

Apoplastic polyesters in *Arabidopsis* surface tissues – A typical suberin and a particular cutin

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Abstract

Cutinized and suberized cell walls form physiological important plant–environment interfaces as they act as barriers limiting water and nutrient loss and protect from radiation and invasion by pathogens. Due to the lack of protocols for the isolation and analysis of cutin and suberin in *Arabidopsis*, the model plant for molecular biology, mutants and transgenic plants with a defined altered cutin or suberin composition are unavailable, causing that structure and function of these apoplastic barriers are still poorly understood. Transmission electron microscopy (TEM) revealed that *Arabidopsis* leaf cuticle thickness ranges from only 22 nm in leaf blades to 45 nm on petioles, causing the difficulty in cuticular membrane isolation. We report the use of polysaccharide hydrolases to isolate *Arabidopsis* cuticular membranes, suitable for depolymerization and subsequent compositional analysis. Although cutin characteristic ω -hydroxy acids (7%) and mid-chain hydroxylated fatty acids (8%) were detected, the discovery of α,ω -diacids (40%) and 2-hydroxy acids (14%) as major depolymerization products reveals a so far novel monomer composition in *Arabidopsis* cutin, but with chemical analogy to root suberin. Histochemical and TEM analysis revealed that suberin depositions were localized to the cell walls in the endodermis of primary roots and the periderm of mature roots of *Arabidopsis*. Enzyme digested and solvent extracted root cell walls when subjected to suberin depolymerization conditions released ω -hydroxy acids (43%) and α,ω -diacids (24%) as major components together with carboxylic acids (9%), alcohols (6%) and 2-hydroxyacids (0.1%). This similarity to suberin of other species indicates that *Arabidopsis* roots can serve as a model for suberized tissue in general.

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1. Introduction

As a protection mechanism from uncontrolled water loss, nutrient depletion and infection by pathogens, plants have developed specialized surface tissues covering all aerial surfaces and the subterranean root organs. These encompassing tissues are characterized by substantial

amounts of the apoplastic aliphatic biopolymers cutin and suberin, respectively.

In vascular plants the plant–atmosphere interface of all primary above-ground organs such as leaves and fruits is formed by the wax encrusted plant cuticle (Kerstiens, 1996). The cuticle is deposited at the outermost part of the epidermis cell wall as a 0.1–10 μm thin membrane like layer (Vogg et al., 2004). The cuticle is composed of a lipophilic polymer matrix, the cuticular membrane, and soluble cuticular lipids, commonly known as waxes that are deposited on and encrusted in the polymer matrix (Jeffree, 1996; Kunst and Samuels, 2003). The occurrence of suberin in

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plants is highly variable. The cell walls of the periderm, the outer tissues of stems in a secondary growth stage, are known to contain the polymer suberin. In the hypodermis and endodermis, the outer and inner sealing tissues of primary roots, suberin is deposited as lamellae inside the primary cell wall close to the plasma membrane (Wilson and Peterson, 1983; Nawrath, 2002). Furthermore, suberin has been detected in the Casparian bands, endodermal cell walls of the bundle sheaths in grass leaves (Espelie and Kolattukudy, 1979) and is formed upon mechanical or pathogen caused damages to plants (Agrios, 1997).

Cutin, the matrix component of the cuticular membrane, is an insoluble biopolymer predominantly build from C₁₆ and C₁₈ aliphatics which are cross-linked via ester bonds, typically between carboxy and ω -hydroxy groups of individual fatty acid derivatives (Deas and Holloway, 1977; Kolattukudy, 1981, 2001a). ω -Hydroxyacids with additional hydroxyl and epoxy groups in secondary positions, mostly mid-chain, are the major products determined after hydrolytic depolymerization. The non-hydrolysable fraction of the cuticular membrane, named cutan (Jeffree, 1996), is poorly understood in its structure and chemical composition (Villena et al., 1999; Heredia, 2003).

Suberin is a more complex biopolymer composed of an aliphatic and an aromatic domain (Kolattukudy, 1980, 2001b; Bernards and Lewis, 1998). The latter is primarily composed of *p*-coumaric and ferulic acid, presumably involved in linking the aliphatic domain to cell wall polysaccharides (Bernards, 2002). The aliphatic domain of suberin is a polyester polymer, comprised of mainly ω -hydroxyacids and α,ω -diacids as long-chain aliphatic constituents with chain-length ranging from C₁₆ to C₃₂ (Schreiber et al., 1999).

Although in-chain and ω -hydroxylation activities and the subsequent oxidation of ω -hydroxy groups have been demonstrated in cell free extracts (Kolattukudy, 1977, 1981), biochemical approaches to isolate enzymes directly involved in cutin or suberin biosynthesis have not succeeded yet (Kunst et al., 2004). A genetic approach to dissect cutin and suberin biosynthesis seems self-evident, however, plants with cutin-rich and suberin-rich organs such as *Lycopersicon esculentum* (fruit), *Hedera helix* (leaf) or *Quercus suber* (bark) that have successfully been used to study structure and physiological functions are inappropriate for molecular genetic approaches.

Recently discovered *Arabidopsis* mutants displaying epidermal fusion phenotypes, increased cuticular permeability or a discontinued cuticular layer in leaf sections observed by TEM, may define loci with diverse roles in cutin biosynthesis and/or deposition. The molecular characterization of the corresponding genes as fatty acid elongase (*FIDDEL-HEAD* (*FDH*); Pruitt et al., 2000; Yephremov et al., 1999; Efremova et al., 2004), fatty acid ω -hydroxylase (*LACER-ATA* (*LCR*); Wellesen et al., 2001), oxidoreductase (*HOT-HEAD* (*HTH*); Krolkowski et al., 2003), desaturase (*WAX2*; Chen et al., 2003; *YRE*; Kurata et al., 2003) and long-chain acyl-CoA synthase (*LACS2*; Schnurr et al.,

2004) suggests these genes are probably involved in the biosynthesis of functionalized aliphatics. However, a direct effect on the amount or composition of leaf cutin could not be established because methods for the analysis of *Arabidopsis* cutin were not available at the time. Recent progress has been made when Xiao et al. (2004) succeeded in the enzymatic isolation of *Arabidopsis* stem cuticles and determined cutin characteristic depolymerization products, including 16-hydroxyhexadecanoic acid, 9/10,16-dihydroxyhexadecanoic acid along with C₁₆ and C₁₈ α,ω -diacids. Shortly after Bonaventure et al. (2004) and the work in our own laboratory, Yephremov et al. (2004) have determined unsaturated C₁₈ α,ω -diacids as the major depolymerization products of solvent extracted *Arabidopsis* leaves, thus providing the first indication of an unusual monomer composition of *Arabidopsis* leaf cutin. These findings were supported by the detection of unsaturated C₁₈ α,ω -diacids in epidermal peels of *Arabidopsis* stems (Bonaventure et al., 2004). However, the methods to isolate and analyze pure cuticles from *Arabidopsis* leaves still need to be established.

An even bigger gap exists in our knowledge about suberin biosynthesis. After extensive compositional and biochemical studies in the 1970s (Kolattukudy, 1977, 1981), research on suberin biosynthesis has mainly focused on the aromatic domain (Bernards and Lewis, 1998; Bernards and Razem, 2001), however, the function of suberin as a transport barrier for water and solutes is determined by the aliphatic domain (Hose et al., 2001; Schreiber et al., 1999). In this context, Agrawal and Kolattukudy (1978) purified a ω -hydroxyacid dehydrogenase to near homogeneity from wound healing potato tuber disks, supposed to be specific for suberin biosynthesis. Details on other catalysts, and the encoding genes, involved in the biochemical pathways to aliphatic suberin are missing since reasonable *forward genetic* screens to isolate mutants with a phenotype consistent with a defect in suberin biosynthesis or deposition have yet to be developed. For *reverse genetic* approaches aimed at elucidating the function of candidate genes in the biosynthesis or deposition of aliphatic suberin monomers, methods to detect and analyze suberin in *Arabidopsis* are needed. In contrast to cutin were recent compositional analysis of the stem cuticle and solvent extracted leaves have been conducted successfully (Xiao et al., 2004; Bonaventure et al., 2004), no one has determined the suberin composition in *Arabidopsis* yet.

Here, we present the aliphatic monomer composition of isolated *Arabidopsis* leaf cuticles and of suberin from *Arabidopsis*. Whereas *Arabidopsis* suberin consists of characteristic suberin monomers, *Arabidopsis* cutin is surprisingly different from what was found in other plants.

2. Results

2.1. Ultrastructure of leaf cuticles of *Arabidopsis*

Ultrastructural studies by transmission electron microscopy revealed that the cuticular membrane of *Arabidopsis*

covers the leaf epidermis as a very fine electron opaque layer that represents only a small fraction of the outer extracellular matrix of the epidermal cell (Fig. 1A). The leaf blades of fully expanded leaves are covered with a cuticle, on average 21.8 nm ($\pm 7.0 \text{ SD}$) in thickness that is even thinner ($17.2 \pm 4.0 \text{ nm}$) in expanding leaves. An internal structure was often not clearly recognizable in these very fine cuticles. The principle cuticular membrane close to the veins tended to be slightly thicker ($30.5 \pm 6.1 \text{ nm}$). This tendency is even more pronounced in petioles ($44.9 \pm 9.9 \text{ nm}$) having a cuticle of up to 60 nm . Well-preserved cuticular membranes of these tissues have a reticulated structure (Fig. 1B) and an ultra-thin second layer covering the principle cuticular membrane. It can, however, not be totally excluded that this second layer derives from environmental factors or might be caused by osmium fixation.

