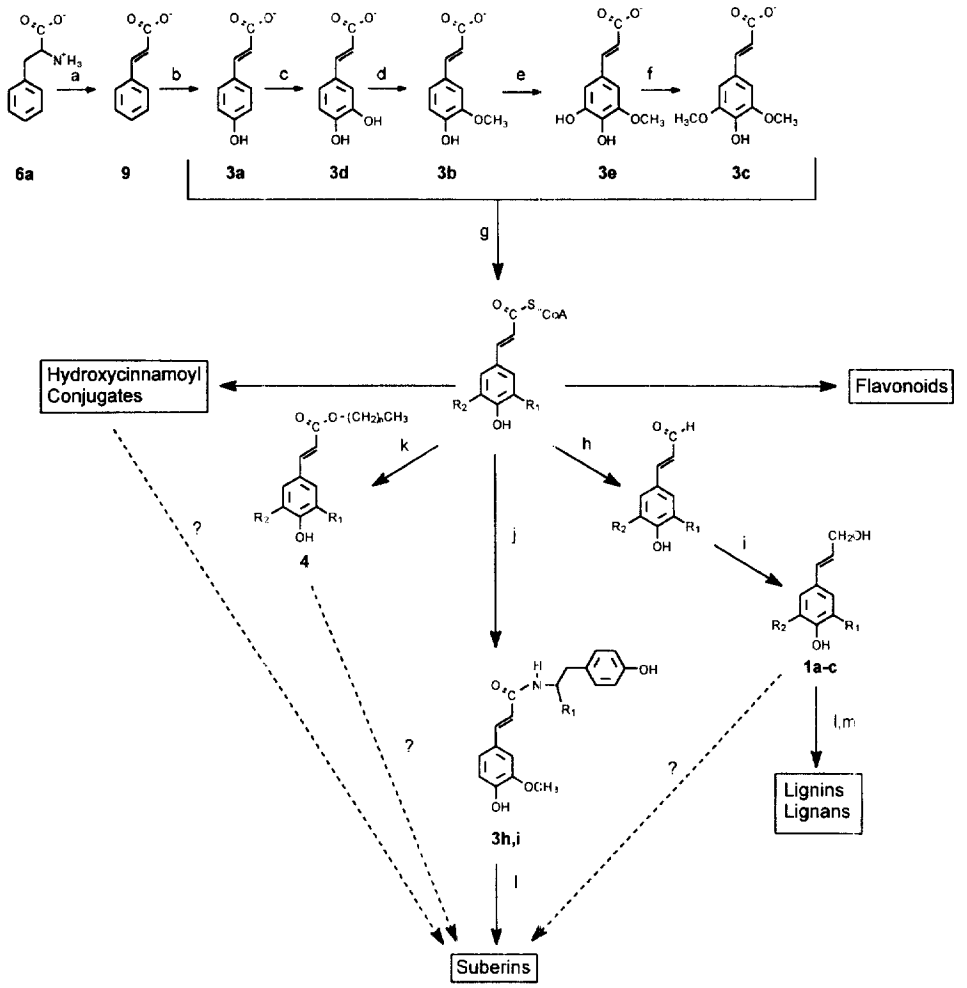


Fig. 4. The central role for hydroxycinnamoyl-CoA derivatives in the biosynthesis of polyaromatic cell wall polymers. The biosynthetic steps leading to the hydroxycinnamic acids **3a**–**e** are indicated, with the route to the coenzyme A derivatives left ambiguous (i.e., no attempt is made to delineate at which stage the coenzyme A derivatization takes place). (Readers are directed to Refs [18, 72 and 73] for a treatment of this aspect of phenylpropanoid metabolism.) The enzymes indicated are: a, phenylalanine ammonia-lyase; b, cinnamate-4-hydroxylase; c, 4-hydroxycinnamate-3-hydroxylase (or 4-hydroxycinnamoyl-CoA-3-hydroxylase); d, caffeate-*O*-methyltransferase (or caffeoyl-CoA *O*-methyltransferase); e, ferulate-5-hydroxylase; f, ferulate *O*-methyltransferase; g, 4-hydroxycinnamoyl-CoA ligase; h, cinnamoyl-CoA oxidoreductase; i, cinnamyl alcohol dehydrogenase; j, hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyltransferase; k, hydroxycinnamoyl-CoA: ω-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase (HHT); l, peroxidase; m, laccase. A question mark (?) indicates possible involvement, but where no enzyme has been described. (Adapted from Refs. [20, 65–67, 73–75].)

of potato tubers not only elicits phenylpropanoid metabolism (see below), but also 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase, the first enzyme of the shikimic acid pathway [78] leading to phenylalanine **6a** and tyrosine **6b**. At this point in time, it is assumed that the remainder of the enzymes of the shikimic acid pathway are also induced under conditions which induce suberization.

