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Cutin and suberin: assembly and origins of specialized lipidic cell wall scaffolds

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Cutin and suberin are hydrophobic lipid biopolyester components of the cell walls of specialized plant tissue and cell-types, where they facilitate adaptation to terrestrial habitats. Many steps in their biosynthetic pathways have been characterized, but the basis of their spatial deposition and precursor trafficking is not well understood. Members of the GDSL lipase/esterase family catalyze cutin polymerization, and candidate proteins have been proposed to mediate interactions between cutin or suberin and other wall components. Comparative genomic studies of charophyte algae and early diverging land plants, combined with knowledge of the biosynthesis, trafficking and assembly mechanisms, suggests an origin for the capacity to secrete waxes, as well as aliphatic and phenolic compounds before the first colonization of true terrestrial habitats.

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Introduction

Since their evolution from aquatic algal ancestors, approximately 500 million years ago, plants have colonized almost every terrestrial habitat; in the process profoundly affecting the planet's geochemical composition, atmosphere, and ecology [1]. This terrestrialization was enabled by evolution of the ability to assemble macromolecules into complex cell wall architectures that provide biomechanical support and regulate water flux both within the plant corpus and with the external environment. Indeed, arguably the most crucial evolutionary innovation that allowed the emergence of embryophytes was the capacity to deposit hydrophobic barriers at the cell surface, thereby providing a means to limit transpiration or uncontrolled water absorption.

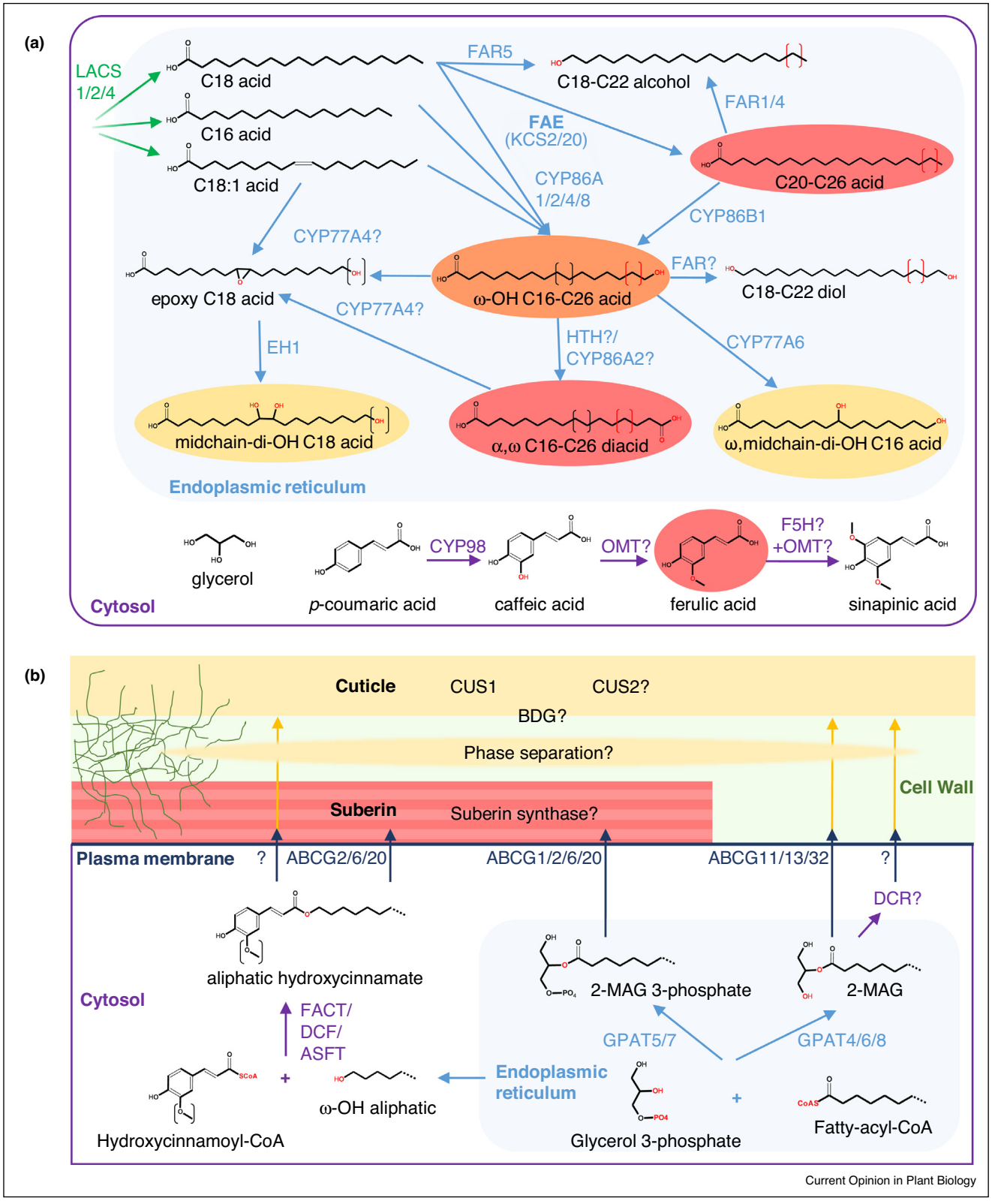
The core scaffolding of two types of these specialized cell wall layers is provided by the lipid polyesters cutin and suberin, which are both non-covalently associated with a diverse range of lipids that are broadly referred to as waxes. A third type of hydrophobic but non-lipidic polymer, lignin, is not discussed in detail here and readers are referred to recent reviews [2–4]. Cutin forms the bulk of the plant cuticle, which is synthesized by epidermal cells and coats the surfaces of aerial organs. In this regard, cutin and waxes collectively play a central role in restricting water loss, as well as other functions, such as providing protection against pathogens and preventing organ fusion during organ development [5–8]. A cuticle was also recently reported as being present on the surface of the root cap and lateral roots, where it has similar protective and barrier functions as the canonical cuticles of aerial organs [9•]. The other major polyester, suberin, also limits water movement and is deposited in the root endodermis, the periderm of roots and tubers and seed coats, as well as in abscission zones and damaged tissues, where it has a protective sealing role [10–12]. Despite similarities in their functions and properties, cutin and suberin are typically described as chemically related, but distinct, polymers, and differences in their composition and patterns of spatial accumulation within the apoplast are generally cited as distinguishing features.