2.2. Isolation of *Arabidopsis* leaf cuticles

Cuticles from many species, ranging in thickness from about 0.7 to $32 \text{ }\mu\text{m}$ (Schreiber and Riederer, 1996), have

been isolated by incubation with cell wall hydrolyzing enzymes due to the fact that the cutin polyester resists this treatment. Subsequent solvent extraction of isolated cuticles enables the chemical analysis of the insoluble cutin polymer separately from the soluble lipid fraction, the intra- and epicuticular waxes. When the above method was applied to freshly harvested *Arabidopsis* leaves all plant material has been macerized using this enzyme treatment, querying whether *Arabidopsis* leaves are suitable for cuticle isolation at all. Given the thickness of *Arabidopsis* leaf cuticles of about 15 – 29 nm , as determined by TEM, could explain why many of our attempts to isolate this very thin and fragile cuticle from *Arabidopsis* using traditional methods have failed. However, when totally extracted, dried leaf discs were incubated with cellulase and pectinase and only carefully agitated, beginning after 5 days it was possible to recover very thin, translucent membranes using a binocular and a black background to contrast the floating membranes. Size and shape of the membranes were identical to that of the leaf disc starting material. In microscopic examinations of those membranes cell wall structures from epidermal cells could not be detected (Fig. 1C). Indicative

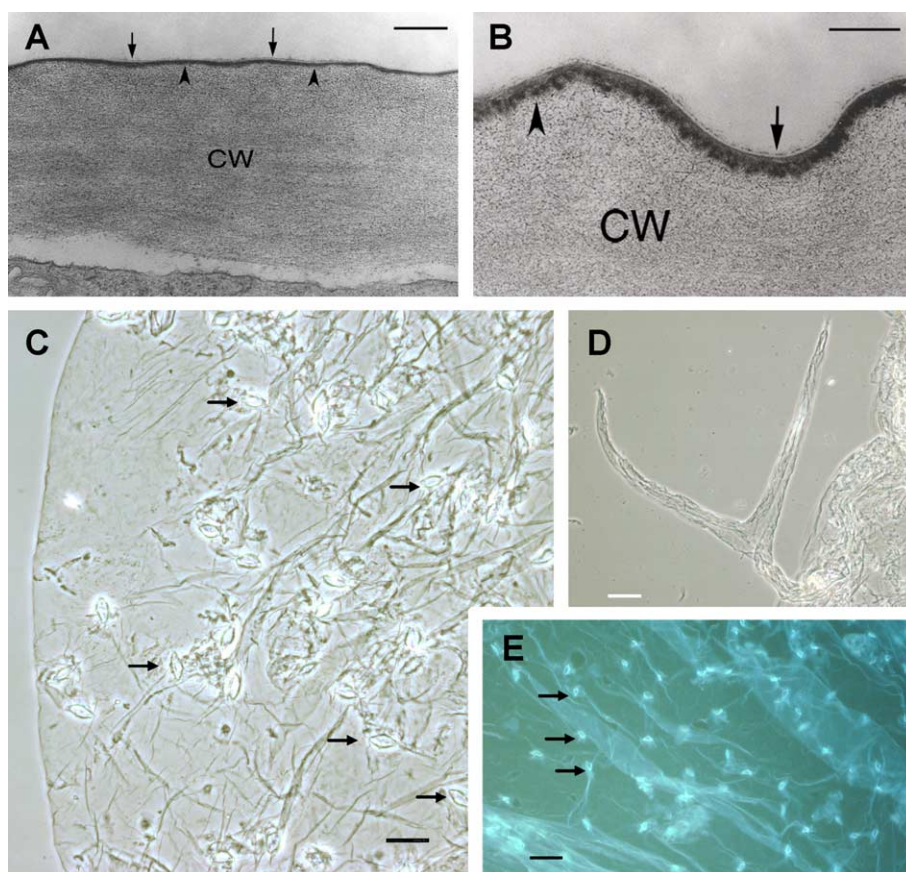


Fig. 1. Leaf cuticle ultrastructure and light microscopic analysis of membranes enzymatically removed from *Arabidopsis* leaf disks. (A) The extracellular matrix of outer epidermal cell walls is represented from the leaf blade and (B) close to the middle vein of a leaf. The principle cuticular membrane is indicated with black arrowheads, while an ultra-fine second layer is indicated with arrows. (C) Bright field microscopic picture in the border region of an isolated cuticular membrane. (D) Different region of (C) indicating an epidermal trichome “envelope”. (E) Autofluorescence of cuticular membranes. Stomates are indicated by arrows (C and E). CW, cell wall. Scale bars are 200 nm , (A) and (B); $20 \text{ }\mu\text{m}$, (C)–(E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of cuticles, stomates can be observed because of the strongly reinforced guard cell walls. Furthermore, the trichome “envelopes” on these transparent membranes demonstrated that we successfully isolated leaf cuticles. No other cell or cell wall structures could be detected on the translucent membranes. Unlike other cuticles (Schönherr and Riederer, 1986), it was not possible to flatten the *Arabidopsis* cuticles without causing the wrinkly appearance of the membrane.

2.3. Determination of ester-linked aliphatic monomers in *Arabidopsis* isolated cuticles

For the chemical analysis a minimum of 30 leaf cuticles of 1 cm diameter were pooled per sample. After depolymerization with MeOH–HCl the monomers were subsequently derivatized to their corresponding TMS derivatives and analyzed by GC–MS. A GC-chromatogram of the monomer mixture released from *Arabidopsis* cuticles is shown in Fig. 2A. As exemplified for the four most prominent peaks compounds were identified based on their EI-MS fragmentation pattern. They were readily identified as acid methyl esters from the characteristic McLafferty rearrangement ion at m/z 74. The fragmentation pattern of compound 1 and compound 3 (data not shown) are identical to the EI mass spectra published by Holloway (1982) for methyl esters of 1,16-hexadecane dioic acid (1) and 1,18-octadecene dioic acid (3), respectively. The fragmentation pattern of compound 2 (data not shown) is very similar to 3 with mass differences of two in most rearrangement ions and in the diagnostic peaks m/z 274 (276 in compound 2) and m/z 306 (308 in compound 2) indicating an additional double bond. These fragments correspond to the $M^+ - 64$ and $M^+ - 32$ of the predicted structure of this compound being the methyl diester of 1,18-octadecadiene dioic acid with a MW of 338, also detected in the mass spectrum. For compound 4, identified as the methylester of 2-hydroxy-tetracosanoic acid, the MS fragmentation pattern is shown in Fig. 3A. The molecular ion m/z 470 is of low abundance, the diagnostic peak at m/z 455 represents the $M^+ - 15$ – loss of a methyl group from the trimethylsilyl ether moiety. The peak at m/z 411 ($M^+ - 59$) results from cleavage between carbon 1 and 2. Similarly more than 30 compounds were identified and quantified from the chromatogram (Table 1).

2.4. The aliphatic monomer composition of *Arabidopsis* cuticles

The methanolysate of *Arabidopsis* cuticles is primarily composed of saturated and unsaturated C_{16} and C_{18} hydroxylated alkanolic acids many of them known to be cutin components (Table 1). These include ω -hydroxyacids, alkanolic acids, alkanols and mid-chain hydroxylated fatty acids. However, the most abundant monomers released from *Arabidopsis* leaf cuticles were α,ω -diacids, of chain length C_{16} and C_{18} and 2-hydroxyacids varying in chain

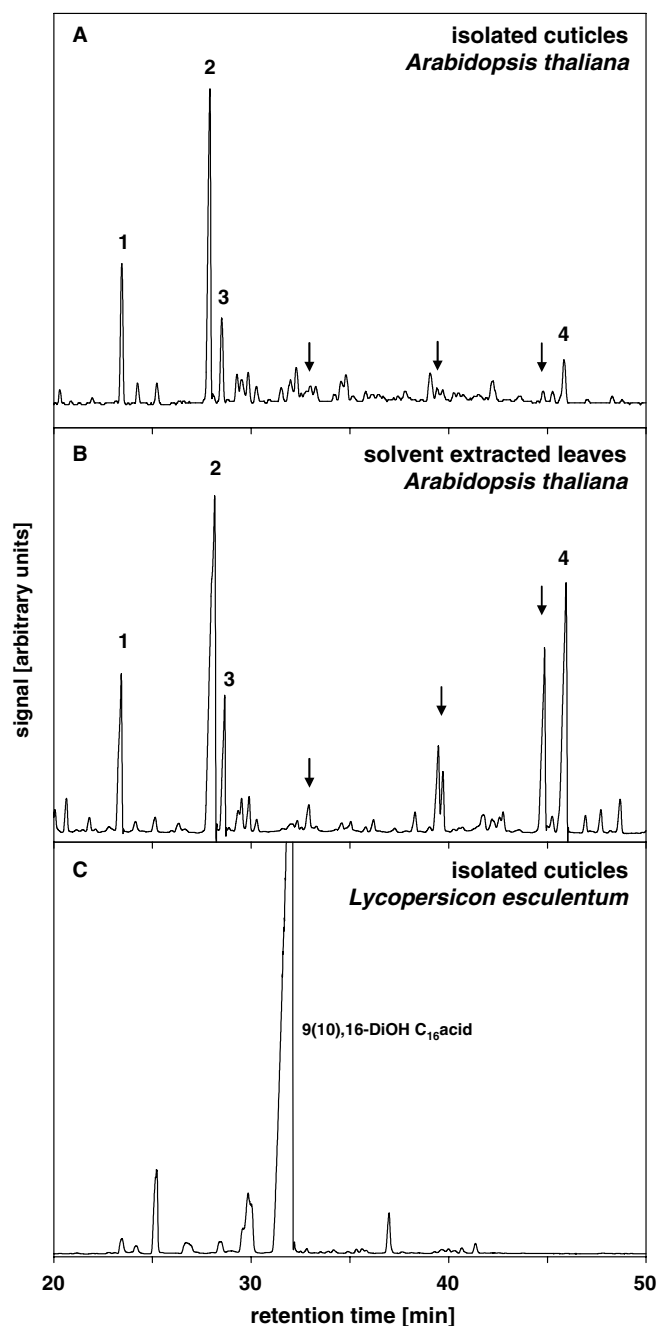


Fig. 2. Gas chromatogram of aliphatic monomers released from *Arabidopsis* leaf polyesters and tomato fruit cuticles by transesterification using MeOH–HCl. (A) Depolymerization products of isolated *Arabidopsis* cuticular membranes. (B) Depolymerization products released from totally extracted *Arabidopsis* leaves. (C) Depolymerization products of isolated cuticular membranes from tomato (*Lycopersicon esculentum*)*, representing a “standard” cutin predominantly comprised of mid-chain hydroxylated fatty acid derivatives. Monomers were extracted and derivatized to their corresponding TMS ether and ester prior to GC analysis. The predominant compounds are marked: 1, hexadecane-1,16-dioic acid**; 2, octadecadien-1,18-dioic acid**; 3, octadecene-1,18-dioic acid**; 4, 2-hydroxy-tetracosanoic acid***; 9(10),16-DiOH C_{16} acid, 9(10),16-Dihydroxyhexadecanoic acid***. Arrows indicate C_{20} , C_{22} and $C_{24}(1)$ 2-hydroxy acid (sorted by increasing retention time). *The detailed monomer composition of tomato cuticles has been analyzed previously (Baker and Holloway, 1970; Graça et al., 2002). **Analyzed as methyl ester. ***Analyzed as methylester, hydroxyl group as trimethylsilyl ether.