Phenylalanine ammonia-lyase (PAL) is essential for suberization [79, 80], since its inhibition *in vivo* by either *S*-carvone [79] or L-AOPP [80] prevents polyaromatic deposition. During wound healing (i.e., suberization) in potato tubers, PAL is rapidly induced (i.e., within 12 h) 10 to 15-fold, from near non-detectable levels. The induction has been measurable both at the mRNA [81] and protein [73, 79, 80, 82, 83] levels. Interestingly, high levels of PAL activity remain for several days after wounding [73, 79, 81], and do not drop back to pre-induction levels until the suberization process is well advanced.

The carboxylic acid moiety of hydroxycinnamic acids **3a**–**e** must be activated prior to further derivatization or modification (i.e., whether for feruloyltyramine or monolignol biosynthesis). The mechanism in potatoes appears to be via synthesis of coenzyme-A thioesters, since 4-coumaryl CoA ligase (4-

CL) is induced during the initial stages of suberin biosynthesis [73]. That is, 4-CL is rapidly induced in potato tubers by wounding, with measurable activity levels rising approximately 15-fold within 12 h of wounding. Interestingly, both *p*-coumaric **3a** and ferulic **3b** acids serve as effective substrates for induced potato 4-CL, whereas sinapic **3c** and caffeic **3d** acids do not [73]. The role for *p*-coumaroyl-CoA in suberization remains unresolved since these units are not found in the final polymer. In addition, the observed lack of 4-CL activity with **3c** raises questions about the origins of syringyl **12c** residues recovered from suberized potato tissues [13, 47]. With respect to the former, derivatization of *p*-coumaric acid **3a** may be required prior to the subsequent hydroxylation and methylation steps common to phenylpropanoid biosynthesis. Similarly, feruloyl-CoA may act as a substrate for ferulate-5-hydroxylase, with subsequent methylation to yield sinapoyl-CoA. Alternatively, 5-hydroxyferulic acid **3e** may be activated by 4-CL. Neither of these hypotheses has been tested experimentally in suberizing tissues. In any case, within 12 h of wound induction, potato tubers appear to have phenylpropanoid metabolism enzymes in place and are capable of generating the coenzyme-A derivatives of the hydroxycinnamic acids **3a, b**.

The metabolic fate of hydroxycinnamoyl-CoA derivatives

Hydroxycinnamoyl-CoA derivatives are central intermediates in the biosynthesis of many phenylpropanoids [20], and can have many competing metabolic fates (e.g., Fig. 4). While Fig. 4 shows some of the possible routes by which hydroxycinnamoyl-CoA derivatives can be metabolized, the key to delineating their metabolic fate during suberization may in part lie in the identity of the (major) phenolic components present in the tissue. Unfortunately, there is not a complete picture detailing the phenolic composition of all suberized tissues. In addition, as alluded to earlier, suberized tissues of some plants (especially the bark of woody plants) are diverse in their chemical composition and are not only suberized. Again, the best defined system is that of the potato tuber, where the phenolic composition of both natural and wound-induced periderm have been described [13, 28, 29, 38, 44, 47], and polyhydroxycinnamic acids/amides, including alkyl–aryl ether linked *N*-feruloyl amides [29] are known to accumulate. As described above, there are a variety of other phenolics associated with induced suberization in potatoes [29, 38, 46, 47], namely chlorogenic acid **3j**, *N*-caffeoylputrescine **3f**, *N*-feruloylputrescine **3g**, *N*-feruloyltyramine **3h**, *N*-feruloyloctopamine **3i**, tyramine **10a**, hydroxybenzaldehydes **2a–c**, hydroxybenzoates **5a, b** and alkylferulates **4**. In this section, however, we wish to attempt to distinguish between the biochemical pathway to feruloyltyramine and that to monolignols.

Since they are unequivocally integral to the aromatic domain of potato suberin, the *de novo* biosynthesis of *N*-(hydroxycinnamoyl) amides (particularly those of tyramine) and to a lesser extent alkyl ferulates **4** in tubers induced to suberize is of particular importance in delineating the fate of hydroxycinnamoyl-CoA derivatives. This has been achieved recently, through the demonstration of feruloyl-CoA as a good substrate for both a wound-induced hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyltransferase (THT) (Fig. 4, step *j*) [65, 68] and a hydroxycinnamoyl-CoA:ω-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase (HHT) (Fig. 4, step *k*) [65, 66, 68], which catalyze the formation of **3h** and alkyl ferulate esters **4**, respectively.

