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REVIEW ARTICLE

The dark side of the cell wall: Molecular genetics of plant cuticle

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Abstract
The deposition of lipid-based polymers makes the outermost layer of the plant epidermis very special. Recent advances in the study of cuticular polymers in *Arabidopsis thaliana*, an established model system for genetic studies, have made it possible to understand how plants protect themselves from the damaging effects of dehydration, toxic chemicals and pathogens. Mutational analysis of relevant pathways has revealed that a surprisingly large set of diverse traits may be affected by defects in the formation of the cuticle. Alterations in cell shape and deformation of organs in some mutants, and the expression of the corresponding genes in roots, suggest that the mutated genes may actually be required for the biosynthesis of a suberin-like material, which is deposited inside epidermal cell walls. Rapid progress in genome sequencing and the availability of chemical inhibitors offer a promising means of exploring cuticle biosynthesis in many other plant species.

Key words: *Epidermis, cuticle, cutin biosynthesis, suberin, cell wall*

Introduction

In multicellular organisms, including plants, only a minority of all cells are exposed to the environment. Specific regulatory and metabolic pathways have to be active in these cells in order to protect them and the whole plant body. The extracellular matrix produced by these cells is specialized, and it includes materials that prevent oxidation and the uncontrolled loss of water, and provide mechanical strength. The major extracellular barriers are made up of hydrophobic molecules, which are cross-linked in such a way as to form a polymeric coat that is almost impermeable to water. In electron micrographs, the osmiophilic, lipid-rich layer of the plant cell wall is not only seen as darkly staining material that delineates the outermost portion of the cell wall: this layer also represents the 'dark side' of the cell wall with regard to our understanding of its structure and development.

The focus of this review is on genes that participate in the biosynthesis of the aliphatic monomers that are the basic units of the biopolymers of the cuticular layer of the plant cell wall, and of cutin, which forms the cuticle proper. The interested reader is also referred to recent reviews covering the biosynthesis of cutin and suberin (Kolattukudy, 2001; Nawrath, 2002) and waxes (Kunst & Samuels, 2003). Our review is intended to highlight areas for future studies of cutin and suberin polymers, and to stimulate discussion and collaboration between researchers working in various aspects of cell wall research.

The borders of the apoplast system are made from suberin-like materials, while cutin is the major polymer in cuticle itself

Fortification of the cell wall by the use of lipids occurs in tissues that restrict or prevent apoplastic transport of water and solutes. Not only epidermal cells fall into this category; it also includes cells inside plant organs — whenever there is a need to build a barrier. One of the best known examples is the endodermis, the cell layer that isolates the central cylinder of the root from the cortex. In cross-sections, radial walls are characterized by Casparian bands, which represent modifications of the walls of endodermal cells. Other examples include bundle sheath cells, the hypodermis of roots, and the

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epidermal cells of the roots and aerial parts of the plant. Suberin, which consists of a polyester blend of aliphatics, aromatics and glycerol, is the main polymer that provides protection. Suberin is normally deposited on the inner surface of, and inside, cell walls, where it can be detected by staining with a number of lipophilic dyes (e.g., Sudan black) or by the autofluorescence of its aromatic components under UV.

The surfaces of the fruits, leaves and stems of higher plants are covered by a cuticular membrane that can be subdivided into the cuticular layer of the cell wall and the cuticle proper, which often has a lamellate appearance in electron micrographs. In some species, the cuticle proper can be detached and isolated in a relatively pure form, and in such cases its chemical composition is well known (Kolattukudy, 2001). Its major component is cutin. Cutin, like suberin, is a polyester but, in contrast to suberin, aromatic compounds are rare in cutin; furthermore, α-,ω-dicarboxylic fatty acids are typically not found among cutin monomers (Kolattukudy, 2001). However, recent data on the chemical composition of *Arabidopsis* cutin (Bonaventure et al., 2004; Xiao et al., 2004; Yephremov et al., 2004) suggest that cutin and suberin may be quite similar with regard to the amounts of α-,ω-dicarboxylic fatty acids that they contain.