This review compares the mechanisms, by which these lipidic plant polyesters are synthesized and undergo extracellular (*sensu stricto* extraprotoplasmic) assembly, and examines their evolutionary origins in the green plant lineage. Such information can help clarify structural and functional differences between cutin and suberin biopolymers, but also highlights how this distinction is becoming increasingly blurred.

Cutin and suberin: twin biopolyesters with similar compositions and partly shared synthetic pathways . . .

Cutin and suberin consist of functionalized saturated and non-saturated fatty acids, fatty alcohols, hydroxycinnamic acids and glycerol (Figure 1a), which are linked by ester-bonds into complex matrices. The proportions and

Figure 1



Cutin and suberin biosynthesis **(a)** Summary of known cutin and suberin monomers, based on *in vitro* chemical depolymerization of the extracted macromolecular complexes, highlighting the subcellular sites of their biosynthesis. Structural changes between steps are shown in red. Fatty acids are first synthesized in the plastid, conjugated to acyl-CoA by the long chain acyl-CoA synthetases LACS1, -2 and -4, and then traffic to the

compositions of these monomers differ substantially between species, and even among organs within the same species. Cutin is mainly composed of derivatives of C16 and C18 fatty acids, with one or more hydroxyl, mid-chain epoxide and end-chain carboxyl functional groups. Like cutin, suberin is mainly derived from long-chain aliphatic acids (>C16); however, it is generally described as having higher proportions of fatty alcohols, hydroxycinnamic acids and glycerol, compared with cutin [13].