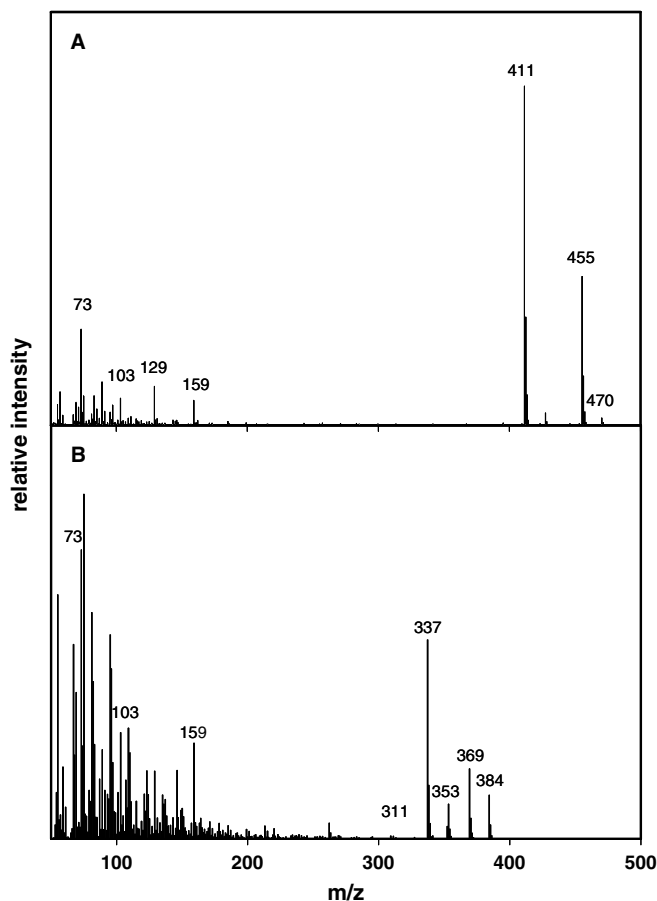


Fig. 3. EI mass spectra of transesterification products released from isolated *Arabidopsis* leaf cuticles (A) and hydrolase digested root cell walls (B), respectively. (A) Compound 4 in Fig. 2 identified as the methyl ester and TMS ether of 2-hydroxy-tetracosanoic acid. (B) Compound C₁₈(1)ω in Fig. 5A identified as the methyl ester and TMS ether of 18-hydroxy-octadecenoic acid.

length from C₁₆ to C₂₈. The latter two-substance classes account for more than 50% of the monomer mixture. Of these the major component of *Arabidopsis* cutin is double unsaturated octadecadien-1,18-dioic acid representing 20% of the monomers released from isolated *Arabidopsis* leaf cuticles. Lesser, but also substantial amounts were detected of hexadecane-1,16-dioic acid (10.6%), octadecene-1,18-dioic acid (6.6%), 2-hydroxy-tetracosanoic acid (4.5%), octadecane-1,18-dioic acid (2.7%), tetracosanoic acid (2.6%), 18-hydroxy-octadecadienoic acid (2.4%), and 2-hydroxy-hexacosanoic acid (2.3%). All other compounds accounted for less than 2% of the monomer mixture.

Unidentified mid-chain hydroxylated fatty acids listed in Table 1 were recognized by their characteristic fragment ions *m/z* 259 and 273, compatible with C₉ or C₁₀ hydroxylated aliphatic acids (Holloway, 1982). Based on retention times compared to other C₁₆ and C₁₈ monomers they presumably include C₁₈ 9(10)-hydroxylated fatty acids. However, due to the low amounts of isolated cuticles, EI-MS fragments interfered with back ground signals hampering the determination of the exact structure. As

Table 1
Monomers released from isolated *Arabidopsis* leaf cuticles by methanolic-HCl catalyzed transesterification

Cutin monomer (chain length (double bonds))	μg/dm ²		Relative amounts (%)	
	Mean	SD	Mean	SD
<i>Alkan-1-oic acids</i>				
Hexadecanoic acid (C ₁₆)	3.53	0.70	8.10	0.15
Octadecanoic acid (C ₁₈)	0.56	0.02	1.32	0.23
Eicosanoic acid (C ₂₀)	0.32	0.12	0.71	0.12
Docosanoic acid (C ₂₂)	0.22	0.05	0.51	0.13
Tetracosanoic acid (C ₂₄)	0.75	0.20	1.71	0.18
Octadecenoic acid (C ₁₈ (1))	1.14	0.43	2.56	0.47
Octadecenoic acid (C ₁₈ (1))	0.19	0.02	0.45	0.11
Octadecadienoic acid (C ₁₈ (2))	0.34	0.05	0.82	0.23
<i>Alkan-1-ols</i>				
Hexadecanol (C ₁₆)	0.68	0.08	1.57	0.18
Octadecanol (C ₁₈)	0.03	0.00	0.06	0.01
Hexacosanol (C ₂₆)	0.18	0.03	0.42	0.01
Octacosanol (C ₂₈)	0.16	0.04	0.37	0.07
Octacosanol (C ₂₈)	0.31	0.01	0.73	0.12
<i>ω-Hydroxyacids</i>				
16-Hydroxy-hexadecanoic acid (C ₁₆)	3.07	0.70	7.01	0.27
16-Hydroxy-hexadecanoic acid (C ₁₆)	0.68	0.07	1.59	0.19
18-Hydroxy-octadecanoic acid (C ₁₈)	0.18	0.03	0.42	0.06
18-Hydroxy-octadecenoic acid (C ₁₈ (1))	0.09	0.01	0.20	0.03
18-Hydroxy-octadecadienoic acid (C ₁₈ (2))	1.03	0.24	2.36	0.15
18-Hydroxy-octadecatrienoic acid (C ₁₈ (3))	0.81	0.28	1.83	0.26
20-Hydroxy-eicosanoic acid (C ₂₀ (1))	0.27	0.12	0.60	0.21
<i>α,ω-Dicarboxylic acids</i>				
Hexadecane-1,16-dioic acid (C ₁₆)	17.70	2.36	40.93	2.91
Hexadecane-1,16-dioic acid (C ₁₆)	4.54	0.40	10.64	2.03
Octadecane-1,18-dioic acid (C ₁₈)	1.17	0.13	2.74	0.51
Octadecene-1,18-dioic acid (C ₁₈ (1))	2.83	0.37	6.58	1.08
Octadecadien-1,18-dioic acid (C ₁₈ (2))	9.17	2.02	20.97	0.67
<i>2-Hydroxyacids</i>				
2-Hydroxy-hexadecanoic acid (C ₁₆)	5.95	0.18	13.93	2.07
2-Hydroxy-hexadecanoic acid (C ₁₆)	0.10	0.00	0.23	0.04
2-Hydroxy-octadecanoic acid (C ₁₈)	0.14	0.02	0.33	0.04
2-Hydroxy-eicosanoic acid (C ₂₀)	0.60	0.07	1.43	0.39
2-Hydroxy-docosanoic acid (C ₂₂)	0.64	0.06	1.50	0.15
2-Hydroxy-tricosanoic acid (C ₂₃)	0.20	0.06	0.48	0.15
2-Hydroxy-tetracosanoic acid (C ₂₄)	1.93	0.08	4.52	0.72
2-Hydroxy-pentacosanoic acid (C ₂₅)	0.18	0.00	0.42	0.08
2-Hydroxy-hexacosanoic acid (C ₂₆)	0.98	0.02	2.30	0.36
2-Hydroxy-octacosanoic acid (C ₂₈)	0.63	0.01	1.47	0.24
2-Hydroxy-tetracosanoic acid (C ₂₄ (1))	0.55	0.08	1.26	0.06
<i>“Mid-chain” oxygenated hydroxyacids</i>				
9(10)-Hydroxy-hexadecane-1,16-dioic acid (C ₁₆)	3.77	0.76	8.64	0.16
9(10)-Hydroxy-hexadecane-1,16-dioic acid (C ₁₆)	0.62	0.10	1.43	0.25
9(10),16-Dihydroxy-hexadecanoic acid (C ₁₆)	0.51	0.10	1.17	0.16
Unidentified ^a “mid-chain” hydroxylated acids	2.65	0.66	6.04	0.56
Unidentified ^b longchain aliphates	8.87	3.76	19.82	4.49
Total	43.57	8.36	100.0	

Cuticles were isolated enzymatically from totally extracted leaf disks of 5-week-old plants. Absolute amounts are given in μg/dm² as mean of three replicates and standard deviation (SD). Each of the three replicates includes 30 cuticular membranes of 1 cm diameter, pooled from 10 to 15 plants. Acids were analyzed as methyl esters, hydroxyl groups as trimethylsilyl ethers.

^a Unidentified mid-chain hydroxylated fatty acids were recognized by their characteristic fragment ions *m/z* 259 and 273, compatible with C₉ or C₁₀ hydroxylated aliphatic acids (Holloway, 1982).

^b Unidentified long-chain aliphates were recognized by EI-MS spectra compatible with saturated and unsaturated long-chain fatty acid derivatives; however, exact structure determination was retarded due to low abundance of molecular weight characteristic fragment ions.

exemplified by the methanolysate of tomato (*Lycopersicon esculentum*) fruit cuticles (Fig. 2C) the complex *Arabidopsis* cutin composition with low relative amounts of mid-chain oxygenated compounds (8.6%) is exceptionally different to the cutin monomer composition of most other species that are mostly dominated (50–90%) by mid-chain oxygenated C₁₆ and C₁₈ fatty acid derivatives (Graça et al., 2002).

