12

Cutin Monomers

Biosynthesis and Plant Defense

ELIZABETH BLÉE

Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France

1 INTRODUCTION

The plant cuticle, which covers virtually all the aerial surfaces of vascular plants, is chemically heterogeneous in nature, because it is generally composed of a mixture of insoluble polymers and waxes, which are deposited on the outer surface (epicuticular waxes) and embedded in the matrix (intracuticular waxes). The soluble cuticular waxes consist of monoesters of very long chains of fatty acids (C-18–C-22), alcohols (C-22–C-26), and a wide variety of other aliphatic and aromatic molecules [1–4]. The insoluble matrix is constituted by (1) cutin, a three-dimensional polymer network of esterified fatty acids [1,5] and (2) cutan, a nondegradable biopolymer made of aliphatic chains presumably cross-linked by ether bonds [6]. In addition, the cuticular layers also contain nonlipid components such as polysaccharides, phenols, and flavonoids. Large variations have been found in cuticular fine structure, wax load, or cutin composition. Indeed, the structure of the cuticle varies considerably depending on the species and on the age of the plant, but also between leaves, fruits, and stems of a single species and even between the upper and lower surfaces of individual leaves [7]. This complexity may explain the wide diversity of functions fulfilled by the cuticular layer. As an interface between the plant and its environment, the cuticle plays a key role in providing protection from mechanical damage, ultraviolet (UV) radiation [8,9], or penetration by fungal hyphae and insect mouthparts [10]. It also constitutes the main barrier limiting the transport across the plant–atmosphere exchange zone, impeding the foliar uptake of xenobiotics but also reducing the uncon-

Copyright © 2002 Marcel Dekker, Inc.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

trolled loss of water and apoplastic solutes from plant tissues [11,12]. It seems that waxes are largely responsible for the low permeability of cuticles, causing a lognormal distribution of transport parameters [13] and therefore contributing for example to the regulation of the cuticular transpiration. Permeability of cuticles differs greatly among plant species and it has been argued that these differences are more likely due to the physical arrangement of waxes, rather than their amounts or composition per se [14]. On the other hand, if the constitutive waxes markedly inhibit sorption of a wide range of organic compounds, they may not always form the main barrier to the penetration of chemicals through plant cuticles [15]. They do favor the penetration of biologically active compounds by acting as a compartment in which lipophilic compounds can accumulate [16]. Moreover, these lipids reduce water retention on the plant surface, which bears important consequences on germination of spores, survival of microorganisms, or deposition of dust, pollen, and so forth. Additionally, the chemical composition of epicuticular waxes may influence the interaction of herbivorous insects, with their plant hosts [17,18] acting as well in tritrophic interactions, by affecting the settling and oviposition behavior of some predators and parasitoids of these herbivores [18]. It was also reported that waxes found in the tryphine layer of pollen grains are essential for proper pollen-stigma signaling required for fertilization [19].

It is generally admitted that the biosynthesis of epicuticular waxes proceeds via multienzymatic complexes; both the proposed metabolic pathways by which wax constituents are synthesized and the genetic studies of mutants were reviewed in the past few years [20–22]. In sharp contrast with the mass of information which became recently available for the cuticular waxes, very little work has been devoted to the cutin matrix since the pioneering work of the groups of Kollatukudy and Holloway. The view of the cutin matrix was classically restricted to an inert scaffolding of the cuticle, but it appears that its components may act more actively as participants for signaling across the divider during the pathogen attack. The aim of this chapter is to summarize the most recent data concerning this quite new role for cutin monomers in plant defense, in addition to the latest insights in their biosynthetic pathways.

2 BIOSYNTHESIS OF CUTIN MONOMERS

2.1 Composition of the Cutin Biopolymer

If the investigations of waxes have been facilitated by their ease of extraction with organic solvents, cutin composition analysis was initially hampered by the difficulty encountered in the depolymerization of this biopolymer, which required quite strong chemical procedures. The first clue to the chemical nature of cutin components emerged from the investigations of Matic [23], who isolated hydroxylated fatty acids from the ether-soluble fraction obtained by extraction of hydrolysis products of the leaf cuticle of *Agave americana*. The hydroxy fatty acids that were characterized included 9,10,18-trihydroxystearate, 10,18-dihydroxystearic acid, 18-hydroxystearic acid, and 10,16-dihydroxypalmitic acids. The trihydroxystearic acid was found at that time to be predominant in most of the mixtures of cutin hydrolysates. Later, the use of milder degradative chemical methods (i.e., alcoholysis) confirmed 9,10-epoxy-18-hydroxyoctadecanoic acid as a common cutin constituent [24]. Since then, partial depolymerization of the cutin polyester has been accomplished using various chemical and enzymatic methods, including hydrogenolysis with $LiAlH_4$, hydrolysis with alcoholic KOH or HCl, and breakdown with cutinase [25]. Cutin

Copyright © 2002 Marcel Dekker, Inc.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

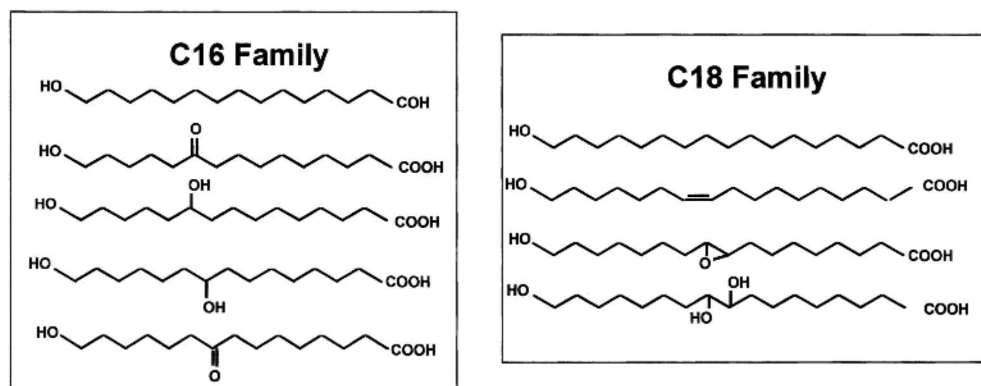

Figure 1 Structure of the major cutin monomers.

analysis of a large number of plants have established that plant cutins are built from a sole series of substituted C-16 and C-18 alkanoic acids (Fig 1). Species differ from one another primarily in the relative proportions of these two groups. For example, the cutin of bean leaves contains mostly C-16, in contrast to Poacae leaf cutin, which contain predominantly C-18 components. Commonly, cutin includes monomers of the two groups.

Although, from early investigations [26], cutin was described as an esterlike material, the details of how the monomers are linked together are still elusive. Clearly, the structural framework of cutin will depend on the chain lengths and on the number and positions of esterifiable groups in the monomers. Elongation of this biopolymer likely involves primary hydroxyl groups, whereas its reticulation comprises the secondary ones.

Such a network of hydroxy fatty acids linked by primary and secondary alcohols derived ester bonds has recently been confirmed in the case of lime cuticle (composed essentially of monomers of the C-16 family), observed by electron-impact mass spectrometry supported by solution-state nuclear magnetic resonance (NMR) spectroscopy [27]. These newly accessible analytical tools should permit a better understanding of the intermolecular structures of cutin biopolymers.

It was reported that the entire cutin biopolymer could not be depolymerized by treatment with BF_3-methanol, which cleaves ester linkages [28]. This was attributed to the presence of large amounts of epoxy fatty acids in the cuticles, which could form ether bonds. The formation and the complexity of the cross-linkages in cutin was found to increase with the age of the leaves [29], possibly reducing their water-permeability capacity.