Although the primary chemical constituents of suberin and cutin have been the subject of intensive research (Kolattukudy, 2001), the polymer structure of suberin and cutin, and of suberized and cutinized cell walls, is still an enigma. It also remains to be seen whether cell type-dependent differences occur in the monomer composition and structure of cell wall-bound polymers. Many mutants defective in cutin biosynthesis have been identified in plants, but no mutants for suberin biosynthesis have been described so far.

Many roads lead to cuticular mutants

A great deal of knowledge about cuticles has been obtained from structural studies and the use of biochemical methods, but the epidermal cuticle now represents an attractive model system for the geneticist also. However, in sharp contrast to what might be expected, finding mutants for genes involved in the biosynthesis of cutin or the formation of the cuticle has not been easy, as mutant phenotypes often give no immediately obvious indications that they have resulted from cuticular defects. However, a number of defects may now be associated with improper cuticle formation in *Arabidopsis* mutants: poor growth and performance, sensitivity to low humidity, increased sensitivity to chemicals such as pesticides and herbicides, morphological irregularities in the shapes of organs and single cells, altered resistance to pathogens, distorted cell differentiation, illicit cell–cell interactions and cell death (Table I).

This list suggests a variety of screening protocols; however, the most direct means of screening for cuticular mutants is based on the staining of plants with toluidine blue (Tanaka et al., 2004). All intact plants can take up this cationic dye provided that they are bathed in it for a sufficiently long time. However, cuticular mutants appear to stain faster and more strongly. For *Arabidopsis* plants growing in liquid medium, exposure to the dye for 2 min allows one to distinguish some mutants, whereas longer exposure periods may be required for plants that have been grown in the greenhouse (A. Y., unpublished data). Although further studies will be required to confirm this, it is likely that the time needed to stain plants is positively correlated with their ability to resist dehydration. Use of other compounds, e.g., fluorescent dyes, will probably allow for more effective high-throughput mutant screening and for the development of assays with which to study the role of the cuticle in the adaptation response.

It has been widely accepted that plant cuticles present barriers to pathogens, and that monomers resulting from the degradation of the cuticle by pathogens may serve as signalling molecules. This issue has recently received renewed attention with the description of the first disease-susceptible or disease-resistant plants with altered cuticle in *Arabidopsis* (Chassot et al., 2004; Xiao et al., 2004). It can be anticipated that this is an area that will develop rapidly over the coming years.

Cutin composition in *Arabidopsis*: An exception to the rule

Analyses of cutin composition in *Arabidopsis* have been attempted only recently. This is mainly because the cuticle of *Arabidopsis* is only 20–30 nm thick — extraordinarily thin compared with all cuticles that had been analysed previously, which have a thickness of 1 μm or more (Nawrath, 2002). Furthermore, the chemical composition of *Arabidopsis* cutin (Bonaventure et al., 2004; Xiao et al., 2004; Yephremov et al., 2004) is very different from those found in other plants. Besides the typical cutin monomers, such as saturated C_{16} and C_{18} hydroxy-fatty acids, one- and two-fold unsaturated C_{18} diacids have been found as major monomers of *Arabidopsis* cutin. These compounds have previously been described as major compounds of suberin and not cutin. In addition, 2-hydroxy fatty acids with chain-lengths up to C_{26} have been identified in *Arabidopsis* cutin (L. S., unpublished data), which are again compounds thought to

Table I. Candidate genes identified by mutation that are directly or indirectly implicated in cutin and suberin biosynthesis in *Arabidopsis thaliana*. Some genes known only by the map position may be allelic to corresponding genes identified in other laboratories.

