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# Apoplastic Diffusion Barriers in Arabidopsis

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**During the development of Arabidopsis and other land plants, diffusion barriers are formed in the apoplast of specialized tissues within a variety of plant organs. While the cuticle of the epidermis is the primary diffusion barrier in the shoot, the Casparian strips and suberin lamellae of the endodermis and the periderm represent the diffusion barriers in the root. Different classes of molecules contribute to the formation of extracellular diffusion barriers in an organ- and tissue-specific manner. Cutin and wax are the major components of the cuticle, lignin forms the early Casparian strip, and suberin is deposited in the stage II endodermis and the periderm. The current status of our understanding of the relationships between the chemical structure, ultrastructure and physiological functions of plant diffusion barriers is discussed. Specific aspects of the synthesis of diffusion barrier components and protocols that can be used for the assessment of barrier function and important barrier properties are also presented.**

## INTRODUCTION

During the evolution of land plants, diffusion barriers located in the apoplast developed to seal plants from their environment and to separate different plant tissues. Different types of diffusion barriers are formed by reinforcing the polysaccharide-based cell wall with additional hydrophobic material (Franke and Schreiber, 2007). The cuticle, which covers the surface of epidermal cells in different organs during the primary growth stage, is the most important diffusion barrier of the shoot. Shoots that have undergone secondary growth are protected by a periderm. In the roots, diffusion barriers (i.e., Casparian strips and suberin lamellae) are formed by cell wall modifications within the endodermis and exodermis in primary tissues and by reinforcement of the periderm during secondary growth. In addition, plants must seal wounds after injury and isolate organs during development (e.g. the abscission zone of siliques). Seeds and pollen have specialized diffusion barriers.

The diverse functions of extracellular diffusion barriers are reflected by the corresponding diversity of their compositions, structures and properties (Schreiber, 2010). Cell wall polysaccharides (e.g., pectins) can interfere with the diffusion of high molecular weight molecules (Liu et al., 2005). However, this chapter will focus on diffusion barriers that are formed by the additional deposition of polymers that have a backbone of lipids or aromatic compounds. Cutin is the aliphatic polyester that is present in the plant cuticle (Kolattukudy, 2001a; Pollard et al., 2008; Li-Beisson et al., 2013). Suberin is an aliphatic polyester that is deposited in a broad spectrum of diffusion barriers, particularly those that form in the roots and in the periderm of shoots (Kolattukudy, 2001b). In cutin, the amount of aromatic components is very low, but this proportion is higher in suberin (Kolattukudy, 2001b; Pollard et al., 2008; Beisson et al., 2012). For both cutin and suberin, the presence of ester linkages between ω-hydroxyacids and glycerol has been confirmed by nuclear magnetic resonance (NMR)- and by gas chromatography-mass spectrometry studies (GC/MS)-based studies (Graça and Santos, 2006; Tian et al., 2008) In addition, linkages between polyhydroxy acids and cell wall sugars have been tentatively identified in cutin (Tian et al., 2008). Lignin, which is a polymer of aromatic components, is usually seen as a structural polymer that is deposited in secondary cell walls (Boerjan et al., 2003). Lignin is present in the Casparian strips of the endo- and exodermis and in the periderm of shoots, but it is also an important component of some cuticles (e.g., in conifers) (Reina et al., 2001). Many asepcts of the aliphatic polyesters that are present in diffusion barriers, such as the polymer size, the linkage to the cell wall, the organization of molecules into domains with

different ultrastructures and the localization and organization of functional domains, are still unknown (Pollard et al., 2008).

Aliphatic waxes and secondary metabolites, such as triterpenoids, sterols and flavonoids, are often associated with polyesters in diffusion barriers (Jetter et al., 2006). The importance of aliphatic wax components for the formation of a functional diffusion barrier has been well established for both the cuticle and the tuber periderm (Schreiber et al., 2005b; Schreiber, 2010).

In this chapter, we will review the current knowledge about the impregnation of the polysaccharide cell wall with the lipids and aromatic compounds that form diffusion barriers, with a focus on *Arabidopsis thaliana* (Arabidopsis) (Table 1). In particular, we will discuss the formation of these diffusion barriers during development and the relationships that exist between the composition, ultrastructure and function of diffusion barriers that have been elucidated up to now. Because the composition and biosynthesis of polyesters has been previously discussed (Li-Beisson et al., 2013), we will provide an overview here. The polymers associated with pollen, such as sporopollenin and tryphine, have been previously discussed (Ariizumi and Toriyama, 2011; Jessen et al., 2011) and fall outside the scope of this chapter.

## THE LOCALIZATION AND ULTRASTRUCTURE OF DIFFUSION BARRIERS

### The Cuticle of the Shoot

The cuticle is a lipidic incrustation of the outer epidermal cell wall of the leaves, stems, fruits and flower organs that efficiently controls the exchange of water, gases and solutes between the plant and its environment. The cuticle may also be found in distinct locations in different plant organs (e.g., in the cell wall of the mesophyll cells adjacent to the sub-stomatal chamber and in the surrounding locular cavities of fruits). In contrast to other diffusion barriers, the cuticle is formed towards the outward facing side of the cell wall and contributes to the structure and properties of the surface of plant organs.

In many plant species, including Arabidopsis, the major structural component of the cuticle is cutin, a polyester that is rich in glycerol and oxygenated fatty acids with chain lengths of 16 or 18 carbons. However, the specific cutin composition varies considerably in different species, different plant organs and different stages of development and may include additional compounds, such as phenolics and flavonoids (Holloway, 1982a). Whether the cutin fraction of an organ consists of several types of polymers with distinct composition and linkages or a single type of polymer with different subdomains is currently unknown.

Cuticular wax, the other principal component of the cuticle, is a complex mixture of hydrophobic material that contains predominantly very long-chain fatty acids (VLCFA) and their derivatives, together with secondary metabolites, such as flavonoids and triterpenoids (Jetter et al., 2006). Cuticular wax is embedded in the cutin matrix (i.e., intracuticular wax) and deposited as a distinct layer on the surface of the plant (i.e., epicuticular wax) (Figure 1A). Triterpenoids have been found preferentially in the intracuticular wax fraction (Jetter et al., 2006).

The cuticle forms a continuum with the cell wall and may be covalently linked to the cell wall (Sitte and Rennier, 1963; Jeffree, 2006; Tian et al., 2008).

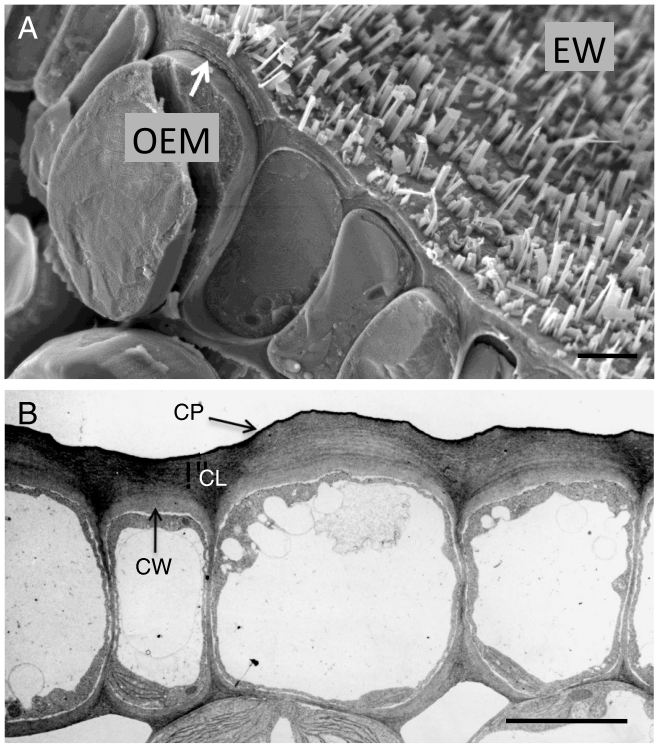


**Figure 1.** Epidermal cell layer of an Arabidopsis inflorescence stem.

**(A)** Cryo-scanning electron microgram. OEM: Outer extracellular Matrix, EW: epicuticular wax. Picture by Lacey Samuels, UBC.

**(B)** Transmission electron microgram. CP: cuticle proper, CL: cuticular layer and CW: cell wall. EW is not preserved with this fixation technique. Picture by Martine Schorderet, University of Fribourg. Scale bars: 5 µm.

#### *The ultrastructure of the cuticle in mature plant organs*

The cuticle is typically organized into distinct layers of variable thickness. There is a large amount of structural diversity among different plant species and organs (Jeffree, 2006; Buda et al., 2009). The outermost layer of the cuticle is formed by epicuticular waxes that may be deposited as an amorphous film or in the form of crystals (Jetter et al., 2006). In many species, a specific hydrophobic layer called the cuticle proper can be distinguished beneath the epicuticular wax layer (Sitte and Rennier, 1963; Schreiber and Schönherr, 2009) (Figure 1B). This layer very hydrophobic and consists mainly of the aliphatic polyester cutin, which is the main structural component, and intracuticular wax. The layer below the cuticle proper is less hydrophobic and is often referred to as the cuticular layer (Sitte and Rennier, 1963; Schreiber and Schönherr, 2009). The cuticular layer contains additional polysaccharides and forms a continuum with the cell wall, potentially with gradually decreasing proportions of lipid material towards the inner side of the cell wall (Figure 1B) (Sitte and Rennier, 1963; Schreiber and Schönherr, 2009). The cuticle has an amorphous, lamellate or reticulate structure, and these features have long served as the primary characteristics for the classification of cuticles (Holloway, 1982b). In species with a thick cuticle, the cuticular layer may form several structurally distinct layers (Jeffree, 2006). The thickness and ultrastructure of the cuticle

changes markedly during the growth and development of organs, and these changes correlate with changes in cutin and wax composition (Riederer and Schonherr, 1988; Hauke and Schreiber, 1998).

The ultrastructure of the cuticle can be visualized by conventional transmission electron microscopy (TEM) of embedded and sectioned tissues. Soluble components, such as wax and other molecules that are not cross-linked, are removed during the embedding procedure that is necessary for TEM (Figure 1B). The surface structure of the cuticle can be visualized by cryo- or environmental scanning electron microscopy (SEM), preserving the structure of the epicuticular wax (Figure 1A).

*The ultrastructure of the cuticle in rosette leaves*

In wild-type (wt) Arabidopsis, the ultrastructure of the cuticle has been characterized in the leaves, inflorescence stems and flower organs. In different accessions, the cuticle of the leaf blade is documented as a thin, electron-dense layer of 20-25 nm (Xiao et al., 2007; Voisin et al., 2009) (Figure 2A, D). A layered reticulate structure was observed at high magnification (Broun et al., 2004). Mutants with a substatial reduction in cutin polyester content (> 50%), such as the *long-chain acyl-CoA synthethase 2* (*lacs2*) (At1g49430) mutant and the *glycerol-3-phosphate acyltransferase* (*gpat*) double mutant *gpat4gpat8* (At1g01610, At4g00400) and transgenic plants expressing a fungal cutinase (CUTE-plants), may not have any an osmiophilic layer on the surface of their cell walls (Sieber et al., 2000; Bessire et al., 2007; Li et al., 2007b) (Figure 2F). The *lacs2* mutant exhibits an unusual gradual darkening of the cell wall, as well as osmiophilic structures within the cell wall. It has been speculated that these structures represent residual cutinization in the absence of a distinct cuticle layer on the surface of the cell wall (Bessire et al., 2007; Li et al., 2007b) (Figure 2B, 2C). Arabidopsis mutants that exhibit alterations in the composition of cutin in the leaves, such as the *lacerata* (*lcr*) (At2g45970) and *bodyguard* (*bdg*) (At1g64670) mutants, typically display visible modifications to the ultrastructure of the leaf cuticle (Kurdyukov et al., 2006a; Voisin et al., 2009). In the *aberrant induction of type three genes 1 (att1*) (At4g00360) mutant, the cuticle is broader and more electron-translucent than that of wt Arabidopsis (Xiao et al., 2004) (Figure 2E), while WAX INDUCER1/SHINE1 (*win1/shn1*) (At1g15360)-overexpressing plants accumulate electron-dense material beneath a broad electron-translucent cuticle proper (Broun et al., 2004). In a few cases, structural changes were not detected by conventional TEM methods, even when the barrier properties were altered (e.g., in the *fiddlehead* (*fdh*) (At2g26250) mutant) (Voisin et al., 2009). Epicuticular wax is deposited as a thin film on the leaf blade of wt Arabidopsis leaves. However, the overproduction of wax, as in activation-tagged WIN1/SHN1-overexpressing plants, may lead to the formation of flakes on the leaf surface (Aharoni et al., 2004; Broun et al., 2004).

*The ultrastructure of the cuticle of the inflorescence stem*

The inflorescence stem of wt Arabidopsis plants has a more substantial cuticle of 80 -120 nm (Sieber et al., 2000; Chen et al., 2003; Bird et al., 2007; Li et al., 2007b; Lu et al., 2009) (Figure 3A, C, E). An outer, less electron-dense cuticle proper can be distinguished from an electron-dense inner cuticular layer at the

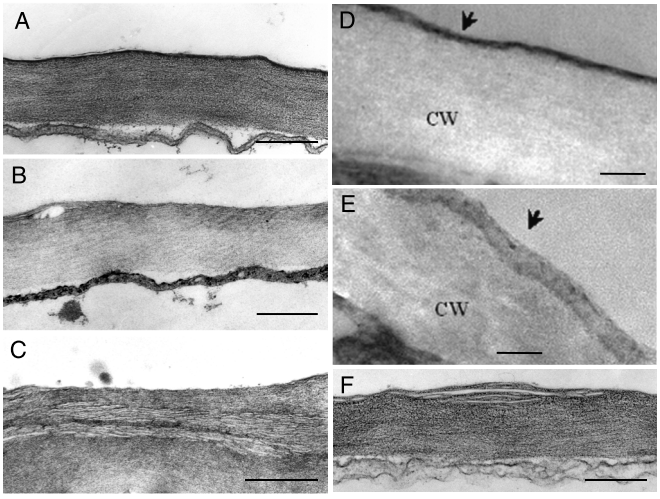


**Figure 2.** Cuticles of Arabidopsis leaves.

Transmission electron micrographs of the cuticle of pavement cells from Col-0 **(A)**, *lacs2-3* **(B, C)** (from Bessire et al., 2007), Col-gl **(D)**, *att1* **(E)** (from Xiao et al., 2004) and transgenic plants expressing a fungal cutinase **(F)** (from Sieber et al., 2000). Scale bars represent 800 nm in A-C, F and 400 nm in D-E.

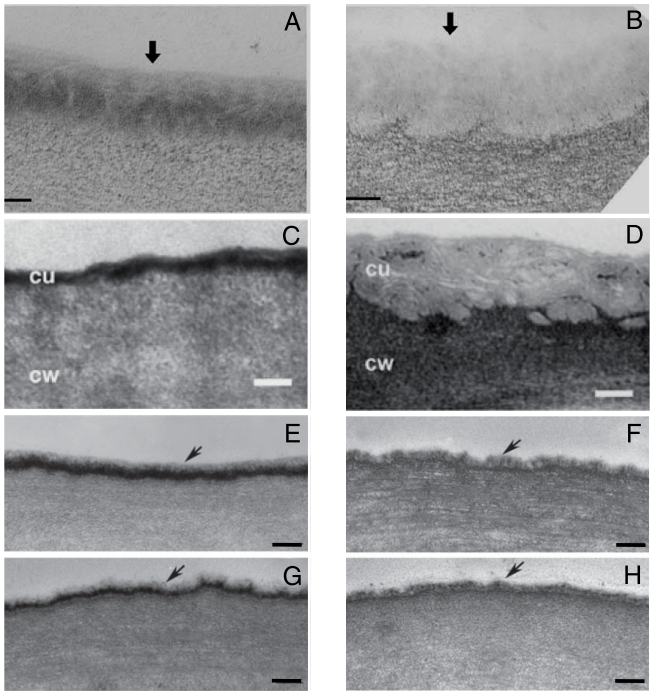


**Figure 3.** Cuticles of inflorescence stems of Arabidopsis.

Transmission electron micrographs of the cuticle of pavement cells of inflorescence stems from C24 **(A)**, *wax2* **(B)** (from Chen et al, 2003), Col-0 **(C)**, *abcg11-3* **(D)** (from Bird et al., 2007), Col-0 **(E)**, *lacs2-3* **(F)**, *lacs1* **(G)**, *lacs1lacs2* **(H)** (from Lü et al., 2009). Scale bars represent 100 nm in A-D and 200 nm in E-H.

cuticle/cell wall interface close to the base of the inflorescence stem of a fully developed wt plant (between the 1st and 2nd internode) (Chen et al., 2003; Lu et al., 2009) (Figure 3A, E). In other preparations, the cuticle is visualized as a single electron-dense layer (Sieber et al., 2000; Bird et al., 2007; Li et al., 2007b; Lu et al., 2011) (Figure 3C). Mutants that have significant reductions in the amount of cutin in the stem usually maintain a distinct cuticle proper. However, the structure of the cuticle proper may vary depending on the genetic lesion. In some mutants, such as the *cer3*/*wax2* (At5g57800*)*, *abcg11-3* (*wbc11-3*) (At1g17840) and *gpat4/gpat8* mutants and transgenic CUTE-plants, the cuticle proper may be thicker with a loose, less osmiophilic structure (Sieber et al., 2000; Chen et al., 2003; Bird et al., 2007; Li et al., 2007b) (Figure 3B, 3D). In other mutants, such as the *glossyhead1* (*gsh1*) (A1g36160) mutant, the cuticle proper may be a thin, strongly osmiophic layer that is more irregular (Lu et al., 2011). When a bilayered cuticle structure could be resolved in TEM preparations of wt Arabidopsis, the organization of the inner cuticular layer was strongly affected by slight decreases in cutin, while the thickness of the entire cuticle was reduced by substantial decreases in polymer content (Lu et al., 2009) (Figures 3F, 3G, 3H). In Arabidopsis, epicuticular wax of inflorescent stems is deposited in the form of crystals that reflect its amount and composition (Jetter et al., 2006). In Arabidopsis and other species, a large number of mutants have been identified by the glossy appearance of their stems, which indicates that these mutants may have reduced wax crystal deposition (Kunst and Samuels, 2003) (Figure 4).