2.5. Aliphatic polyester of totally extracted leaves

The isolated cuticles were very thin, and fragile, very frequently disintegrated or collapsed when touched with steel forceps or when transferred to different containments. Therefore, and due to the labor intensity of the cuticle isolation procedure this method is not applicable in experiments requiring high numbers of samples and multiple replicates. Furthermore, many cuticle mutants have leaf phenotypes like misshaping, organ fusions, abnormal leaf development or a patchy cuticle layer in TEM slides (Kunst et al., 2004; Yephremov and Schreiber, 2005) making cuticle isolation for chemical analysis from severe mutants or the application of this method in screening processes not practicable. In search for a more simple and feasible method we analyzed totally extracted leaves, free of soluble unbound lipids by exhaustive solvent extraction, and compared them to the isolated cuticles. As shown in Fig. 2B, the monomeric composition and the relative abundance of the compounds is very similar in isolated cuticles and solvent extracted leaves. Despite the quantitative difference in 2-hydroxy acids (Fig. S1), all compounds identified in the methanolysate of isolated leaf cuticles can also be detected in the monomer mixture released by MeOH–HCl from totally extracted leaves.

2.6. Spatial distribution of suberized tissue in *Arabidopsis* roots

The major monomers in *Arabidopsis* cutin, α,ω -diacids and 2-hydroxy acids are commonly thought to be suberin characteristic constituents (Matzke and Riederer, 1991; Schreiber et al., 1999), however, there are no compositional data on suberin in *Arabidopsis* in the literature. We started the characterization of *Arabidopsis* suberin with a histochemical investigation using exposure to long-wave UV-light to visualize aromatic domains and Sudan III, a lipophilic dye commonly used to mount aliphatic cell wall domains, to localize the suberized cell layers in *Arabidopsis* roots. Microscopic observation of cross-sections of freshly harvested *Arabidopsis* roots in their primary developmental stage (Fig. 4A) revealed a strong autofluorescence of the cell walls in the central cylinder and the endodermis when exposed to UV-light (365 nm). The autofluorescence is very intense in the radial endodermal cell walls indicating the presence of suberized Casparian bands. Fig. 4B shows that the Sudan III staining resulted in an intensive red color only of endodermis cell walls, indicating the presence of aliphatic suberin in these cells. Intensive staining in radial

walls and inner tangential walls close to the endodermis-pericycle boundary are indicative for a stronger suberin depositions typical for an endodermis in a secondary developmental stage (Schreiber et al., 1999). The autofluorescence in the Sudan III negative central cylinder is most likely caused by lignin phenolics. When root sections of a secondary developmental stage were observed under UV-light again an autofluorescence from lignin aromatics was detectable in the central cylinder (Fig. 4C). Different to roots in the primary developmental stage a fluorescing endodermis was no longer detectable, but a strong autofluorescence could be observed in the periderm, an outer border tissue resulting from secondary thickening. Staining with Sudan III resulted in an intense red colorization of the peridermal cell walls, indicating significant suberization of the peridermal cell walls (Fig. 4D).

Investigating the ultrastructure of the sudan-staining tissues using TEM confirmed the deposition of suberin in the casparian bands of the endodermis of primary roots (data not shown) and suberization of the whole endodermal cells as well as peridermal cells during secondary root growth (Fig. 4E). In all cases, suberin was recognized by its typical lamellae structure (Bernards and Lewis, 1998; Schmutz et al., 1996) of alternating electron-opaque and electron-translucent layers when the tissue was cut perpendicularly to the suberin layers (Fig. 4F).

2.7. Isolation of a suberized cell wall fraction from *Arabidopsis* roots

The chemical composition of root suberin from different species has been determined from enzymatically isolated, suberin enriched endodermal or rhizodermal cell walls (Schreiber et al., 1999). Due to the branching and small size of *Arabidopsis* roots, isolation and purification of suberized endodermal or peridermal cell walls for detailed suberin analysis would be, if feasible, very labor intensive. We therefore used a treatment with polysaccharide hydrolases to reduce the back ground of nonsuberized tissue in the chemical analysis of whole root cell walls. A gravimetric determination revealed that 62.98% (± 1.42 SD) of the polymeric cell wall material was removed by this treatment. After incubation of *Arabidopsis* roots with cellulase and pectinase cortex cells are no longer detectable. In addition to the lignified cells in the central cylinder, only the Sudan III positive cell layers from the periderm remains, indicating that most of the presumably polysaccharide dominated unmodified parenchyma cell walls were removed (data not shown). Since lignin monomers do not interfere with the chemical analysis of the long-chain aliphatic components of suberin, this pre-purified cell wall fraction was used for the chemical analysis of the monomer composition of aliphatic suberin.

2.8. Aliphatic suberin monomer composition

The GC–MS analysis of the TMS-derivatized monomers in the methanolysate revealed a complex monomer

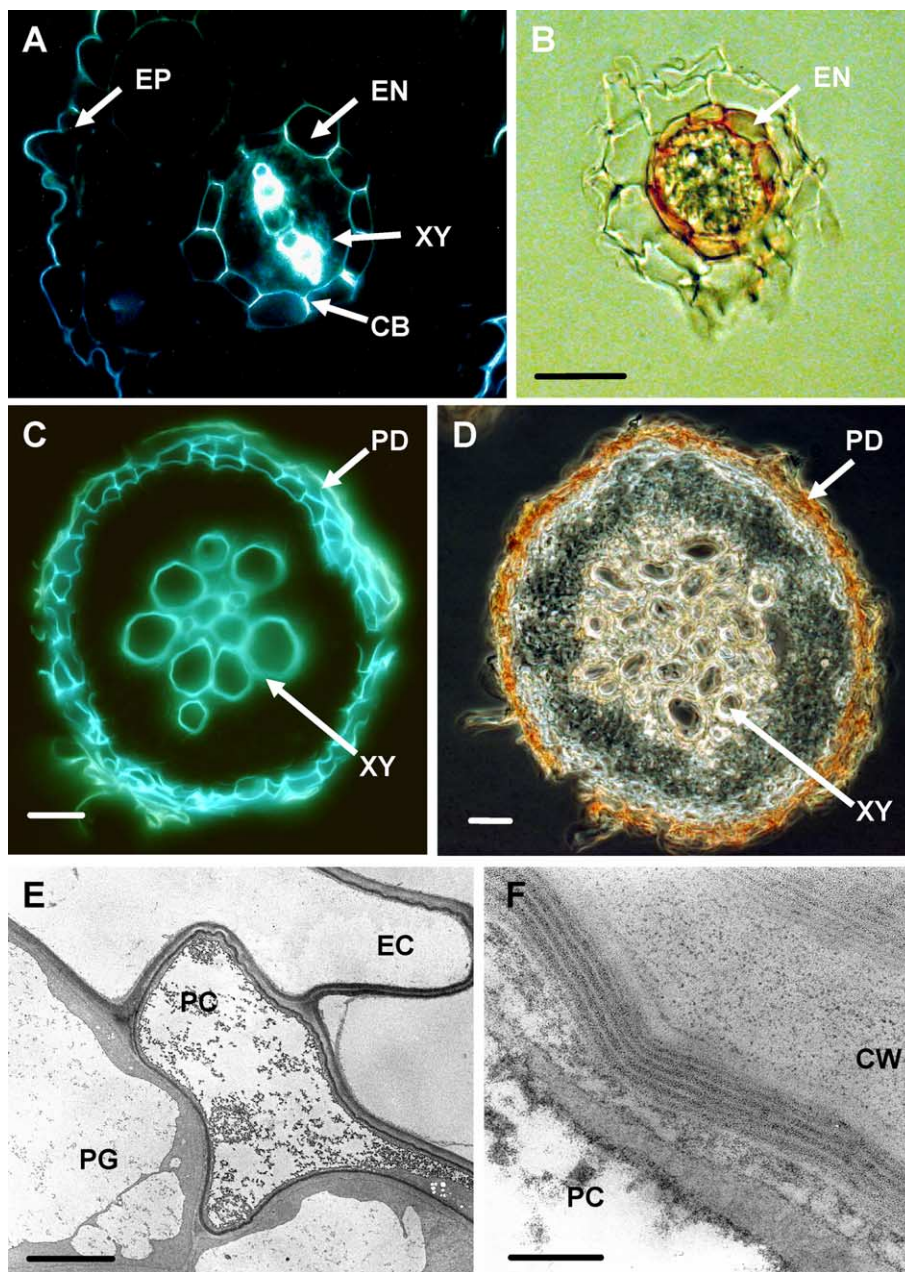


Fig. 4. Histochemical and TEM examination of *Arabidopsis* roots. For light microscopy (A–D) 20 μm cross-sections were taken from root bundles of 5-week-old plants to obtain multiple developmental stages. (A) Autofluorescence in a cross-section of a primary root after excitation with UV-light ($\lambda = 365 \text{ nm}$). Casparian bands (CB) are indicated by arrows. (B) Bright field microscopic picture of a primary root section after staining with the lipophilic dye Sudan III. Suberin, stained with Sudan III (red), is deposited in the endodermal cell walls. (C) Autofluorescence micrograph of a mature root section where secondary growth has occurred. Strong autofluorescence in the peridermal cell walls indicates aromatic domains. (D) Sudan III stained suberin in the periderm of a mature root section. Bars = 20 μm (A–D). (E–F) Ultrastructure of suberized root tissues of *Arabidopsis* plants at the beginning of the secondary thickening of the root. (E) Overview of suberized endodermal and peridermal cells in the root. The fully suberized peridermal cells typically collapse during the dehydration and embedding procedures necessary for TEM because of the low permeability of the suberized cell walls. Bar = 1 μm . (F) Fine structure of suberin. The structure of the lamellae with an alternation of electron-opaque and electron-translucent layers of suberin is clearly visible when the specimen is cut perpendicularly to the suberin layers. Bar = 100 nm. EP, epidermis; EN, endodermis; PD, periderm; XY, xylem; CB, Casparian band; EC, endodermal cell; PC, peridermal cell; PG, phellogen; CW, cell wall. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

composition of the hydrolase resistant *Arabidopsis* root cell wall fraction. Twenty-four compounds detected in the GC chromatogram (Fig. 5A) were identified based on their EI-mass spectra. The most prominent peak ($\text{C}_{18}(1)\omega$ in Fig. 5) was identified as the methylester of the trimethylsilylated 18-

hydroxy-octadecenoic acid (Fig. 3B). The m/z 384 peak represents the mol-ion of the predicted molecule structure. The peak at m/z 103 is diagnostic for the loss of $\text{CH}_2\text{O-Si}(\text{CH}_3)_3$ from the TMS-derivatized ω -hydroxy group. The m/z 369, 353 and 337 represent the $\text{M}^+ - 15$ (loss of