Gene names and AGI Number	Status	Putative function	Reference
FIDDLEHEAD (*FDH*); *PERMEABLE LEAVES4* (*PEL4*); At2g26250	Cloned	Putative β-ketoacyl-CoA synthase, long chain fatty acid elongase	Lolle et al., 1992; Yephremov et al., 1999; Pruitt et al., 2000; Tanaka et al., 2004
HIGH CARBON DIOXIDE (*HIC*); At2g46720	Cloned	Putative β-ketoacyl-CoA synthase, long chain fatty acid elongase	Gray et al., 2000
LACERATA (*LCR*); CYP86A8; At2g45970	Cloned	Cytochrome P_{450}, fatty acid ω-hydroxylase	Wellesen et al., 2001
ADHESION OF CALYX EDGES (*ACE*); *HOTHEAD* (*HTH*); APB24; At1g72970	Cloned	Unknown enzyme	Lolle et al., 1998; Araki & Nakatani-Goto, 1999; Yephremov et al., unpublished results
LONG-CHAIN ACYL-COA SYNTHETASE2 (*LACS2*); At1g49430	Cloned	Long-chain acyl-CoA synthetase	Schnurr et al., 2004
ABERANT INDUCTION OF TYPE THREE GENES1 (*ATT1*) CYP86A2; At4g00360	Cloned	Cytochrome P_{450}, fatty acid ω-hydroxylase	Xiao et al., 2004
BODYGUARD (*BDG*)	Cloned	Putative carboxyesterase/synthase	Yephremov et al., unpublished results
ABNORMAL LEAF SHAPE (*ALE*); At1g62340	Cloned	Putative serine protease	Tanaka et al., 2001
ARABIDOPSIS CRINKLY4 (*ACR4*); At3g59420	Cloned	Receptor kinase	Tanaka et al., 2002; Gifford et al., 2003; Watanabe et al., 2004
PASTICCINO2 (*PAS2*); *PEPINO* (*PAP*); At5g10480	Cloned	Putative anti-phosphatase	Faure et al., 1998; Bellec et al., 2002; Haberer et al., 2002
ACETYL-COA CARBOXYLASE1 (*ACC1*); *GURKE* (*GK*); *PERMABLE LEAVES2* (*PEL2*); At1g36160	Cloned	Acetyl-CoA carboxylase	Baud et al., 2003; Tanaka et al., 2004; Kajiwara et al., 2004
WAX2; *YORE-YORE* (*YRE*); *PERMABLE LEAVES6* (*PEL6*); At5g57800	Cloned	Unknown enzyme	Jenks et al., 1996; Chen et al., 2003; Kurata et al., 2003; Tanaka et al., 2004
WAX INDUCER1 (*WIN1*); *SHINE* (*SHN*); At1g15360	Cloned	AP2/EREBP transcription factor	Broun et.al., 2004; Aharoni et al., 2004
CER10	Mapped		Koornneef et al., 1989
CER13	Mapped		Koornneef et al., 1989
WAX1	Mapped		Jenks et al., 1996
AIRHEAD (*AHD*)	Mapped		Lolle et al., 1998
BULKHEAD (*BUD*)	Mapped		Lolle et al., 1998
CONEHEAD (*COD*)	Mapped		Lolle et al., 1998
DEADHEAD (*DED*)	Mapped		Lolle et al., 1998
POTHEAD (*PHD*)	Mapped		Lolle et al., 1998
THUNDERHEAD (*THD*)	Mapped		Lolle et al., 1998
PERMEABLE LEAVES1 (*PEL1*)	Mapped		Tanaka et al., 2004
PERMEABLE LEAVES3 (*PEL3*)	Mapped		Tanaka et al., 2004
PERMEABLE LEAVES5 (*PEL5*)	Mapped		Tanaka et al., 2004

be typical of suberin (Schreiber et al. 1999). It remains to be seen whether these findings are specific for *Arabidopsis* cutin. However, the combination of biochemical and genetic approaches in *Arabidopsis* now offers an opportunity to understand the biosynthesis and assembly of the cuticular barrier in the cell wall.