The biosynthetic pathways of both cutin and suberin aliphatic and aromatic monomers have been extensively studied, mostly in *Arabidopsis thaliana*, and much of the genetic framework has been resolved (Table 1). The initial lipid precursors of both cutin and suberin are C16:0, C18:0 and C18:1 fatty acids, which are synthesized in the plastid and traffic to the endoplasmic reticulum [14]. Here, they can undergo further modifications (Figure 1a): aliphatic chain elongation by the fatty acid elongase (FAE) enzyme complex, of which β-ketoacyl-CoA synthase (KCS) has definitively been related to suberin formation [10]; reduction of carboxyl groups to alcohols by fatty acyl-CoA reductase (FAR); and hydroxylation by cytochrome P450 enzymes (CYP86, CYP77) or epoxide hydrolase (EH). For additional information, see legend for Figure 1. In most cases, it is not currently known whether individual enzymes in these families are specifically involved in either cutin or suberin synthesis, or whether they contribute to both. However, following monomer biosynthesis, some of the subsequent steps to generate cutin or suberin lipid precursors are known to involve different enzymes from the same superfamily, but which have distinct mechanisms of action (Figure 1b). One example is the glycerol-3-phosphate acyltransferase (GPAT) enzymes, which convert fatty acid monomers to 2-monoacylglycerides (2-MAGs). GPAT4, GPAT6 and GPAT8 enzymes are involved in cutin precursor formation and catalyze a dephosphorylation reaction during condensation between glycerol and fatty-acyl co-A chains. In contrast, this dephosphorylation reaction is absent from GPAT5 and GPAT7 activities during suberin precursor synthesis [15]. Both classes of MAG precursors are transported across the plasma membrane by ATP-binding cassette transporter subfamily G proteins (ABCGs), but again by different protein family members, resulting in the deposition of cutin and suberin building blocks in the apoplast [16].

Another feature of the precursor biosynthesis involves the action of BAHD-type acyltransferases that link hydroxycinnamic acids to ω-hydroxy fatty acids or ω-alcohols in the cytosol for the synthesis of suberin (FACT, ASFT) or cutin (DCF) precursors [17,18]. However, several other key steps in the synthesis of these phenolic precursors, as well as their mechanisms of export, have yet to be characterized (Figure 1b).

. . . but distinct spatial patterns of accumulation

While the intracellular biosynthetic pathways have been relatively well defined, far less is known about processes that determine cutin and suberin polymerization and distribution. One of the key features that is cited as differentiating cutin and suberin is the spatial pattern of localization of the polymer within the apoplast, and specifically with respect to the polysaccharide cell wall. Suberin is typically described as accumulating adjacent to the plasma membrane, often appearing in transmission electron microscopic images in the form of lamellae [19] (Figure 1b). It has been proposed that the monomer composition, and particularly the high phenolic content, of suberin is responsible for its lamellate structure and macromolecular organization [11]. However, a causal relationship between monomer composition and spatial patterns of cutin or suberin deposition has yet to be demonstrated, and another study has suggested that phenolic content is not a major determinant of lamellae formation [20]. It is also notable that studies of the cuticle substructures of hundreds of plant species have revealed examples of lamellate, reticulate, or amorphous architectures, and some outer epidermal cuticles have distinct layers [21]. Attempts to associate specific chemical features with these classes of cuticle organization, including analyses of intracuticular wax composition and cutin polymeric structure, have not provided conclusive results [22].

Suberin is likely polymerized immediately after cell export, whereas cutin monomeric units must traverse the cell wall to the cell surface before polymerization (Figure 1b). The mechanistic basis of this monomer trafficking is a key question in plant cell wall biology, and has yet to be resolved. It is often suggested that lipid transport proteins (LTPs) act as chaperones to mediate the movement of both cutin monomers and waxes across the hydrophilic wall to its outer surface (e.g. Ref. [23]). However, while this possibility cannot be excluded, in the

(Figure 1 Legend Continued) endoplasmic reticulum (ER). The biosynthesis of the phenolic monomers that are incorporated into cutin or suberin includes the conversion of *p*-coumaric-CoA to caffeic derivatives by CYP98 [50], and likely includes enzymatic reactions that also contribute to lignin biosynthesis through the generation of ferulic acid and sinapinic acid [2–4]. Biosynthetic enzymes localized in the ER are indicated in blue, and those in the cytosol in purple. Enzymes specifically related to cutin or suberin biosynthesis that have yet to be characterized are shown with a question mark. Monomers that are particularly abundant in cutin or suberin are indicated with yellow and red ellipses, respectively, while ω-OH C16-C26 acid (orange ellipse) is abundant in both. Red parentheses indicate elongation of the carbon chain (number of carbons is specified in compound name) and black parentheses indicate variation in different functional groups at that molecular location. **(b)** Key steps in the biosynthesis and export of cutin and suberin precursors and in their extracellular trafficking and assembly. The polysaccharide cell wall is shown in green, together with glycans extending into the cutinized or suberized layers. Dashed lines on the compound structures indicate extended aliphatic chains. Uncharacterized enzymes or steps are shown with a question mark.