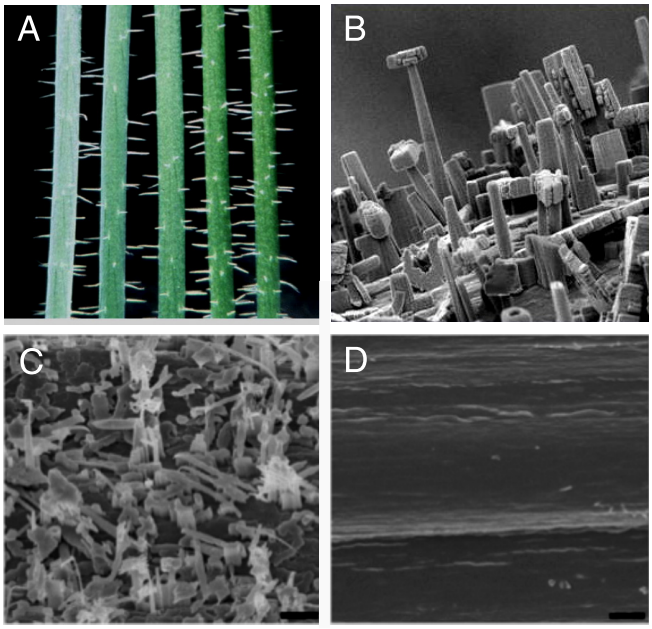


**Figure 4.** Epicuticular waxes on inflorescence stems of *Arabidopsis thaliana*.

**(A)** Arabidopsis influorescent stems, with decreasing wax amounts from left to right: WS, *cer3, cer4, cer1, cer2* (from Mc Nevin *et al.,* 1993*)*. **(B–C)** Cryo-SEM pictures of wax crystals of Col-0. **(D)** Residual wax on the *cer2* mutant in the form of a wax film (from Haslam *et al.,* 2012). Scale bars (C, D): 2 µm.

*The ultrastructure of the cuticle in floral organs*

The surface of the cuticle of Arabidopsis petals and sepals is characterized by the presence of nanoridges (Aharoni et al., 2004; Li-Beisson et al., 2009; Panikashvili et al., 2009) (Figure 5). Nanoridges are formed by an undulating electron-lucent cuticle proper of 60-80 nm that has an amorphous structure and an electron-opaque cuticular layer that underlies the cuticle proper and fills the nanoridges (Figures 5A, 5H) (Aharoni et al., 2004; Li-Beisson et al., 2009; Panikashvili et al., 2009; Bessire et al., 2011). Structural alterations of cuticle in the petals of the *permeable cuticle1* (*pec1*) (At2g26910) mutant suggest that the cuticular layer also contributes to the formation of nanoridges (Bessire et al., 2011) (Figure 5B, 5D, 5E, 5G). The absence of nanoridges in mutants that exhibit a large reduction in the amount of 9(10), 16-dihydroxyhexadecanoic acid, such as the *gpat6* (At2g38110), *cyp77A6* (At3g10570) and *defective in cuticular ridges* (*dcr*) (At5g23940) mutants, demonstrates the essential contribution of cutin and the role of this fatty acid in nanoridge formation (Li-Beisson et al., 2009; Panikashvili et al., 2009) (Figure 5I, 5K). In preparations that preserve epicuticular waxes, the nanoridges of the petals remained visible, indicating that flower waxes are deposited as a thin film (Aharoni et al., 2004).

In summary, in Arabidopsis and other plants, both wax and cutin contribute to the formation of the surface texture of different organs in a diverse and complex manner.

## The Cuticle of the Embryo and in Emerging Organs

A cuticle is formed early during embryo development. In Arabidopsis, the presence of the cuticle has been demonstrated experimentally using fluorescent staining of cuticular lipids at the globular stage, when the embryo consists of a few dozen cells (Szczuka and Szczuka, 2003). Mutant phenotypes associated with impairments in cuticle formation have been reported at the torpedo stage in the *gassoh1/gassoh2* (*gso1/gso2*) (At4g20140/At5g44700) double mutant, indicating that at this stage of development, the diffusion barrier is usually functional (Tsuwamoto et al., 2008). Similarly, the expression of ABCG11, a transporter that is involved in cutin and wax secretion, suggests that the cuticle is present in walking stick embryos (Panikashvili et al., 2010).

The embryo is further protected by additional permeability barriers that are present in the seed coat. The seed coat contains several polyesters of different compositions that are difficult to characterize separately (Molina et al., 2006). ATT1, a cytochrome P450-dependent fatty acid ω-hydroxylase that is encoded by *CYP86A2* (At4g00360) and necessary for cutin biosynthesis, is exclusively expressed in the inner integument, while *GPAT5* (At3g11430), a glycerol-3-phosphate acyltransferase necessary for suberin formation, is expressed in the outer integument (Molina et al., 2008), suggesting that these polyesters are localized in different layers of the seed coat. The seed coat will not be discussed further in this chapter.

After the cuticle has been formed during the establishment of the L1 layer, it is maintained during plant development. Thus, when new organs are formed at the shoot and floral apical meristem, they are from the beginning covered by an electron-opaque layer called the procuticle (Heide-Jorgensen, 1991). The composition of

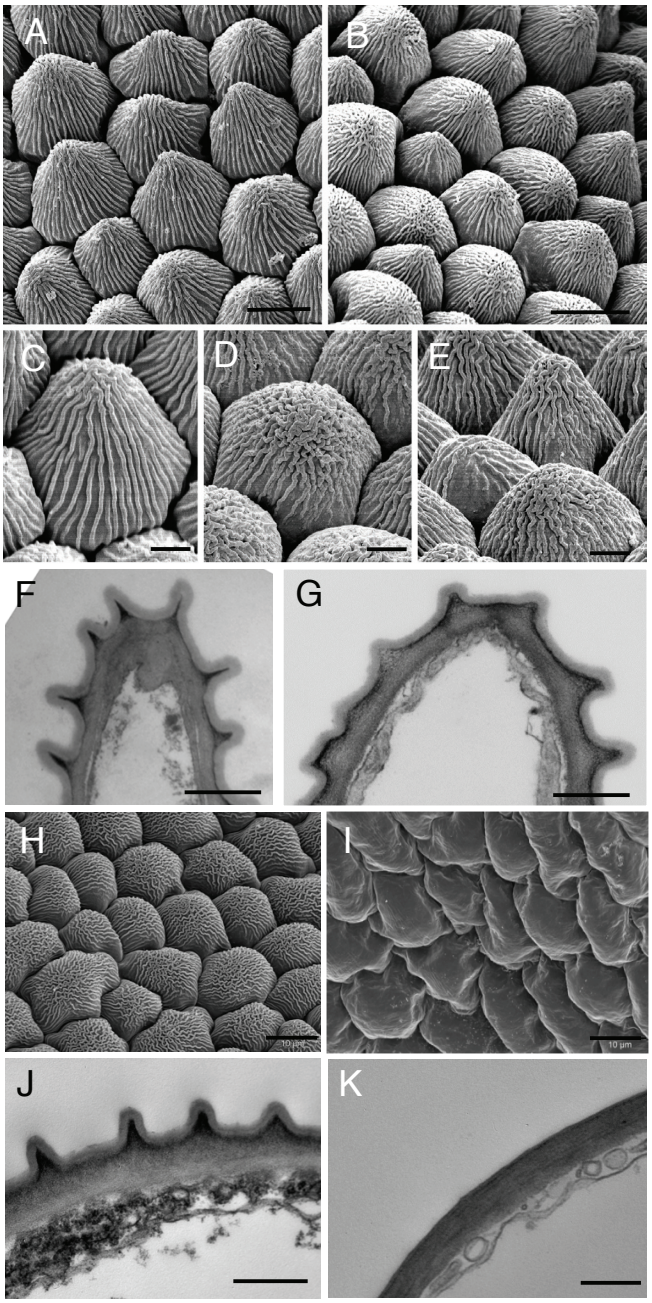


**Figure 5.** Cuticles of epidermal cells of Arabidopsis petals.

SEM **(A-E)** and **(H-I)** and TEM **(F-G)** and **(J-K)** analyses of Arabidopsis petals. On the adaxial side of the petal **(A-G)** and the abaxial side of the petal **(H-K)**, epidermal cells display nanoridges on their surface. Col-0 **(A, C, F, J, H)**, *pec1* **(B, D, E, G)** (from Bessire et al.), *gpat6* **(I, K)** (from Li-Beisson et al., 2009). Scale bars (A, B, H, I): 10 µm; (C-E): 2 µm; (F, G, J, K): 500 nm.

the procuticle is not known in Arabidopsis, but this layer likely has a different composition than the cuticle of mature organs, as some cutin biosynthesis mutants form organ fusions, while others do not (see below). Once the cuticle is damaged during the life of a plant, it does not regenerate; instead, suberin is formed to heal the wound.

After the emergence of the seedling, the cuticle undergoes compositional and structural changes that are highly coordinated with the growth and development of the plant. In barley leaves, cutin synthesis occurs during cell elongation, while wax synthesis occurs after the cells reach their final length (Richardson et al., 2007). The exact timing of the deposition of cutin and wax has not yet been determined in Arabidopsis leaves. However, the induction of the transcription factor SHINE leads to the initial expression of genes involved in cutin biosynthesis and the subsequent induction of genes involved in wax biosynthesis 10 hours later, suggesting that cutin is formed first and then impregnated with wax (Kannangara et al., 2007). In stems, cutin and wax are present 3 mm below the meristem of elongating stem segments, indicating that cutin and wax are likely deposited in close sequence (Suh et al., 2005). The wax load remains constant at 32 µg/cm$^2$ from 1 cm below the meristem to the base of the stem. The amount of polyester that is susceptible to depolymerization is much lower in the oldest segment (i.e., 2 µg/cm$^2$) than in the youngest segment (i.e., 8 µg/cm$^2$), suggesting that the polymer undergoes structural changes when elongation ceases, such as increased crosslinking within itself or to other molecules of the extracellular matrix (Suh et al., 2005).

## Diffusion Barriers of the Root

The simple structure of the primary Arabidopsis root contains most of the cell types that make up protective diffusion barriers in roots. The root epidermis, which is also called the rhizodermis, is the outermost cell layer of young roots, and the endodermis surrounds the vascular cylinder of young roots (Figure 6A). The periderm replaces both the epidermis and the endodermis in older roots that have undergone secondary thickening (Figure 6B). The root cap is comprised of short-lived, highly secretory cells that surround and protect the root meristems, including both established meristems and those of young, emerging lateral root primordia. The pectin-like mucilage produced by the root cap cells can continue to cover the epidermal cell layer, potentially affecting the diffusion of solutes and the movement of bacteria (Hawes et al., 2000). However, the diverse roles and composition of this mucilage polymer are outside the scope of this chapter, which focuses on polymers composed of lipids and aromatic compounds. Arabidopsis lacks the hypodermis/exodermis, a specialized cell layer that is present immediately below the epidermis in many plants (Wilson and Peterson, 1983) and can be extensively suberized and lignified. This hypodermal cell layer can have localized ring-like cell wall impregnations equivalent to the Casparian strips of the endodermis, in which case it is called the exodermis. This type of cellular barrier will not be addressed in this chapter, as it is absent in Arabidopsis.

### *The root epidermis and its elusive cutin/suberin fortifications*

Less than a millimeter from the tip of the root meristem, the last root cap cells are sloughed off, and the epidermal cells constitute the outermost cell layer of the root. The Arabidopsis root epidermis is patterned in two distinct cell files of alternating root hair and non-root hair cells (Dolan et al., 1994). Root hairs emerge quickly upon the exit of cells from the meristem and begin to perform their role in nutrient uptake. Thus, in contrast to the shoot

epidermis, the epidermis of primary roots is a cell layer that is actively involved in solute acquisition from the soil. As a result, the root epidermis cannot feature a pronounced, hydrophobic cell wall modification. However, it is conceivable that the soil-facing cell walls at the base of the root hairs contain polymers that alter the epidermal cell wall properties for increased resistance to pathogens or specialized diffusive functions. Consistent with their physiological function, Arabidopsis root epidermal cell walls do not represent a significant barrier to the diffusion of solutes, especially in early developmental stages. This characteristic is demonstrated by the rapid diffusion of fluorescent tracer molecules, such as propidium iodide (PI), into inner tissue layers, which is only blocked once the dye reaches the differentiated endodermal layer (see below). Modification of the root epidermal cell wall (e.g., by the presence of suberin or lignin-like polymers) may possibly increase resistance without completely blocking the diffusion of solutes. It has been reported that dyes that detect lignin/polyaromatic substances or suberin-like components stain epidermal cell walls. This finding was observed in a number of species (Wilson and Peterson, 1983) and has been investigated in more detail in maize, onion and soybean (Peterson et al., 1978; Schreiber et al., 1999; Thomas et al., 2007). Confirmation of the presence of lignin and/or suberin in the epidermis by chemical analysis is complicated, because many species possess a highly suberized hypodermal/exodermal cell layer immediately below the epidermis, which cannot be separated from the epidermis. Chemical analysis performed in soybean (a species without a hypodermis) and ultrastructural analysis of the onion epidermis support the theory that “diffuse suberin” is present in epidermal cell walls in the root (Thomas et al., 2007). In Arabidopsis, the chemical analysis of epidermal cells would be technically challenging, and we are unaware of ultrastructural studies reporting specialized cell wall features of the root epidermal cell wall. However, increased autofluorescence and an increased resistance to PI penetration can be observed in differentiated epidermal cells (Franke et al., 2005) (Naseer *et al.,* unpublished observation). Thus, it is possible that Arabidopsis also deposits some aliphatic and/or aromatic polymers in its root epidermal cell walls. In addition, studies of the expression patterns of cutin biosynthetic genes detected the expression of a number of relevant genes in root epidermal cells. For example, *CUTICLE DESTRUCTING FACTOR 1* (*CDEF1*) (At4g30140) is a gene that encodes an esterase involved in the degradation of the cuticle during pollen penetration. Promoter GFP-fusions revealed a specific upregulation of *CDEF1* in the root epidermis during lateral root emergence (Takahashi et al., 2010). This process requires the separation of epidermal cells and is accompanied by an induction of cell wall remodeling enzymes (Swarup et al., 2008). Thus, the expression of *CDEF1* in root epidermal cells suggests that the protein encoded by this gene functions in the remodeling or degradation of standard cell wall components in this tissue. Alternatively, this finding could suggest the presence of aliphatic polymers within root epidermal cell walls (Takahashi et al., 2010). Other examples of root epidermis-expressed genes involved in cell wall polyester formation are *BDG,* which encodes a hydrolase involved in cuticle production, *LCR,* which encodes a cytochrome P450 enzyme, and *ADHESION OF CALYX EDGES/HOTHEAD* (*ACE/HTH*) (At1g72970), which encodes a protein thought to be involved in the biosynthesis of dicarboxylic acids (Wellesen et al., 2001; Kurdyukov et al., 2006a; Kurdyukov et al., 2006b). Interestingly, although a polyester composition typical of suberin was found in other species, the proteins that were found to be upregulated in the Arabidopsis root epidermis were previously demonstrated to be involved in cutin

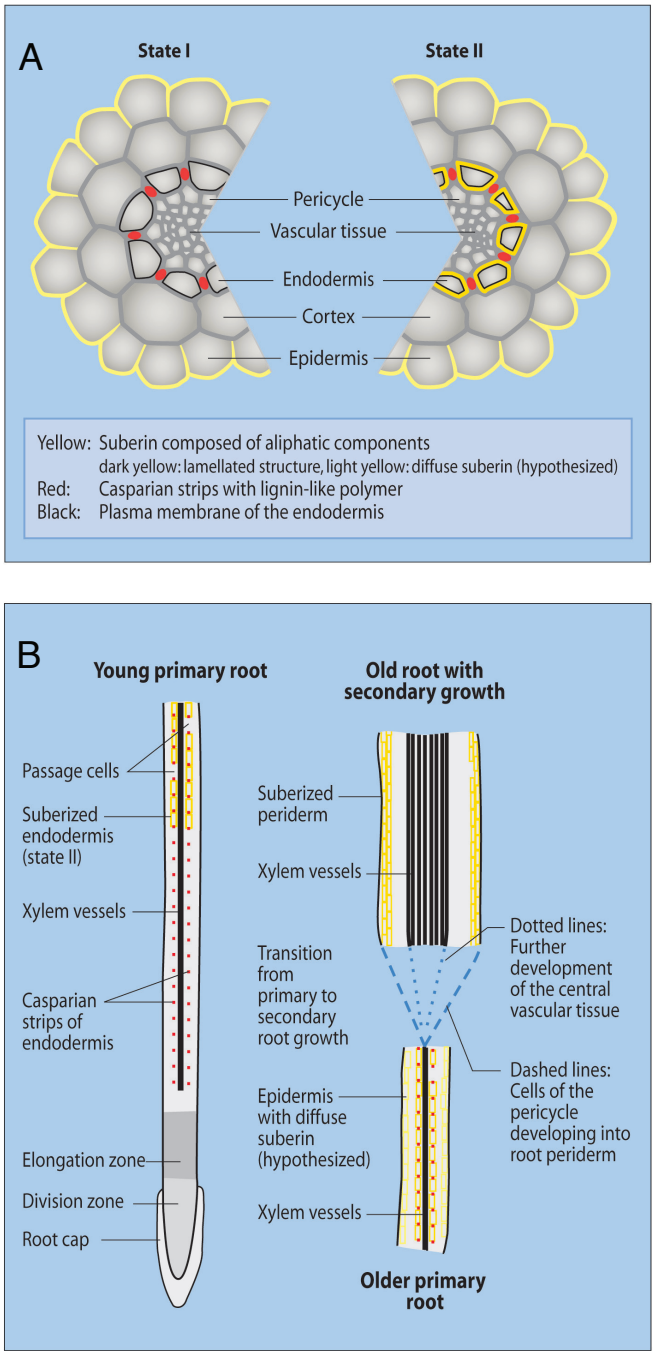


**Figure 6.** Deposition of diffusion barrier polymers in Arabidopsis roots.

Schematic diagrams of Arabidopsis roots. Cross-sections through the roots of 5-day-old Arabidopsis plants approximately 1 mm and 3 mm from the root tip **(A)**, illustrating the endodermal differentiation states I and II, respectively. Longitudinal sections of Arabidopsis roots **(B)** from different growth stages, representing different types of diffusion barriers. Root hairs and lateral roots are not depicted.

biosynthesis (Schreiber et al., 1999). Arabidopsis root epidermal cells are likely reinforced by aliphatic polymers, but the exact nature of these polymers remains unknown (Figure 6A).