While both of these biosynthetic enzymes are associated with induced suberization, only THT shows a temporal relationship. That is, within 24 h of wounding, THT is induced 50-fold over near non-detectable, constitutive levels [65], and maintained at high levels for two or more days, while HHT does not reach maximum activity levels until several days after wounding [66]. However, the time course of HHT induction is consistent with the accumulation of its putative *in vivo* product(s) [38], and the indirect role these metabolites may play in suberization.

Hydroxycinnamoyl transferases are widespread in plants (e.g. [18, 67, 69–72]), as they are found in gymnosperms and angiosperms. A recent survey of 17 plant species demonstrated the presence of HHT in 10 of 11 families examined, including Solanaceae, Compositae, Umbelliferae, Chenopodiaceae, Cucurbitaceae, Cruciferae, Liliaceae, Palmae, Gramineae and Pinaceae [67]. Only trace amounts of activity were measured in non-induced Leguminosae. By contrast, an apparent HHT activity was induced in elicitor-treated *Phaseolus vulgaris* [18], coincident with an induced laurate ω-hydroxylase. The *P. vulgaris* hydroxycinnamoyl transferase was partially purified and reported to yield three bands (Mr 28 kD, 40 kD and 70 kD) on an SDS-PAGE gel. By contrast, the purified HHT from tobacco (*Nicotiana tabacum*) yielded a single polypeptide of 55 kD, which showed a strong preference for feruloyl-CoA as hydroxycinnamoyl donor [70]. The potato THT enzyme has also been purified to apparent homogeneity [71, 72], and appears to be a hetero dimer of approx. 49 kD, comprising two approx. 25 kD polypeptides [72]. Interestingly, the apparent K_M for tyramine for this enzyme varies with hydroxycinnamoyl-acceptor, such that the enzyme appears to favour feruloyl-CoA (K_M for tyramine = 20 μM) over *p*-coumaryl-CoA (K_M for tyramine = 174 μM). Thus a role for hydroxycinnamoyl transferases in "suberizing" tissues is beginning to emerge. However, an analysis of hydroxycinnamoyl transferase activities in a variety of plant species, coupled with a demonstration of concomitant suberization, is required to unequivocally establish this role.

In lignifying tissues, where the polyaromatic domain comprises covalently linked monolignols **1a**-

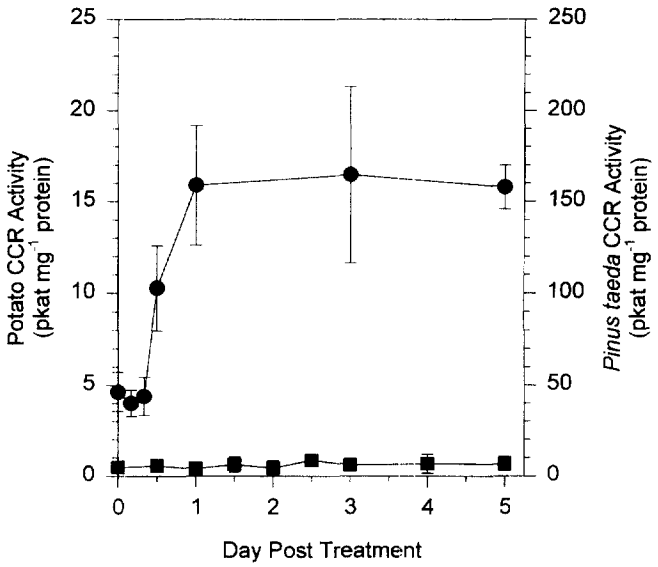


Fig. 5. Role of cinnamoyl Co-A oxidoreductase (CCR) in suberization and lignification. The time course induction of CCR under conditions which promote suberization in potato (■) and lignification in *P. taeda* cell cultures (●) are indicated. (Adapted from Ref. [73].)

c, the CoA derivatives of hydroxycinnamic acids undergo a two-step, enzyme-catalyzed reduction to afford the monolignols **1a–c** [20, 84, 85]. In this reductive sequence, hydroxycinnamoyl-CoA:NADP$^+$ oxidoreductase (CCR) catalyzes the putative commitment step toward the biosynthesis of hydroxycinnamyl alcohols **1a–c** [86] via the physiologically irreversible reduction of hydroxycinnamoyl-CoA derivatives to their corresponding aldehydes (Fig. 4, step *h*). The connection between induced CCR activity and "lignification" has recently been shown using cell cultures of *Pinus taeda*. In this system, which has been demonstrated to deposit "lignin" under specific culture conditions [26], a 3 to 4-fold induction in measurable CCR activity, over an already high constitutive level (i.e., approx. 50 pkat mg^{-1} protein) occurs [73] coincident with lignin-like formation (Fig. 5) [26]. By contrast, during wound-induced formation of the polyaromatic domain of suberizing potato tissue, CCR does not appear to be induced at all (Fig. 5). Under these conditions, CCR activity levels remain both very low and unchanged (i.e., <1 pkat mg^{-1} protein) throughout the time period in which suberization takes place [73].