Table 1

Genes involved in cutin and suberin formation as described in *Arabidopsis thaliana*. Numbers represent the number of orthologs in a given species for the corresponding *A. thaliana* gene. *O. tauri, Ostreoccocus tauri; C. variabilis, Chlorella variabilis; C. reinhardtii, Chlamydomonas reinhardtii; K. flaccidum, Klebsormidium nitens; C. braunii, Chara braunii; S. muscicola, Spirogloea muscicola; M. endlicherianum, Mesotaenium endlicherianum; P. margaritaceum, Penium margaritaceum; M. polymorpha, Marchantia polymorpha; P. patens, Physcomitrella patens; S. moellendorffii, Selaginella moellendorffii; A. filiculoides, Azolla filiculoides; G. montanum, Gnetum montanum; A. trichopoda, Amborella trichopoda; O. sativa, Oryza sativa*

Gene family	Gene name	GeneID	Function	*O.tauri*	*C.reinhardtii*	*C.variabilis*	*K. nitens*	*C.braunii*	*S.muscicola*	*M.endlicherianum*	*P.margaritaceum*	*M.polymorpha*	*P.patens*	*S.moellendorffii*	*A.filiculoides*	*G.montanum*	*A.trichopoda*	*O.sativa*	Cutin related genes	Suberin related genes	References
Biosynthesis																					
Long chain acylCoA synthetase	LACS1	AT2G47240	Attachment of CoA to free fatty acids														3	1	C		[8]
	LACS2	AT1G49430															1	1	C		[8]
	LACS4	AT4G23850			1	2	2	1	2	1	2	1	4	1	2	3	1	3	C		[55]
βketoacylCoA synthase	KCS2	AT1G04220	Fatty acid elongase complex												1	1		2		S	[10]
	KCS20	AT5G43760													1					S	[10]
Fatty acylCoA reductase	FAR1	AT5G22500	Fatty acid to alcohol reduction													6		7		S	[10]
	FAR4	AT3G44540															1	1		S	[10]
	FAR5	AT3G44550			1															S	[10]
Cytochrome P450	CYP86B1	AT5G23190	ω-hydroxylase				2		6	5	14		2	1	10	5	2	1		S	[10]
	CYP86A1	AT5G58860															1	1		S	[10]
	CYP86A2	AT4G00360																	C		[8]
	CYP86A4	AT1G01600																	C		[8]
	CYP86A8	AT2G45970																	C		[8]
	CYP77A6	AT3G10570	Inchain hydroxylase										1						C		[8]
Glycerol-3-phosphate acylCoA transferase	GPAT4	AT1G01610	Synthesis of 2-MAGs												1				C		[8]
	GPAT6	AT2G38110										4	7	8	8	3	3	6	C		[8]
	GPAT8	AT4G00400																	C		[8]
	GPAT5	AT3G11430												3	3	1	1	1		S	[10]
	GPAT7	AT5G06090																		S	[10]
BAHD acyltransferase family protein	FACT	AT5G63560	CaffeoylCoA acyltransferase													3				S	[10]
	ASFT	AT5G41040	FeruloylCoA acyltransferase	1			1					8	1	17	2	4	4	5		S	[10]
	DCF	AT3G48720										1		5		1			C		[8]
	DCR	AT5G23940	Cutin precursor synthesis (speculative)											5	1	2	1	2	C		[40•]
None	HTH	AT1G72970	Diacid synthesis (speculative)		1		3		3				2	1		2	1	2	C		[8]
α/βHydrolase superfamily protein	EH1	AT3G05600	Epoxide hydrolase				2							1	1				C		[56]
	BDG1	AT1G64670	Unknown							1			2						C		[8]
	BDG3	AT4G24140					1			1	3		1			1	1	2	C		[32]

Table 1 (*Continued*)