A systematic study of the expression of the gene families involved in cutin and suberin biosynthesis in the root epidermis is currently feasible. Such a study could facilitate studies that clarify the presence and function of these polymers in root epidermal cell walls in Arabidopsis.

### *The endodermis: the Casparian strip and suberin lamellae*

As mentioned above, the role of the root in nutrient uptake does not allow for the formation of highly protective, essentially impermeable barriers. Instead, a compromise between protection and interaction with the environment must be attained. To overcome this problem, many multicellular organisms have developed epithelial sheets, in which cells within a layer are tightly attached to each other through ring-like junctions that seal the extracellular space (Cereijido et al., 2004). This strategy leaves the rest of the cellular surface unprotected, but it prevents the uncontrolled entry of substances across the cell layer through the intercellular spaces. The outer and inner cellular surfaces (relative to the ring) can then engage in the selective uptake of substances from the environment. This solution developed independently in both the polarized epithelia of animals and the root endodermis of vascular plants.

In the endodermis, the sealing of the extracellular cell wall space between cells is achieved by the Casparian strip, which was named after its discoverer, the German botanist Robert Caspary (Caspary, 1865) (Figure 6). The Casparian strip is a hydrophobic impregnation of the primary cell wall that is distinct from the widespread deposition of secondary cell walls that is observed in other cells (Esau, 1977) (Figure 7A). In addition, at the Casparian strip, the plasma membrane adheres tightly to the cell wall and suppresses the lateral diffusion of plasma membrane proteins (Behrisch, 1926; Karahara and Shibaoka, 1992; Alassimone et al., 2010; Bornette and Puijalon, 2011). This specialized cell wall-plasma membrane (PM) junction effectively blocks the penetration of substances through the extracellular space from the cortex into the central cylinder of the root and *vice versa*, performing a function similar to that of tight and adherens junctions in animals. Endodermal cell walls are further impregnated by suberin in their secondary developmental stage (Krömer, 1903) (Figure 7C).

### *The suberized periderm*

The roots of dicots, such as Arabidopsis, undergo considerable radial thickening during their lifetime, leading to the elimination of the endodermis, cortex and epidermis and the replacement of these layers by a periderm. This secondary dermal tissue develops in the pericycle, representing the first cell layer in the central cylinder of the root (Figure 6B). In Arabidopsis, the root periderm is composed of 1-2 layers of suberized cells. In their secondary developmental state, roots must facilitate the longitudinal transport of water and solutes in the xylem from the root to the shoot and the transport of sugars in the phloem from the shoot to the root. Thus, in contrast to primary roots, roots in their secondary developmental state do not significantly contribute to the radial uptake of water and dissolved nutrients. Ultrastructural investigations of the root periderm in Arabidopsis revealed the occurrence of characteristic lamellae that were previously described in suberized cells of the potato periderm and in cork (Figure 8). Typical of suberin depositions, these lamellae are formed next to the plasma membrane at the inner side of the cell wall. The localization of the diffusion barrier within the extracellular matrix is one of the features that clearly distinguish cutin from suberin depositions.

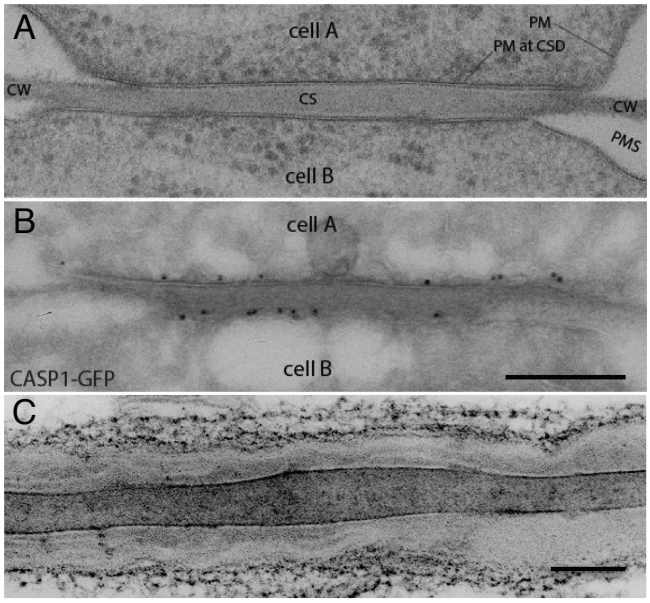


**Figure 7.** Casparian strips between endodermal cells of *Arabidopsis thaliana.*

**(A, B)** Casparian strip between two endodermal cells of a 5-day-old Arabidopsis root. **(C)** Casparian strip in the cell wall next to the periderm of a 5-week-old Arabidopsis root. **(B)** Localization of Casparian strip protein 1 (CASP1) in the plasma membrane beneath the Casparian strip (CS) (from Ropollo et al. 2011). **(C)** Casparian strips reinforced with suberin lamellae and secondary cell wall next to the periderm (picture by Martine Schorderet, University of Fribourg). Scale bars (A, B): 250 nm; (C):100 nm. CW: Cell wall, PM: plasma membrane, CSD: Casparian strip domain.

## THE COMPOSITION OF DIFFUSION BARRIERS

### The Cuticle

### *Cuticle preparation and cutin monomer composition*

The standard procedure for the determination of cutin monomer composition in different plant species is based on the isolation of the cuticle by digestion of the tissue with cellulases and pectinases (Schönherr and Riederer, 1986). In addition, solvent treatments are necessary to extract the wax components of the cuticle (see below). The chloroform-insoluble fraction contains the cutin polyester and a residual fraction that is composed primarily of polysaccharides. After extensive solvent extraction, cutin can be depolymerized by base- or acid-catalyzed procedures that cleave ester bonds. While reductive cleavage with lithium aluminum hy-

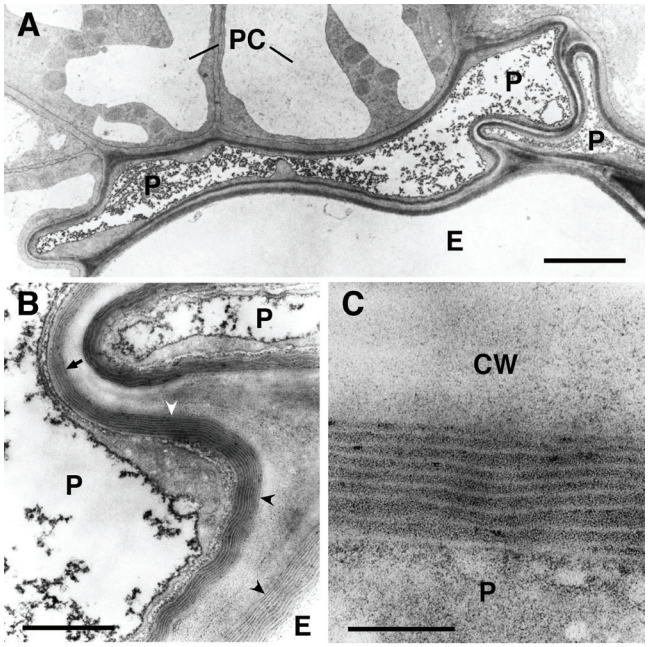


**Figure 8.** Ultrastructure of suberized root tissues of wild type Arabidopsis plants at the beginning of the secondary thickening of the root.

**(A)** Overview of suberized endodermal and peridermal cells in the root. Suberin deposition is visible as electron-opaque layer inside the primary cell wall. The fully suberized peridermal cell layer typically collapses during the dehydration and embedding procedures necessary for TEM because of the low permeability of the suberized cell walls. Scale bar: 2.5 µm. **(B)** Enlargement of **(A)**. Fine structure of suberin. The structure of the lamellae, with an alternation of electron-opaque and electron-translucent suberin layers, is clearly visible when the specimen is cut perpendicularly to the suberin layers (concave arrowheads). However, the lamellate structure of suberin is barely visible when the specimen is not cut perpendicularly to the suberin layers (arrow). Scale bar: 500 nm. **(C)** Enlargement of **(B)**. The thickness of the electron-opaque and electron-translucent suberin layers is very regular and characteristic for the tissue sample (pictures by Martine Schorderet, University of Fribourg). Scale bar: 100 nm. P: peridermal cell, E: endodermal cell: pericycle cell, CW: cell wall.

dride is the classic method (Walton and Kolattukudy, 1972; Kolattukudy, 2001a), the cutin of Arabidopsis has been characterized by transesterification with methanol containing boron trifluoride, sodium methoxide or hydrochloric acid (Bonaventure et al., 2004; Franke et al., 2005; Li-Beisson et al., 2013). Different methods produce similar results, although acid catalysis releases more 2-hydroxy acids, which may be derived from sphingolipids rather than polyester (Franke et al., 2005). The resulting polyester monomers must be converted into derivatives (e.g., with *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)) before being subjected to GC-MS for identification based on their characteristic fragmentation patterns (Walton and Kolattukudy, 1972).

Because the cuticle of Arabidopsis leaves is very thin, the polyester composition of isolated cuticles has only been reported once (Franke et al., 2005). A comparison of the polyester composition of isolated cuticles with the aliphatic monomers released by the transesterification of total leaf material that has been extensively extracted with methanol-chloroform revealed qualitatively identical composition. The analysis of exclusively solvent-extracted plant material has the disadvantage of detecting monomers that include material that is solubilized from the cell walls of internal tissues. However, this procedure also has several advantages and is commonly used. The composition of the polyester can be obtained from nearly any tissue, even from mutants with reduced or fragmented cuticles. In addition, material at the interface between the cell wall and the cuticle is not lost during preparation.

Typical cutin monomers are hydroxy and epoxy fatty acids with a backbone of 16 or 18 carbons, such as 10(9),16-dihydroxyhexadecanoic acid, 9,10,18-trihydroxyoctadecanoic acid and 9,10-epoxy-18-hydroxyoctadecanoic acid. Minor monomers may also be fatty acids (FA), fatty alcohols, fatty acids that contain aldehyde and carbonyl groups, fatty diacids and hydroxycinnamic acids. In Arabidopsis, the generic compositional predominance of C16 and C18 in-chain hydroxylated FA derivatives applies only to floral cutin (Li-Beisson et al., 2013). The leaf and stem cutin is remarkably different, as it consists of more than 40% of unsaturated α,ω-dicarboxylic acids (DCAs) (Bonaventure et al., 2004; Franke et al., 2005; Li-Beisson et al., 2013), which were previously reported as minor cutin monomers (Holloway, 1982a). Whether these compositional differences reflect differences in structure and functional properties remains to be determined. A comparison of the cutin load and composition in different organs, including rosette leaves, stems and flowers, is provided in Table 13 of the TAB chapter on acyl-lipid metabolism (Li-Beisson et al., 2013). Mutants that lack hydroxylated fatty acids in the cell wall-residue have not yet been identified, indicating that the incorporation of hydroxylated fatty acids in the epidermal extracellular matrix is necessary for the survival of the plant.

### *Cuticular wax extraction and composition*

#### *Total cuticular wax*

The entire wax of the cuticle may be obtained by surface extraction of intact plant organs with organic solvents of intermediate to low polarity. The choice of solvent is important for maximizing the solubility of all wax components, which range from extremely non-polar hydrocarbons to more polar compounds that contain different functional groups. The preferred solvent is chloroform, which was shown to generate high and reproducible wax yields from diverse plant species (Holloway, 1984). The duration of extraction depends on the thickness and the structure of the cuticle, but in most cases, extraction is complete after 20 s. Standard wax extraction protocols that include two 30-s extraction steps are assumed to result in exhaustive extraction. Prolonged exposure to solvent may result in contamination of samples by internal lipids, which is usually indicated by the presence of chlorophyll.

The major wax components are long-chain aliphatic compounds derived from saturated VLCFAs. Chemical profiling of cuticular waxes identified a set of compounds that are present in the cuticles of virtually all investigated plant species. These ubiquitous constituents are unbranched, fully saturated and contain one primary (terminal) functional group. These compounds include fatty acids, primary alcohols, aldehydes, and alkanes with chain lengths between 20 and 40 carbons, as well as alkyl

esters composed of alkyl and acyl moieties between 36 and 70 carbons in length. In Arabidopsis, aliphatic constituents typically range from 26 to 34 carbons (Samuels et al., 2008), whereas the predominant chain lengths of wax esters are in the C42 to C46 range (Lai et al., 2007). A large number of taxon-specific compounds and compound classes have been identified in cuticular wax mixtures (Jetter et al., 2006). Bifunctional components, such as alkanediols and ketols, have been detected as minor constituents of Arabidopsis stem wax (Wen and Jetter, 2009). In addition to aliphatic constituents, plants also contain non-aliphatic components in their cuticular wax. In most cases, these components are present in trace amounts, but in some plant species, they accumulate to very high levels. Most of the prominent, non-fatty acid-derived components detected in cuticular wax are triterpenoids, phytosterols, flavonoids and alkaloids (Jetter et al., 2006). An overview of the load and composition of waxes of rosette leaves and stems in Arabidopsis has been reviewed in Table 8 of the TAB Chapter on acyl-lipid metabolism" (Li-Beisson et al., 2013).

*Specific cuticular wax fractions*

In addition to the extraction of total cuticular waxes, it might be desirable to sample specific wax fractions either from specific sides of the leaf or to distinguish between epicuticular and intracuticular waxes. Waxes from one side of the leaf surface are best collected by pressing small glass cylinders filled with solvent against the leaf surface (Jetter et al., 2000; Gniwotta et al., 2005). In addition, it is often of interest to compare the composition of epicuticular wax to that of intracuticular wax. Sampling techniques employing cryo-adhesive (Jetter et al., 2000) or aqueous gum arabic (Jetter and Schaffer, 2001) allow for the selective removal of epicuticular wax, followed by chemical extraction and accurate qualitative and quantitative analyses of both cuticular wax layers. Such analyses, which were initially performed with *Prunus laurocerasus* leaves, revealed distinct compositional differences between epicuticular wax film and intracuticular wax, with the epicuticular wax mixture containing only aliphatic components (e.g., alkanes, alcohols, aldehydes fatty acids and alcohol acetates) and the intracuticular wax containing large amounts of alicyclic triterpenoids in addition to the aliphatic components (Jetter and Schaffer, 2001). Similar segregation of wax constituents into different cuticular layers was detected in a number of other species (Buschhaus and Jetter, 2011).

A detailed quantitative chemical analysis of leaf wax and a corresponding functional analysis of the water barrier properties of the cuticles of the Arabidopsis *glabra1* (*gl1*) (At3g27920) mutant were recently performed (Buschhaus and Jetter, 2012). This study demonstrated that the upper (adaxial) and lower (abaxial) leaf surfaces differed in their wax loads and had distinct wax compositions. The adaxial surface had a wax load of 0.8±0.1 µg $cm^{-2}$, with alkanes being the most abundant wax constituent, whereas the abaxial surface contained only 0.4±0.1 µg $cm^{-2}$ of wax, with primary alcohols being the most abundant class. Further analyses of the adaxial wax also revealed compositional differences between the epicuticular and intracuticular wax layers. The major wax constituents of the epicuticular layer were alkanes (60%), while the primary alcohols represented the most abundant wax component of intracuticular wax (40%). The wax formed a barrier against non-stomatal water loss, with the epicuticular wax layer contributing 66% of the total wax barrier and the intracuticular wax layer contributing only 34%.