2.2 Biosynthesis of the C-16 Cutin Monomer Family

Research on the biosynthesis of cutin components has been neglected during the past two decades. Until a few years ago, nearly all of our knowledge about cutin biosynthesis was based on the initial work of the group of Kolattukudy in the 1970s. Using labeled acetate, palmitate, and 16-hydroxypalmitate, these researchers proposed a major biosynthetic pathway for the C-16 cutin monomers, starting from palmitic acid and involving first the hydroxylation of this unsaturated fatty acid at the ω-position, followed by its in-chain (on carbon-9 or carbon-10) oxidation [30]. However, the recently identified 10-hydroxyhexa-

Copyright © 2002 Marcel Dekker, Inc.

decanoic acid in lime fruit cutin indicates that ω-hydroxylation of palmitic acid is not a requisite, as hydroxylation of palmitic acid may also take place at other chain positions during formation of the polyester matrix [31].

Oxidation of the C-16 extremity, studied in *Vicia faba* microsomal fractions, exhibited features of cytochrome P-450-dependent catalysis (i.e., dependence on molecular oxygen, requirement of NADPH as cosubstrate, inhibition by CO) although the reversion of this inhibition by light was not obtained [32]. It should be noted that such a reversion by photons of the binding of CO to the ferryl-oxo complex can be difficult to achieve (Werck-Reichhart, personal communication). At this time, purification of the enzyme responsible for ω-hydroxylation of palmitic acid was not reported. Recently, *Arabidopsis thaliana* express sequence tag (EST) databases were screened with consensus motifs derived from families of P-450 catalyzing the ω-hydroxylation of fatty acids and alkanes in *Candida* and in mammals. Hereby, a gene, CYP86A1, encoding a similar enzyme was obtained [33]. Except for stearic acid, which was not metabolized, lauric, (C-12) myristic (C-14), and palmitic (C-16) acids were efficiently oxidized at their terminal position, the latter being the best substrate. However, no physiological role has yet been attributed to this new plant P-450 family. In parallel, another plant cytochrome P-450-dependent fatty acid ω-hydrolylase (CYP94A1) was cloned and expressed in *Saccharomyces cerevisae* over-producing a reductase from *Arabidopsis thaliana* [34]. This cytochrome P-450-dependent enzyme was tagged in *Vicia faba* microsomes, with a radiolabeled suicide inhibitor allowing its isolation and cloning. As expected, lauric acid was shown to be the best substrate for the expressed CYP94A1, but this enzyme is also able to oxidize other saturated fatty acids ranging from C-10 to C-16. The K_m value for palmitate was lower than that measured for the other substrates, and its was suggested that CYP94A1 could be involved in the biosynthesis of cutin monomer [34]. Unfortunately, no experimental data were provided in this work, confirming the claimed accumulation of CPY94A1 transcripts during plant development or wounding. This could have constituted a first indication of the implication of such a cytochrome P-450 in cutin formation or cuticle repair.

In addition to 16-hydroxypalmitic acid, 9,16- and 10,16-hydroxypalmitic acids are also present in plant cutins. It has been suggested that in-chain hydroxylation reactions result from cytochrome P-450 catalysis. In this regard, the first indication of a P-450-dependent in-chain hydroxylase was reported recently in *Helianthus tuberosus* tuber [35]. Such P-450, designed CPY81B1, catalyzed the hydroxylation of medium-chain saturated fatty acids, the major site of attack being at carbon-9 (capric and lauric acids) or carbon-10 (myristic acid), depending on the length of the aliphatic chains. In addition, a complex mixture of in-chain monohydroxylated derivatives was generated by this enzyme, denoting that saturated medium-chain fatty acids are probably not the physiological substrates. No longer-chain fatty acids such as palmitic or oleic acids were substrates for CYP81B1, ruling out the participation of this enzyme in cutin biosynthesis.

Thus, at present, data on the involvement of the already cloned cytochrome P-450-dependent hydroxylases in C-16 cutin monomer formation are not conclusive, but, hopefully, the systematic cloning of such plant enzymes will lead to better candidates in the future.

It should also be emphasized that in addition to cytochrome P-450, nonheme hydroxylases have been characterized in recent years. For example, ricinoleic acid is synthesized by direct hydroxy substitution at the 12-position of oleic acid by a diiron protein, related to a fatty acid desaturase family, which requires oxygen, NADH, and cytochrome b_5 for its activity [36]. Such a 12-hydroxylase can produce lesquerolic acid, a 14-hydroxylated

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

eicosanoic acid, but the possibility that this oxidase may catalyze in-chain hydroxylations at other positions with appropriate substrates is far from proven.

2.3 Biosynthesis of the C-18 Cutin Monomer Family

2.3.1 Epoxidation Step Catalyzed by Cytochrome P-450s?

In the early seventies, Kolattukudy and colleagues made the seminal finding that the biosynthesis of the C-18 family of cutin monomers proceeds through formation of epoxy derivatives [37]. [1-^{14}C] Oleic acid was rapidly incorporated into 9,10-epoxy-18-hydroxystearic acid, as well as 18-hydroxystearic and 9,10,18-trihydroxystearic acids by skin slices of young apples, grape and berry skins, and apple leaves. Likewise, 18-hydroxyoleic acid was transformed into its corresponding epoxy and dihydroxy derivatives leading to the conclusion that the sequence yielding the major C-18 cutin monomers was ω-hydroxylation, epoxidation of the double bond, and hydration of the epoxide. Trichloropropene oxide (TCPO), a noncompetitive inhibitor of microsomal epoxide hydrolase [38], was found to reduce incorporation of [1-^{14}C] oleic acid into 9,10,18-trihydroxystearic acid but also, surprisingly, into the epoxide [39]. A preparation from spinach leaves centrifuged at low speed (3000*g* and therefore probably enriched with nucleus, chloroplast fragments, unbroken cells, pieces of tissues, as well as cell walls and cutin biopolymer) was able to epoxidize the double bond of 18-hydroxyoleic acid. In contrast, oleic acid was a very poor substrate [40]. This epoxidase required molecular oxygen and NADPH for its activity, and it was inhibited by CO (this inhibition was completely reversed by light), Consequently, it was identified as a cytochrome P-450-dependent oxidase. Because CoA and ATP were needed for this catalysis, it was proposed that the CoA ester was the physiological substrate for this epoxidase [41]. Since this initial study, purification and further characterization of this protein have, however, not been accomplished.

This last decade, several oxidases have been demonstrated to be capable of oxidizing double bonds of unsaturated long-chain fatty acids. In particular, linoleate, linked to phosphatidylcholine in microsomes from developing seeds of *Euphorbia lagascae*, was epoxidized into vernolate in the presence of NADPH [42]. The formation of this derivative exhibited all of the characteristics of a cytochrome P-450-catalyzed reaction, including its inhibition by an anti-NADPH cytochrome P-450 reductase antibody. This cytochrome P-450-dependent epoxidation was characterized by a remarkable regioselectivity and enantioselectivity, [i.e., only the 12(*S*), 13(*R*)-enantiomer was formed in the endosperm of the seeds] [43]. Moreover, no compounds derived from 9,10-epoxy-12-octadecenoic acid were found to accumulate, no 9-epoxystearate was formed from [^{14}C] oleate, and only one of the three double bonds of linolenate was epoxidized, indicating that this epoxidase was specific for the C-12,13-position [42]. Because the biosynthesis of C-18 cutin monomers starts from 9-unsaturated octadecanoic acid, this cytochrome P-450 appears as an unlikely candidate for the formation of cutin epoxides.

2.3.2 Epoxidation Step Catalyzed by Diiron Proteins?

Another oxidase was recently shown to catalyze the epoxidation of the C-12,13 bond of linoleic acid. This epoxygenase, isolated from *Crepis palaestina* (related to the castor bean), required NADH or NADPH for its activity but was unaffected by carbon monoxide or antibodies to cytochrome P-450 reductase [44]. The gene encoding this epoxygenase was cloned and the deduced amino acid sequence was similar to that of other nonheme diiron proteins, such as the Δ12-hydroxylase (mentioned in Sec. 2.2), Δ12-desaturase,

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

and Δ12-acetylenase, all of them being integral membrane proteins. It has been shown that minor changes (as few as six amino acids) resulted in the conversion of an oleate hydroxylase to a desaturase [45], and it can be speculated that few changes will be needed for the recognition of an oleate instead of a linoleate substrate. Nevertheless, it seems that such enzymes are restricted to certain classes of higher plants such as *Euphorbeacae*, because neither vernolic acid nor ricinoleic acid is, for example, ever present in soybean or *Arabidopsis* seeds [46].