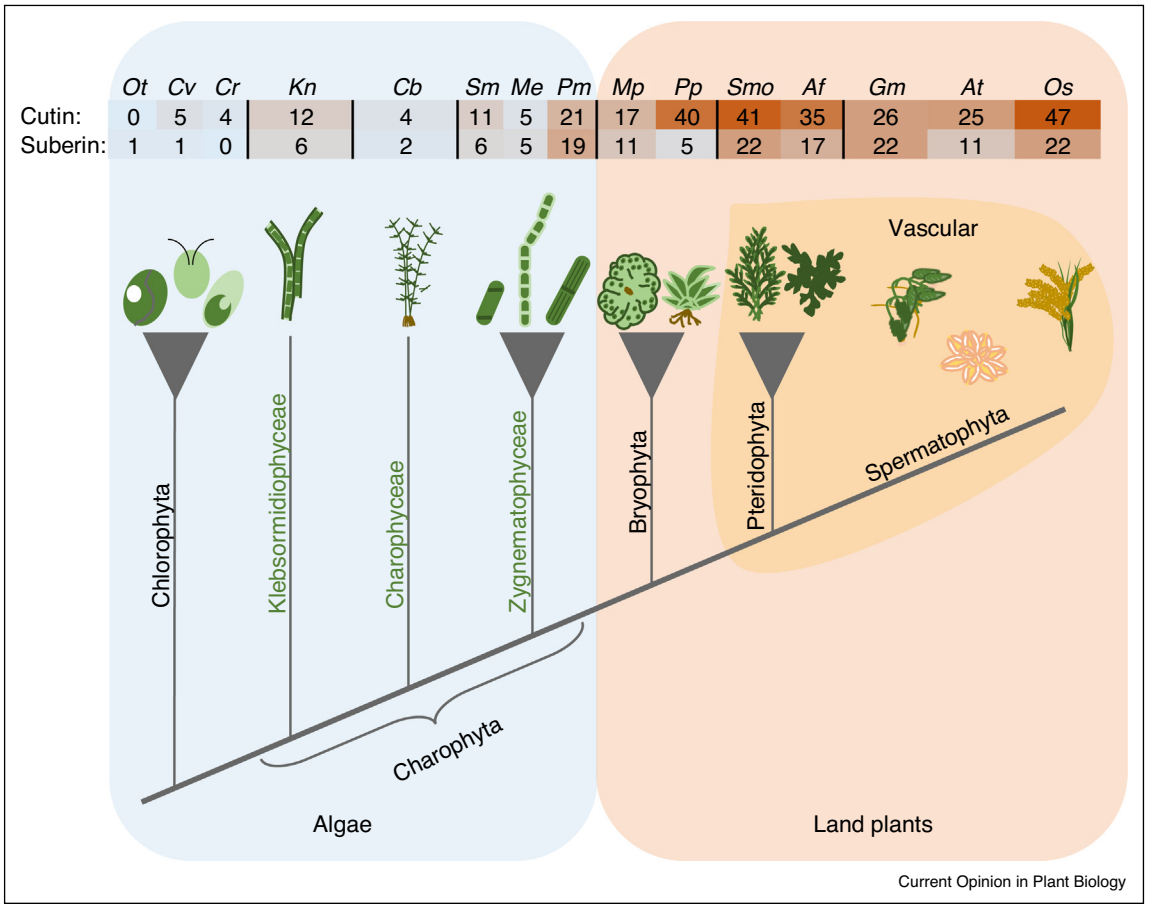
Gene family	Gene name	GeneID	Function	*O.tauri*	*C.reinhardtii*	*C.variabilis*	*K. nitens*	*C.braunii*	*S.muscicola*	*M.endlicherianum*	*P.margaritaceum*	*M.polymorpha*	*P.patens*	*S.moellendorffii*	*A.filiculoides*	*G.montanum*	*A.trichopoda*	*O.sativa*	Cutin related genes	Suberin related genes	References
Cutin Synthase	CUS1	AT3G04290	Polymerization of 2-MAG monomers				1					1				1			C		[8]
	CUS2	AT5G33370	Unknown								1	1	1	1	1			6	C		[35]
Dirigentlike protein	ESB1	AT2G28670																		S	[10]
Secretion																					
ATP binding cassette transporter family protein	ABCG11	AT1G17840	Transport across the plasma membrane		1		1	1			1	6	6	17	14	5	6	10	C		[8]
	ABCG13	AT1G51460																	C		[8]
	ABCG32	AT2G26910				1					1					1	1	1	C		[8]
	ABCG1	AT2G39350					1					1					1	1		S	[57]
	ABCG2	AT2G37360					2					2	2	1		1				S	[10]
	ABCG6	AT5G13580																		S	[10]
	ABCG20	AT3G53510						2												S	[10]
Regulation																					
Zinc Finger transcription factor	NFXL2	AT5G05660	Negative regulator		1	1	1	1	3	1	3	1	1	1	1	1	2	1	C		[8]
WW domain protein	CFL1	AT2G33510													1			1	C		[8]
	ANL2	AT4G00730														2		4	C		[8]
AP2 transcription factor	SHN1	AT1G15360															1	2	C		[8]
	SHN2	AT5G11190																	C		[8]
	SHN3	AT5G25390														1			C		[8]
MYB transcription factor	MYB16	AT5G15310	Positive regulator									1	7		2	1	1	2	C		[8]
	MYB106	AT3G01140											2		1			1	C		[8]
	MYB9	AT5G16770																3		S	[58]
	MYB107	AT3G02940																		S	[58]
	MYB41	AT4G28110	Response to Abiotic stress								5					1	1		C	S	[10,32]
Post-translational regulation	HUB1	AT2G44950	Histone H2B Monoubiquitination						1		1	1	3		1		1	1	C		[59]
	HUB2	AT1G55250			1		1	1	2	1	4		3	1	1	1	1	1	C		[59]
Total number of polyester related genes				1	6	4	18	6	17	10	35	28	45	63	52	47	35	69			