Enzymic functions responsible for cuticle biosynthesis

Many of the enzymic activities involved in cuticle formation may be inferred from an analysis of the composition of cutin and suberin, but a detailed characterization of individual enzymes is either lacking altogether or incomplete. The WAX2/YRE protein is a particularly interesting example, because both cuticle structure and wax biosynthesis appear to be affected in a wax2/*yre* mutant (Chen et al., 2003; Kurata et al., 2003). It is conceivable that the two pathways (cutin biosynthesis and wax biosynthesis) may be linked, if they share an intermediate compound in a chain of enzymic reactions. On the basis of its sequence alone, it is difficult to discern at what junction point WAX2/YRE might act. FDH, a member of the FATTY ACID ELONGATION1

family is another enigmatic protein whose enzymic function remains unknown, despite indications that it may be a β-ketoacyl-CoA synthase and efforts to express it in yeast and in *Arabidopsis* seeds (Efremova et al., 2005; Trenkamp et al., 2004).

Genes that control cuticle development

Table I lists a number of genes that may be directly or indirectly involved in cutin formation in *Arabidopsis*. So far, most efforts have been concentrated on genes that are likely to be required for particular steps in the biosynthesis of cutin. However, regulatory aspects of this pathway and its relationship to the biosynthesis of other polymers and constituents of the cell wall are now coming into focus. Thus, *SHN1*/*WIN1*, which encodes an AP2/EREBP transcription factor, may be involved in regulating the metabolism of lipid and/or cell wall components, although its precise biological function has still to be determined (Aharoni et al., 2004; Broun et al., 2004). Candidates for transcription factors that may participate in the regulation of *FDH* have been found in a yeast one-hybrid screen (A. Y., unpublished results) based on conserved DNA motifs identified in the promoters of *FDH* and the orthologous *ANTIRRHINUM FIDDLEHEAD* (*AFI*) gene (Efremova et al., 2005). Table I does not include the genes *AtML1* and *PDF*, which code for homeobox proteins that are required to maintain the identity of epidermal cells (Abe et al., 2003). However, these genes may function upstream from specific transcription factors, such as SHN1/WIN1, that control the expression of cuticular enzymes in the epidermis.

Many mutants listed in Table I were not originally classified as having cuticular defects, but have been included on the basis of their phenotypic similarities to known cuticular mutants. For example, the *pasticcino* (*pas*) mutants were described as cell differentiation and proliferation mutants, but *pas2* produces post-genital organ fusions (Bellec et al., 2002) very much like those seen in a well-known class of cuticular mutant (Lolle et al., 1998). Therefore it will be interesting to see whether the cuticle of *pas* mutants is actually defective.

Time to move beyond *Arabidopsis*?

Arabidopsis is not the only plant in which genes involved in the formation of cuticle are known. A number of candidate mutants can be found in the Maize Genetics and Genomics Database (http://www.maizegdb.org/) (Yephremov et al., 1999) but only two of these, *ad1* and *cr4*, have been characterized in any detail. It seems highly desirable to extend genetic and molecular studies on cuticle to other species, including crop plants, to address biodiversity issues. The sequencing of the rice genome and ongoing projects in several other species provide unprecedented opportunities for mutant screening using reverse genetics.