The absence of inducible CCR activity in suberizing potatoes, coupled with the emerging role for hydroxycinnamoyl transferases, may provide further explanation for the monomeric differences between the polyaromatic domains of lignified and suberized tissues. Clearly the relatively low levels of CCR activity could influence the fate of hydroxycinnamoyl-CoA derivatives formed during suberization, with the bulk being channelled through pathways mediated by hydroxycinnamoyl transferases. Nevertheless, the measurable amounts of CCR activity in wound induced potato tubers may account for the minute amounts of lignin found there [13, 27–29, 47].

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the next reaction in the two-step reduction of hydroxycinnamic acids **3a–c** to monolignols **1a–c** (Fig. 4, step *i*), by reducing the corresponding hydroxycinnamaldehydes to the monolignols **1a–c** [20, 84, 85]. In contrast to CCR, however, there is a relatively high constitutive level of CAD activity in potato tubers (approx. 40 pkat mg^{-1} protein), the level of which is induced 2- to 3-fold by wounding [65,73]. A similar temporal pattern of CAD activity is observed in *P. taeda* cells induced to "lignify" by culture treatment [26, 73]. However, it should be kept in mind that the spectrophotometric assay used to measure CAD activity is not necessarily specific, and the measured dehydrogenase activity may include contributions by other dehydrogenases. For example, four isoforms of CAD have been isolated from the periderm of *Eucalyptus gunnii* (eucalyptus) [87]. However, within crude extracts there were also two aliphatic and two aromatic alcohol dehydrogenases that could interfere with the assay. The presence of CAD isoforms in eucalyptus periderm underscores the biochemical complexity of woody "bark" tissues, and may also help explain the isolation of an apparently typical lignin from milled *Q. suber* bark [49, 50].

Suberin macromolecular assembly

The perceived assembly of the aromatic cell wall polymers has been reviewed extensively for lignins (e.g. [20, 22, 88, 89]). The final polymerization step is often viewed to proceed via either a peroxidase/H_2O_2 and/or laccase/O_2 mediated free radical coupling pro-

cess. Indeed, H_2O_2 scavengers such as KI can effectively inhibit lignification in cell cultures of *P. taeda* [90]. Even though the final lignin polymer is heterogeneous [20, 22, 88], uncontrolled free radical coupling is not likely. The polymerization of aromatic moieties into the cell walls of suberizing tissues has been hypothesized to occur in an analogous fashion to that for lignification [5, 6]. Thus, it has been proposed that the assembly of the polyaromatic domain during suberization also follows a peroxidase/H_2O_2-mediated process [5, 6, 91].

However, the macromolecular assembly of cell wall polymers involves more than the oxidation of monomers by a peroxidase. In its simplest form, the macromolecular assembly process involves (a) the synthesis and/or release (from storage) of the appropriate monomers, (b) transport of the monomers to the site of polymerization, and (c) control of polymerization (i.e., assembly). Clearly each of these components must be both spatially and temporally regulated, including the co-ordinate regulation of the enzymes involved, to ensure that the correct monomers arrive at the appropriate site(s) and are then subsequently polymerized in a controllable, reproducible manner.

For the suberization process, very little is known about any of these steps. However, it seems clear that within 12–24 h of wound induction, suberin monomer synthesis is under way in potato tubers. Histochemical and ultrastructural changes are evident within 1–3 d. Within 2–3 d, a functional water resistant barrier is in place [92], and the "suberized" layer on the surface of wound healing potatoes can be mechanically removed intact (unpublished observation). All of these observations reveal a highly orchestrated, rapid deposition of polymeric materials during suberization.