2.3.3 Epoxidation Step Catalyzed by Peroxygenases?

A third enzyme, a membrane-bound hemoprotein, has been reported likewise to catalyze the epoxidation of the C-12,13 double bond of linoleic acid. Characterized first as a hydroxylase [47] and then as a sulfoxidase [48], the peroxygenase was shown to actively catalyze, in the presence of alkylhydroperoxides as cosubstrates, the epoxidation of mono unsaturated and polyunsaturated fatty acids such as oleic acid or linoleic acid. The epoxidase activity was first discovered in soybean seedlings [49] and then in broad beans [50], and similar results were obtained later with another bean species [51]. However, this enzyme seems ubiquitous and is not only confined to the Leguminosae. Octadecenoic acids were found to be better substrates than shorter monounsaturated fatty acids (C-16 or C-14), but the position of the double bond (at position 6, 9, or 11) had little effect on the rates of oxidation. Only cis unsaturations were oxidized with retention of configuration resulting in *cis*-epoxide. Linolenic acid was oxidized with important regiofacial and enantiofacial selectivities [52] into the two positional monoepoxides and, as a minor product, the diepoxide. Analysis of the composition of various cutins had revealed that they contain epoxy and hydroxy derivatives at C-9 but also at C-12, yielding pentahydroxy acids. For example, 9,10,18-trihydroxy-12,13-epoxystearate and 9,10,12,13,18-pentahydroxystearate were found in *Rosamarinus officinalis*, probably arising by epoxidation and subsequent hydration of both the C-9,10 and C-12,13 epoxides of ω-hydroxylinoleic acid [53], although the order of the sequence of the reactions could differ. Because the peroxygenase did not present a strict regioselectivity, it should therefore be a favorable candidate for epoxidizing both oleic and linoleic acids into the precursors of cutin monomers. Using soybean microsomes, we have demonstrated that peroxygenase associated with a membrane-bound epoxide hydrolase and a cytochrome P-450 can catalyze, in vitro, the formation of the major cutin monomers of the C-18 family [54], but we did not establish real biological connections to cutin formation. Now we can provide (Blée et al., unpublished data) unequivocal proof for the involvement, in vivo, of plant peroxygenase in cutin biosynthesis. We have specifically altered peroxygenase activity via a mechanism-based inhibitor. No effect of this compound on other enzymes potentially involved in the formation of cuticle was noticed, such as membrane-bound and soluble lipoxygenases, epoxide hydrolases, cytochrome P-450, or fatty acid elongases. The specific inhibition, *in planta*, of the peroxygenase led to a dramatic decrease of content of cuticular epoxides from maize leaves, which could be vizualized by using a specific ultrahistochemical reaction [55]. This effect was restricted to plants, which are coated with a cutin rich in C-18 monomers. Accordingly, cuticles of treated plants which possess a majority of cuticular C-16 acids, such as pea, soybean, or vetch, remained unchanged. Moreover, we have established the existence of a strict relationship between the inhibition of the peroxygenase activity and the modification of the cuticle that is triggered by different molecules closely related to the inhibitor initially used. Altogether, these results constitute the first evidence of the physiological implication of possible biosynthetic enzymes in cutin formation but, most

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

importantly, confirm the plant peroxygenase as a key oxidase for the epoxidation of cutin monomers.

Consequently, because peroxygenase activity is dependent on alkyl-hydroperoxides, the biosynthesis of cutin monomers will be also under the control of the formation of such hydroperoxides. Fatty acid hydroperoxides are formed in the plant via chemical and/or enzymatic reactions; it is well known that oxidative stress due, for example, to injury can lead to chemical lipid peroxidation in membranes and that hydroperoxides are products of lipoxygenase-catalyzed oxidation of linoleic and linolenic acids. Heinen and Brand [56] had already reported stimulation of lipoxygenase activity during synthesis of cutin in wounded leaves. Moreover, an increasing number of publications deals with the induction of lipoxygenase activities during stress conditions, such as infection, injury, germination, and growth, some of which are known to lead to the biosynthesis of cutin. We have shown that a membrane-associated lipoxygenase was involved in the oxidative metabolism of oleic acid. However, we cannot exclude completely the participation of H_2O_2 in cutin formation. The level of this hydroperoxide is strongly enhanced by stress conditions, constituting with other activated oxygen radicals the so-called oxidative burst and may act as cosubstrate for peroxygenase during cutin repair. Nevertheless, it seems now that under conditions where alkyl-hydroperoxides are available, the biosynthetic pathways of cutin monomers involve peroxygenase activity as a potent epoxidase of unsaturated fatty acids. When hydroperoxides are limiting, one cannot a priori exclude a basal pathway involving also a cytochrome P-450-dependent epoxidase, as suggested previously by Croteau and Kolattukudy [39]. We and others [54,57] have, however, been unable to detect such an activity in bean-cell-free extracts. Related to this point, it is interesting to note that Croteau and Kolattukudy have observed incorporation of labeled fatty acid precursors into cutin only with rapidly growing tissues, such as young fruits or developing leaves i.e., precisely under conditions where high levels of lipoxygenase activity are classically found [57]. Curiously, it was reported that a spinach preparation capable of epoxidizing oleic acid via a cytochrome P-450-like activity showed no measurable ω-hydroxylase activity with this acid as the substrate [39]. This observation is in agreement with a subsequent report, which pointed out that in-chain and ω-hydroxylation P-450-dependent activities do not coexist in a single plant [58].

2.3.4 Epoxide Hydration Step

The presence of a vicinal diol in the C-18 cutin skeleton most likely results from hydration of the corresponding epoxide by an epoxide hydrolase. Among the first authors to suggest the presence of such an activity in plants were Croteau and Kolattukudy, who described the hydration of 9,10-epoxy-18-hydroxystearic acid to *threo*-9,10,18-trihydroxystearic acid by a particulate fraction prepared from the skin of young apples [59]. More recently, we have been able to purify and characterize a soluble form of an epoxide hydrolase from soybean that preferentially catalyzes the hydration of unsaturated fatty-acid-derived epoxides [60]. Furthermore, the isolation and expression of cDNAs encoding soluble epoxide hydrolases from soybean and two other plant species have been reported [61–63] and the enzymes tentatively ascribed as members of the HYL3 family [64]. It is clear now that multiple forms of epoxide hydrolase exist in plants, depending on the species and on the subcellular fractions examined. For example, besides the well-characterized cytosolic fatty acid epoxide hydrolase found in soybean seedlings, there also exists a membrane-bound isoform, which we have proposed to be involved in cutin synthesis [54]. This latter isoform hydrated preferentially the epoxide enantiomers formed prevalently by peroxygenase whereas 9,10-

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

epoxy-18-hydroxystearic acid seemed to be a poor substrate for this enzyme. Immunolocalization and *in situ* hybridization experiments revealed that the epoxide hydrolase is localized mainly in the epidermis of young leaves, substantiating their significant involvement in the formation of hydroxy cutin acids (Blée et al, unpublished data).

2.3.5 ω-Hydroxylation Step

Hydroxylation of oleic acid and of its epoxy and dihydroxy derivatives were catalyzed by a cytochrome P-450 [54,57]. Recently, it was reported that CYP94A1, initially proposed to be involved in C-16 cutin monomer formation (*vide supra*), oxidized 9,10 epoxy-stearate very efficiently with a K_m value of 1 μ*M*, thus in the physiological range [65]. Interestingly, this cytochrome P-450 prevalently ω-hydroxylated the 9(*R*),10(*S*) enantiomer [65] (i.e., the isomer preferentially formed by peroxygenase) [49].