## Diffusion Barriers in Roots

Several kinds of diffusion barriers are formed in roots that contain components of different chemical nature that may be isolated by specific isolation procedures. While the suberin polymer present in endodermis and peridermis of the root has classically been characterized in roots, recently root waxes have been identified in Arabidopsis and the chemical nature of Casparian strips has been reassessed.

### *Suberin preparation and suberin monomer composition*

Similar to cutin, the preferred sample preparation method for the analysis of the chemical composition of suberized cell walls of the root includes incubation with polysaccharide hydrolases and manual separation of suberin-containing cell walls, which are resistant to cellulases and pectinases, followed by a solvent extraction for the removal of unbound components. Suberized periderms, such as potato tuber peels, are well suited for this approach, whereas suberized root tissues, such as the mostly monolayered exodermis, have only been isolated from certain species (Schreiber et al., 2005a, 2007). In Arabidopsis, the isolation and separation of suberized tissues from roots has not yet been achieved, and hydrolase digestion and/or extraction of whole root sections is a commonly used method (Franke et al., 2005; Li-Beisson et al., 2013).

Standard depolymerization methods (i.e., base- or acid-catalyzed transmethylation) of suberin-enriched Arabidopsis root cell wall material revealed a monomer composition similar to that of potato suberin, the long-time suberin model. Bifunctional C18 monounsaturated ω-hydroxyacids and α,ω-DCA are the major monomers, followed by saturated ω-hydroxyacids and α,ω-DCAs that vary in chain-length from C16 to C24 (Franke et al., 2005). Monofunctional C18 to C24 FA and alcohols comprise 15% of the Arabidopsis root suberin polyester.

Suberin-rich roots and seeds can also be extracted without prior enzymatic digestion (Molina et al., 2006; Li-Beisson et al., 2013). Typically, in an optimized chromatography system for long-chain aliphatics, contamination with a small amount of carbohydrates does not cause significant interference. A systematic evaluation of the advantages, disadvantages and yields of the different base- and acid-catalyzed depolymerization methods for Arabidopsis root suberin has not yet been reported. However, suberin profiles generated using different methods are qualitatively similar (i.e., in the composition and ratio of the aliphatic monomers). Some quantitative differences that have been reported in the literature, even within a single publication, can be attributed to seasonal, developmental and cultivation variations. It is reasonable to assume that, like cutin, no significant differences in suberin composition will be observed between base- and acid-catalyzed transmethylation (Franke et al., 2005 suppl.). An overview of suberin monomer load and composition are presented in Tables 9 and 10 of the TAB chapter acyl-lipid metabolism (Li-Beisson et al., 2013).

### *Identification and characterization of root waxes*

While cuticles, which cover leaf and fruit surfaces, always occur together with wax that efficiently seals the cutin polymer, suberin does not necessarily contain wax molecules (Schreiber, 2010). In suberized endodermal and hypodermal cells isolated from the roots of a number of different species, typical wax compounds are absent or present in trace amounts. Conversely, in suberized tissues directly exposed to the atmosphere (e.g., potato tubers, aerial roots, etc.), which experience a lower water potential than tissues in the soil, wax amounts and wax compositions similar to those observed in cuticles have been described. Like the cuticle, wax also efficiently seals the periderm, and wax extraction from the potato periderm increases the peridermal permeability by approximately 2 orders of magnitude (Schreiber et al., 2005b).

Extraction methods similar to the methods employed for the collection of total cuticular waxes have recently been applied to whole roots of fully developed Arabidopsis plants. In addition to sterols, free fatty acids, primary alcohols, secondary alcohols and alkanes that resemble aerial waxes, the soluble lipids in these extracts also included monoacylglycerols and alkyl hydroxycinnamates (Li et al., 2007a; Molina et al., 2009). These lipids have been termed "root waxes" and are located in the tap root (Kosma et al., 2012). The taproot wax is particularly rich in alkyl hydroxycinnamates, which comprise approximately 50% of the extracted lipids, including esters of C18-C22 alcohols with the hydroxycinnamic acids p-coumarate, caffeate and ferulate (Kosma et al., 2012). The presence of C18-C22 alkyl hydroxycinnamates on root surfaces was also confirmed using laser desorption-ionization mass spectrometry (Jun et al., 2010). Alkyl hydroxycinnamates in potato tuber periderms (e.g., C16-C32 ferulate esters) contribute substantially to the barrier properties of this suberized tissue (Schreiber et al., 2005b). The roles of these waxy compounds in diffusion barrier formation and other physiological functions in Arabidopsis remain to be determined. This complex mixture of waxy components may possibly also be the result of pooling compounds that originate from the apoplast of different cell types, such as the epidermis, endodermis and periderm; thus, these components may mediate multiple physiological functions. Cell type-specific expression analysis and the localization of biosynthetic enzymes in different cell types may provide answers to these questions (Kosma et al., 2012).

### *The composition of the Casparian strip*

Despite extensive research, considerable uncertainty as to the chemical nature of this localized wall impregnation remains. Caspary himself was unable to conclude whether the Casparian strips are composed of "Holzstoff" (i.e., wood substance, or lignin) or "Korkstoff" (i.e., cork substance, or suberin). Krömer later concluded that the Casparian strip shows a typical "wood staining reaction" and found no convincing evidence for the presence of suberin (Krömer, 1903). Despite this finding, suberin was often proposed to be the major constituent of Casparian strips. In 1961, Vanfleet discussed these divergent views and concluded that although none of the studies "satisfy the question, lignin or suberin, they do suggest the presence of phenolic material" (Vanfleet, 1961). However, Vanfleet also cites studies that suggest that the "Casparian strip is probably made of lignin plus fatty acid." Regardless, the textbook "Anatomy of Seed Plants" by Esau describes the Casparian strip as being composed of suberin and does not mention lignin (Esau, 1977). One major obstacle that must be overcome when investigating this issue is that after the establishment of the Casparian strip, Arabidopsis endodermal cells rapidly form suberin lamellae on all cell surfaces, making it difficult to exclusively analyze Casparian strips. Pure samples of Casparian strips are difficult to obtain in sufficient amounts for chemical analysis. In addition, a survey by Wilson and Peterson that used a number of histochemical stains on more than 30 plant species suggested that there is a considerable degree of natural variation in the chemical composition of the Casparian strip (Wilson and Peterson, 1983).

Schreiber *et al.* used natural variations in endodermal development to analyze the endodermis in its primary developmental state, which is characterized by the sole presence of Casparian strips, in three species: *Clivia miniata*, *Pisum sativum* and *Monstera deliciosa* (Schreiber, 1996). This study demonstrated that lignin is the major polymer in the Casparian strips of these species. However, suberin was also detected in small amounts. Histochemical investigations of Casparian strips in a variety of species typically reveal the presence of both lignin (via staining with phloroglucinol/HCl) and suberin (via staining with Sudan). The lignin monomer ratios observed in the endodermal cell wall are similar to the lignin of xylem vessels, but a specialized form of Casparian strip lignin, which may represent an adaptation to the function of the Casparian strip as a solute diffusion barrier, cannot be excluded (Naseer et al., 2012). In another study, maize roots were dissected at different developmental stages (Zeier et al., 1999). The first stage, which included only Casparian strips, was observed to contain a distinct lignin component and smaller amounts of suberin (Schreiber et al., 1999). Recent developmental studies in Arabidopsis, with a higher spatial resolution along the length of the developing root, indicate that lignification of the primary cell wall occurs in parallel with the tight attachment of the plasma membrane to the cell wall site of the Casparian strip, approximately 12 cells after the onset of elongation. In contrast, suberization appears to occur much later, when functional Casparian strips have already been formed. Histochemically detectable suberin appears approximately 38 cells after the onset of elongation (Naseer et al., 2012). Importantly, this study employed genetic and pharmacological manipulations, which demonstrated that interference with suberin deposition has no observable effect on the development of functional Casparian strips. Conversely, blocking lignin biosynthesis using an inhibitor of the phenylpropanoid pathway abrogates the formation of Casparian strips and leads to a completely permeable endodermis. In addition, monolignol feeding experiments and chemical analysis of root tips that contain only Casparian strips as potentially lignified cell layers both indicated that the initial Casparian strips of Arabidopsis are composed of a polymer closely related to the "canonical" lignin of xylem vessels. These findings strongly suggest that in Arabidopsis, suberin is confined to the formation of suberin lamellae during the secondary developmental state of endodermal cells (Figure 6A, Figure 7A, C). Currently, the spatial resolution of sampling methods for the analysis of root cell walls does not allow for the chemical determination of whether suberin is added to older Casparian strips at the time of suberin lamellae formation.

To answer this question, Casparian strip cell walls and the cell walls from the inner and outer tangential walls of the endodermis would have to be isolated in sufficient amounts for quantitative compositional analysis; such an experiment is not currently realistic in Arabidopsis.

In the past, suberin has been described as a polymer that not only contains ferulate esterified to the aliphatic polymer but also contains a phenylpropanoid-derived polyaromatic domain. The definition of the polyaromatic domain in suberin is specifically based on studies with the potato wound periderm (Bernards, 2002). Recent studies used an inhibitor of the phenylpropanoid pathway in Arabidopsis that blocks both monolignol formation and the formation of other monomers needed for the synthesis of the aromatic polymer of suberized tissues (Naseer et al., 2012). As mentioned above, the application of this drug to Arabidopsis seedling roots led to a complete block of lignin formation without affecting the formation of (aliphatic) suberin in distal endodermal cell walls. This finding demonstrated that in Arabidopsis, the aliphatic polyester (suberin) could form independently of an aromatic polymer. The finding that suberin can exist independently of the presence of a polyaromatic domain raises the question of whether suberin should be in general viewed as a polymer with an aliphatic and a polyaromatic domain or a polymer containing only an aliphatic domain with esterified aromatic compounds. The data described above clearly demonstrate that the Casparian strip of 5-day-old Arabidopsis seedlings is a functional diffusion barrier that is composed predominantly of a lignin polymer. This finding is also supported by the recent identification of cell wall biosynthetic genes involved in Casparian strip formation (Lee et al., 2013) (see below). Additional model systems must be investigated to answer important questions concerning the general structure of suberin and the importance of lignin for the formation of the Casparian strip in other species. It will also be important to obtain better read-outs for barrier functionality. The use of fluorescent tracers, such as PI, is very convenient. However, large, charged molecules might behave differently from relevant monovalent or divalent ions and polar water molecules. It seems reasonable to assume that a defect that allows for the penetration of PI would also allow for the penetration of elemental nutrients and water. The reverse conclusion, however, does not apply. Many mutants with subtle defects that do not exhibit penetration of PI may display significant penetration of other elements or water.

### Lipid-based Diffusion Barriers as Part of the Cell Wall Continuum

A substantial drawback of biochemical preparation methods using cell wall degradation enzymes is that essential information about the relation of the polymers in the diffusion barriers to the polysaccharide cell wall is lost. In addition, complete depolymerization of the polyester destroys polyester structure, including the linkages between different aliphatic and non-aliphatic cutin monomers.

Chemical functions of the intact cutin polymer have been characterized using different techniques, including solid-state nuclear magnetic resonance (NMR) and Fourier transform infrared (FT-IR) spectroscopy (Stark and Tian, 2006). In addition, partial polymerization followed by liquid state NMR or GC/MS provides information about the linkages between different monomers within the polymer (Graça et al., 2002; Graça and Santos, 2006; Tian et al., 2008; Graça and Lamosa, 2010). However, these techniques require quantities of material that cannot be easily obtained in Arabidopsis. The feasibility of analyzing aliphatic polyesters in the petals of Arabidopsis using FTIR spectroscopy has been recently demonstrated, providing new opportunities for the characterization of the relationship between the cuticle and the cell wall (Mazurek et al., 2013).

## THE BIOSYNTHESIS OF THE BUILDING BLOCKS OF DIFFUSION BARRIERS

The significance of aliphatic components in the formation of diffusion barriers was recognized over 100 years ago (Sitte and Rennier, 1963). A portion of this chapter will be devoted to the biosynthesis of specific aliphatic molecules that form the cuticle and the suberin lamellae in the endodermis and the periderm of the root. Although the topic was debated for years, it was only recently demonstrated that the early Casparian strip is composed of lignin (see above). In recent decades, lignin production has been characterized in detail in the context of secondary wall formation (Boerjan et al., 2003; Liu, 2012). The biosynthesis of monolignols has been previously reviewed (Vogt, 2010; Fraser and Chapple, 2011; Liu, 2012). In contrast, the mechanisms by which the formation of the Casparian strip occurs remain unknown and cannot be covered here. This chapter reviews recent advances in our understanding of the transport processes that are involved in diffusion barrier formation, as well as the localized deposition of lignin in Casparian strips, which is orchestrated by endodermal cells.

Aliphatic monomers that are included in aliphatic polyesters and aliphatic wax components branch off of the general lipid biosynthetic pathway and are discussed in the TAB chapter on acyllipid metabolism (Li-Beisson et al., 2013). Aromatic components of aliphatic diffusion barriers (i.e., cinnamic acid derivatives) arise from the general phenolpropanoid pathway that has been previously reviewed (Vogt, 2010; Fraser and Chapple, 2011).

### The Elucidation of Cutin and Suberin Monomer Biosynthesis

For several reasons biochemical approaches gave only limited information on cutin and suberin biosynthesis. As discussed above, cutin and suberin are cell wall modifications that occur in distinct dermal tissues. Therefore, in homogenized plant material, the proteins involved in cutin and suberin formation are underrepresented in comparison with those that act in parenchymatic tissues. In addition to the limited availability of suitable substrates for these proteins and the hydrophobicity and membrane-associated nature of key enzymes in this process, this issue severely limits the application of biochemical approaches to analyses of cutin and suberin biosynthesis. As a result, only few attempts to biochemically characterize cutin- and suberin-related proteins have made significant contributions to our current knowledge of the enzymology of cutin and suberin formation. Using microsomal

fractions from *Vicia sativa* surface tissues, Kolattukudy and colleagues (Soliday and Kolattukudy, 1977, 1978) demonstrated that cytochrome P450 monooxygenases (P450) are involved in the ω-hydroxylation and mid-chain hydroxylation of C16 and C18 fatty acid precursors. Additionally, studies using "wound healing" potato tuber disks as a source of suberizing tissue resulted in the characterization of an NADP-dependent oxidoreductase activity that is required for the formation of diacids from ω-hydroxyacids (Agrawal and Kolattukudy, 1978) and provided evidence for the involvement of a $H_2O_2$-dependent peroxidase in the synthesis of aromatic suberin components (Razem and Bernards, 2002; Razem and Bernards, 2003). Finally, the observed properties and fatty acid elongase (FAE) activity in corn root microsomes correlated with the expected qualitative (i.e., chain-length profile) and quantitative requirements for suberin biosynthesis along the root axis (Schreiber et al., 2005a).

Forward and reverse genetic approaches resulted in a broad elucidation of cutin and suberin biosynthesis in Arabidopsis as well as in tomato fruits and potato tubers representing excellent model systems for cutin and suberin biosynthesis, respectively.

### *Core reactions common to cutin and suberin monomer biosynthesis*

Based on the predominant classes of compounds detected in other plant species, namely ω-hydroxy acids, α,ω-DCA, alcohols, carboxylic acids and fatty acids, and the presence of glycerol in both cutin and suberin, it is reasonable to assume that the core reactions in cutin and suberin biosynthesis are catalyzed by the same or similar enzymes. The identification of candidate genes was facilitated by epidermal and endodermal transcriptomics (Birnbaum et al., 2003; Suh et al., 2005; Brady et al., 2007), which allowed for the identification of transcripts whose predicted functions aligned with the proposed metabolic activities deduced from the known chemical composition of these polyesters. Four biosynthetic steps are common to the biosynthetic pathways of these polyesters, namely the oxygenation, reduction and activation of fatty acids and their subsequent transfer to glycerol-3-phosphate.

#### *Oxygenation of fatty acids*

In Arabidopsis, oxygenated FA derivatives (e.g., ω-hydroxyacids and α,ω-DCA) represent 70-90% of the detectable cutin and suberin monomers (Bonaventure et al., 2004; Franke et al., 2005; Li-Beisson et al., 2013). These oxygenated FAs are generated by the enzyme-catalyzed insertion of oxygen into the carbon chain, which is catalyzed by cytochrome P450 oxygenases (P450s). The majority of FA hydroxylases belong to the CYP86 and CYP94 families of P450s; however, FA hydroxylases have recently been identified in other P450 families, including CYP77, CYP703, CYP704 and others (Pinot and Beisson, 2011). Thus, P450 genes represent prime candidates for involvement in polyester biosynthesis. Compositional polyester analysis and TEM visualization of the epidermal ultrastructure of mutants in *CYP86A8/LCR* (Wellesen et al., 2001), *CYP86A2/ATT1* (Xiao et al., 2004) and *CYP86A4* (At2g45970) (Li-Beisson et al., 2009) demonstrated the involvement of these P450s in cutin formation in the leaf, stem and flower, respectively. These results were corroborated by biochemical studies that demonstrated ω-hydroxylase activity for palmitate (C16:0) in the microsomes of yeast expressing CYP86A4 or CYP86A8 and for oleate (C18:1) in yeast expressing CYP86A8. Similar to CYP86A8, palmitate and oleate hydroxylation activity was also demonstrated for recombinant CYP86A1, a FA ω-hydroxylase involved in suberin biosynthesis (Benveniste et al., 1998). The *horst* mutant, which carries a T-DNA insertion in the CYP86A1 (At5g58860) gene, has reduced levels of 16-hydroxy palmitate and 18-hydroxy oleate, which are the predominant monomers in Arabidopsis root suberin (Li et al., 2007a; Höfer et al., 2008). Very-long-chain ω-hydroxyacids (C22-C24) in *horst* suberin are not affected. However, these oxygenated fatty acids are nearly absent in the suberin of *ralph*, a mutant in CYP86B1 (At5g23190). Although the biochemical activity of recombinant CYP86B1 could not be determined, the *ralph* chemical phenotype underlines the importance of CYP86B1 in the ω-oxygenation of polyester precursors (Compagnon et al., 2009; Molina et al., 2009).