absence of a mechanism to recycle the transport proteins once they have deposited their lipid cargo, this would be energetically expensive. Such a unidirectional movement of transport proteins is particularly difficult to reconcile with the large amounts of material needed for thick cuticles, such as those of many fleshy fruits (e.g. cutin deposition up to 200 mg m^{-2}.day^{-1} in apple fruit [24]). An alternative explanation involves the migration of hydrophobic cutin precursors and waxes through the hydrophilic environment of the cell wall and their accumulation on the outer face as a result of passive phase-separation (Figure 1b). Such a physicochemical process has been associated with cell wall self-assembly [25•,26,27] and may underlie many aspects of extracellular matrix heterogeneity. In the case of suberin and cutin monomers, the relative extent and rate of movement within the apoplast may be influenced by other wall components. For example, it has been proposed that cutin polymerization is facilitated by stabilization involving interaction with non-polar waxes, or with specific methylated and acetylated polysaccharides that become embedded in the cutin matrix [28,29••].

The assembly of lipid polyester scaffolding

To date, cutin synthase 1 (CUS1), belonging to the GDSL-lipase/esterase family, is the only enzyme known to be involved in the polymerization step of plant polyesters [30–32] (Figure 2). *In vitro*, CUS1 was reported to have high selectivity toward cutin monomers in their 2-MAG precursor form [33], and to generate linear polymers by transesterification of the end-chain hydroxyl groups [30]. However, the transesterification activity of CUS1 *in vivo* was found, using *in situ* chemical labeling, to occur at mid-chain hydroxyl groups, as highlighted in CUS1 deficient tomato fruit [34••]. The basis of this discrepancy has yet to be resolved, but it may reflect constraints on end chain transacylation that exist in the unique environment of the cell wall-cuticle interface,

Figure 2



Evolutionary emergence of cutin and suberin associated genes across the green plant lineage. Species were selected based on availability of full genome sequences. *Ot*, *Ostreoccocus tauri*; *Cv*, *Chlorella variabilis*; *Cr*, *Chlamydomonas reinhardtii*; *Kn*, *Klebsormidium nitens*; *Cb*, *Chara braunii*; *Smo*, *Spirogloea muscicola*; *Me*, *Mesotaenium endlicherianum*; *Pm*, *Penium margaritaceum*; *Mp*, *Marchantia polymorpha*; *Pp*, *Physcomitrella patens*; *Sm*, *Selaginella moellendorffii*; *Af*, *Azolla filiculoides*; *Gm*, *Gnetum montanum*; *At*, *Amborella trichopoda*; *Os*, *Oryza sativa*. Numbers above the tree refer to gene homologs found when using *Arabidopsis thaliana* genes known to be involved in cutin or suberin biosynthesis as queries (see Table 1). Colors from white to dark brown represent a heatmap from 0 to 47 homologs found.

where tomato CUS1 has been shown to accumulate [30,31]. It is notable that CUS1-deficient tomato fruit still form a thin cuticle, indicating that other enzymes are involved and, indeed, CUS1 is typically a member of a multi-member cutin synthase-like clade within the GDSL-lipase/esterase superfamily [32]. It is also possible that the branched structure of cutin results from the activities of multiple CUS proteins acting simultaneously, or sequentially, to generate complex three-dimensional architectures. Cutin polymerizing activity has been demonstrated *in vitro* by CUS1 proteins from both angiosperms and the moss *Physcomitrella patens* [32], indicating that the capacity to polymerize cutin is ubiquitous in land plants and was likely an early evolutionary adaptation.