2.4 Biosynthetic Schemes

The biosynthetic scheme that we propose (Fig. 2) involves the peroxygenase pathway and includes most of the results related to this subject, as well as earlier reports which showed that cutin monomers could derive from reactions catalyzed by ''fatty acid oxidases'' and ''lipoxidase'' [56].

At the onset, oleic acid could be a substrate for ω-hydroxylation by a cytochrome P-450 and an epoxidation by a peroxygenase. The latter enzyme seems to be constitutively expressed; in contrast, cytochrome P-450 isoenzymes generally need to be induced, their basal activity being particularly low in plants. It follows that in the presence of endogenous fatty acid hydroperoxides, peroxygenase, an oxidase which compared with cytochrome

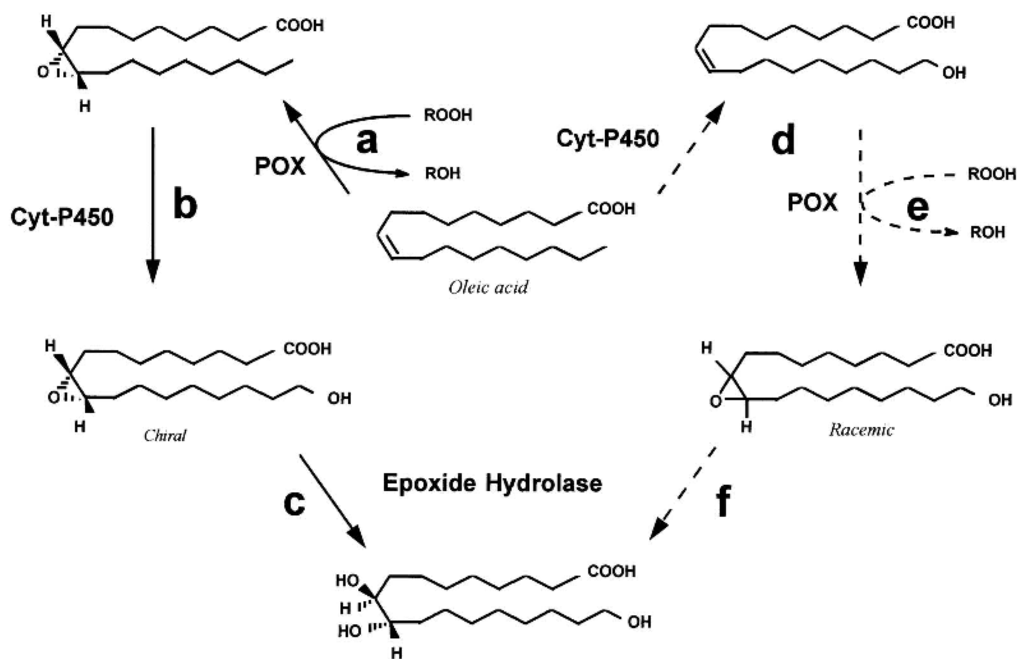

Figure 2 Biosynthetic scheme proposed for C-18 cutin monomers.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

P-450-dependent enzymes are characterized by a high turnover number [52], should actively metabolize oleic acid (Fig. 2a). By reasoning on the specificity expressed by both the peroxygenase and the ω-hydroxylase [51,65], it appears that the favored route to 9,10-epoxy-18-hydroxy stearate from oleic acid would be pathway a–b. In comparison, pathway d–e involves a succession of least preferred substrates [54]. We also hypothesized that the first pathway, a–b, will yield to chiral 9,10-epoxy-18-hydroxystearate, in line with the strong stereoselectivity of both peroxygenase and ω-hydroxylase, which lead to such compounds in vitro. In contrast, pathway d–e will give a racemic epoxy–hydroxy derivative. From our scheme (Fig. 2), we also predict that the stereochemistry of the cutin monomers should predominantly be determined by the stereoselectivity of the peroxygenase and the epoxide hydrolase, and thus a single stereoisomer [i.e., 9(*R*), 10(*R*), 18-trihydroxystearic acid] should be formed. This stereochemistry corresponds to the one reported for a compound already described in *Chamaepeuce* seed oil [66], but, unfortunately, the absolute stereochemistry of most cutin components remains unknown to date.

It should be emphasized that the biosynthesis and composition of cutin will not only depend on the presence of specific substrates but also on the relative expression, activities, and compartmentation of the biosynthetic enzymes at a given time of development. For example, 9,10,18-trihydroxystearate was found in older leaves rather than in young ones [7]; this observation is in agreement with the detection of gene expression for soluble epoxide hydrolase (*Arabidopsis thaliana*) in aged leaves and stems [62]. Likewise, a particularly low amount of this trihydroxy derivative in spinach [5] could be correlated with a very low level of fatty acid epoxide hydrolase in this plant [60].

In summary, the scheme given in Figure 3 represents a *dynamic* combination of possible pathways which are modulated by the presence and levels of the involved biosyn-

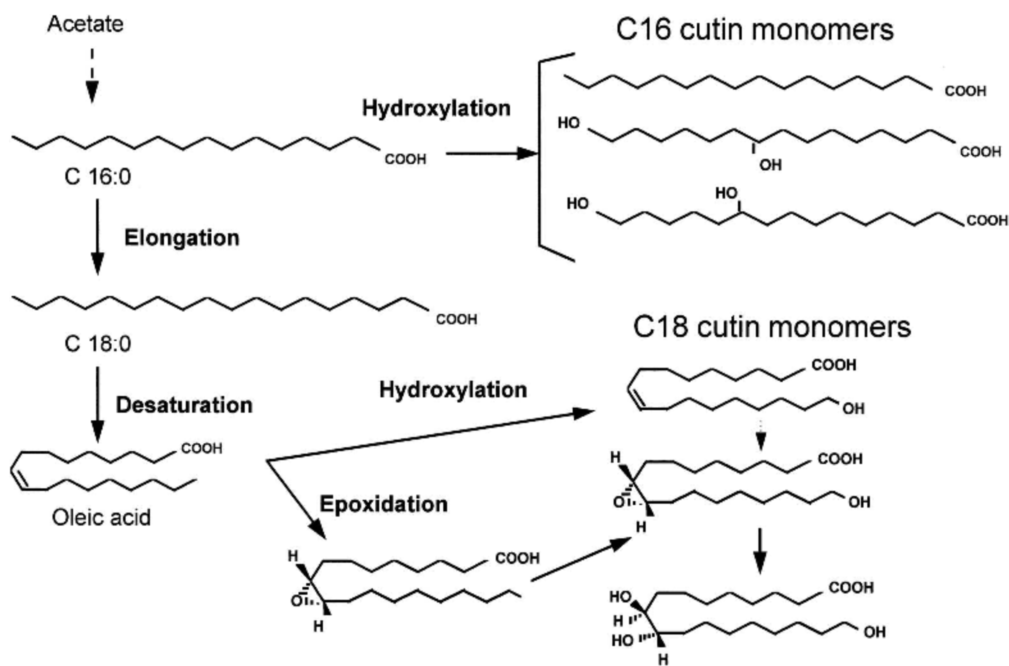

Figure 3 Hypothetical scheme for the biosynthesis of cutin monomers.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

thetic enzymes reflecting the diverse composition of cutins between plants grown under a variety of environmental conditions. It should be emphasized that some of the crucial enzymes involved in the biosynthesis of C-18 cutin monomers, such as peroxygenase, ω-hydroxylase, or epoxide hydrolase, appeared to be constitutively present in plants that are coated with cuticles poor in these C-18 monomers, such as soybean [54]. This raises the question of the regulation of the biosynthetic scheme (Fig. 3) in such plants. Besides compartmentation of such enzymes, one could imagine that the bottlenecks of the cutin biosynthetic steps are specific elongases or desaturases, which could be absent or inactive (inhibitors or regulatory elements) in epidermal cells, rendering plants unable to synthesize C-18 precursors of cutin, thus yielding peroxygenase and ω-hydroxylase functionally orphaned. At present, we have no experimental evidence for this attractive idea.