While the biosynthesis of suberin monomers was discussed above, virtually nothing is known about the cellular site of synthesis. Additionally, the mechanisms involved in transporting monomers to the cell wall and the control of polymerization remain undefined. As alluded to above, the best characterized component of the macromolecular assembly of suberin is the putative "final" step involving the polymerization of monomers. Thus, over a decade ago it was demonstrated that wound-induced suberization in potato tubers was coincident with the induction of a highly anionic (pI approx. 3.2) peroxidase [93], which could be immunocytochemically localized to the site of wound healing [93, 94]. However, a definitive role for this "suberin-associated" anionic peroxidase in the macromolecular assembly of the aromatic domain in suberized tissue remains to be demonstrated, and the literature contains conflicting results. For example, the suberin-associated peroxidase has not yet been fully characterized at the biochemical level; that is, with respect to either its substrate specificity or the nature of the products it produces. It has, however, been cloned from potato [95, 96], and subsequently shown to be expressed in tomato [95, 97–99] and tobacco [100]. In addition to its expression during wound- and pathogen-induced suberization [97–99], this peroxidase is also developmentally expressed in tomato fruit [97, 99]. This observation suggests that the "suberin specific" description for the enzyme may not reflect its complete role in plants, especially since tomato fruits are not normally suberized, unless wounded (e.g. [101]). In addition, when tomatoes were transformed with anionic peroxidase antisense constructs, effectively nullifying the suberin-associated anionic peroxidase, the incorporation of phenolics into the cell wall of suberizing tomato fruits was unabated [101]. Unfortunately, the authors relied only on non-quantitative autofluorescence to gauge the deposition of phenolics, and any compositional changes in wound-induced antisense tomatoes remains unknown. It may be that another peroxidase isoform present in the tissue was capable of cross-linking the aromatic moieties. Alternatively, the phenolics deposited during suberization were not polymerized normally into the cell wall matrix.

Thus, the question as to whether the polymerization of phenolics occurs via a peroxidase-mediated process remains open. However, the recovery of ether-linked *N*-feruloyltyramine **3h** units from the cell walls of suberized potatoes via thioacidolysis [29] lends support, since their incorporation into the cell walls may occur via oxidative processes.

An intriguing aspect of the peroxidase-mediated macromolecular assembly of the aromatic domain in suberizing tissues is the question of where the H_2O_2 necessary for peroxidative coupling comes from. Several mechanisms for H_2O_2 generation in plants have been presented in the literature, including a cell wall bound malate dehydrogenase [102–104], polyamine oxidase [105 and references therein], oxalate oxidase [106], plasma membrane-associated NAD(P)H,H^+-dependent oxidase [106–109] and peroxidases themselves (e.g., reviewed in [106]). All are able to generate active oxygen species, including H_2O_2 [102–109], especially in the presence of a reductant (e.g., phenolics), but none have yet been implicated in the suberization process. In potatoes (for which suberization is best described), activated oxygen species are generated in response to wounding and pathogen attack [110–112], although no connection has yet been made to suberization. It remains to be seen whether any of the phenylpropanoids that accumulate in suberizing tissues play a role in the macromolecular assembly of the polyaromatic domain by acting as substrates for H_2O_2 generation at the cell wall/plasmalemma interface.

CONCLUSIONS

One of the problems encountered in reviewing the literature on "suberin", is that there is no clear definition of what it is. While it is well established that suberized tissues contain both polyaromatic and polyaliphatic domains [5, 6], it is not clear from the literature whether these two domains are inextricably

linked. Consequently, the notion of a heteropolymer, called suberin, must be questioned and it is perhaps more prudent to describe tissues as suberized, rather than as containing suberin *per se*.

It is now clear that suberized tissues are distinct from both lignified and cutinized tissues, and that the domains that comprise "suberin" are unique and distinct. It should be cautioned, however, that the bulk of the experimental work describing suberized tissues utilizes potato tubers as a model system. There is a critical need to establish whether the potato periderm findings extend to other plant systems. There is also a need to identify a chemical marker for the polyaromatic domain of suberized tissues, much the same as α,ω-dioic acids are markers for the aliphatic domain.

Nevertheless, on the basis of the work using potato periderm as a model for suberized tissues, the following can be surmized:

(i) the polyaromatic domain of suberized tissues comprises covalently cross-linked hydroxycinnamic acids and their derivatives, principally amides and
(ii) the two domains of suberized tissues (i.e., the polyaromatic and polyaliphatic domains) are spatially separate.

One of the morphologically distinct features of suberized tissues is the presence of multiamellar bands in TEM micrographs. These lamellae have long been thought to arise from the alternation of aromatic and aliphatic domains in the tissue. An important question raised by the apparent spatial separation of the aromatic and aliphatic domains of suberized tissues concerns the origin of the lamellae.

With respect to the biosynthesis of the aromatic domain of suberized tissues, there is an apparent channelling of hydroxycinnamic acids, via their coenzyme-A derivatives, into the formation of "suberin", and away from the biosynthesis of monolignols (i.e., the precursors of lignin). The macromolecular assembly of the polyaromatic domain of suberin remains very poorly defined. An anionic peroxidase has been implicated in the final steps of the polymerization, but has yet to be shown to be essential. With an emerging picture of hydroxycinnamoyl-derivatives as precursors to the polyaromatic domain, and some progress toward the details of their biosynthesis, new directions in delineating the macromolecular assembly process will present themselves, and chart the course for future research.