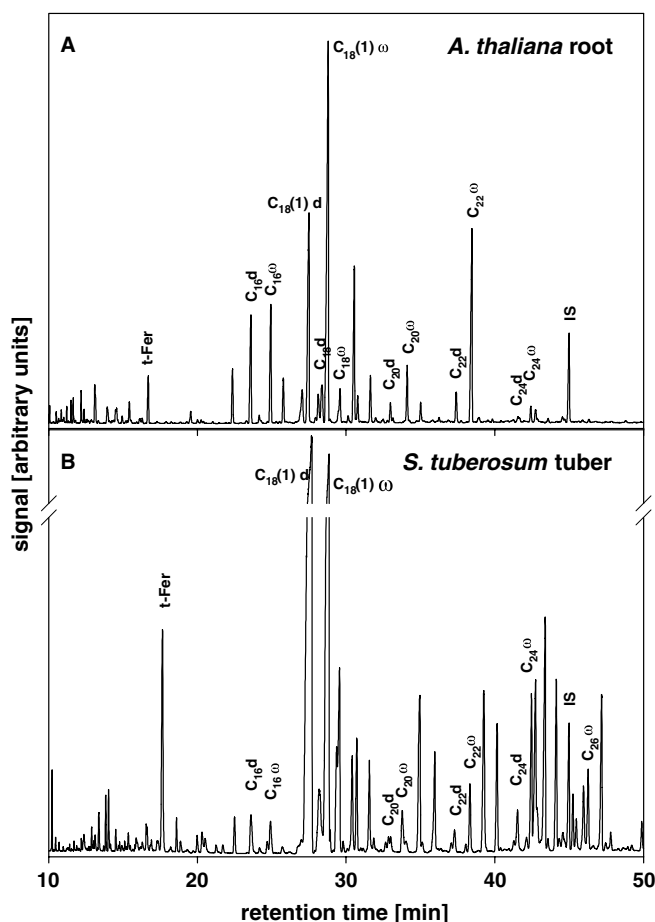


Fig. 5. Gas chromatogram of suberin monomers obtained after BF_3/MeOH depolymerization of hydrolase digested *Arabidopsis* root cell walls (A) and potato (*Solanum tuberosum*) tuber periderm (B). The depolymerization products were extracted and TMS derivatized before GC separation. The two most prominent suberin monomers 18-hydroxyoctadecanoic acid and octadecene-1,18-dioic acid are labeled as $\text{C}_{18}(1)\omega$ and $\text{C}_{18}(1)d$, respectively. To exemplify the chain-length distribution in *Arabidopsis*, ranging from C_{16} to C_{24} , the homologous series of α,ω -diacids is marked with d and the homologous series of ω -hydroxy-acids is marked with ω .

methyl group), M^+-31 (loss of methoxy group) and M^+-47 (CH_3-O_2), respectively.

All carboxylic groups in the methanolsate are methylated, indicating that they were formerly esterified in the polymer. GC-MS identification of the other compounds revealed that the root suberin-enriched cell wall fraction predominantly comprised saturated and monounsaturated ω -hydroxy acids (43%) and α,ω -dicarboxylic acids (24%), but also carboxylic acids (10%), alcohols (6%) and 2-hydroxyacids (<1%) (Table 2). Aromatic constituents (5%) detected were the *cis* and *trans* isomers of ferulic acid and *p*-coumaric acid. This substance class composition is very similar to the suberin composition of other species (Fig. 5B; Schreiber et al., 1999, 2005a). Similar to potato tuber periderm, the two most abundant monomers released from hydrolase digested and solvent extracted root cell wall fractions, are the monounsaturated C_{18} ω -hydroxy acid (23%) and α,ω -diacid (11%). The chain length distribution

Table 2

Monomers released from hydrolase resistant *Arabidopsis* root cell walls by BF_3/MeOH transesterification

Suberin monomer (chain length (double bonds))	$\mu\text{g}/\text{mg}$		Relative amounts (%)	
	Mean	SD	Mean	SD
<i>Alkan-1-oic acids</i>				
Octadecanoic acid (C_{18})	6.27	1.19	9.64	0.77
Eicosanoic acid (C_{20})	0.08	0.03	0.12	0.03
Eicosanoic acid (C_{20})	1.43	0.66	2.23	0.82
Docosanoic acid (C_{22})	3.82	0.83	6.18	0.70
Tetracosanoic acid (C_{24})	0.94	0.82	1.11	0.52
<i>Alkan-1-ols</i>				
Octadecanol (C_{18})	4.06	1.39	6.43	1.06
Eicosanol (C_{20})	1.39	1.08	2.11	1.46
Eicosanol (C_{20})	1.56	0.41	2.49	0.13
Docosanol (C_{22})	1.12	0.31	1.83	0.53
<i>ω-Hydroxyacids</i>				
16-Hydroxy-hexadecanoic acid (C_{16})	27.10	7.73	43.19	4.14
16-Hydroxy-hexadecanoic acid (C_{16})	3.63	1.26	5.77	1.20
18-Hydroxy-octadecanoic acid (C_{18})	1.55	0.47	2.73	0.88
20-Hydroxy-eicosanoic acid (C_{20})	1.93	0.60	3.07	0.44
22-Hydroxy-docosanoic acid (C_{22})	4.82	1.06	7.79	0.76
24-Hydroxy-tetracosanoic acid (C_{24})	0.76	1.09	0.71	0.24
18-Hydroxy-octadecenoic acid ($\text{C}_{18}(1)$)	14.40	3.77	23.11	2.26
<i>α,ω-Dicarboxylic acids</i>				
Hexadecane-1,16-dioic acid (C_{16})	15.53	6.26	24.21	4.69
Hexadecane-1,16-dioic acid (C_{16})	3.12	1.24	4.91	1.30
Octadecane-1,18-dioic acid (C_{18})	4.05	3.92	5.87	4.79
Eicosane-1,20-dioic acid (C_{20})	0.65	0.25	1.01	0.20
Docosane-1,22-dioic acid (C_{22})	0.86	0.21	1.39	0.15
Tetracosane-1,24-dioic acid (C_{24})	0.24	0.20	0.35	0.26
Octadecene-1,18-dioic acid ($\text{C}_{18}(1)$)	6.62	1.50	10.68	0.76
<i>2-Hydroxyacids</i>				
2-Hydroxy-tetracosanoic acid (C_{24})	0.08	0.07	0.12	0.10
<i>Hydroxycinnamic acids</i>				
<i>cis-p</i> -Coumaric acid	3.11	1.84	5.15	3.58
<i>cis-p</i> -Coumaric acid	0.22	0.16	0.38	0.34
<i>trans-p</i> -Coumaric acid	0.71	0.29	1.13	0.35
<i>cis</i> -Ferulic acid	0.95	1.14	1.64	2.24
<i>trans</i> -Ferulic acid	1.23	0.52	2.01	0.91
Unidentified ^a longchain aliphates	6.55	3.21	11.26	3.89
Total	62.71	16.02	100.0	

Root cell walls were prepared from 5-week-old plants. Amounts are given as μg suberin per mg cell wall. Mean and SD was determined from 10 replicates each representing the roots of 5–7 plants. Acids were analyzed as methyl esters, hydroxyl groups as trimethylsilyl ethers.

^a See unidentified long-chain aliphates in footnote b of Table 1.

within most substance classes ranges from C_{16} to C_{24} with C_{18} being the most prominent members (Fig. 6). Different to cutin a second peak with compounds of 22 carbon units exists among the suberin substance classes.

3. Discussion

3.1. Cuticles can be isolated from *Arabidopsis* leaves

Leaf cuticles have been isolated from many plant species and used in physiological transport studies or for detailed chemical analysis (Schreiber and Riederer, 1996; Kolattukudy, 2001a). However, the investigated species are neither good biochemical nor molecular genetic model systems leaving a great gap in our knowledge about cutin

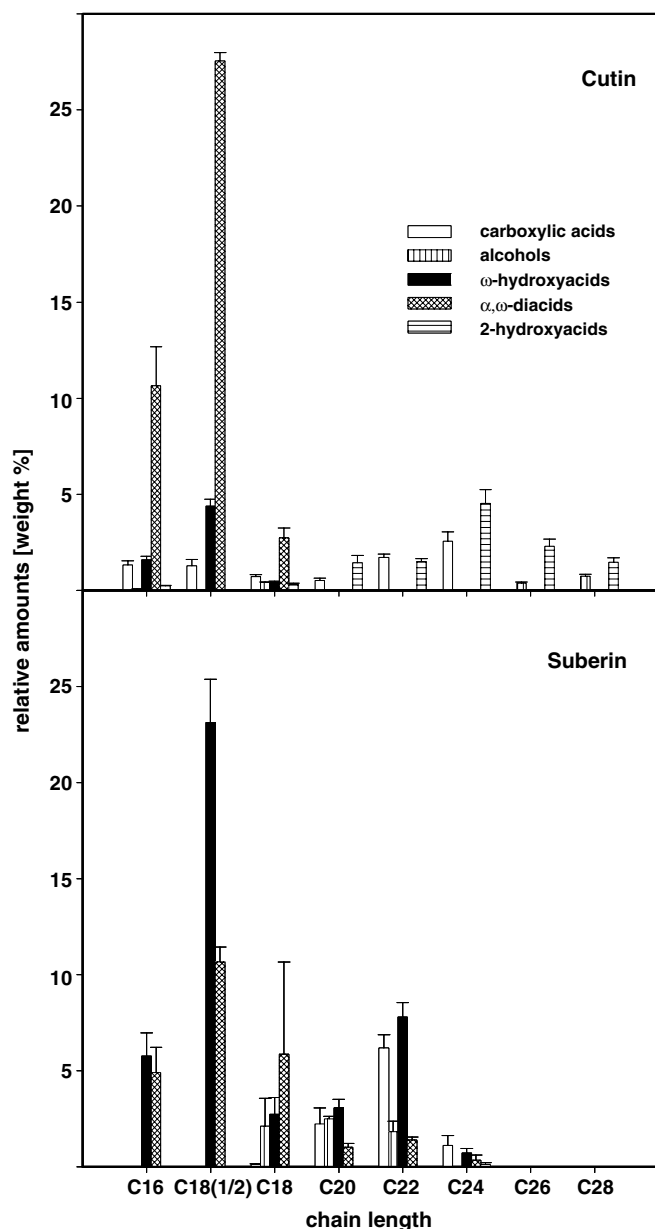


Fig. 6. Chain length distribution of aliphatic monomers released by transesterification from *Arabidopsis* cutin and suberin, respectively.

biosynthesis and deposition, its regulation and structure–function relationships. On the other hand, genetic approaches have led to the discovery of many putative cutin mutants in *Arabidopsis*, but for most of the mutated genes a role in cuticle biosynthesis or deposition is assumed based on database annotation only (Yephremov and Schreiber, 2005). A direct involvement of these genes in cutin biosynthesis could not be demonstrated because methods for the isolation and chemical analysis of very thin cuticles, like in *Arabidopsis*, have not been established yet.