2.5 Formation of Cuticle

Little is known about the biochemical mechanisms underlying the polymerization steps involved in the synthesis of cutin and the transport of the monomers to the surface of the cells. Whereas epicuticular waxes may crystallize spontaneously at the surface of the leaves or self-assemble into lamellae [67], polymerization of cutin requires enzymatic catalysis. The unique work in this field comes from the group of Kollatukudy, which showed that particulate enzyme preparations from two species were able to catalyze the incorporation of labeled C-16 cutin monomers into cutins, with ATP and CoA as the required cofactors. Such incorporation was stimulated by increasing the number of free hydroxyl groups in the cutin primer. Consequently, an extracellular transacylase was proposed to transfer CoA esters of the incoming monomers to the hydroxyl groups of the growing polymer [68]. No further characterization of this acyl-CoA–cutin transacylase was accomplished.

Hydrophobic cutin monomers and waxes undoubtedly must cross the aqueous environment of the apoplast from the epidermal cells, where they are synthesized, to reach the site of cutin synthesis and wax assembly in and outside of the cell wall. Lipid transfer proteins (LTPs) were recently suggested to facilitate the transfer of these cuticular components. Such proteins were initially characterized by their ability to catalyze the exchange of a large number of lipid molecules between natural and artificial membranes in vitro [69,70]. They also exhibit antibacterial and antifungal properties [71] and may, likewise, act in defense against pathogens in accordance with their high concentrations in waxes of some leaves [72], making them the major proteins in surface wax of some plants [73]. Their extracellular location [74], their capacity to bind palmitic and oleic acids [75], as well as their acyl-CoA derivatives [76], and the expression pattern of their encoding genes [74,77–79] led to the proposal that LTPs could play a role in the transport of cutin monomers through the extracellular matrix to sites of cutin synthesis. The fact that heavy metals, such as cadmium, triggered expression of barley LTP genes, followed by a thicker cuticle wax layer, adds circumstantial evidence to the hypothesis that LTP could function in the transfer of wax/cutin monomers from the site of their synthesis to the cuticle [80]. The binding mode of these proteins is still not fully understood. It appears that even if sequences and three-dimensional structures are very similar among plant LTPs, the shape and the volume of their internal cavity could vary considerably. For example, barley LTP was able to expand its hydrophobic cavity significantly upon binding of a ligand [81], whereas LTP from maize could bind easily only fatty acids of 16–19 atoms of carbon [76].

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

Moreover, although palmitate could bind to both barley and maize LTPs, the orientation of this fatty acid into the two proteins was exactly opposite [82]. Thus, the mechanism in situ for the transport of cutin monomers has yet to be assessed.

The compartment where cutin monomers are synthesized within the cells and the mechanism of their assembly at the surface of, for example, the leaves with the other cuticular components are also still unknown. Likewise, the regulation of the biosynthesis of cutin and waxes during growth or membrane repair is an open question. We have observed a decrease in the global cuticular thickness, paralleled with a fading of the epoxides in plants treated with an inhibitor of the peroxygenase, suggesting that regulation of the cotransport and/or the association of cutin monomers to waxes may occur. Finally, the nature of the attachment of cutins to cell walls remains unknown, probably the cutins are covalently bound to polysaccharides of the cell walls [83].

3 CUTIN MONOMERS AND PLANT DEFENSE

When intact, the cuticle will play its role of a protective layer against mechanical damage by covering all the aerial parts of a plant. Wounds, cracks, and breachs represent sites where cutin components will be released and act as a signal used both by the microbe to facilitate its penetration and by the plant to resist pathogen aggression.

3.1 Cutin as Structural Component of Cuticle

Cutin is known to be the chief structural component of the cuticle and is regarded as its strengthening constituent. One of the scarce reports on mechanical cuticular properties confirmed that cuticles are of importance for plants as reinforcing elements, especially in the case of organs which contain little fibers or those with a cuticle thicker than the epidermal cell wall [84]. The thickness of cuticles could be one of the factors causing leaves to be better protected against all kinds of mechanical injury because they will be harder to pierce, tougher to tear, and, therefore, difficult to consume [85]. However, no correlation between thickness and breaking stress has been found for fruit cuticles.

Cuticle is elastic and can be under tension and, therefore, may be part of the growth-limiting structures, which include epidermal cell walls, contributing to the restriction of cell expansion [86]. Its viscoelasticity is mainly due to its cutin matrix, whereas waxes confer its rigidity [87]. The toughness of the cuticle is therefore likely to be dependent on the distribution and composition of cutin, but it also depends on the nature of its association with other cuticle components.

3.2 Cutin Monomers as Signal Molecules Used by Pathogens

The cuticle acts as a prime mechanical barrier through which pathogenic fungi and insect mouthparts must breach for direct penetration of the epidermis, even if they may invade plants through natural openings such as stomata or through wounds [88]. Direct penetration through the cuticle could occur via two mechanisms. One implies the physical force of the growing hyphal tip due to increasing hydrostatic pressure by melanin in appressoria of certain pathogens such as *Magnaporthe grisea* [89,90]. The second involves the weakening or the desintegration of the protective barrier, resulting from the hydrolysis of the esters bonds present in the cutin matrix by cutinases. Fungal pathogens typically produce

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

such enzymes, and the first cutinase purified came from *Fusarium solani*, the asexual form of *Nectria haematococca*, saprophytically grown on cutin as the sole carbon source [91]. A cutinase was detected immunologically at the site of penetration of the fungus into the host *Pisum sativum* [92]. In parallel, the structure of the cuticle was modified after the contact with the fungus, probably as the result of enzyme activity [93]. Because expression and release of cutinase were triggered by monomers of cutin, it was postulated that conidia of virulent fungi can sense the contact with plants via cutin monomers that are released by the small amounts of cutinase carried on the conidia. These cutin monomers then amplify the production of cutinase in the germinating spores to assist the penetration into the host [94]. Among the cutin components, 10,16-dihydroxypalmitic and 9,10,18-trihydroxystearic acids were found to be the best inducers of cutinase transcription, which is believed to be due to the phosphorylation of cutinase transcription factor(s) [95–98]. Several lines of evidence supported this scenario. Disrupted cutinase genes as well as inhibitors and antibodies toward cutinase prevented fungal infection [94,99–101]. Mutants with poor content of cutinase presented low virulence, which could be restored by addition of exogenous cutinase [102–104]. Pathogens unable to infect plants devoid of wounds or breaches could penetrate intact surfaces of papaya fruits once a cutinase gene from a virulent fungus has been inserted by genetic engineering [105]. Nonetheless, the requirement of cutinase in pathogenic processes, and therefore the involvement of cutin monomers, has been a matter of debate over these last years [106–110]. Concerning this point, the findings that distinct classes of cutinases function either during the saprophytic mobilization of cutin or during early stages of plant infection may reconciliate the different theories [111]. Until now, disruption or mutation of cutinase genes were mainly applied to genes encoding for enzymes predominantly expressed during saprophytic stages of the respective pathogens, leaving intact significant amounts of cutinases which may play decisive roles in pathogenicity [112]. Accordingly, different cutinases–esterases have recently been shown to be induced in fungi and therefore be implicated in the initiation of the infection process [113–116]. For example, it was shown that a serine esterase, related to a lipase, was secreted by *Botrytis cinerea*, and it possessed strong cutinolytic activity [117]. Antibodies raised against this protein inhibited its catalytic activity but also prevented the infection of tomato leaves by conidia of this fungus. Because the enzyme was stimulated by cuticle components, it was postulated that it was probably involved in the penetration of the host surface during plant infection [118].