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REFERENCES

1. Stebbins, G. J. and Hill, G. J. C., *Am. Nat.*, 1980, **115**, 342.
2. Manhart, J. R. and Palmer, J. D., *Nature*, 1990, **345**, 268.
3. Lewis, N. G. and Davin, L. B., *ACS Symp. Series*, 1994, **562**, 202.
4. Hahlbrock, K. and Scheel, D., *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1989, **40**, 347.
5. Kolattukudy, P. E., *Science*, 1980, **208**, 990.
6. Kolattukudy, P. E., *Can. J. Bot.*, 1984, **62**, 2918.
7. Kolattukudy, P. E., in *The Biochemistry of Plants*, Vol. 9. Academic Press, NY, 1987, pp. 291.
8. Rittinger, P. A., Biggs, A. R. and Peirson, D. R., *Can. J. Bot.*, 1987, **65**, 1886.
9. Biggs, A. R., *Phytopathology*, 1987, **77**, 718.
10. Knobloch, I., Kahl, G., Landré, P. and Nougarède, A., *Can. J. Bot.*, 1989, **67**, 3090.
11. Schmitt, U. and Liese, W., *Holzforschung*, 1991, **45**, 313.
12. Trockenbrodt, M., *Flora*, 1994, **189**, 131.
13. Borg-Olivier, O. and Monties, B., *C.R. Acad. Sci. Paris*, 1989, **308 III**, 141.
14. Thomson, N., Evert, R. F. and Kelman, A., *Can. J. Bot.*, 1995, **73**, 1436.
15. Hawkins, S. and Boudet, A. M., *Protoplasma*, 1996, **191**, 96.
16. Newcombe, G. and Robb, J., *Physiol. Mol. Plant Pathol.*, 1989, **34**, 55.
17. Robb, J., Lee, S.-W., Mohan, R. and Kolattukudy, P. E., *Plant Physiol.*, 1991, **97**, 528.
18. Bolwell, G. P., Gerrish, C. and Salaun, J.-P., *Phytochemistry*, 1997, **45**, 1351.
19. Esau, K. in *Anatomy of Seed Plants*, 2nd edn. John Wiley and Sons, NY, 1977, pp. 527.
20. Davin, L. B. and Lewis, N. G., *Rec. Adv. Phytochemistry*, 1992, **26**, 325.
21. Bernards, M. A., Lopez, M. L., Zajicek, J. and Lewis, N. G., *J. Biol. Chem.*, 1995, **270**, 7382.
22. Lewis, N. G. and Yamamoto, E., *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1990, **41**, 455.
23. Monties, B. in *Methods in Plant Biochemistry*, ed. J. B. Harbone, 1989, **1**, 113.
24. Lewis, N. G., Newman, J., Just, G. and Ripmeister, J., *Macromolecules*, 1987, **20**, 1752.
25. Lewis, N. G., Yamamoto, E., Wooten, J. B., Just, G., Ohashi, H. and Towers, G. H. N., *Science*, 1987, **237**, 1344.
26. Eberhardt, T. L., Bernards, M. A., He, L., Davin, L. B., Wooten, J. B. and Lewis, N. G., *J. Biol. Chem.*, 1993, **268**, 21088.
27. Lapierre, C., Pollet, B., Monties, B. and Rolando, C., *Holzforschung*, 1991, **45**, 61.
28. Lapierre, C., Pollet, B. and Négrel, J., *Phytochemistry*, 1996, **42**, 949.