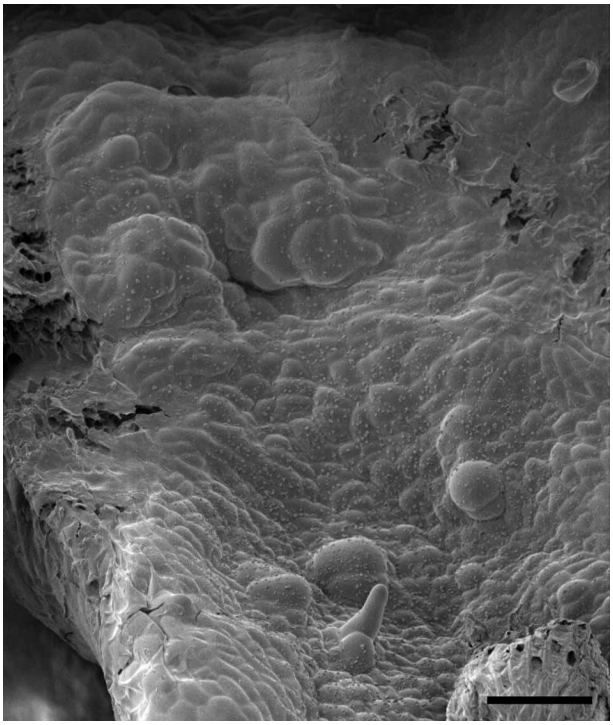

Figure 1. Scanning electron micrograph of the adaxial leaf surface of the *lcr* (*lacerata*) mutant of *Arabidopsis*. Bulging of epidermal cells in *lcr* (Wellesen et al., 2001) may be similar to that seen in cell wall biosynthesis-deficient mutants, e.g. *rsw1* (Williamson et al., 2001). Does this mean that cell wall-bound lipid polymers, cutin and suberin, play a role in cell shape maintenance? Scale bar = 100 μm.

Chemical genetics or the use of chemical inhibitors offers an alternative for exploring cuticle biosynthesis in various plant species where relevant mutants are not yet available (Lequeu et al., 2003; Efremova et al., 2005).

Do gene expression studies suggest rethinking gene functions?

Thirteen of the genes listed in Table I have been cloned, and some, like *FDH, WAX2*/*YRE, BDG* and *APB24*, have been shown to be epidermis-specific. Surprisingly, these studies revealed expression of the genes in root cells as well, although roots are considered to synthesize suberin rather than cutin, suggesting either that this tenet of plant biology does not hold or that the genes are involved in the biosynthesis of both cutin and suberin. If the latter is the case, then the intriguing questions are whether epidermal cell walls in the relevant mutants are defective in the accumulation of suberin, which is a functional component of cell walls, and whether

these defects might not be responsible for at least some of the mutant phenotypes. In particular, a number of mutants listed in Table I (e.g., *fdh*, wax2/*yre, lcr, bdg*) exhibit leaf deformations and irregularities in epidermal cell shape, which, in some cases, are reminiscent of those in mutants that are unable to carry out particular steps in cell wall biosynthesis (e.g., *radially swollen1*(*rsw1*)/*cellulose synthase A1*(*cesA1*) (Williamson et al., 2001) and *root epidermal bulger1*(*reb1*)/*root hair deficient1*(*rhd1*) (Seifert et al., 2002).

Other tissues which should be analysed in this respect are the papillar region and the septum of the pistil and the endothelium of the ovule, because these tissues participate in cell – cell interactions with pollen and the pollen tube, or border the nucellus and embryo sac. Isolating properties of the cell wall or cuticle in these cases may be critical for the regulation of cell – cell communication and development. It has been shown that *FDH* is expressed in the inner integument (Efremova et al., 2005), corroborating the earlier finding by transmission electron microscopy that an electron-dense layer can be seen facing the embryo sac on inner cell walls of the inner integument (Beeckman et al., 2000).

Conclusions

Plant cells are surrounded by the cell wall, which forms a relatively strong envelope. However, it is not sufficiently robust to provide protection against hostile environmental factors. The development of extracellular barriers to water- and solute-flow has allowed plants to bolster their defences against such factors and to regulate apoplastic transport. The extracellular framework of several cell types is strengthened by the deposition of hydrophobic lipid-based biopolymers. An integral view of how extracellular barriers are constructed needs to take into account all cell wall materials, including polysaccharides, proteins and lipid-based cell wall-bound polymers. This area, also important from a practical perspective, is ideally suited for collaborative research interactions, and will no doubt stimulate such cooperative projects in the coming years.

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