In addition to their general controversial role in cuticle penetration, involvement of cutinases in other steps of the infection process has been suggested. For example, it was proposed that these hydrolytic enzymes contribute to the adhesion of spores to host surfaces [115,119] (i.e., a process often associated with enzymatic modification of the host cuticle [120]). Moreover, release of cutin monomers may affect germination of fungal spores because it was shown that they could act as signals to trigger appressorium formation [121]. Among the major cutin monomers, 9,10-epoxy-18-hydroxy-octadecanoic acid was, by far, the most effective inducer of this infection structure [122].

3.3 Cutin Monomers as Signal Molecules Used by Plants

When a plant is challenged by an aggressor, a series of complex events are triggered upon perception of the transduced signals. This includes production of active oxygen species (‘‘oxidative burst’’), ion fluxes, changes in extracellular pH and in membrane potentials,

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

and activation of several phosphorylase and kinase cascades, all these events ultimately lead to various defense responses. The mechanism by which a plant perceives an aggression has been a challenging question these last years. Fungal pathogens that attempt to penetrate into leaf surfaces have to cope with the plant cuticle. Therefore, it has been considered that this first frontier could play a significant role in signaling. Indeed, evidence has been presented that free cutin monomers can be perceived by cultured potato cells and act as endogenous signal molecules [123]. They were found to trigger the alkalinization of the medium, this effect being paralleled by changes in the phosphorylation state of specific proteins. Moreover, this treatment also stimulated the production of the plant hormone ethylene and activated defense-related genes at the mRNA level [123]. In such experiments, cutin monomers of C-16 and C-18 families varied considerably in their potential to induce the alkalinization. For example, the major constituents of the potato leaf cutin n-16-dihydroxypalmitic acid ($n = 8, 9$, or 10) are less active than 9,10-epoxy-18-hydroxystearic acid. Furthermore, hydroxylation at the ω-position seemed to be an absolute requisite for this eliciting activity when the molecule possessed an epoxy function (such as 9,10-epoxy stearate versus 9,10-epoxy-18 hydroxy stearate), but not when it contained a vicinal diol. Thus, 9,10-dihydroxystearic acid is more active than 9,10,18-trihydroxystearic acid. Altogether, it clearly appears that structural features of cutin monomers are required for biological activity of such phytooxylipins, suggesting their specific interaction with one or several perceptive structures, such as receptor molecules, rather than unspecific binding.

In addition, cutin monomers were found to enhance elicitation of H_2O_2, the most thermodynamically stable state of active oxygen species [124]. Hydroxy and epoxy groups seemed important features for this eliciting potential. A recently characterized cutin monomer from cucumber, namely dodecan-1-ol, was also active in eliciting H_2O_2 via both a constitutive and an inducible generating system, this latter involving protein phosphorylation, Ca^{2+} influx, and NAD(P)H oxidase [125]. Furthermore, topical application of free cutin monomers partially protected the treated leaves from pathogen attack [126,127]. Because the cutin monomers exhibited no apparent fungicidal effect, the observed protection was suggested to be due to acquired resistance by the plants. Here, also, specific structural requirements were needed for the prevention of infection. Interestingly, the *cis*-epoxy or the *threo*-hydroxy group(s) in the middle of the C-18 molecules was necessary for protection, and replacement by a cis double bond yielded a completely inactive molecule. Strikingly, 9,10-epoxy-18-hydroxystearate and 9,10,18-trihydroxystearate, which are among the major C-18 cutin monomers, exhibited the strongest effect in eliciting defense mechanisms and protection against fungal aggression, whereas 16-hydroxy palmitic acid, which is present in low amounts in barley cuticle (Blée, unpublished result), showed no protective effect. This argues against a foliar surface modification provoked by the coating of the leaf with oxygenated fatty acids and is more likely in favor of a specific effect of cutin monomers. In this context, it was reported recently that cutinase and other lipolytic esterases protected bean leaves from fungal infection through an unknown mechanism not likely to involve the released cutin monomers [128]. However, before excluding any action of cuticular oxygenated fatty acids in the protective effect of hydrolases, the notion of threshhold for eliciting effects should be pointed out. Clearly, a dose of cutin monomers unable to trigger late defense responses, such as accumulation of antimicrobial proteins (i.e., ‘‘pathogenesis-related proteins’’) and tissue necrosis, could perfectly enhance the rapid onset of primary defense mechanisms [129].

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

4 CONCLUSION

For a long time, cutin was regarded only as an inactive reticulated framework filled up with waxes and other components of the cuticle. This limited-role conception has now evolved with the astonishing finding that cutin monomers could act as primary signals at the site of aggression. The identification of the components involved in signaling is just starting. Clearly, a good knowledge of their biosynthesis will be indispensable for biotechnological applications. Only recently, some of the enzymes involved in the biosynthetic pathway of the major cutin components have been characterized at the molecular level. For most of them, many issues remain to be solved such as tissue and cellular localizations and changes in expression levels under biotic or abiotic stress. Additionally, we ignore most of the enzymes responsible for the biosynthesis of minor cutin components, which, despite being present in low amounts, could prove to be very important from a physiological point of view.

The molecular structure of the precursors of the cutin monomers conditions the nature of the cross-linkage between these different constituents. The cutin network is primarily established via ester bonds which are susceptible to the enzymatic action of hydrolases (i.e., cutinases, esterases, lipases) secreted from some fungi. The presence in cuticles of linkages other than ester bonds may, therefore, protect plants from invasion by certain pathogens. For example, ether bonds, due to the reaction between epoxy groups and hydroxy substituents of adjacent monomers, have been shown to contribute to the reticulation of cutin. One can envision that the number of such ether bonds, which are resistant to the action of cutinase, could be increased by suppression of the hydrolysis of the epoxy groups present in the cutin monomers. Such a result could be attained by inhibition of the epoxide hydrolase, which also would decrease the amount of mid-chain hydroxy functions susceptible to form ester bonds. These last years, cDNAs coding for epoxide hydrolases of *Arabidopsis* and potato were obtained; however, these enzymes are soluble and may not participate in cuticle formation. Obviously, further work is needed to clone the membrane-bound epoxide hydrolase in order to construct transgenic plants expressing antisense RNA to this hydrolase.

Agrochemical molecules, which are very active in vitro, sometimes are useless under field conditions because they cannot cross the leaf cuticle. Therefore, modifying the composition of cutin may change the physical properties of this layer and especially its permeability. Such goals could be achieved by inhibition, for instance, of peroxygenase, one of the key enzymes in the biosynthetic pathway of cutin monomers. Specific mechanism-based inhibitors of this enzyme are presently under investigation in our laboratory to test their influence on the permeability of the cuticle to xenobiotics and to pathogens.

In addition, recent evidence has shown that epoxy and hydroxy derivatives of fatty acids produced by the peroxygenase pathway play a primary role in plant disease resistance. First, it was reported that two cutin monomers, which lack fungitoxic activity, act as endogenous inducers of acquired resistance in cereals, possibly through a mechanism involving transcription in the host. Second, epoxides formed from linoleic and linolenic acids were shown to induce resistance of rice to infection by *Magnaporthe grisea*. Moreover, infection of rice by the fungus triggers the activation of the peroxygenase pathway in plants. Thus, peroxygenase appears to be a key enzyme in such resistance mechanisms, and one could expect that overexpression of this enzyme could result in a better defense of the transformed plant against fungal attack. Presumably, researchers will clone this enzyme in the near future and establish its location in plant tissues.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

Undoubtedly, our vision of cutin monomers is far from being complete: It is just beginning.

ACKNOWLEDGMENTS

Many thanks to Rachel Kahn and Francis Schuber for critically reading this manuscript.