As indicated by the presence of stomates and trichomes, the lack of cell walls and the transparency of the enzymatically isolated membranes from *Arabidopsis* leaves (Fig. 1), we succeeded in the isolation of cuticular membranes suit-

able for depolymerization and subsequent compositional analysis. Although the light-microscopic investigation cannot finally exclude associated cell wall or plasma membrane contaminants, Table 1 shows that C₁₆ and C₁₈ carboxylic acids, typical for membrane phospholipids are very minor components in the methanolysate. Similarly, membrane characteristic sterols such as β -sitosterol (Schaeffer et al., 2001) were only detected in traces and cell wall carbohydrate characteristic pyranose and furanose peaks were not detectable in the methanolysate of isolated cuticles.

3.2. The thickness of the *Arabidopsis* cutin layer is in the nanometer range

The methanolysis of isolated *Arabidopsis* cuticles yielded in 0.44 μg aliphatic monomers per cm^2 (Table 1). Based on an average cuticle density of $0.94 \times 10^{-3} \text{ kg m}^{-3}$ (Schreiber and Schönherr, 1990), the total cutin monomers correspond to a leaf cuticular membrane of 4.1 nm compared to 15–29 nm as determined by TEM on leaf blades. This might indicate a so far undiscovered structural organization below TEM resolution in cuticles of leaf blades but detectable in regions of thicker cuticles. The well-preserved cuticle membrane of the veins and petioles have a reticulate structure, similar as seen in stems of *Arabidopsis* analyzed by Chen et al. (2003). A less electron-opaque outer layer has not been observed in our studies. However, a second ultra-thin electron-dense layer was regularly observed in samples evidencing well-preserved internal structure of the cuticular membrane.

With regard to the potential deficit of the cuticle thickness determined by analytical chemistry, a none-hydrolysable cutan fraction was reported for *Arabidopsis* stem cuticles (Xiao et al., 2004), but was not detectable in our analysis of leaf cuticles. Furthermore, osmium tetroxide stained lipid membranes in TEM pictures are not necessarily pure lipid fractions and our quantification is based on the internal standard dotriacontane since standards for most cutin monomers are not available. Therefore, monomer-specific response factors, which have been reported to be higher for ω -hydroxyacids and α,ω -diacids (Graça and Pereira, 2000a) could not be applied.

3.3. The *Arabidopsis* cutin is unusually rich in α,ω -dicarboxylic acids

Arabidopsis leaves are covered by a mixed C₁₆ and C₁₈ type cuticle (Fig. 6) according to the chain length based classification by Holloway (1982). Compared to “control” samples for cutin characteristic mid-chain hydroxy fatty acids (*Lycopersicon esculentum* cuticles, Fig. 2C), epoxides (*Prunus avium* cuticles, data not shown) and ω -oxo-acids (16-oxo-hexadecanoic acid standard), epoxides or aldehydes (ω -oxo-acids) could not be detected in the methanolysate of *Arabidopsis* cuticles. Although C₁₆ and other mid-chain hydroxylated fatty acids, are present in significant amounts, Table 1 and Fig. 2 show that the

Arabidopsis cutin methanolysate is mostly comprised of α,ω -dicarboxylic acids, monomers that have only been reported as very minor leaf cutin components in *Brassica oleracea* and *Spinacia oleracea* (Holloway, 1982). Recently, Bonaventure et al. (2004) indicated very nicely that α,ω -diacids might be considerable leaf cutin components by a similar distribution of depolymerization products, particularly α,ω -diacids, obtained from *Arabidopsis* extracted leaves and mechanically isolated stem epidermal peels. In agreement with their analysis of leaf polyester, our analysis of isolated cuticles demonstrated that saturated and unsaturated C₁₆ and C₁₈ dicarboxylic acids contribute to more than 40% of the cutin monomers, indicating that α,ω -diacids are major structural components in leaf cuticles of *Arabidopsis*. Consistent with an important role of α,ω -diacids, in cutin cross-linking and proper cuticle structure in *Arabidopsis*, *lcr* and *att1*, mutants in P450-genes encoding fatty acid ω -hydroxylases presumably involved in the pathway to α,ω -diacids, show patchy or thicker cuticular membranes in TEM observations (Wellesen et al., 2001; Xiao et al., 2004). In the cutin polymer α,ω -diacids could function as ester-linker between hydroxyl groups in the cutin polymer and hydroxyl groups in other cell wall polymers such as carbohydrates.

The high amounts of α,ω -diacids and low amounts of mid-chain hydroxyl groups could cause fewer cross-links via secondary hydroxyls and might lead to parallel aligning structures resulting in a polymer assembly of high density, allowing *Arabidopsis* to live with a very thin cuticle compared to other species. Similar structure models have been proposed for the aliphatic domain of suberin, that is also characterized by substantial amounts of diacids and a moderate content of secondary functional groups (Kolattukudy, 2001b; Bernards, 2002). This unusual, rather suberin-like α,ω -diacid content in the cutin might be unique to *Arabidopsis*, but could also be a biochemical property of very thin cuticular membranes. However, cuticles below 50 nm thickness have not been isolated and analyzed before.

Although response factors to determine molecular ratios are not available, the high amounts of α,ω -diacids implicates an excess of carboxyl groups, indicated by an carboxyl:hydroxyl ratio of 3.8:1, based on the quantification results. The triol glycerol, that has only recently been identified as a cutin component (Graça et al., 2002), has been proposed to replace the role of ω -hydroxyl groups in ester-linkages. Glycerol (0.0015 $\mu\text{g}/\text{cm}^2$) could be detected in the *Arabidopsis* cuticle methanolysate, however, hydrophylic compounds like glycerol are rather discriminated by the applied protocol; therefore the glycerol determination is not quantitative.

3.4. Long-chain 2-hydroxy fatty acids are exceptional cutin components in *Arabidopsis*

Even though polymer-bound 2-hydroxy acids have been described as constituents (2–12%) of leaf polyester (Matzke and Riederer, 1990, 1991), these compounds have frequently

been reported as monomers of suberin (Schreiber et al., 1999), most prominent, with up to 28%, in suberin of endodermal cell walls in roots (Zeier et al., 1999b). The higher amounts of 2-hydroxy acids in extracted leaves compared to isolated cuticles (Figs. 2 and S1), gives raise to the question whether they are cutin components only or if they are also components of other apoplastic polymers co-isolated with the cuticular membranes. Structurally, the major 2-hydroxy acid, 2-hydroxy-tetracosanoic acid, is identical to the acyl chain of ceramide derived membrane sphingolipids (Lynch and Dunn, 2004). As stated above the enzymatically isolated cuticles, exhaustively extracted with chloroform–methanol, are almost free of membrane contaminants, indicating that 2-hydroxy acids are true cutin components in *Arabidopsis*, with so far unknown function. This amphiphatic molecules could act as cutin *primers* anchored in the plasma membrane with their hydrophobic *tail*, providing polymerization sites by their bifunctional hydrophilic *head* of carboxyl and hydroxyl substituents; for example in the anticlinal epidermis cell walls where the cuticle proper and the plasma membrane are close together. Because such a fine structural element might get disrupted during incubation with hydrolases the 2-hydroxy acid content in cuticles is lower compared to extracted leaves. 2-Hydroxy acids could also function as hydrophobic *end caps* esterified to other cutin monomers. Despite the difference in the distribution of 2-hydroxyacids, qualitatively the monomer composition of isolated cuticles and solvent extracted leaves were identical (Figs. 2 and S1), demonstrating that for compositional analysis, leaf tissue exempt from soluble lipids by organic solvents can be employed as cuticle equivalent.

3.5. Endodermis and peridermis are suberized tissues in *Arabidopsis* roots

Because the major cutin monomers in *Arabidopsis* have been described in the literature as characteristic suberin constituents (Kolattukudy, 1981; Matzke and Riederer, 1991; Zeier et al., 1999a; Schreiber et al., 1999), a second aim of this study was to investigate the occurrence and chemical composition of suberin in *Arabidopsis*. Histochemical staining with Sudan III and TEM observations indicated that in *Arabidopsis* roots suberin is deposited in the cell wall of the endodermis of younger root sections and the peridermal cells at the beginning of secondary growth and in the periderm of fully developed roots, respectively. These findings suggest profound changes in tissue specific suberin deposition during root maturation and could reflect a dual role of suberin during root development. In young roots, suberin depositions function as an inner barrier guarding the central cylinder from significant water and nutrient losses, supported by stronger suberin depositions on inner tangential and radial walls in the endodermis (Fig. 4B). In mature roots, suberin builds an outer barrier protecting the organ from pathogens in the rhizosphere, incorporation of toxic solutes and strengthens the cell wall and the whole root organ.