In addition to potential differences in enzyme activity, divergent CUS genes have been associated with specific biological roles. For example, the *A. thaliana cus2* mutant shows defects in the formation of petal nanoridges and a decrease in cutin monomer content [35]. Similar abnormalities in petal nanoridges have been observed in other mutants with deficiencies in known cutin biosynthesis genes [36,37], suggesting that this phenotype may provide a means to identify less characterized cutin associated enzyme families. An example is the cuticle and petal nanoridge ultrastructural defects in the *A. thaliana bdg1* and *bdg3* mutants, respectively, with mutations encoding α/β hydrolase BODYGUARD proteins [37,38]. The *A. thaliana* mutant *defective in cuticular rid*ges (*dcr*), affected in a BAHD acyltransferase family gene, also shows defects in the cuticular organization [39]. The polysaccharide cell wall-cuticle interface is disrupted in both the *bdg1* [38] and *dcr* mutant [40•], suggesting that the corresponding BDG and DCR proteins may play a role in establishing cutin-polysaccharide interactions. Although the activity of BDG has not been identified, DCR may have a role in cutin precursor biosynthesis following 2-MAG formation, as suggested by its intracellular location, *in vitro* activity, and study of *gpat6*/*dcr* double mutants [40•,41].

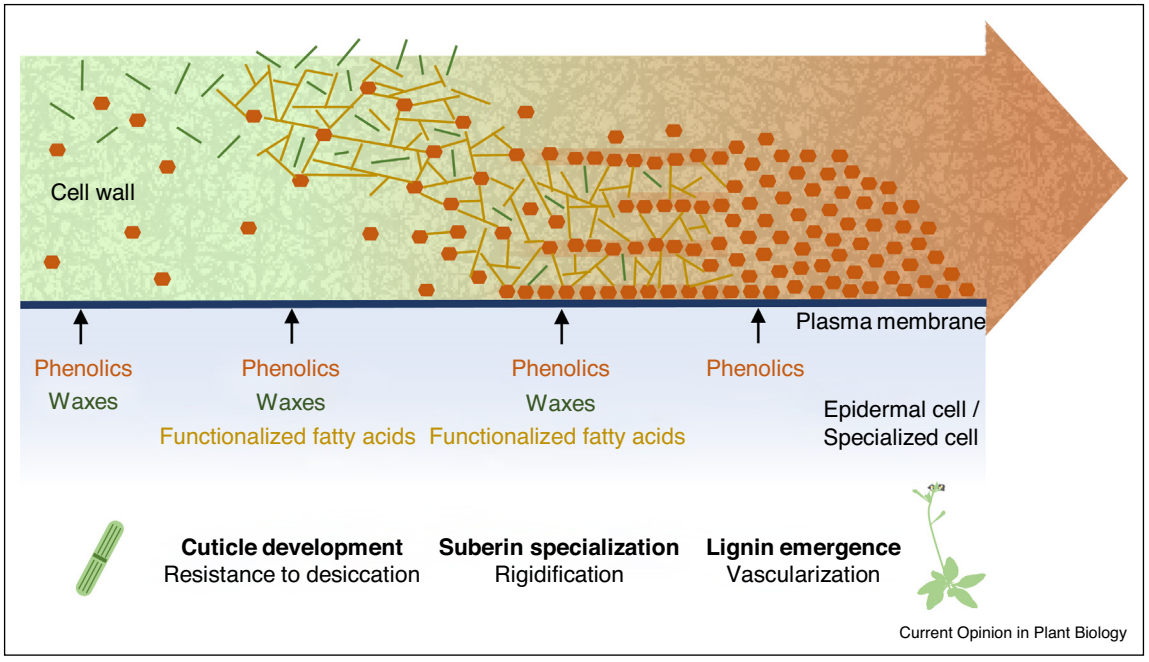
Like cutin, suberin assembly requires enzymatic transesterification of its 2-MAG precursors. A mechanism of suberin polymerization has yet to be identified, but given the similarities in polymer structures, it probably involves similar enzymes to those that catalyze cutin polymerization. Accordingly, it seems likely that one, or more, of the members of the large GDSL-lipase super family encodes a suberin synthase, and strong candidates are apparent among the GDSL genes whose expression coincides with suberin deposition in various species. In this regard, it is also notable that the cutinase CUTICLE DESTRUCTING FACTOR 1 (CDEF1), which is also a member of the GDSL-lipase superfamily, can depolymerize both suberin and cutin *in vivo*, further highlighting the structural similarity and shared associated enzymology of the two polymer types [42,43].