REFERENCES

1. PE Kollatukudy. In: PK Stumpf, ed. The Biochemistry of Plants: Lipids. Structure and function, Cutin, Suberin and Waxes. New York: Academic Press, 1980, pp 571–646.
2. EA Baker. In: DF Cutter, KL Alvin and CE Price, eds. The Plant Cuticle: Chemistry and Morphology of Plant Epicuticular Waxes. London: Academic Press, 1982, pp 136–166.
3. TJ Walton. In: JL Harwood and JR Bowyer, eds. Lipids, Membranes and Aspect of Photobiology: Waxes, Cutin and Suberin. London: Academic Press, 1990, pp 105–158.
4. G Bianchi. In: RJ Hamilton, ed. Plant Waxes: Waxes: Chemistry, Molecular Biology and Functions. Dundee: The Oily Press, 1995, pp 177–222.
5. PJ Holloway. In: DF Cutler, KL Alvin, CE Price, eds. The Plant Cuticle: The Chemical Constitution of Plant Cutins. London, New York: Academic Press, 1982, pp 45–82.
6. JF Villena, E Dominguez, D Stewart, A Heredia. Planta 208:181–187, 1999.
7. M Riederer, J Schönherr. Planta 174:121–138, 1988.
8. JB Clarck, GR Lister. Plant Physiol 55:407–413, 1975.
9. DA Reicosky, JW Hanover. Plant Physiol 62:101–104, 1978.
10. MA Jenks, RJ Joly, PJ Peters, PJ Rich, JD Axtell, EA Ashworth. Plant Physiol 105:1239–1245, 1994.
11. P Luque, S Bruque, AA Heredia. Arch Biochem Biophys 317:417–422, 1995.
12. J Schönherr. Planta 131:159–164, 1976.
13. P Baur. Plant Cell Environ 20:167–177, 1997.
14. L Schreiber, J Riederer. Plant Cell Environ 19:1075–1082, 1996.
15. S Santier, A Chamel. Plant Physiol Biochem 36:225–231, 1998.
16. MJ Bukovac, PD Petracek, RG Fader, RD Morse. 38:289–298, 1990.
17. KE Espelie, EA Bernays, JJ Brown. Arch Insect Biochem Physiol 17:223–233, 1991.
18. SD Eigenbrode, KE Espelie. 40:171–194, 1995.
19. D Preuss, B Lemieux, G Yen, BW Davis. Genes Dev 7:974–985, 1993.
20. P von Wettstein-Knowles. In: RJ Hamilton, ed. Waxes: Chemistry, Molecular Biology and Functions: Biosynthesis and Genetic of Waxes. Dundee: The Oily Press, 1995, pp 91–129.
21. D Post-Beittenmiller. Ann Rev Plant Physiol Plant Mol Biol 47:405–430, 1996.
22. B Lemieux. Trends Plant Sci 1:312–318, 1996.
23. M Matic. Biochem J 63:168–176, 1956.
24. PJ Holloway, AHB Deas. Phytochemistry 12:1721–1735, 1973.
25. HC Gerard, WF Fett, SF Osman, RA Moreau. 17:181–189, 1993.
26. EA Baker, JT Martin. Nature 199:1268–1270, 1963.
27. A Ray, RE Stark. Phytochemistry 48:1313–1320, 1998.
28. HW Schmidt, J Schönherr. Planta 156:380–384, 1982.
29. CE Crisp. The Biopolymer Cutin. Ph. D. dissertation, University of California, 1965.
30. PE Kolattukudy, TJ Walton. Biochemistry 11:1897–1907, 1972.
31. AK Ray, YY Lin, HC Gerard, ZJ Chen, SF Osman, WF Fett, RA Moreau, RE Stark. Phytochemistry 38:1361–1369, 1995.
32. CI Soliday, PE Kolattukudy. Plant Physiol 59:1116–1121, 1977.
33. I Benveniste, N Tijet, F Adas, G Phillips, J-P Salaun, F Durst. Biochem Biophys Res Commun 243:688–693, 1998.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

34. N Tijet, C Helvig, F Pinot, R Le Bouquin, A Lesot, F Durst, J-P Salaun, I Benveniste. Biochem J 332:583–589, 1998.
35. F Cabello-Hurtado, Y Batard, J-P Salaun, F Durst, F Pinot, D Werck-Reichhart. J Biol Chem 273:7260–7267, 1998.
36. FJ van de Loo, P Broun, S Turner, C Sommerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
37. PE Kollatukudy, TJ Walton, RP Kuswaha. Biochemistry 12:4488–4498, 1973.
38. F Oesch, N Kaubisch, DM Jerina, JW Daly. Biochemistry 10:4858–4866, 1971.
39. R Croteau, PE Kollatukudy. Arch Biochem Biophys 162:471–480, 1974.
40. R Croteau, PE Kollatukudy. Arch Biochem Biophys 170:61–72, 1975.
41. PE Kollatukudy. In: G Kerstein, ed. Plant Cuticles, an Integrated Functional Approach: Biosynthetic Pathways of Cutin and Waxes, and Their Sensitivity to Environmental Stress. Oxford: BIOS Scientific Publishers, 1996, pp 83–108.
42. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Arch Biochem Biophys 303:145–151, 1993.
43. E Blée, U Stahl, F Schuber, S Stymne. Biochem Biophys Res Commun 197:778–784, 1993.
44. M Lee, M Lenman, A Banas, M Bafor, S Singh, M Schweizer, M Nilsson, C Lijenberg, A Dahlqvist, PO Gummeson, S Sjodahl, A Green, S Stymne. Science 280:915–918, 1998.
45. P Broun, J Shanklin, E Whittle, C Somerville. Science 282:1315–1317, 1998.
46. B Mazur, E Krebbers, S Tingey. Science 285:372–375, 1999.
47. A Ishimaru, I Yamazaki. J Biol Chem 252:6118–6124, 1977.
48. E Blée, F Schuber. Biochemistry 28:4962–4967, 1989.
49. E Blée, F Schuber. J Biol Chem 265:12667–12894, 1990.
50. M Hamberg, G Hamberg. Arch Biochem Biophys 283:409–416, 1990.
51. F Pinot, J-P Salaun, H Bosch, A Lesot, C Mioskowski, F Durst. Biochem Biophys Res Commun 164:183–193, 1992.
52. E Blée, F Schuber. Biochem Biophys Res Commun 173:1354–1360, 1993.
53. R Croteau, PE Kollatukudy. Arch Biochem Biophys 162:458–470, 1974.
54. E Blée, F Schuber. Plant J 4:113–123, 1993.
55. PJ Holloway, GA Brown, J Wattendorff. J Exp Bot 32:1051–1066, 1991.
56. W Heinen, IVD Brand. Z Naturforsch 18b:67–79, 1963.
57. JN Siedow. Annu Rev Plant Physiol Plant Mol Biol 42:145–188, 1991.
58. J-P Salaun, C Helvig. Drug Metabolism Drug Interact 12:261–283, 1995.
59. R Croteau, PE Kollatukudy. Arch Biochem Biophys 170:73–81, 1975.
60. E Blée, F Schuber. Biochem J 282:711–714, 1992.
61. T Kiyosue, JK Beetham, F Pinot, BD Hammock. Plant J 6:259–269, 1994.
62. A Stapleton, JK Beetham, F Pinot, JE Garbarino, DR Rockhold, M Friedman, BD Hammock, WR Belknap. Plant J 6:251–258, 1994.
63. T Katsube, M Adachi, N Maruyama, K Ichise, Y Takenada, S Utsumi. Plant Physiol 109:722–723, 1995.
64. JK Bettham, D Grant, M Arand, J Garbarino, T Kiyosue, F Pinot, F Oesch, WR Belknap, K Shinozaki, BD Hammock. DNA Cell Biol 14:61–71, 1995.
65. F Pinot, I Benveniste, J-P Salaun, O loreau, J-P Noel, L Schreiber, F Durst. Biochem J 342:27–32, 1999.
66. LJ Morris, MI Crouchman. Lipids 7:372–379, 1972.
67. E Dominguez, A Heredia. Z Naturforsch 54c:141–143, 1999.
68. PE Kollatukudy. Annu Rev Plant Physiol 32:539–567, 1981.
69. DG Rueckert, K Schmidt. Chem Phys Lipids 56:1–20, 1990.
70. JC Kader. Subcell Biochem 16:69–110, 1990.
71. A Molina, A Segura, F Garcia-Olmedo. FEBS Lett 316:119–122, 1993.
72. J Pyee, PE Kollatukudy. Plant J 7:49–59, 1995.
73. J Pyee, H Yu, PE Kollatukudy. Arch Biochem Biophys 311:460–468, 1994.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