3.6. The monomer composition of hydrolase resistant *Arabidopsis* root cell walls represents a typical suberized plant tissue

Suberin monomers equivalent to 6.3% of the enzyme resistant cell wall fraction were released by transesterification. Similar amounts have been determined in enzymatically isolated endodermal cell walls of monocotyledonous species (Zeier and Schreiber, 1998; Zeier et al., 1999b). Compared to dicotyledonous plants this is lower than the 9.8% of *Ricinus communis* L. and 19.5% of *Cicer arietinum* root endodermal cell walls (Zeier et al., 1999a) probably due to the fact that our starting material also contained the lignified cell walls of the xylem. These comparisons, complicated by the fact that different cell wall fractions have been isolated from different species, points out that comparisons of suberin amounts referring to the dry weight of the isolated cell wall material is not accurate. Based on the function of suberin as an apoplastic barrier, Schreiber et al. (2005b) suggested to relate the suberin amount to surface area for comparison of different species. In our experiments, 1 mg isolated root cell wall material correlated to 1.18 cm² root surface resulting in 50.5 µg aliphatic suberin per cm², similar to species with a multilayered hypodermis or a root periderm (Schreiber et al., 2005a). Although this surface amount is representative due to the analysis of mature roots predominantly suberized in the periderm (Fig. 4D), methods to isolate endodermal cell walls representing an inner suberized surface needs to be established for actual tissue specific surface amounts.

The substance class composition with high relative amounts of ω-hydroxyacids and α,ω-diacids and a high proportion of C₁₈ unsaturated aliphatics is in good agreement with the composition of lamella suberin from other dicotyledonous and monocotyledonous species (Fig. 5; Zeier et al., 1999a; Zeier and Schreiber, 1998; Schreiber et al., 2005a). Furthermore, the C₁₆ and C₁₈ aliphatics were the major suberin monomers, which showed an overall chain length distribution ranging from C₁₆ to C₂₄ (Fig. 6). Similar to the analysis of *Q. suber* and *Pseudotsuga menziesii* suberin, glycerol (0.025–0.079 µg mg⁻¹) could also be detected in the methanolysate of the enzyme resistant cell wall fraction, indicating polymer-bound glycerol. This is consistent with the recently proposed structure models of the suberin polyester containing glycerol as a “linker” between carboxylated suberin monomers (Graça and Pereira, 2000b; Bernards, 2002). Thus, there are no significant quantitative and qualitative differences in the aliphatic monomer composition of *Arabidopsis* root cell walls and suberin of other dicotyledonous and monocotyledonous species. Therefore, *Arabidopsis* root cell walls can serve as a good model for suberized plant tissues in general.

3.7. Similar monomers in *Arabidopsis* cutin and suberin

In the direct comparison of the aliphatic monomer composition *Arabidopsis* cutin and suberin is strikingly similar.

Only minor differences can be detected concerning chain-length distribution, saturation of C₁₈ monomers and dominance of substance classes (Fig. 6). In addition to the maximum of C₁₈ aliphatics, the chain-length distribution of suberin is characterized by a second maximum of very long-chain molecules with carbon chain length of 22 whereas in cutin only carboxylic acids and 2-hydroxyacids are elongated beyond C₁₈. In suberin the major C₁₈ monomers are monounsaturated ω-hydroxy acids, whereas in cutin double unsaturated α,ω-diacids are the major C₁₈ derivatives. Despite this differences in the relative amounts of individual aliphatic monomers *Arabidopsis* cutin and suberin comprised the same substance classes suggesting that the biosynthesis of aliphatic cutin and suberin monomers in *Arabidopsis* requires similar enzyme activities and presumably share common pathways. This is supported by the fact that *HTH*, *LCR*, *KCSI* and *LACS2* genes presumably involved in cuticle biosynthesis are also expressed in roots (Krolikowski et al., 2003; Wellesen et al., 2001; Todd et al., 1999; Schnurr et al., 2004).

3.8. The very long-chain fatty acid modification pathway

Although the ubiquity in all higher plants suberin and cutin biosynthesis has not been elucidated in much detail and no enzyme directly involved in cutin and/or suberin biosynthesis has been characterized yet. Based on our compositional analysis we propose a hypothetical pathway (Fig. 7) for suberin and cutin biosynthesis in *Arabidopsis*, providing the basis for upcoming *reverse genetic* approaches. The precursors of this pathway are supplied by the pool of C₁₆ and C₁₈ carboxylic acids derived from plastidic fatty acid biosynthesis (Kunst et al., 2004; Bernards, 2002). The biosynthetic backbone is an ω-oxidation pathway that functionalize the ω-position of long-chain fatty acid derivatives. The ω-hydroxylation reaction is typically catalyzed by oxygen- and NADPH-dependent cytochrome P450 monooxygenases (Schuler and Werck-Reichhart, 2003), particularly of the Cyp86 and Cyp94 subfamilies (Duan and Schuler, 2005). Some of them have been demonstrated to catalyze the ω-hydroxylation of fatty acids (Benveniste et al., 1998; Le Bouquin et al., 1999, 2001) but it is still a matter of debate whether any of these hydroxylases is involved in cutin and suberin biosynthesis or not. The *LCR* gene, cloned from a putative cutin mutant with organ fusion phenotype, encoding a P450 (Cyp86A8) capable of the ω-hydroxylation of C₁₂–C₁₈ fatty acids, has been proposed to be a candidate (Wellesen et al., 2001) as well as *ATT1*, encoding CYP86A2 and also cloned from a cutin mutant (Xiao et al., 2004). The *HTH* gene, isolated from another organ fusion mutant (Krolikowski et al., 2003), encoding a putative oxidoreductase might be involved in the subsequent formation of α,ω-diacids catalyzed by a ω-hydroxy fatty acid dehydrogenase and ω-oxo fatty acid dehydrogenase (Agrawal and Kollattukudy, 1978). Recently, the tobacco P450 Cyp94A5 has been characterized to be able to catalyze the oxidation of ω-hydroxy fatty acids to α,ω-diacids, indicating that this

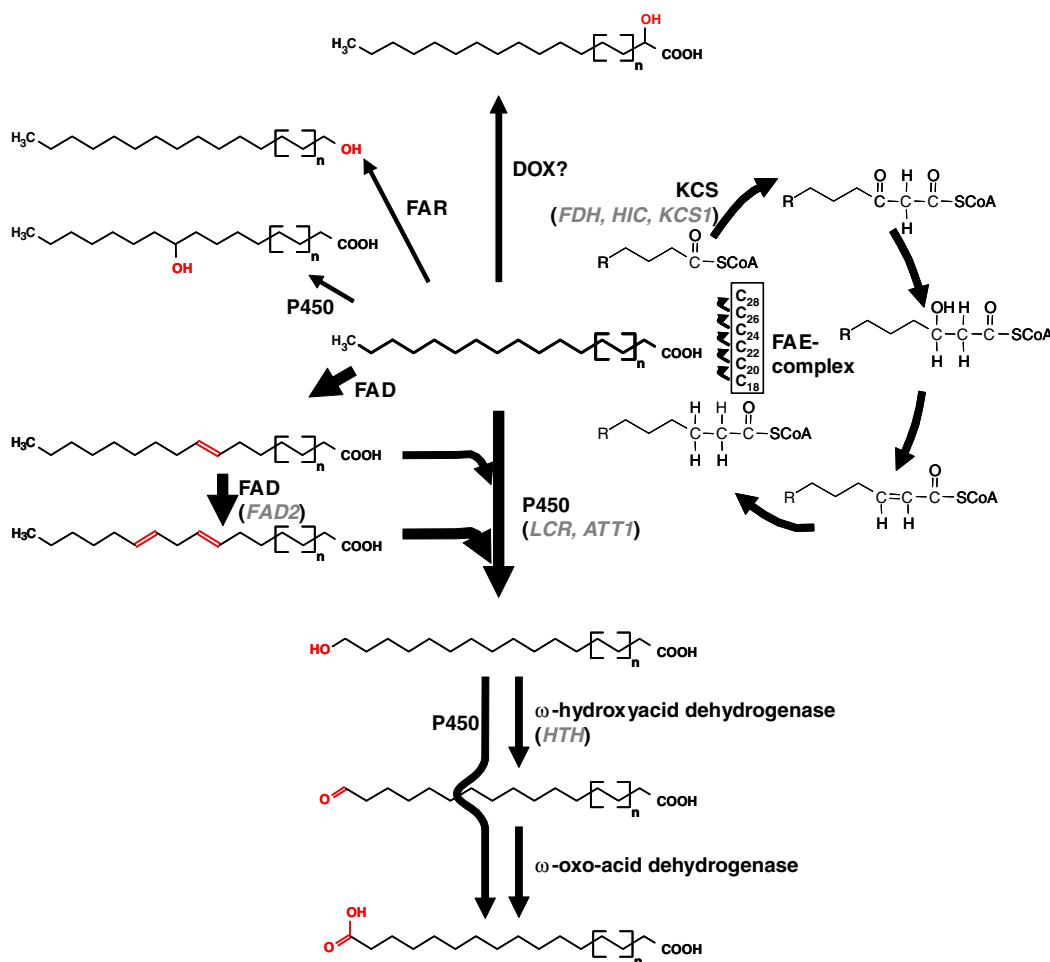


Fig. 7. Model of the biosynthetic pathways for cutin and suberin monomers in *Arabidopsis*. Enzymes presumably involved are indicated as follows: DOX, dioxygenase; FAD, fatty acid desaturase; FAE, fatty acid elongase; FAR, fatty acid reductase; KCS, β -ketoacyl-CoA-synthase; P450, Cytochrome P450 monooxygenase. *Arabidopsis* candidate genes are given in parenthesis as published. The various chain length is indicated by n ($n = 1-7$). Arrow thickness indicates the extend of carbon flux.

two-step reaction can also be catalyzed by a single enzyme (Le Bouquin et al., 2001). The mid-chain hydroxylation is another P450 catalyzed reaction that was demonstrated in *Vicia faba* leaf extracts (Kolattukudy, 1981) indicating that P450 are key enzymes in aliphatic suberin and cutin biosynthesis.

Based on the content of unsaturated C_{18} aliphatics, fatty acid desaturases (FAD) are involved in the biosynthesis of 30–35% of the cutin and suberin monomers. This was indicated by a decrease of double unsaturated C_{18} α,ω -diacids in leaf polyester of the *fad2* mutant (Bonaventure et al., 2004). Since in-chain modification seems not to be critical for the substrate specificity of the ω -hydroxylase(s) (Benveniste et al., 1998), desaturated and mid-chain oxygenated fatty acids can enter the core ω -oxidation pathway again. Alternatively, it has also been shown that both, desaturation and hydroxylation can be catalyzed by the same enzyme (Broadwater et al., 2002).