In the *cyp86a2*/*att1*, *cyp86a4*, *cyp86a1*/*horst* and *cyp86b1*/*ralph* mutant polyesters, not only the ω-hydroxyacids but also the corresponding α,ω-DCAs are greatly reduced (Xiao et al., 2004; Höfer et al., 2008; Compagnon et al., 2009; Li-Beisson et al., 2009; Molina et al., 2009; Li et al., 2010). It remains to be determined whether this reduction is a consequence of the depletion of ω-hydroxyacids, the direct precursor of α,ω-DCA, or the consequence of a catalytic function in the subsequent oxidation of ω-hydroxyacids to α,ω-DCA, as was demonstrated for CYP94C1 (Kandel et al., 2007) and CYP94A5 (Le Bouquin et al., 2001). Alternatively α,ω-DCA can be produced by a two-step oxidation that involves a ω-hydroxyacid and a ω-oxoacid dehydrogenase activity. The epidermal expression of *ADHESION OF CALYX EDGES*/*HOTHEAD* (*ACE/HTH*) (At1g72970) and the reduced level of α,ω-DCA in the leaf cutin of the *ace*/*hth* mutant suggest that ACE/HTH is an enzyme that catalyzes the first step of this ω-hydroxyacid oxidation pathway (Krolikowski et al., 2003; Kurdyukov et al., 2006b).

#### *Fatty acid reduction*

The generation of long chain fatty alcohols is required for cutin and suberin monomer biosynthesis, although these modifications are less abundant in shoot polyesters. The reduction of the FA carboxyl group is catalyzed by fatty acid reductases (FAR), an eight-member protein family in Arabidopsis. *FAR2* (At3g11980) and *FAR3/CER4* (At4g33790) are involved in sporopollenin and epidermal wax production, respectively (Rowland et al., 2006; Chen et al., 2011b). The analysis of mutants of endodermis-expressed FARs demonstrated chain-length-specific reductions in the alcohols of root suberin (Domergue et al., 2010). C18 alcohol was reduced in the *far5* (At3g44550) mutant, C20 alcohol was reduced in the *far4* (At3g44540) mutant and C22 alcohol was reduced in the *far1* (At5g22500) mutant. The observed phenotypes correlated with the reported chain length specificities for these FARs when they were expressed in yeast and demonstrated that Arabidopsis FAR1, FAR4, and FAR5 produce the alcohols for root suberin and root waxes (Kosma et al., 2012).

*Fatty acid activation*

Prior to entering the cutin- and suberin-specific pathways, FAs synthesized in plastids are activated to the corresponding acyl-CoA esters by long chain acyl-CoA synthases (LACS). Similar to the other FA modification reactions, FARs require CoA-activated FAs. Changes in the ultrastructure and compositional analysis of the polyester in the cuticle revealed that *LACS1* (At2g47249) and *LACS2* are involved in cutin formation (Schnurr et al., 2004; Bessire et al., 2007; Lu et al., 2009; Weng et al., 2010). In addition, a mutation in *LACS9* (At1g77590) leads to a permeable cuticle, indicating a role for the protein encoded by this gene in cutin formation (Xia et al., 2010). The characterization of the encoded proteins showed that these enzymes activate FAs of various chain lengths, including those present in cutin and suberin. In addition, recombinant LACS2 was demontrated to prefer ω-hydroxy palmitate to free palmitic acid, suggesting that ω-hydroxyacids are this protein's primary *in vivo* substrates. Accordingly, α,ω-DCAs are strongly reduced in cutin from the *lacs2* mutant. Although CoA-activation is likely to be involved in suberin biosynthesis and *LACS1* and *LACS2* are expressed in the roots, direct evidence for the involvement of LACS in suberin formation is currently lacking.

*Acyl transfer to glycerol-3-phosphate*

The formation of acylglycerols, which is catalyzed by members of the glycerol-3-phosphate acyltransferase (GPAT) family, is another FA conjugation reaction required for cutin and suberin biosynthesis (Zheng et al., 2003). Monomer-specific changes in polyester composition, as determined by reverse genetic approaches, demonstrated that GPAT4, GPAT8 and GPAT6 are required for cutin formation in leaves and flowers (Li et al., 2007b; Li-Beisson et al., 2009), whereas GPAT5 is involved in suberin biosynthesis (Beisson et al., 2007). Interestingly, *in vitro* biochemical studies revealed that these GPATs catalyze the acyl transfer to the *sn-2* position of glycerol in contrast to the classical GPAT-catalyzed *sn-1* acylation (Yang et al., 2010). Therefore, an important branch point between membrane lipid synthesis and polyester synthesis may have evolved within the GPAT family (Yang et al., 2012). In addition, differences in the activity of the GPAT4/8 and GPAT5 are hypothesized to be critical for the synthesis of cutin and suberin; these enzymes are discussed in detail below (Yang et al., 2012).

### *Characteristic reactions in the biosynthesis of aliphatic cutin monomers*

*Formation of sn2-monoacylglycerols*

The GPATs involved in cutin biosynthesis, GPAT4, GPAT6 and GPAT8, are bifunctional enzymes. In addition to an acyltransferase domain, they possess a second domain that has phosphatase activity. The end products of the reactions catalyzed by these enzymes are *sn-2* monoacylglycerols (Yang et al., 2010). GPAT5 lacks this dual functionality and generates 2-acyl-glycerol-3-phosphate. The synthesis of cutin from *sn-2* monoacylglycerols *in vitro* (see below) demonstrates that this *sn-2* acylation and the phosphatase activity of the GPAT4/GPAT8/GPAT6-branch may be a central step of extracellular cutin biosynthesis (Yeats et al., 2012a; Yeats et al., 2012b). GPAT4 and GPAT8 act redundantly in the formation of leaf and stem cutin, which are rich in diacids (Li et al., 2007b), and only the *gpat4 gpat8* double mutant displays a reduced level of leaf cutin (65% of the wt level).

*Synthesis of 10,16-dihydroxyhexadecanoic acid-rich cutins*

Over 55% of the floral cutin that can be transesterified is comprised of 10(9),16-dihydroxyhexadecanoic acid, an in-chain hydroxylated monomer of low abundance in leaf and stem cutin (see above). Therefore, Arabidopsis flowers offer a unique opportunity for studying the biosynthesis of cutin that is rich in a typical cutin monomer that has a wide distribution in other plant species. Complementary reverse genetics and biochemical approaches identified *CYP77A6* (At3g10570), which encodes the P450 in-chain hydroxylase that catalyzes the 10-hydroxylation (and the 8 and 9 hydroxylations *in vitro*) of 16-hydroxyhexadecanoic acids in flower cutin biosynthesis (Li-Beisson et al., 2009). In *CYP77A6* mutants, the absence of 10(9),16-dihydroxy hexadecanoic acid is compensated by an over-accumulation of other C16 monomers (Li-Beisson et al., 2009). *GPAT6* mutants exhibit a substantial reduction in all C16 monomers in flower cutin, including a 90% reduction in 10(9),16-dihydroxy hexadecanoic acid. Both mutants are characterized by an absence of nanoridges on the surface of petal epidermal cells, demonstrating the significance of 10(9),16-dihydroxy hexadecanoic acid in nanoridge formation (see above). An absence of nanoridges has also been observed in *dcr*, a mutant in an acyltransferase of the BAHD protein family (Panikashvili et al., 2009). Although a biochemical study characterized DCR as a diacylglycerol acyltransferase *in vitro,* the role of DCR in cutin precursor biosynthesis *in vivo* remains elusive (Rani et al., 2010).

*Other reactions*

In some species, epoxy fatty acids are also present in cutin, and they may be present in Arabidopsis in low quantities in order to cross link the cutin monomer (Suh et al., 2005; Beisson et al., 2012). Enzymes that perform epoxidation have been identified in the cytochrome P450 family of enzymes (Sauveplane et al., 2009). However, there is currently no *in vivo* evidence for their involvement in this process.

### *Characteristic reactions in the biosynthesis of aliphatic suberin monomers*

The major chemical differences between cutin and suberin aliphatic polyesters are: i) the abundance of very long-chain fatty acid (VLCFA) derivatives and ii) the presence of up to 5% aromatic components, mainly ferulic acid, in Arabidopsis suberin.

*The biosynthesis of VLCFAs in suberin*

A prerequisite for the synthesis of suberin is the production of VLCFAs by fatty acid elongation, which is also essential for wax biosynthesis (see below). In Arabidopsis, the initial step of FAE is catalyzed by 21 members of the 3-keto acyl-CoA synthase (KCS)

family. The enzymes encoded by *KCS2/DAISY* (At1g04220) and *KCS20* (At5g43760) have been demonstrated to be involved in the production of >C20 suberin constituents (Franke et al., 2009; Lee et al., 2009). The moderate chemical phenotypes and the substantial amounts of very long-chain suberin monomers observed in these mutants indicate the participation of one or more additional KCS enzymes in suberin biosynthesis. Accordingly, five other KCSs have strong expression in internal root tissues (Franke et al., 2012). However, the identification of other suberin-related KCSs is hampered by the functional redundancy within this protein family. Expression of Arabidosis KCS in heterologous systems has identified five KCSs that may participate in the elongation of C22 monomers (Trenkamp et al., 2004; Blacklock and Jaworski, 2006; Paul et al., 2006; Tresch et al., 2012). The role of enzymes that generate malonyl-CoA, the 2-carbon donor for FAE, in suberin biosynthesis requires further study.

### *Open questions regarding the biosynthesis of polyester intermediates*

Many key enzymes involved in cutin and suberin monomer biosynthesis have been identified through the characterization of Arabidopsis mutants. However, inferring enzymatic activity from chemical phenotypes of the polymer is difficult, as plants are complex organisms that often compensate for the loss of a gene function by activating other pathways, and biochemical verification is only available for a subset of proteins. The organization of individual reactions into pathways, the *in vivo* substrates of the required enzymes, and the relevant metabolic intermediates must be elucidated. For example, it is not known whether FAE precedes ω-hydroxylation. It also remains to be determined at which steps of FA modification CoA-activation and acylglycerol formation take place (Beisson et al., 2012).

The compositional analysis of cutin in *cyp86a4* and *cyp77a6* mutants and the finding that recombinant CYP77A6 hydroxylates 16-hydroxyhexadecanoic acids indicate that in flower cutin biosynthesis, ω-hydroxylation is required for subsequent in-chain hydroxylation. In contrast, yeast-expressed CYP86B1 did not generate any hydroxylated products from the provided FAs. To determine whether conjugated FA, such as acyl-CoAs and glycerolipids, which are potentially generated by LACS or GPAT, and not free FA are the endogenous substrates further study is required. For CYP86A22 from *Petunia sp.,* it has been demonstrated that saturated and unsaturated acyl-CoA derivatives are the substrates for ω-hydroxylation (Han et al., 2010). A strong partnership of GPAT with CYP86 has been demonstrated by co-expression of the suberin-associated genes GPAT5 and CYP86A1 (Li et al., 2007a; Li-Beisson et al., 2009). Co-expressing lines incorporated C20 and C22 ω-hydroxyacids and α,ω-DCAs, which are typically present in root polyesters, into shoot polyesters (Li et al., 2007a). These findings and other metabolic evidence concerning the sequential order of reactions has been previously discussed (Yephremov and Schreiber, 2005; Li-Beisson et al., 2013; Beisson et al., 2012).

Many open questions concern the mechanisms by which the biosynthesis of cuticular lipids interacts with general acyl-lipid metabolism, as a number of mutants affected in general lipid biosynthesis have cuticular phenotypes, including *fatty acid desaturase2* (*fad2*) (At3g12120), *fatty acyl-ACP thioesterase b* (*fatb*) (At1g08510), *acyl carrier protein4* (*acp4*) (At4g25050), *acyl CoA binding protein 3* (*acbp3*) (At4g24230), *acbp4* (At3g05420), and *acbp6* (At1g31812) (Bonaventure et al., 2004; Xia et al., 2009; Xia et al., 2012).

## The Biosynthesis of Aliphatic Wax Components

Characterization of numerous mutants isolated from several plant species, including barley, *Brassica napus*, maize, and Arabidopsis, has revealed that a multitude of gene products are involved in wax biosynthesis, as well as the regulation of this process and the transport of wax to the plant surface. Because wax biosynthetic enzymes are membrane- and possibly cell wall-associated, biochemical approaches have not been successfully employed for their identification. In contrast, genetic approaches using Arabidopsis *eceriferum* (*cer*) mutants and maize *glossy* (*gl*) mutants have proven invaluable for isolating genes involved in wax production. The biosynthesis of aliphatic wax components involves two types of pathways: those required for the synthesis of VLCFAs and those required for the modification of VLCFAs to form diverse wax products. The 16- and 18-carbon fatty acids that are supplied by the plastid are further extended into VLCFA wax precursors by fatty acid elongase (FAE) complexes associated with the endoplasmic reticulum (ER) (Kunst and Samuels, 2009). VLCFAs are used by the acyl-reduction and alkane-forming pathways to yield all the major wax constituents, which is reviewed in the TAB chapter on acyl-lipid metabolism (Li-Beisson et al., 2013). The biosynthesis of triterpenoids that are non-aliphatic wax components has been previously described (Wang et al., 2011a).

### *The biosynthesis of VLCFAs*

FAE complexes are heterotetramers of monofunctional proteins that catalyze a series of four reactions to elongate plastid-generated C16 or C18 fatty acids by the sequential addition of two carbons from malonyl-CoA. The condensing enzyme, or β-ketoacyl-CoA synthase (KCS), catalyzes the first of the four reactions and is both rate-limiting and specific for the chain length of acyl-CoA synthesized (Millar and Kunst, 1997). Two highly divergent families of KCSs have been identified in Arabidopsis: an FAE1-type family homologous to the first condensing enzyme demonstrated to be involved in seed oil biosynthesis (Kunst et al., 1992; James et al., 1995), and ELOs, which have homology to the *Saccharomyces cerevisiae* ELO family that is responsible for sphingolipid synthesis (Dunn et al., 2004). Of the 21 FAE1-type KCS enzymes in Arabidopsis (Joubes et al., 2008), 11 have been shown by microarray analysis to be upregulated in the stem epidermis (Suh et al., 2005). Only one of these proteins, ECERIFERUM 6/3-KETOACYL-COA SYNTHETASE 6/CUTICULAR WAX 1, which is encoded by *CER6/KCS6/CUT1* (At1g68530) (Millar et al., 1999; Fiebig et al., 2000; Joubes et al., 2008), has a dominant role in the elongation of VLCFAs for cuticular wax synthesis, as *CER6* suppression causes a dramatic reduction in wax monomers longer than C24 and a 90-95% depletion of cuticular wax on Arabidopsis inflorescence stems (Millar et al., 1999). The only Arabidopsis ELO protein functionally characterized to date is HIGH EXPRESSION OF OSMOTICALLY

RESPONSIVE GENES 3, encoded by *HOS3* (At4g36830), which was shown to be involved in the synthesis of VLCFAs ranging from C22 to C26 in length. Disruption of VLCFA production in the *hos3* mutant causes ceramide deficiency and affects ABA-related stress responses, such as root growth inhibition, seed germination with ABA treatment and water loss from leaves. These results implicate HOS3 and VLCFA pathway products in several aspects of ABA-mediated stress signaling (Quist et al., 2009).

Recently, an additional protein has been demonstrated to be involved in the elongation of VLCFAs to 30 carbons, which is critical for the production of most wax constituents. Functional characterization of KCS enzymes from Arabidopsis in yeast revealed that the CER6 enzyme, the key KCS associated with cuticular wax synthesis, is unable to elongate VLCFAs beyond C28. Mutant screens have not identified other condensing enzymes involved in VLCFA elongation beyond C28. The only known mutant defective in the conversion of C28 to C30 VLCFAs is *eceriferum2* (*cer2*) (At4g24510), indicating that CER2, annotated as a BAHD acyltransferase based on sequence homology, is required for C28 elongation. Biochemical analysis of CER2 demonstrated that CER2 does not share the catalytic mechanism that has been confirmed for other members of the BAHD family and that concerted activity of CER2 and CER6 in *Saccharomyces cerevisiae* is necessary and sufficient for C30 VLCFA synthesis (Haslam et al., 2012). Recent evidence also indicated that a *CER2-like* gene called *CER26* encodes an enzyme involved in the elongation of C30 to C32 for leaf cuticular wax production (Pascal et al., 2013).