74. P Sterk, H Booij, GA Schellekens, A van Kammen, S de Vries. Plant Cell 3:907–921, 1991.
75. EA Meijer, SC de Vries, P Sterk, DWJ Gadelle, KWA Wirtz, T Hendriks. Mol Cell Biochem 123:159–166, 1993.
76. A Zachowki, F Guerbette, M Grosbois, A Jolliot-Croquin, JC Kader. Eur J Biochem 257: 443–448, 1998.
77. L Sossountzov, I Ruiz Avila, E Vignols, A Jolliot, V Arondel, F Tchang, M Grosbois, F Guerbette, E Miginiac, M Delseny, P Puigdomenach, J-C Kader. Plant Cell 3:925–933, 1991.
78. S Thoma, Y Kanedo, C Sommerville. Plant J 3:427–436, 1993.
79. S Thoma, U Hecht, A Kippers, J Botella, S de Vries, C Sommerville. Plant Physiol 105: 35–45, 1994.
80. B Hollenbach, L Schreiber, W Hartung, KJ Dietz. Planta 203:9–19, 1997.
81. MH Lerche, BB Krageland, LM Bech, FM Poulsen. Structure 5:291–306, 1997.
82. MH Lerche, FM Poulsen. Protein Sci 7:2490–2498, 1998.
83. PJ Holloway. In:E Percy, ed. Air Pollutants and the Leaf Cuticle: Plant Cuticles, Physico-chemical and Biosynthesis. Berlin: Springer-Verlag, 1994, pp 1–13.
84. P Wiedeman, C Neinhuis. Bot Acta 111:28–34, 1998.
85. G Kerstein. Trends Plant Sci 1:125–129, 1996.
86. S Hoffman-Benning, H Kende. Plant Physiol 104:719–723, 1994.
87. PD Petracek, MJ Bukowac. Plant Physiol 109:675–679, 1995.
88. RJ Howard, B Valent. Annu Rev Microbiol 50:491–512, 1996.
89. B Valent, FG Chumley. Annu Rev Phytopathol 29:443–467, 1991.
90. RJ Howard, MA Ferrari. Exp Mycol 13:403–418, 1989.
91. RE Purdy, PE Kollatukudy. J Biol Chem 270:11,753–11,756, 1995.
92. M Shaykh, C Soliday, PE Kollatukudy. Plant Physiol 60:170–172, 1977.
93. H Xu, K Mendgen. Mol Plant Microbe Interact 10:87–94, 1997.
94. PE Kollatukudy. Annu Rev Phytopathol 23:223–250, 1985.
95. CP Woloshuk, PE Kollatukudy. Proc Natl Acad Sci USA 83:1704–1708, 1986.
96. GK Podilla, MB Dickman, PE Kollatukudy. Science 242:922–925, 1988.
97. A Bajar, MB Dickman, PE Kollatukudy. Proc Natl Acad Sci USA 88:8208–8212, 1991.
98. D Li, PE Kollatukudy. J Biol Chem 270:11,753–11,756, 1995.
99. W Köller, CR Adam, PE Kollatukudy. Phytopathology 81:1375–1379, 1991.
100. LM Rogers, MA Flaishman, PE Kollatukudy. Plant Cell 6:935–945, 1994.
101. K Tanabe, S Nishimura, K Kohmato. Ann Phytopathol Soc Japan 54:483–492, 1988.
102. W Köller, DM Parker, CM Becker, Phyopathology 81:1375–1379, 1991.
103. AH Danzig, SH Zuckerman, MM Andonov-Roland. J Bacteriol 168:911–916, 1986.
104. MB Dickman, SS Patil. Physiol Mol Plant Pathol 28:235–242, 1986.
105. MB Dickman, GK Podila, PE Kollatukudy. Nature 342:446–448, 1989.
106. DJ Stahl, W Schäfer. Plant Cell 4:621–629, 1992.
107. B Oeser, OC Yoder. Mol Plant Microbe Interact 7:282–288, 1993.
108. DJ Stahl, A Theuerkauf, R Heitefuss, W Schäfer. Mol Plant Microbe Interact 6:713–725, 1994.
109. JAL van Kan, JW Klooster, CAM Wagenmakers, DCT Dees, CJB van der Vlugt-Bergmans. Mol Plant Microbe Interact 10:30–38, 1997.
110. RN Crowhurst, SJ Binnie, JK Bowen, BT Hawthorne, KM Plummer, J Rees-Gearge, EHA Rikkerink, MD Templeton. Mol Plant Microbe Interact 10:355–368, 1997.
111. C Yoa, W Köller. Mol Plant Microbe Interact 8:122–130, 1995.
112. JA Sweigard, FG Chumley, B Valent. Mol Gen Genet 232:174–182, 1992.
113. RL Nicholson, H Yoshida, N Yamaoka, H Kunoh. Exp Mycol 12:336–349, 1988.
114. SF Pascholati, H Yoshioka, H Kunoh, RL Nicholson. Physiol Mol Plant Pathol 41:53–59, 1992.
115. SF Pascholati, H Deisig, B Leite, D Anderson, RL Nicholson. Physiol Mol Plant Pathol 42: 37–51, 1993.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.

116. JA Suggi, SF Pascholati, H Kunoh, RJ Howard, RL Nicholson. Physiol Mol Plant Pathol 52:213–221, 1998.
117. P Comménil, L Belingheri, B Dehorter. Physiol Mol Plant Pathol 52:1–14, 1998.
118. P Comménil L, Belingheri, G Bauw, B Dehorter. Physiol Mol Plant Pathol 55:37–43, 1999.
119. H Delsing, RL Nicholson, M Haug, RJ Howard, K Mendgen. Plant Cell 4:1101–1111, 1992.
120. RL Nicholson, L Epstein. In: GT Cole, HC Hoch, eds. The Fungal Spore and Disease Initiation in Plants and Animals. New York: Plenum Press, 1991, pp 3–23.
121. SA Francis, FM Dewey, SJ Gurr. Physiol Mol Plant Pathol 49:201–211, 1996.
122. RD Gilbert, AM Johnson, RA Dean. Physiol Mol Plant Pathol 48:335–346, 1996.
123. P Schweizer, G Felix, A Buchala, C Muller, J-P Métraux. Plant J 10:331–341, 1996.
124. M Fauth, P Schweizer, A Buchala, C Markstader, M Reiderer, T Kato, H Kauss. Plant Physiol 117:1373–1380, 1998.
125. H Kauss, M Fauth, A Merten, W Jeblick. Plant Physiol 120:1175–1182, 1999.
126. P Schweizer, A Jeanguenat, E Mösinger, J-P Métraux. In: MJ Danils, JA Downie, AE Osbourn, eds. Advance in Molecular Genetic of Plant–Microbe Interactions. Dordrecht: Klewer Academic, 1994, pp 371–374.
127. P Schweizer, A Jeanguenat, D Whitacre, J-P Métraux, E Mösinger. Physiol Mol Plant Pathol 49:103–120, 1996.
128. DM Parker, W Köller. Mol Plant Microbe Interact 11:514–522, 1998.
129. L Costet, S Cordelier, S Dorey, F Bailleuil, F Fritig, S Kauffman. Mol Plant Microbe Interact 12:655–662, 1999.

Downloaded by [Alma Mater Studiorum - Università di Bologna] at 08:04 30 April 2015

Copyright © 2002 Marcel Dekker, Inc.