29. Négrel, J., Pollet, B. and Lapierre, C., *Phytochemistry*, 1996, **43**, 1195.
30. Schmidt, H. W. and Schönherr, J., *Planta*, 1982, **154**, 76.
31. Matzke, K. and Riederer, M., *Planta*, 1991, **185**, 233.
32. Holloway, P. J., *Phytochemistry*, 1983, **22**, 495.
33. Gérard, H. C., Pfeffer, P. E. and Osman, S. F., *Phytochemistry*, 1994, **35**, 818.
34. Lulai, E. C. and Morgan, W. C., *Biotechnic, Histochem.*, 1992, **67**, 185.
35. Barnabas, A. D. and Peterson, C. A., *Can. J. Bot.*, 1992, **70**, 2233.
36. Peterson, C. A., Murrmann, M. and Steudle, E., *Planta*, 1993, **190**, 127.
37. Adamovics, J. A., Johnson, G. and Stermitz, F. R., *Phytochemistry*, 1977, **16**, 1089.
38. Bernards, M. A. and Lewis, N. G., *Phytochemistry*, 1992, **31**, 3409.
39. Laver, M. L. and Fang, H. H.-L., *J. Agric. Food Chem.*, 1989, **37**, 114.
40. Baldé, A. M., Claeys, M., Pieters, L. A., Wray, V. and Vlietinck, A. J., *Phytochemistry*, 1991, **30**, 1024.
41. Tanaka, R., Inosiri, A., Yoneda, M., Ishida, T., Numada, A. and Matsunaga, S., *Phytochemistry*, 1990, **29**, 3263.
42. Schmutz, A., Jenny, T., Amrhein, N. H. and Ryser, U., *Planta*, 1993, **189**, 453.
43. Schmutz, A., Jenny, T. and Ryser, U., *Phytochemistry*, 1994, **36**, 1343.
44. Cottle, W. and Kolattukudy, P. E., *Plant Physiol.*, 1982, **69**, 393.
45. Ramamurthy, M. S., Maiti, B., Thomas, P. and Nair, P. M., *J. Agric. Food Chem.*, 1992, **40**, 569.
46. Malmberg, A., *Acta Chem. Scand.*, 1984, **B38**, 153.
47. Borg-Oliver, O. and Monties, B., *Phytochemistry*, 1993, **32**, 601.
48. Zimmermann, W., Nimz, H. and Seemüller, E., *Holzforschung*, 1985, **39**, 45.
49. Marques, A. V., Pereira, H., Meier, D. and Faix, O., *Holzforschung*, 1994, **48** (supp.), 43.
50. Marques, A. V., Pereira, H., Meier, D. and Faix, O., *Holzforschung*, 1996, **50**, 393.
51. Perra, B., Haluk, J.-P. and Metche, M., *Holzforschung*, 1993, **47**, 486.
52. Björkman, A., *Svensk Papperstidn.*, 1956, **59**, 477.
53. Whitmore, F. W., *Phytochemistry*, 1978, **17**, 421.
54. Hammerschmidt, R., *Potato Res.*, 1985, **28**, 123.
55. Garbow, J. R., Ferrantello, L. M. and Stark, R. E., *Plant Physiol.*, 1989, **90**, 783.
56. Stark, R. E., Zlotnick-Mazori, T., Ferrantello, L. M. and Garbow, J. R., *ACS Symp. Series*, 1989, **399**, 214.
57. Stark, R. E. and Garbow, J. R., *Macromolecules*, 1992, **25**, 149.
58. Stark, R. E., Sohn, W., Pacchiano, R. A., Al-Bashir, M. and Garbow, J. R., *Plant Physiol.*, 1994, **104**, 527.
59. Pereira, H., *Wood Sci. Technol.*, 1988, **22**, 211.
60. Schreiber, L., *Planta*, 1996, **199**, 596.
61. Zeier, J. and Schreiber, L., *Plant Physiol.*, 1997, **113**, 1223.
62. Bland, D. E. and Logan, A. F., *Biochem. J.*, 1965, **95**, 315.
63. Pinot, F., Salaün, J.-P., Bosch, H., Lesot, A., Mioskowski, C. and Durst, F., *Biochem. Biophys. Res. Comm.*, 1992, **184**, 183.
64. Pinot, F., Bosch, H., Alayrac, C., Mioskowski, C., Vendais, A., Durst, F. and Salaün, J.-P., *Plant Physiol.*, 1993, **102**, 1313.
65. Négrel, J., Javelle, F. and Paynot, M., *J. Plant. Physiol.*, 1993, **142**, 518.
66. Lotfy, S., Négrel, J. and Javelle, F., *Phytochemistry*, 1994, **35**, 1419.
67. Lotfy, S., Javelle, F. and Négrel, J., *Phytochemistry*, 1995, **40**, 389.
68. Négrel, J., Lotfy, S. and Javelle, F., *J. Plant. Physiol.*, 1995, **146**, 318.
69. Lotfy, S., *Plant Physiol. Biochem.*, 1995, **33**, 423.
70. Lotfy, S., Javelle, F. and Négrel, J., *Planta*, 1996, **199**, 475.
71. Hohlfeld, H., Schürmann, W., Scheel, D. and Strack, D., *Plant Physiol.*, 1995, **107**, 545.
72. Hohlfeld, H., Scheel, D. and Strack, D., *Planta*, 1996, **199**, 166.
73. Bernards, M. A., Susag, L. M., Bedgar, D. L., Anterola, A. M. and Lewis, N. G., *Phytochemistry* (in preparation).
74. Koukol, J. and Conn, E. E. *J. Biol. Chem.* 1961, **236**, 2692.
75. Neish, A. C., *Phytochemistry*, 1961, **1**, 1.
76. Razal, R. A., Ellis, S., Singh, S., Lewis, N. G. and Towers, G. H. N., *Phytochemistry*, 1996, **41**, 31.
77. van Heerden, P. S., Towers, G. H. N. and Lewis, N. G., *J. Biol. Chem.*, 1996, **271**, 12350.
78. Dyer, W. E., Henstrand, J. M., Handa, A. K. and Herrmann, K. M., *Proc. Natl. Acad. Sci. U.S.A.*, 1989, **86**, 7370
79. Oosterhaven, K., Hartmans, K. J., Scheffer, J. J. C. and van der Plas, L. H. W., *J. Plant Physiol.*, 1995, **146**, 288.
80. Street, P. F. S., Robb, J. and Ellis, B. E., *Protoplasma*, 1986, **132**, 1.
81. Ishizuka, M., Yamada, F., Tanaka, Y., Takeuchi, Y. and Imaseki, H., *Plant Cell Physiol.*, 1991, **32**, 57.
82. Borchert, R., *Plant Physiol.*, 1978, **62**, 789.
83. Cottle, W. and Kolattukudy, P. E., *Plant Physiol.*, 1982, **70**, 775.
84. Lüderitz, T. and Grisebach, H., *Eur. J. Biochem.*, 1981, **119**, 115.
85. Lüderitz, T., Schatz, G. and Grisebach, H., *Eur. J. Biochem.*, 1982, **123**, 583.