The specialization of extracellular biopolymers through plant evolution

Another perspective of the relationship between cutin and suberin, and the origins of these hydrophobic polyesters can be gained through studies of plant genomes and gene family structures. These can allow inferences regarding both the presence of specific mechanisms underlying the synthesis, transport and polymerization of cutin and suberin, but also their evolutionary trajectories (Table 1; Figure 2). The genomes of species that span the transition of plants from exclusively aquatic environments, to semi-aquatic aeroterrestrial habitats, to true land plants, which require more robust hydrophobic barriers will likely be particularly informative (Figure 2). These include *Klebsormidium nitens* [44] and *Chara braunii* [45], representatives of two of the six major lineages of charophyte algae, from which land plants emerged. The two earlier diverging charophyte algae, *K. nitens* and *C. braunii*, show minimal evidence of cutin or suberin-related biosynthetic systems, suggesting the absence of these polymers, although it has been reported that *K. nitens* develops hydrophobic layers consisting of wax-like lipids deposited on a glycoprotein framework [46]. However, sequences of representatives of the Zygnematophyceae [47,48••], the sister charophyte lineage to land plants, suggest that components of the pathways leading to cutin and suberin formation evolved before the first land plants (Table 1; Figure 2). These algal species live at the margins of terrestrial and aquatic habitats, and experience transient exposure to desiccating conditions.

It is notable that cutin and suberin share biochemical features with lignin, a ubiquitous phenolic biopolymer in vascular land plants that is deposited in the secondary walls of specific tissues and cell types. Like the cuticle, appearance of lignin represents a major innovation in land plant evolution, providing structural reinforcement to allow protection against pathogens as well as formation of plant vascularization [49]. Although steps from the lignin biosynthetic pathway and lignin-like compounds have been identified in early diverging land plants, true lignin evolved with the emergence of vascular plants (Figure 3) [4]. However, the capacity to synthesize and secrete phenolic compounds is apparently a more ancient evolutionary innovation. For example, the cuticle of the bryophyte *P. patens* contains large amounts of the phenolic compound caffeic acid, which is important for the function of its cuticle [50,51], establishing an association in early land plants of the pathways giving rise to the aliphatic and aromatic moieties of cutin. However, notably, phenolic compounds have also been detected in the cell walls of several species of charophyte algae [52–54], suggesting that synthesis and secretion of at least some of the building blocks of all the major hydrophobic biopolymers predated the emergence of plants onto land.

Figure 3



Model of the evolution of extracellular hydrophobic biopolymers. Phenolic compounds are represented by hexagons, waxes by green lines and functionalized fatty acids by orange lines. An archetypal alga and land plant are shown on the left and right, respectively.

The 'ancestral' extracellular hydrophobic biopolymers likely involved the assembly of aliphatic precursors, with a lower phenolic content, to form a cutin-like protective layer, associated with an array of wax compounds, which prevented desiccation during the colonization of increasingly terrestrial habitats (Figure 3). This may then have diverged to form denser, more rigid and protective phenolic-rich suberin-like layers with additional cell wall anchoring points. This in turn led to lignin-like polymers, and subsequently true lignin composed almost exclusively of phenolic compounds, which is deposited in the secondary walls of vascular plants. The sequential emergence of cutin and then suberin polymers is supported by studies of the GPAT family [15], and as additional biosynthetic/assembly genes and protein activities are characterized similar information will likely provide important insights into the emergence of hydrophobic biopolymers.

Conclusion

The idea of distinct suberin and cutin polymers with unique compositions, patterns of distribution and functions, arose from analyses of relatively few extant angiosperms, which highlighted such differences, and these became archetypes. However, as biochemical studies have extended into a broader range of plant taxa, it has become apparent that exceptions to canonical suberin and cutin are not uncommon [8]. Indeed, the leaf and stem cutin of *A. thaliana*, which has provided the experimental model for most studies of cutin and suberin, is sometimes described as being atypical, as is the recently identified *A. thaliana* root cutin [9•]. It is clear that polyesters with varying degrees of phenolic composition, monomer chain lengths and spatial patterns of distribution are present in different specialized cell wall types. However, it seems likely that increasingly sensitive biochemical analyses, extensive exploration of compositional diversity in many more plant species, coupled with additional molecular information, will continue to reveal 'unusual' examples of these polyesters. Accordingly, canonical cutin and suberin may represent points on a continuum that has, through more than half a billion years of evolution, given rise to a remarkable range of biopolymers. The relationship between their structures, functions, and properties represents a fascinating area for future discovery.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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