### *The synthesis of different wax components from VLCFAs*

All aliphatic wax components are generated from VLCFAs by two biosynthetic pathways: the alcohol-forming acyl reduction pathway, and the alkane-forming decarbonylation pathway. The enzymes and reactions of the acyl reduction pathway that generate primary alcohols and alkyl esters have been well characterized in Arabidopsis (Samuels et al., 2008). The alkane formation pathway is more obscure, and it has only recently been demonstrated that co-expression of two well known wax-related Arabidopsis proteins, ECERIFERUM 1, encoded by *CER1* (At1g02205), and ECERIFERUM 3, encoded by *CER3* (At5g57800) (Li-Beisson et al., 2013), in yeast results in the production of Arabidopsis-characteristic alkanes (Bernard et al., 2012). Although the exact biochemical role of these proteins in alkane formation remains unresolved, the CER1 and CER3 proteins were shown to physically interact in the endoplasmic reticulum in both yeast and Arabidopsis, suggesting that they associate in a complex. Both CER1 and CER3 contain tripartite His clusters, which are essential for the catalytic activity of hydroxylase and desaturase enzymes. Site-directed mutagenesis of these His-rich motifs revealed that all three His clusters were required for CER1 activity but not CER3 activity. CER1 was also demonstrated to interact with endoplasmic reticulum-localized isoforms of cytochrome b5 (CYTB5), a proposed electron donor to nonheme irons associated with His clusters. Co-expression of CYTB5 with CER1 and CER3 in yeast resulted in a two-fold increase in alkane yield, suggesting that CYTB5s are CER1 electron donors and that alkane synthesis is a redox-dependent process (Bernard et al., 2012). Alkanes are further metabolized in the Arabidopsis stem epidermis to yield secondary alcohols and ketones. A midchain alkane hydroxylase, which is encoded by *MIDCHAIN ALKANE HYDROXYLASE1 (MAH1)* (At1g57750), catalyzes the conversion of odd-chain alkanes to secondary alcohols and is likely to catalyze the conversion of secondary alcohols to ketones (Greer et al., 2007).

### Linking Aromatic Components with Aliphatic Polyesters and Wax Components

While suberin is rich in ester-bound aromatic cinnamic acid derivatives (i.e., ferulic acid and coumaric acid), cutin contains only small amounts of these compounds. In roots, alkyl hydroxycinnamates have been identified, while cuticular wax does not contain significant amounts of these compounds. The transfer of these aromatic compounds to aliphatic molecules depends on different members of the BAHD family of acyltransferases.

For the incorporation of ferulate into the suberin polyester, the *ALIPHATIC SUBERIN FERULOYL TRANSFERASE/HYDROXYCINNAMOYL-COA:ω-HYDROXYACID O-HYDROXYCINNAMOYL TRANSFERASE* (*ASFT/HHT*) (At5g41040) is required. In *asft*/*hht* mutants, suberin is strongly depleted of ferulate (Gou et al., 2009; Molina et al., 2009). Although the composition of aliphatic root suberin is not affected in *asft/hht* mutants, in the seed coat polyester, a reduction in ω-hydroxylated aliphatic compounds was detected in similar molar amounts to the ferulate reduction. Biochemical studies confirmed that recombinant ASFT/HHT catalyzes an acyl transfer from feruloyl-CoA to ω-hydroxyacids and alcohols. Another member of the BAHD family, DEFECTIVE IN CUTIN FERULATE, which is encoded by *DCF* (At3g48720), has been implicated in the feruloylation reaction of the lipid polyester cutin. In contrast to other species, the polysaccharide cell wall of Arabidopsis is not feruylated (Rautengarten et al., 2012). The BAHD family member *FATTY ALCOHOL:CAFFEOYL-CoA CAFFEOYL TRANSFERASE* (*FACT*) (At5g63560) encodes the enzyme responsible for the synthesis of a subset of alkyl hydroxycinnamate esters, the alkyl caffeates, in root waxes (Kosma et al., 2012).

## TRANSLOCATION OF DIFFUSION BARRIER COMPONENTS

Key enzymes involved in the biosynthesis of polyester monomers, as well as aliphatic wax components, are localized at the endoplasmic reticulum (ER). Major challenges in studying the formation of plant diffusion barriers involve the elucidation of the mechanisms that underlie the transport of cuticular lipid precursors from the ER across the cytoplasm to the plasma membrane (PM), across the PM, and within the cell wall, as well as the subsequent polymerization of the cutin or suberin macromolecule and the coordinated assembly of the components into a functional diffusion barrier. In contrast, monolignol biosynthesis takes place in the cytoplasm. As a result, no additional transport steps are necessary prior to the export of monolignols to the apoplast (Liu et al., 2011; Liu, 2012).

## Transport of Aliphatic Compounds

### *Transport of aliphatic compounds across the plasma membrane*

One mechanism for the delivery of wax and polyester components to the PM involves a classical secretory pathway in which secretory vesicles carry the biosynthetic intermediates and export them into the cell wall by exocytosis (Franke and Schreiber, 2007; Pollard et al., 2008). In addition, direct transfer of lipids from the ER to the PM has been hypothesized (Kunst and Samuels, 2003). Interestingly, some of the enzymes of the BAHD family, such as DCR and DCF, which are involved in the formation of cutin intermediates, have been localized to the cytoplasm, which necessitates the presence of their substrates in this compartment or at the PM/cytoplasm interface (Panikashvili et al., 2009; Rautengarten et al., 2012). Thus, additional transport mechanisms may be involved (Panikashvili et al., 2009).

While the wax molecules that must be transported are relatively well characterized, the nature of the exported molecules for polyester formation (i.e., monomeric acyl lipids, esterified lipids, such as acylglycerol and feruloyl conjugates, or intracellular preformed oligomers) remains largely unknown. However, there is increasing evidence that free fatty acids and *sn-2* monoacylgylcerols are exported (Li et al., 2007b). In GPAT5-overexpressing lines, new free fatty acids and suberin-specific monoacylglycerols appear in cuticular waxes and are incorporated into cutin when CYP86A1 is co-expressed with GPAT5 (Li et al., 2007a). Because it is unlikely that monoacylglycerols are the only precursors of polyester monomers, other intermediates are likely synthesized by additional acyltransferases, some of which may have broader substrate specificities and may also be involved in general acyl lipid metabolism.

#### *ABCG half transporters*

Plasma membrane-localized transporters of the G-family of ATP binding cassette proteins (ABCG-transporters) have been postulated to play a role in the export of wax molecules, as well as cutin and suberin building blocks (Kunst and Samuels, 2009). ABCG12/CER5 (formerly WBCG12) (At1g51500) was the first ABC transporter identified during the characterization of the *cer5* mutant; this finding defined a potential clade of half transporters involved in the export of cuticular lipids (Pighin et al., 2004). The functions of ABCG12/CER5, ABCG11/DESPERADO (DSO) (formerly WBC11) (At1g17840) and ABCG13 (Atg51460) in lipid export have been substantiated by reductions in the extracellular lipid material and unusual intracellular sequestration of lipids in the corresponding mutants (Bird et al., 2007; Luo et al., 2007; Panikashvili et al., 2007; Panikashvili et al., 2010; Panikashvili et al., 2011). Mutations in *ABCG11* have the broadest impact on the export of lipidic molecules, and the knockout of this gene causes an extreme dwarf phenotype (Bird et al., 2007; Panikashvili et al., 2007). The pleiotropic phenotypes in the *abcg11* mutant may be linked to ABCG11 expression in a number of different tissues and to the ability of ABCG11 to homodimerize or heterodimerize with other half transporters of the ABCG family. Genetic and molecular studies provided evidence that heterodimerization of ABCG11 with ABCG12 is required for its function in wax deposition (Bird et al., 2007; Mc Farlane et al., 2010). Whether ABCG11 homodimerizes or heterodimerizes with other ABC half transporters for its function in cutin and suberin formation remains to be determined. In addition, partners of ABCG13 that are necessary for its function in cutin export in flowers remain unidentified. Thus, different combinations of the dimeric half-size transporters may underlie specificity for different transported molecules. ABCG11 knockout resulted in a decrease in a broad range of cutin and suberin polyester monomers in several organs (Bird et al., 2007; Panikashvili et al., 2007; Panikashvili et al., 2010). The function of ABCG13 is flower-specific and its knockout results in an approximately 50% decrease of all floral cutin monomers (Panikashvili et al., 2011).

#### *ABCG transporters of the PDR family*

In addition to the ABCG clade of half transporters, full-size ABCG transporters of the PLEIOTROPIC DRUG RESISTANCE (PDR) family have been implicated in cuticular lipid export following characterization of the *pec1* mutant of Arabidopsis and the *eibi* mutant of barley, which have been designated PEC1/AtABCG32 and HvABCG31, respectively (Bessire et al., 2011; Chen et al., 2011a). Interestingly, the AtABCG32 transporter is highly conserved between monocots and dicots, exhibiting 70% amino acid identity between Arabidopsis and rice. The closest homologue of AtABCG32 within Arabidopsis has only 55% identity (Bessire et al., 2011). To what degree this evolutionary conservation reflects functional conservation remains unknown. The characterization of AtABCG32 revealed a polar localization to the plant surface side of epidermal cells, supporting the hypothesis that PEC1/ABCG32 is directly involved in the export of cutin building blocks. The AtABCG32 knockout caused a specific reduction of distinct oxygenated monomers, particularly 10(9), 16-dihydroxyhexadecanoic acids (Bessire et al., 2011). The unique ultrastructural alterations of the cuticular layer under the nanoridges in petals (see above) suggest a role for this protein in the transport of particular cutin monomers.

### *Transport of aliphatic compounds within the apoplast*

Once transported across the plasma membrane, cutin precursors and wax molecules must reach the surface of the plant to form the cuticle, while suberin precursors form the polyester that is close to the plasma membrane. Lipid transfer proteins (LTPs) have long been postulated to play a role in the transport of these molecules across the cell wall, but no *in vivo* evidence is available (Kader, 1996). Recently, a glycosylphosphatidylinositol-ancored LTP (LTPG1), which is encoded by *LTPG1* (At1g17840), has been demonstrated to be involved in cuticle formation. However, the specific function of LPTG1 in this process has not been determined (DeBono et al., 2009).

## Transport of Aromatic Compounds

Similar to the export of the aliphatic components of diffusion barriers, different models exist for the export of monolignols to the extracellular space, including exocytosis via ER-derived vesicles,

passive diffusion through the plasma membrane via hydrophobic reactions and active transport via plasma membrane-located transporters or facilitators (Liu et al., 2011; Liu, 2012).

#### *Monolignol export*

Several lines of evidence have demonstrated that the full-size ABC-transporter ABCG29 of Arabidopsis, which belongs to the PDR family (At3g16340), can act as a p-coumaryl alcohol transporter *in yeast* and *in planta* (Alejandro et al., 2012). *abcg29* mutant lines have reduced levels of p-hydroxyphenyl and guaiacyl monomers and a slight reduction of synringyl monomers in the lignin, supporting this hypothesis. Although ABCG29 was expressed in endodermal cells at the plasma membrane, it was excluded from the Casparian strip, consistent with the theory that localized export of monolignols is not necessary for Casparian strip formation (Alejandro et al., 2012) (see below).

## EXTRACELLULAR SYNTHESIS OF POLYMERS

### Assembly of Aliphatic Polyesters

Based on known polymer linkages and *in vitro* polymerization studies (Olson and Sheares, 2006; Olsson et al., 2007), esterases and lipases represent candidate proteins for polyester condensation (Beisson et al., 2012). The $\alpha/\beta$ hydrolase BDG is involved in cutin formation and has been shown to be cell wall-localized (Kurdyukov et al., 2006a). Some members of the glycine-aspartic acid-serine-leucine motif lipase/hydrolase (GDSL lipase) gene family, such as At5g33370 and At3g04290, are co-regulated with genes involved in cutin biosynthesis (Kannangara et al., 2007; Yeats et al., 2010; Shi et al., 2011), and RNAi plants exhibiting simultaneous down-regulation of these two genes have a strongly defective petal cuticle (Shi et al., 2011). The strongest evidence that GDSL lipases are cutin synthases comes from the characterization of the *cd1* mutant of tomato, which has a much thinner fruit cuticle and only 5-10% of the cutin of the wild type (M82 cultivar). CD1/GDSL1 is a protein encoded by the gene Solyc11g006250, and it is extracellularly localized in the cuticle at the time of cuticle formation (Girard et al., 2012; Yeats et al., 2012b). Recombinant CD1/GDSL1 has been demonstrated to use 2-mono (10,16-dihydroxyhexadecanoyl) glycerol as a substrate for acyl transfer to either another 2-mono(10,16-dihydroxyhexadecanoyl) glycerol molecule or a growing cutin oligomer (Yeats et al., 2012b). These experiments emphasize the importance of *sn-2* monoacylglycerols in cutin formation and demonstrate that the cutin polymer is formed by transesterification.

### Synthesis of Lignin in the Casparian Strip

Lignin biosynthesis has been previously discussed (Boerjan et al., 2003; Fraser and Chapple, 2011). Further investigation of lignin biosynthesis during Casparian strip formation in the root endodermis of Arabidopsis could provide new opportunities for improving our understanding of the localization of lignin-type cell wall modifications in plants. Relevant findings are described below.

## THE FORMATION OF MACROMOLECULAR SUPERSTRUCTURES

### The Formation of the Cuticle

One of the most fascinating and least investigated aspects of the cuticle is the formation of superstructures within the extracellular matrix.

Nanoridges are superstructures on the surface of petal cells. The lack of nanoridges in plants with 60-80% reductions in cutin demonstrated the important contribution of cuticular polyester to these structures (Li-Beisson et al., 2009; Panikashvili et al., 2009). Moderate reductions in hydroxylated cutin monomers lead to the broader structure of nanoridges having a less electron-opaque cuticular layer. This observation has been interpreted as evidence of the participation of these cutin monomers in the formation of nanoridges (Bessire et al., 2011). The mechanisms by which different molecules are organized to form the different layers of these ridges remain unclear. The formation of stomatal ledges, the cutin-rich extensions of the extracellular matrix that are formed on the surface of guard cells and largely cover the stomata, is also poorly understood (Li et al., 2007b).

The mechanism by which waxes are incorporated into the cutin is also elusive. In a number of mutants, defects in cutin can be only partially compensated by wax deposition. In addition, wax biosynthesis is induced after cutin biosynthesis in transgenic plants expressing the WIN/SHINE transcription factor in an inducible manner, supporting speculation that cutin is necessary for the organization of wax within the cuticle (Kannangara et al., 2007). Wax crystal formation on the surface of the shoot is hypothesized to arise by spontaneous crystallization (Jetter et al., 2006).

### The Formation of Suberin Lamellae

The structure of suberin lamellae has led to speculation that the light and dark bands could be formed by the aliphatic and aromatic domains of suberin, respectively (Bernards, 2002; Graça and Santos, 2007) (Figure 8C). This was also believed to be the case in Arabidopsis, in which suberin appears as an ordered structure of electron-opaque and electron-lucent layers. However, knocking out the BAHD feruloyl transferase responsible for the transfer of ferulic acid onto $\omega$-fatty acids or alcohols, which virtually eliminates ferulic acid from suberin in Arabidopsis and potato, has no impact on the appearance of suberin lamellae in either of these plant species (Gou et al., 2009; Molina et al., 2009; Serra et al., 2010). While this enzyme should not be necessary for the formation of the aromatic polymers *per se*, it has been argued that the ester-bound ferulic acid has an important function in connecting the two suberin domains. Because there is currently no experimental evidence in Arabidopsis suggesting that suberin is comprised of a polyaliphatic and a polyaromatic domain, none of these hypotheses appear to be supported.

In accordance with these results, studies using inhibitors of fatty acid elongation that lead to alterations in the thickness of the suberin lamellae in cotton fibers indicate that the electron-translucent bands derive from fatty acid precursors (Schmutz et al., 1996). The chemical identity of the electron-opaque bands remains to be determined.