86. Goffner, D., Campbell, M. M., Compargue, C., Clastre, M., Borderies, G., Boudet, A. and Boudet, A. M., *Plant Physiol.*, 1994, **106**, 625.
87. Hawkins, S. W. and Boudet, A. M., *Plant Physiol.*, 1994, **104**, 75.
88. Higuchi, T., in *Biosynthesis and Biodegradation of Wood Components*, ed. T. Higuchi. Academic Press, 1985. pp. 141.
89. Dean, J. F. D. and Eriksson, K.-K. L., *Holzforschung*, 1994, **48**(supp.), p. 21.
90. Nose, M., Bernards, M. A., Furlan, M., Zajicek, J., Eberhardt, T. L. and Lewis, N. G., *Phytochemistry*, 1995, **39**, 71.
91. Kolattukudy, P. E., Mohan, R., Bajar, M. A. and Sherf, B. A., *Biochem. Soc. Trans.*, 1992, **20**, 333.
92. Soliday, C. L., Kolattukudy, P. E. and Davis, R. W., *Planta*, 1979, **146**, 607.
93. Espelie, K. E. and Kolattukudy, P. E., *Arch. Biochem. Biophys.*, 1985, **240**, 539.
94. Espelie, K. E., Franceschi, V. R. and Kolattukudy, P. E., *Plant Physiol.*, 1986, **81**, 487.
95. Roberts, E., Kutchan, T. and Kolattukudy, P. E., *Plant Mol. Biol.*, 1988, **11**, 15.
96. Roberts, E. and Kolattukudy, P. E., *Mol. Gen. Genet.*, 1989, **217**, 223.
97. Mohan, R. and Kolattukudy, P. E., *Plant Physiol.*, 1990, **92**, 276.
98. Mohan, R., Vijayan, P. and Kolattukudy, P. E., *Plant Mol. Biol.*, 1993, **22**, 475.
99. Sherf, B. A. and Kolattukudy, P. E., *Plant J.*, 1993, **3**, 829.
100. Mohan, R., Bajar, A. M. and Kolattukudy, P. E., *Plant Mol. Biol.*, 1993, **21**, 341.
101. Sherf, B. A., Bajar, A. M. and Kolattukudy, P. E., *Plant Physiol.*, 1993, **101**, 201.
102. Elstner, E. F. and Heupel, A., *Planta*, 1976, **130**, 175.
103. Gross, G. G., *Phytochemistry*, 1977, **16**, 319.
104. Gross, G. G. and Janse, C., *Z. Pflanzenphysiol.*, 1977, **84**, 447.
105. Angelini, R., Federico, R. and Bonfante, P., *J. Plant Physiol.*, 1995, **145**, 686.
106. Wojtaszek, P., *Biochem. J.*, 1997, **322**, 681.
107. Vianello, A. and Macri, F., *J. Bioenerget. Biomem.*, 1991, **23**, 409.
108. Cross, A. R. and Jones, O. T. G., *Biochim. Biophys. Acta*, 1991, **1057**, 281.
109. Vianello, A. and Macri, F., *Biochim. Biophys. Acta*, 1989, **980**, 202.
110. Chai, H. B. and Doke, N., *Phytopathology*, 1987, **77**, 645.
111. Doke, N., *Physiol. Plant Path.*, 1985, **27**, 311.
112. Murai, A., Yoshizawa, Y., Toida, T., Sakamoto, M., Monden, T. and Masamune, T., *Chem. Lett.*, 1995, 171.