The production of 2-hydroxy acids is a totally unknown branch of this pathway. Dioxygenases that have been shown to catalyze a two step oxidation of unsaturated C_{16} and C_{18} fatty acids via hydroperoxides (Hamberg

et al., 1999) have been proposed to be involved in the production of unsaturated 2-hydroxy fatty acids in plants. However, the process of α -oxidation of saturated very long-chain fatty acids is not clear.

The long-chain carbon backbone of C_{20} - C_{28} 2-hydroxy acids in the cutin and the C_{20} - C_{24} monomers in suberin requires the elongation of the fatty acid precursors catalyzed by fatty acid elongase (FAE) complexes. β -ketoacyl-CoA-synthases (KCS) encoded by the so-called *FAE* genes catalyze the rate-limiting step in the production of very long-chain fatty acid precursors (Miller and Kunst, 1997). The *FAE* genes *FDH* and *HIC* have been suggested to be involved in cuticle formation (Pruitt et al., 2000; Yephremov et al., 1999; Gray et al., 2000) and *KCS1* a root expressed FAE is involved in the biosynthesis of fatty acid precursors in roots (Todd et al., 1999) supporting the presumed pathway model.

3.9. Future perspectives

The methods for the quantitative and qualitative determination of cutin and suberin in *Arabidopsis* enable us to study the deposition of this protective polymers during normal

plant development and in response to biotic and abiotic stress conditions and relate it to global gene expression studies to identify genes presumably involved in these processes. In addition, the array of identified mutants with altered leaf and root morphology, pathogen susceptibility or sensitivity to drought, humidity, herbicides and nutrient availability can now be subjected to biochemical phenotypic analysis. Finally, transgenic plants with altered cutin and/or suberin composition can be generated to study the physiological importance of cutin and suberin as an apoplastic barrier and to evaluate the biotechnological potential of cutin and suberin modification in agriculturally important plants.

4. Experimental

4.1. Plant material

Arabidopsis thaliana L. Heynh. ecotype Columbia plants were cultivated in Floradur potting mix (Floraguard, Germany) at a light intensity of $100 \mu\text{E m}^{-2} \text{s}^{-1}$ at 22°C under a photoperiod of 16 h light/8 h dark. 5-week-old fully developed flowering plants have been used for analysis.

4.2. Cuticle isolation

Cuticular membrane isolation was basically performed as described by Schönherr and Riederer (1986) with some modifications. Leaf disks of 1 cm diameter, intensively extracted with chloroform:methanol (1:1; v/v) to remove soluble lipids and waxes, were used instead of native leaf disks. For the incubation with polysaccharide hydrolases (1% v/v cellulase (Celluclast, Novo Nordisk, Denmark), 1% v/v pectinase (Trenolin, Erbslöh, Germany) in 10^{-2} M citric buffer pH 3, containing 10^{-3} M NaN_3) leaf disks were not vacuum infiltrated to avoid damages. After 5–10 days with occasional agitation, leaf cuticles were collected with a micro spatula and transferred into 10^{-2} M borate buffer pH 9 to remove soluble lipophilic compounds possibly resorbed to the cuticle. After several days with renewal of the borate buffer, cuticles were finally washed with deionized water and 30 cuticles per sample were pooled and directly transferred into reaction vials for depolymerization.

4.3. Totally extracted leaves

20–50 rosette leaves were collected and scanned to determine the surface area using imaging software. The leaves were then exhaustively extracted with chloroform:methanol (1:1; v/v) with daily change of solvent over a period of two weeks. After drying 30–50 mg of extracted leaves were used for the depolymerization reaction.

4.4. Isolation of suberized root cell wall material

Roots were carefully dug out of the potting mixture and rinsed with tap water to remove all soil contaminants before they were finally washed with deionized water. 4–7

roots per sample were cut in 5 mm sections, transferred into the above enzyme solution containing polysaccharide hydrolases, vacuum infiltrated and incubated for 3 weeks with a weekly change of the enzyme solution. The remaining cell wall fraction was then washed using borate buffer (10^{-2} M, pH 9) and water, and unbound lipids were removed by prolonged extraction in chloroform:methanol (1:1; v/v). 3–6 mg of the cell wall material was used for depolymerization.

4.5. Chemical degradation of polyesters

Phytopolyesters can be depolymerized by any reagent used to cleave ester bounds. Acid catalyzed transesterification using borontrifluorid in methanol (BF_3/MeOH), releasing solvent extractable methylesters suitable for GC and GC–MS analysis has often been used because it results in less background from other cell wall components and is more practicable compared to KOH or reduction with $\text{LiAlH}_4/\text{LiAlD}_4$ (Kolattukudy, 1980; Schreiber et al., 1999). As indicated by the disappearance of ester-associated absorbance bands, quantitative FT-IR-analysis has shown that BF_3/MeOH quantitatively dissolves all aliphatic esters in suberized cell walls (Zeier and Schreiber, 1999; Schreiber et al., 2005a). Alternatively transmethylation with methanolic-HCl (MeOH/HCl) can be used giving similar results (Holloway, 1982; Fig. S1).

4.5.1. Suberin depolymerization

The isolated root cell wall fraction was depolymerized by transesterification in a 10% BF_3/MeOH (Fluka) solution. To 3–6 mg cell walls 1 ml BF_3/MeOH was added and incubated in a teflon-sealed screw cap tube for 16 h at 70°C . The reaction was stopped by adding 2 ml saturated $\text{NaHCO}_3/\text{H}_2\text{O}$ to the methanolysate that was then extracted with chloroform (3 times) after 10 μg of dotriacontane (Fluka) has been added as an internal standard. The combined organic phase was dried over NaSO_4 and then evaporated in a stream of nitrogen. Free hydroxyl and carboxyl groups were converted into their trimethylsilyl (TMS) ethers and esters with bis-(*N,N*-trimethylsilyl)-tri-fluoroacetamide (Machery-Nagel, Dueren, Germany) in pyridine for 40 min at 70°C prior to GC–MS analysis.

4.5.2. Cutin depolymerization

Cutin monomers were solubilized by transesterification of 30 isolated cuticular membranes (1 cm diameter) or 30–50 mg totally extracted leaves with 1 ml or 6 ml 1 N MeOH/HCl (Supelco) for 2 h at 80°C . After addition of 2 ml saturated $\text{NaCl}/\text{H}_2\text{O}$ the hydrophobic monomers were subsequently extracted in hexane (3 times) containing 20 μg internal standard (Dotriacontane). The combined extracts were evaporated and derivatized as described above.

This method was chosen due to lower background levels in GC and GC–MS chromatographic analysis improving identification and quantification of low abundant

compounds. Optimization studies comparing MeOH/HCl transesterification to the above BF₃/MeOH transesterification revealed no significant qualitative and quantitative differences. Furthermore, using enzymatically isolated *Lycopersicon esculentum* fruit cuticles, which are to >80% comprised of 9(10),16-dihydroxy-hexadecanoic acid (Graça et al., 2002), we verified that on the applied scale hexane extraction of polyhydroxylated cutin depolymerization products is quantitative.

4.6. GC and GC–MS analysis of depolymerization products

Monomers were identified from their EI-MS spectra (70 eV, *m/z* 50–700) after capillary GC (DB-1, 30 m × 0.32 mm, 0.1 μm (J&W), on-column-injection at 50 °C, oven 2 min at 50 °C, 10 °C min⁻¹ to 150 °C, 1 min at 150 °C, 3 °C min⁻¹ to 310 °C, 30 min at 310 °C and He carrier gas with 2 ml min⁻¹) on an Agilent 6890N gas chromatograph combined with a quadrupole mass selective detector 5973N (Agilent Technologies, Böblingen, Germany). Quantitative determination of cutin components was carried out with an identical GC-system equipped with a flame ionization detector based on the internal standard. The analyses were conducted with three (Table 1) or 10 (Table 2) replicates. Data are presented as means ± standard deviation (SD).

4.7. Light-microscopic techniques

Whole root bundles, similar to the material used for suberin analysis, were washed and fixed in a mixture of glutaraldehyde (1.75%; v/v) and formaldehyde (2%; v/v) in phosphate buffer saline (10⁻² M NaPO₄, 0.137 M NaCl, 27 × 10⁻⁴ M KCl, pH 7.4) for 24 h. The samples were frozen at -50 °C and 20 μm sections were cut using a cryo microtome (Cryostat H 500). Sections were transferred to microscope slides, embedded in glycerol:water (1:1; v/v) and examined with an Axioplan microscope (Zeiss, Germany) with brightfield illumination or fluorescence excitation at 365 nm using filter LP397 (Zeiss). For Sudan staining according to Gerlach (1984) a saturated solution of Sudan III in 92% (v/v) ethanol was applied to the sections and heated to 70 °C for 30 s, washed with water and examined after embedding in glycerol–water. Enzymatically isolated cuticular membranes were carefully transferred to glycerol–water and examined using the same microscope.

4.8. Transmission electron microscopy of cell wall ultrastructure

For the analysis of the cuticle ultra structure, rosette leaves of different age of 4- to 5-week-old plants, grown under a 12 h light/dark cycle were investigated. For the analysis of suberization of root tissues, the primary roots of 3-week-old agar-plate grown plants were analyzed as well as the oldest root parts of 8-week-old soil-grown plants. Small pieces of *Arabidopsis* leaves were fixed for

2 h with 2% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at room temperature. Small pieces of roots were fixed in paraformaldehyde (2%) and glutaraldehyde (1.5%) in 0.066 M potassium phosphate buffer (pH 7.4) under the same conditions. Both materials were post-fixed with OsO₄ (1%) at 4 °C overnight in the corresponding buffers. After dehydration with acetone at room temperature, the material was embedded in Spurr's standard epoxy resin and the resin was polymerized at 70 °C for 18 h. Thin sections were stained with 2% uranyl acetate (in 50% acetone) and alkaline lead citrate for 0.5 h each. Micrographs were taken with a Philips CM100BIOTWIN electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). For measurements of cuticle thickness (mean ± SD) 14 independent samples were measured for leaf blade, 11 for veins and 6 for petioles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.phytochem.2005.09.027](https://doi.org/10.1016/j.phytochem.2005.09.027).

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