### The Formation of the Casparian Strip

The main characteristic of the Casparian strip is that it is located at a specific position in the endodermal cell wall. Casparian strip assembly must be therefore a strongly guided process. A family of previously uncharacterized proteins with four transmembrane-spanning domains is specifically expressed in differentiating endodermal cells. These proteins localize precisely to the plasma membrane region at the Casparian strips (Casparian Strip membrane Domain, CSD) (Roppolo et al., 2011) (Figure 7B) and represent the first markers for the CSD. Accordingly, these proteins were named CASPARIAN STRIP DOMAIN PROTEINS (CASPs) (encoded by *CASP1*, At2g36100; *CASP2*, At3g11550; *CASP3*, At2g27370; *CASP4*, At5g06200; and *CASP5*, At5g15290). CASPs have all the expected features for structural proteins of the CSD. They show extremely low lateral diffusion and high stability. In addition, CASPs are able to self-interact and sediment at very low speeds under native extraction conditions. Together, these features support a role for CASPs in the formation of a polymeric lattice within the endodermal plasma membrane, which spans the entire cell as a centrally located belt. This membrane protein platform is speculated to recruit additional biosynthetic machinery for Casparian strip formation and to directly or indirectly mediate the observed tight attachment to the cell wall. A double mutant disrupted in two of the five *CASP* genes shows clear alterations in Casparian strip morphology, demonstrating the importance of CASPs in the establishment of this cell wall modification. Recently, a forward genetic screen for Casparian strip barrier mutants identified an NADPH oxidase (Respiratory Burst Oxidase Homolog F), which is encoded by *RBOHF* (At1g64060), as a crucial factor for the formation of Casparian strips (Lee et al., 2013). It was demonstrated that RBOHF is not required for the formation of the CSD, but RBOHF has the unique capacity to accumulate at the CSD, which excludes most other membrane proteins. In addition, a highly localized production of apoplastic ROS at the sites of Casparian strip formation was observed. It was subsequently demonstrated that a specific peroxidase (PEROXIDASE64), encoded by *PER64* (At5g42180), co-localizes precisely with CASP1-GFP and that its localization depends on the CASPs. These findings led to a model whereby the role of the CASPs is to assemble a complex of a specific NADPH oxidase and peroxidases (Lee et al., 2013). Such an assembly would then mediate the local polymerization of lignin, which is known to be accomplished by ROS-dependent peroxidases. Several lines of evidence indicate that mono-lignols themselves are not required for the localized formation of Casparian strips (Alejandro et al., 2012; Naseer et al., 2012). These recent findings represent additional support for the hypothesis that the Casparian strip is composed of lignin and not suberin.

## REGULATION OF DIFFUSION BARRIER FORMATION

The correct spatial distribution of cutin and suberin in dermal tissues requires a highly coordinated control of tissue-specific biosynthesis and deposition of these polyesters. In addition, wax must be integrated into the polyester matrix to form a functional diffusion barrier. Large-scale gene expression analyses during stem and root development have led to the identification of central enzymes in cutin, suberin and wax biosynthesis, as well as the identification of proteins that organize the Casparian strip (Birnbaum et al., 2003; Suh et al., 2005). However, relatively little is known about the regulation of polyester and wax biosynthesis, and only a small number of genes involved in this processes have been identified to date.

Current knowledge concerning the regulatory pathways involved in cuticle formation is discussed below and summarized in a schematic diagram (Figure 9).

### The Regulation of Cuticular Lipid Biosynthesis During Development

#### *The regulation of cutin biosynthesis*

The first transcription factors shown to regulate cuticle formation were WIN1/SHN1 and its homologs (Aharoni et al., 2004; Broun et al., 2004). These transcription factors primarily control cutin biosynthesis, as demonstrated by binding of the WIN1/SHN1 transcription factor to the promoter of the *LACS2* gene, and indirectly affect wax accumulation (Kannangara et al., 2007) (Figure 9). The WIN/SHN family of transcription factors, encoded by *SHN1* (At1g15360), *SHN2* (At5g11190) and *SHN3* (At5g25390), also regulates the expression of a number of genes involved in the formation of cell wall polysaccharides and proteins of the epidermal extracellular matrix in Arabidopsis flowers (Shi et al., 2011). Gene expression studies revealed that the WIN/SHN transcription factors are regulated by gibberellic acid (GA) via DELLA proteins (Shi et al., 2011). In addition, cuticle formation is integrated into the network of epidermal cell differentiation by TRANSPARENT TESTA GLABRA1 (GL1), which is encoded by *TTG1* (At5g24520), and GLABRA3, which is encoded by *GL3* (At5g41315). These transcription factors are key players in the network of proteins involved in trichome differentiation. The mutants *gl1*, *ttg1* and *gl3* have permeable cuticles that are likely due to a reduction in cutin amount, as determined for *gl1* (Xia et al., 2010). The observed deficiencies in cuticle formation in the *gl1* mutant were restored by the application of GA, supporting the notion that these factors act early in the cutin biosynthetic pathway (Shi et al., 2011) (Figure 9). In contrast, the lack of trichomes in these mutants was not restored by the application of GA, demonstrating an absolute requirement for GL1, TTG1 and GL3 during trichome differentiation.

Wu *et al.* (2011) reported the isolation of the *CURLY FLAG LEAF 1* (*CFL1)* gene (At2g33510) and demonstrated that it encodes a WW domain protein involved in cuticle development in Arabidopsis and rice (Wu et al., 2011). This study provided biochemical evidence that AtCFL1 interacts with HOMEODOMAIN GLABROUS1 (HDG1), encoded by *HDG1* (At3g61150), a class IV homeodomain-leucine zipper transcription factor that regulates the cuticle development-related genes *BDG* and *FDH* (Figure 9).

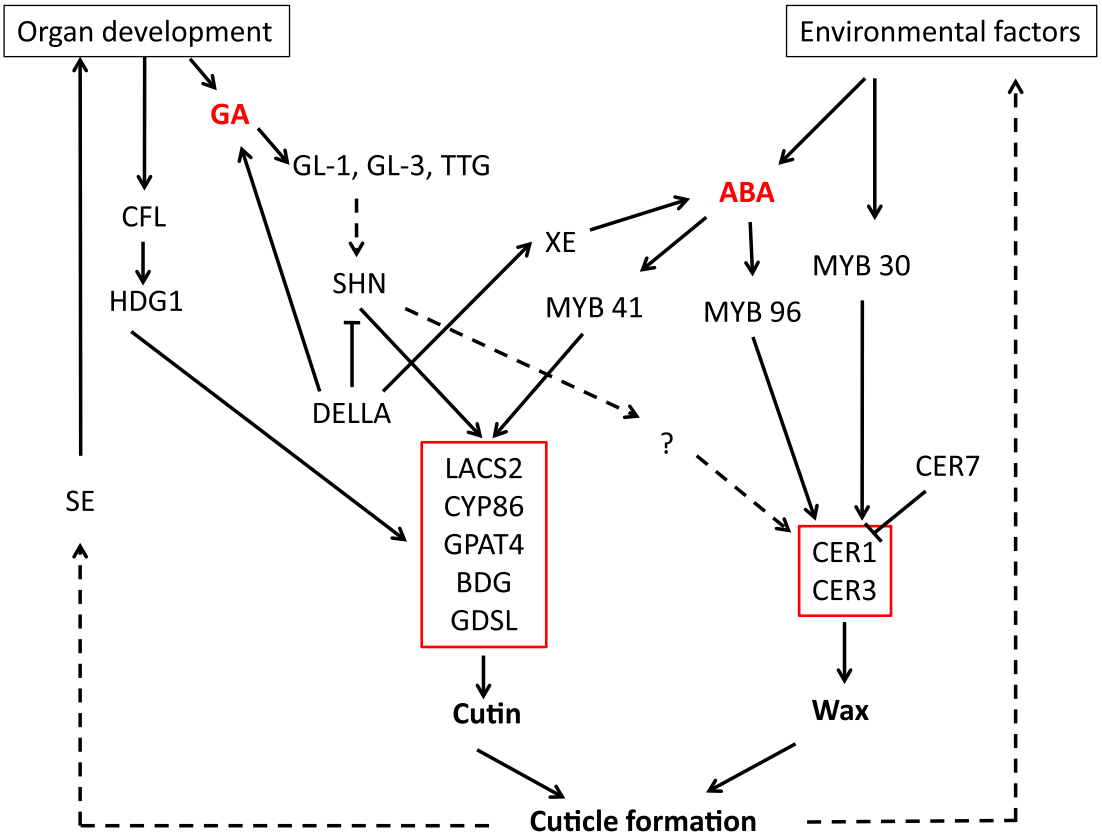


**Figure 9.** Schematic diagram of the regulatory circuits involved in cuticle formation.

Cuticle formation is regulated during organ development and by environmental factors. The hormones GA and ABA (in rouge) are involved in this regulation. Regulatory elements that have been identified are shown. Genes encoding biosynthetic enzymes are also presented (red boxes). Postulated regulatory pathways are indicated by dashed lines.

Thus, the regulation of the deposition of cutin polyester is part of the transcriptional network involved in epidermal cell differentiation.

### *The regulation of wax biosynthesis*

To date, no transcription factors have been demonstrated to be involved in the direct activation of wax biosynthetic genes during cuticle development. Instead, characterization of the wax-deficient *cer7* mutant revealed that wax production in Arabidopsis stems is controlled by the CER7 ribonuclease, which is encoded by *CER7* (At3g60500), a core subunit of the exosome that is responsible for the 3' to 5' degradation of RNA (Hooker et al., 2007) (Figure 9). Functional characterization of the CER7 enzyme demonstrated that it positively regulates mRNA levels of *CER3*, a wax biosynthetic gene whose protein product is required for wax formation via the alkane pathway (Hooker et al., 2007; Rowland et al., 2007). Because CER7 is a ribonuclease, it likely acts indirectly by degrading a repressor of *CER3* transcription. To identify the putative repressor, a genetic screen for mutations that suppress the stem wax deficiency of *cer7* was performed; a series of *wax restorer* (*war*) mutants were identified, which had mutations in genes distinct from *CER7*. Map-based cloning of two of the *WAR* genes identified them as *RDR1* (At1g14790), which encodes RNA-DEPENDENT RNA POLYMERASE 1, and *SGS3* (At5g23570), which encodes SUPPRESSOR OF GENE SILENCING 3. Both of these proteins are involved in the synthesis of small RNAs. These results led to the hypothesis that uncharacterized small RNA species directly or indirectly mediate posttranscriptional silencing of *CER3* to control cuticular wax deposition in developing Arabidopsis inflorescence stems (Lam et al., 2012).

## The Regulation of Cuticular Lipid Biosynthesis by Environmental Stimuli

### *The regulation of polyester biosynthesis*

Genes involved in both cutin and suberin formation are induced by environmental stimuli. For example, localized suberization is modified in response to drought, salinity or wounding. Consistent with these observations, the suberin biosynthetic genes are transcriptionally activated by the same environmental factors (Franke et al., 2009; Lee et al., 2009; Domergue et al., 2010). The *BDG*, *LACS2* and *PEC1* genes that are involved in cutin deposition are also transcriptionally upregulated by drought and abscisic acid

(ABA) treatment (van den Brule and Smart, 2002; L'Haridon et al., 2011). Interestingly, cutin accumulation is only induced by drought stress and ABA, while wax deposition is also affected by water deficit and mild salt treatments, suggesting that the regulation of wax production may be more complex than the control of cutin biosynthesis (Kosma et al., 2009). The ABA biosynthetic mutants *aba2* and *aba3* have permeable cuticles, similar to those of the *bdg* and *lacs2* mutants, underscoring the importance of ABA for cutin biosynthesis (L'Haridon et al., 2011) (Figure 9). A functional cuticle is also required for osmotic stress-mediated regulation of ABA biosynthesis by a mechanism that is impaired in a mutant with a lesion in *9-CIS EPOXYCAROTENOID DIOXYGENASE DEFICIENT1* (*CED1/BDG*) (At2g38110) (Kurdyukov et al., 2006a; Wang et al., 2011b) and in several other cutin biosynthesis mutants, including *att1*, *lacs2* and *lcr,* but not in the wax-deficient mutants *mah1* and *cer7* (Wang et al., 2011b). Surprisingly, this feedback regulation may be independent of cuticular permeability. Additional studies are required to elucidate the mechanism underlying this phenotype (Wang et al., 2011b).

The transcription factor MYB41, encoded by *MYB41* (At4g28110), is specifically involved in the negative regulation of cutin biosynthesis, as indicated by reduced expression of *LACS2* and *ATT1* in *MYB41*-overexpressing lines (Cominelli et al., 2008). These transgenic lines show typical phenotypes associated with a permeable cuticle, including sensitivity to desiccation and abnormal cell expansion. MYB41 has been shown to be involved in general responses to osmotic stress (Lippold et al., 2009) (Figure 9).

#### *The regulation of wax biosynthesis*

Two MYB domain-containing transcription factors have been demonstrated to control cuticular wax biosynthesis in response to environmental stimuli. The MYB96 transcription factor, which is encoded by *MYB96* (At5g62470), was demonstrated to promote cuticular wax accumulation under drought conditions by binding directly to conserved sequences in the promoters of wax biosynthetic genes and subsequently activating their transcription (Seo et al., 2011). MYB30, which is encoded by *MYB30* (At3g28910), was demonstrated to activate the expression of wax biosynthetic genes in response to attacks by pathogens, but the extent to which this transcription factor participates in wax biosynthesis under normal conditions remains to be determined (Raffaele et al., 2008) (Figure 9).

A complex regulatory network is involved in cuticle formation, and the well-coordinated activity of all involved proteins is required for the generation of a functional diffusion barrier. Although some plants, including the *bdg* mutant and WIN/SHN-overexpressing plants, have a larger amount of cutin, this feature does not result in a better-sealed cuticle. In contrast, these plants have disorganized and more permeable cuticles (Aharoni et al., 2004; Broun et al., 2004; Kurdyukov et al., 2006a; Kannangara et al., 2007). In this context, the function of the CER9 protein, which is encoded by *CER9* (At4g34100), a homologue of the E3 ubiquitin ligase DoA10 in yeast, which was previously demonstrated to target ER proteins for proteasomal degradation, is of great interest. Loss of CER9 activity leads to coordinated alterations in cutin and wax biosynthesis and improved sealing properties of the cuticle (Lu et al., 2012).

### Open Questions

Much remains to be learned about the intricacies of both global and tissue-specific regulation of diffusion barrier deposition. These questions include the identity of additional transcription factors and the downstream regulatory networks that they control during development and under environmental stress conditions. This information is particularly important for improving our understanding of suberization and Casparian strip formation, as no transcription factors involved in these processes have been identified. Interestingly, a potential link between suberin biosynthesis and sphingolipid metabolism has been identified, as mutations in the gene TCS10A (At3g06060), which encodes an enzyme with 3-ketodihydrosphinganine reductase activity, is important for normal suberin deposition (Chao et al., 2011). In addition, ENHANCED SUBERIN1, which is encoded by *ESB1* (At2g28670) and annotated as a disease resistance response protein, affects suberin composition and accumulation in roots (Baxter et al., 2009). In seeds, suberization and deposition of a cuticle layer are influenced by a mutation in the UDP-glucose:sterol glycosyltransferase UGT80B1, which is encoded by *UGT80B1* (At1g43620) (DeBolt et al., 2009). The direct or indirect involvement of these proteins in the metabolic processes leading to suberin deposition have not yet been clarified.

## PHYSIOLOGICAL FUNCTIONS OF DIFFUSION BARRIERS

### The Cuticle

#### *Diffusion processes across the cuticle*

The plant cuticle acts as an efficient barrier that protects the shoot from uncontrolled water loss. Gas exchange and transpiration are primarily regulated by stomata. When stomata are closed, residual water loss from the plant surface occurs by cuticular transpiration and non-functional stomata (Kerstiens, 1996). To exclusively study cuticular transpiration, many researchers investigating cuticular water permeability have used enzymatically isolated astomatous cuticles as their experimental system. This approach allows for the quantitative determination of cuticular barrier properties, which are described by the permeance, P, in units of m $s^{-1}$. P is thus a measure of velocity, which describes the speed at which water diffuses across the cuticular membrane (Schreiber and Schönherr, 2009). On the basis of the P value of a barrier, cuticular transpiration can be directly compared with the water permeability of other biological and technical membranes (Riederer and Schreiber, 2001). In addition, P can also be measured for solutes dissolved in water (e.g., herbicides and sugars), which allows for the correlation of solute permeability with water permeability.

##### *Quantitative assessment of cuticle permeability*

Although quantitative characterization of cuticular barrier properties has been performed for a variety of species, this characterization has not been performed for Arabidopsis. Direct application of these well-established experimental approaches to Arabidopsis was not possible. Even if cuticle isolation were feasible, which is not the case, as the isolated cuticle is extremely fragile and easily disintegrates (Franke et al., 2005), Arabidopsis has sto-

mata on both sides of the leaf, and astomatous cuticles are not available. However, because this problem also applies to the cuticles of many other species, approaches have been developed to allow for the quantitative characterization of the cuticular permeability of intact stomatous leaf surfaces. This characterization was possible because (i) it could be demonstrated experimentally that the permeability of isolated cuticles and intact leaves was similar (Kirsch et al., 1997), and (ii) the cuticular permeability of solutes (e.g., organic solutes and herbicides) was highly correlated with water permeability (Niederl et al., 1998). Thus, the cuticular transpiration of stomatous leaf surfaces can be predicted from the measured cuticular permeability of non-volatile, radiolabeled solutes dissolved in water. If radiolabeled water were used instead of radiolabeled solutes with these stomatous leaf surfaces, there is a possibility that a substantial fraction of the water that entered the leaf via open stomata could not be excluded, resulting in an erroneous determination of cuticular transpiration.

This approach has recently been successfully applied to Arabidopsis leaves (Ballmann et al., 2011). Experiments using isolated cuticles and astomatous intact leaf surfaces from a number of species demonstrated that the cuticular permeability of $^{14}$C-epoxiconazole was highly correlated with $^{3}H_2O$ permeability. This correlation was used to predict the cuticular water permeability of intact Arabidopsis leaves from measurements of $^{14}$C-epoxiconazole permeability (Ballmann et al., 2011). The permeance of the Arabidopsis leaf cuticle was 4.55 x $10^{-8}$ m·s$^{-1}$. This value can be directly compared to the permeances established for cuticles isolated from other species (Schreiber and Schönherr, 2009). The permeability of the Arabidopsis cuticle is one to two orders of magnitude higher than that of most other cuticles. Thus, it is in the upper range of permeances. However, previous permeance measurements were primarily obtained using cuticles isolated from evergreen species that had a thickness of approximately 1-3 µm (Schreiber, 2010). Considering that the Arabidopsis leaf cuticle is approximately 100-times thinner, the high permeance is not surprising. In addition, it has been speculated that the unusual chemical composition of the Arabidopsis cuticle, which is dominated by unsaturated monomers, contributes to its relatively high permeance.

Previous studies demonstrated that aliphatic wax molecules improve the sealing of the cuticle, while the role of other wax components was uncertain (Schreiber and Schönherr, 2009). The effect of triterpenoids on the sealing properties of the Arabidopsis cuticle has recently been addressed by overexpression of *LUPEOL SYNTHASE 4* (LUP4) (At1g7895) in Arabidopsis leaves that do not naturally contain β-amyrin (Buschhaus and Jetter, 2012). The increased permeability of the cuticle in the transgenic lines, which accumulated up to 4% β-amyrin in their intracuticular wax layer, suggests that triterpenoids negatively affect the sealing of the cuticle. Alternatively, the unnatural β-amyrin accumulation could also disturb the cutin/wax association in the intracuticular wax layer. Thus, a general conclusion regarding the effect of triterpenoids on cuticle permeability cannot be drawn at this time.

*Semi-quantitative assessment of cuticle permeability*

Although the approach described above allows for quantitative comparisons of the cuticular barrier properties of Arabidopsis wt plants to mutants, it is not convenient for fast and efficient screening of cuticle mutants because it is time consuming and requires radioactivity. Water loss assays, drought susceptibility tests using intact plants or excised leaves, and assays of dye diffusion through the cuticle have been established as alternatives (Tanaka et al., 2004; Kurdyukov et al., 2006a; Bessire et al., 2007). Toluidine blue (TB) is a dye that binds to anions, such as those present in pectin, while calcofluor white binds to polymeric α– or β-glucans of the cell wall (i.e., callose and cellulose, respectively). Thus, for these water-soluble dyes to stain the leaf, they must be able to cross the lipophilic cuticular barrier and bind to the polar cell wall. The uptake of TB can also be determined semi-quantitatively in relation to the chlorophyll content of the plant using a spectrometric assay (Xing et al., 2013). Similarly, herbicide penetration assays can be used to characterize mutants with an altered/defective cuticle, with lower concentrations of herbicide causing tissue damage in the mutants but not the wt (Bessire et al., 2007; Bessire et al., 2011).

The application of these assays for testing the diffusion barrier properties of the leaf cuticle led to the identification of mutants defective in cutin formation and the subsequent identification of many genes involved in this process (Kurdyukov et al., 2006a; Bessire et al., 2007; Bird et al., 2007; Bessire et al., 2011). Some of these mutants have a lesion in both wax and cutin deposition (Bird et al., 2007), but none of the mutants have a wax-specific phenotype. This may be due to the fact that the applied assays detect only strongly permeable cuticles. It remains to be seen whether the same assays can also be used to identify minor changes in cuticle permeability by altering assay conditions (e.g., through the use of long incubation periods). Chemical characterization of the cutin mutants identified to date did not reveal a clear relationship between cutin/wax amount and cuticle permeability. Even plants that have significantly more wax and cutin than wt plants may exhibit increased cuticle permeability, indicating that the organization of cutin and wax is crucial in establishing a functional barrier at the leaf/atmosphere interface. However, a co-regulated increase of wax and cutin may indeed improve cuticular properties, as demonstrated by the *cer9* Arabidopsis mutant (see above) (Lu et al., 2012).

***The role of the cuticle in abiotic stress protection***

Mutants with a permeable cuticle are sensitive to different types of abiotic stress, including drought and treatment with chemicals, because molecules can either be quickly lost from the apoplast or can quickly enter the plant tissue. Drought stress and water loss assays demonstrate the increased transpiration rate in these mutants (Schnurr et al., 2004; Kurdyukov et al., 2006a; Bessire et al., 2007; Tang et al., 2007; Lu et al., 2009; Weng et al., 2010; Bessire et al., 2011; Chen et al., 2011a). Therefore, rapid changes in humidity (e.g., 100% to 60%) may be fatal for Arabidopsis cutin mutants (Tang et al., 2007). Consistent with the effects of a non-functional diffusion barrier, cutin mutants also have an increased susceptibility to herbicides (Kurdyukov et al., 2006a; Bessire et al., 2007; Bessire et al., 2011) and increased chlorophyll leaching (Lolle et al., 1992; Lolle et al., 1998; Sieber et al., 2000; Kurdyukov et al., 2006a). In addition, some cutin mutants, such as the *lacs2* mutant, exhibit an extreme susceptibility to treatments with weak salt solutions, such as 10 mM $MgCl_2$ and NaCl, that results in in-

creased ion leakage, while distilled water and osmotically active compounds do not cause these effects (Tang et al., 2007). The increased permeability of the cuticle is also responsible for differences in lateral root formation induced by mild osmotic stress, such as the uptake of sucrose through the leaf cuticle of mutants of *lateral root formations 2*, an allele of *lacs2*, grown in culture media (Macgregor et al., 2008). Finally, the sensitivity to diffusible compounds also influences the interaction of Arabidopsis mutants with permeable cuticles with different pathogens, as discussed in detail below. In contrast, a mechanism independent of cuticle permeability was proposed for the regulation of osmotic stress tolerance and abscisic acid biosynthesis (see above) (Wang et al., 2011b).

### *The role of the cuticle in biotic interactions*

Since the first report describing Arabidopsis as a viral host in 1981 (Balazs and Lebeurier, 1981), significant advances have been made in our understanding of the mechanisms by which this model plant defends itself against a plethora of pathogens (Nishimura and Dangl, 2010). In the past two decades, the use of Arabidopsis mutants has contributed enormously to the advancement of our knowledge of the molecular basis of plant resistance. Reviews on this topic can be found in other chapters of TAB (Katagiri et al., 2002; Knepper and Day, 2008; Compagnon et al., 2009). Because the plant cuticle is a specialization of the extracellular matrix of epidermal cells in aerial tissues, any alteration of cell wall metabolism in these cells could affect the cuticle and *vice versa*. The role of the cell wall in pathogen interactions has been recently reviewed (Huckelhoven, 2007; Cantu et al., 2008), so this topic will not be addressed in this section. Here, we will discuss the complex relationship between plant-pathogen interactions and cuticle biology.

#### *Cuticular waxes and biotic interactions*

Cuticular waxes are the first frontier between plants and their invaders. They act as substrates for the attachment of colonizing organisms to the leaf surface (Clement et al., 1994; Tucker and Talbot, 2001), and they also play crucial roles in plant-pathogen and plant-pest signaling (Kolattukudy et al., 1995; Chassot and Métraux, 2005; Reina-Pinto and Yephremov, 2009). It is well established that some wax components, such as triterpenoids and aldehydes, are important in pre-invasion processes, including spore germination and appressorium formation, in different plant species (Podila et al., 1993; Gniwotta et al., 2005; Reisige et al., 2006; Inada and Savory, 2011). Inada and Savory (Inada and Savory, 2011) recently demonstrated in Arabidopsis that pre-penetration processes in the biotrophic fungus *Golovinomyces orontii* were suppressed on cauline leaves, stems, siliques and roots compared to rosette leaves. Pre-penetration processes were also inhibited on *cer1* mutant stems but not *cer3* mutant stems. Because both mutants have a similar amount of crystalline waxes on their stems, the authors suggest that these differences could be due to differences in the composition of their cuticular waxes or differences in cuticle permeability (Inada and Savory, 2011). However, on the *lacs2* mutant, which has a highly permeable cuticle, pre-penetration processes were not altered with respect to the wild type (Bessire et al., 2007), suggesting that cuticle permeability is not a determining factor in this process. In contrast, plants overexpressing *CER1* (*CER1ox*), which accumulate high levels of VLC alkanes in their waxes, are more susceptible to both the virulent bacterium *Pseudomonas syringae* DC3000 and the necrotrophic fungus *Sclerotinia sclerotiorum* (Bourdenx et al., 2011). The authors of this study suggest that increased production of VLC alkanes in this line may result in a depletion of the VLCFAs that are required for the synthesis of sphingolipids, which are key molecules in the plant immune response. However, altered wax composition in the *CER1ox* line may also be an important determinant affecting pathogen invasion.

Studies performed with *Medicago truncatula* have demonstrated that a mutation in the *PALM1/IRG1* (*IRG1/PALM1*) gene (Medtr5g014400), which encodes a Cys(2)His(2) zinc finger transcription factor, causes reduced expression of the Arabidopsis *CER4* (At4g33790) and *MYB96* homologs and negatively affects the formation of preinfection structures by the rust fungi *Phakopsora pachyrhizi* and *Puccinia emaculata* and the anthracnose pathogen *Colletotrichum trifolii* (Uppalapati et al., 2012). In this mutant, altered chemical signaling caused by a reduction in C30 primary alcohols and an increase in C29 and C31 alkanes, but not a change in the hydrophobicity of the abaxial leaf surface, appears to be the cause of the failure of these pathogens to progress, because *P. emaculata* urediniospores were able to germinate and form long germ tubes on a hydrophilic (glass) surface and on host leaf surfaces where cuticular waxes were removed. Unfortunately, in Arabidopsis, asymmetric (adaxial/abaxial) inoculations have not been performed.

The physical properties of waxes, particularly the molecular structure of epicuticular waxes (EW), can promote or prevent the attachment and locomotion of insects (Eigenbrode, 2004). In general, a leaf surface covered with crystalline waxes reduces the locomotive ability of insects (Borodich et al., 2010). Although there are many studies investigating the mechanisms by which EWs influence insect survival on diverse plant surfaces, little is known about plant-insect interactions in Arabidopsis. A screen of *eceriferum* mutants for aphid fecundity revealed that wax alterations in the Arabidopsis mutant *cer3* resulted in reduced feeding and reproduction of the cabbage aphid *Brevicoryne brassicae* L. This finding is likely due to the presence of elevated amounts of the C30 primary alcohol triacontanol in the EWs. When applied to wt leaves, this compound produced the same effects as waxes isolated from the mutant (Rashotte and Feldmann, 1996; Rashotte, 1999; Rashotte et al., 2001; Jenks et al., 2002). Reproduction of the diamondback moth, *Plutella xylostella* L., also appears to be affected by the chemical composition of Arabidopsis waxes, as moths lay more eggs on *cer4* plants than on *cer2* plants (Jenks et al., 2002). This behavior may be due to compositional differences between the waxes of these two mutants, but structural properties of the plant surface may also play a role, as the chemical composition of EWs affects their physical structure. Another possible explanation for this observation is that individual EW components directly affect insect behavior by acting as molecular signals.

In summary, the chemical composition, structure and amount of epicuticular waxes in Arabidopsis are important determinants of both plant-microbe and plant-pest interactions, with the ability to affect different aspects of the pre-invasion processes of fungi, as well as insect behavior.

*Cuticle mutants in fungal and oomycete interactions*

Cutin, the major structural component of plant cuticles, was initially viewed as a physical barrier against pathogens. Consistent with this notion, fungal pathogens produce cutin-hydrolases for the breakdown of plant cutin during the infection process (Walton, 1996). Several studies provided evidence that cutinases significantly contribute to the virulence of pathogens. However, the knockout of a cutinase does not always result in reduced pathogenicity, potentially because of redundancy in the cutinase gene family (Tudzynski and Kokkelink, 2009). Other explanations for this observation may also be possible, as discussed below.

Because the breakdown products of cutin, such as hydroxylated fatty acids, can act as fungal signals for the induction of a number of pre-penetration processes (Boller, 1995), these products have also been tested as signals for the induction of defense responses in plants. Although cutin monomers induce early defense responses in plants, neither a receptor for cutin monomers nor a downstream signal transduction cascade has been identified (Schweizer et al., 1996; Fauth et al., 1998). However, transgenic CUTE plants do not show defense responses that can be specifically attributed to the release of cutin monomers; instead, these responses are due to an increase in cuticle permeability, because the observed phenotypes are similar to those observed in a number of mutants with impaired cutin biosynthesis (Sieber et al., 2000).

Consistent with the finding that cutin plays an important role in plant-pathogen interactions, a number of Arabidopsis mutants with altered cutin deposition have been identified in screens for pathogen defense-related phenotypes (Xiao et al., 2004; Bessire et al., 2007; Tang et al., 2007). In addition, in recent years, a number of Arabidopsis mutants with defects in different steps of cutin formation became available. These mutants were analyzed for their resistance to different types of pathogens (Bessire et al., 2007; Chassot et al., 2007; Li et al., 2007b; Tang et al., 2007; Voisin et al., 2009; Xia et al., 2009; Xia et al., 2010; Bessire et al., 2011; L'Haridon et al., 2011).

*lacs2* and transgenic CUTE plants inoculated with a variety of powdery mildew strains did not display any differences in penetration rate or conidia formation when compared to wt (Bessire et al., 2007; Tang et al., 2007; Chassot et al., 2008). Similarly, no differences in resistance to the obligate biotroph *Hyaloperonospora parasitica* and the non-obligate biotroph *Phytophthora brassicae* have been observed in CUTE plants (Chassot et al., 2007, 2008). Thus, cutin content does not appear to be a major determinant of host specificity in biotrophic fungi. In addition, the *lacs2* mutant and CUTE plants did not have increased susceptibility to the hemi-biotrophic fungus *Plectosphaerella cucumerina* (Bessire et al., 2007; Chassot et al., 2007). However, in the case of necrotrophs, the available data are more complex. While unchanged susceptibility to the necrotroph *Alternaria brassicicola* has been reported for *lacs2* plants (Tang et al., 2007), an increased susceptibility to the same pathogen has been observed in the *gpat4gpat8* double mutant (Li and Beisson, 2009). Whether these differences in susceptibility reflect differences in pathogen defense mechanisms in these genotypes or differences in the environmental conditions used during inoculation remains to be determined. A number of different laboratories have reported that mutants with a permeable cuticle (i.e., CUTE plants and *lacs2*, *bdg*, *lcr*, *fdh*, *pec1* and *gl1* mutants) are resistant to the necrotroph *Botrytis cinerea* (Calo et al., 2006; Bessire et al., 2007; Chassot et al., 2007; Tang et al., 2007; Voisin et al., 2009; Bessire et al., 2011). In a number of cases, the degree of cuticle permeability correlated positively with the degree of resistance. However, the *fdh* mutant, which mounted a relatively weak resistance response, had a highly permeable cuticle (Voisin et al., 2009). Careful analyses of the inoculation protocols revealed that in all cases where resistance has been reported, the inoculation was performed in the presence of Potato Dextrose Broth (PDB). PDB was necessary and sufficient for the accumulation of antifungal compounds on the plant surface that inhibited the growth of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Monilia laxa in vitro* and protected Arabidopsis and tomato leaves against infection with *Botrytis in vivo* (Asselbergh et al., 2007; Bessire et al., 2007; Chassot et al., 2007). Thus, *Botrytis* resistance in the cuticle mutants may be actually triggered by chemicals or elicitors present in PDB. In the only study in which *Botrytis* was applied not in PDB but in water, the *gl1* mutant was more susceptible to the pathogen than the wt (Xia et al., 2010) (Kachroo, personal communication), not more resistant (Calo et al., 2006), supporting the former explanation. In addition, gene expression analyses of CUTE plants revealed an upregulation of defense genes after mock inoculation (Chassot et al., 2007). However, the expression level of these genes was even higher in the presence of the PDB/*Botrytis* mixture, indicating that elicitors from the fungus (microbe-associated molecular patterns (MAMPs)) or from the plant (damage-associated molecular patterns (DAMPs)) added to the defense response in the later stages (i.e., 24 and 36 hours after inoculation) (Chassot et al., 2007). Further studies are necessary to dissect the mechanisms that lead to effective resistance to this devastating pathogen.

The unexpectedly strong resistance to *Botrytis* may illustrate the importance of the cuticle as diffusion barrier in plant-pathogen interactions because of its role in determining the fluxes of signal molecules. However, the outcome of the interaction of plants with defects in cutin formation with different pathogens likely depends not only on cuticle permeability but also on other alterations (i.e., reinforcement and remodeling) of the cell wall (Voisin et al., 2009; Shi et al., 2011). In addition, abiotic defense responses, such as those induced by wounding, drought stress, and ABA, influence cuticle formation (L'Haridon et al., 2011). The production of reactive oxygen species (ROS) has also been implicated in a variety of processes, including plant-defense signaling and the cross-linking of cell wall polymers that are present in diffusion barriers in Arabidopsis and tomato (Asselbergh et al., 2007; L'Haridon et al., 2011). Thus, in Arabidopsis, the cuticle is integrated into a complex regulatory network of plant defense reactions against pathogens and abiotic stresses, as defects in the thin Arabidopsis cuticle directly affect cuticle permeance to a number of low molecular weight compounds. Tomato mutants with reduced fruit cuticle deposition that does not influence transpirational water loss revealed that the thick cuticle protects the tomato fruit against infection with fungal pathogens (Isaacson et al., 2009).

*Cuticle mutants in interactions with bacteria*

While a role for the cuticle in the interaction with fungal pathogens can be easily explained, particularly for fungal pathogens

that directly penetrate the cuticle to attack the plant, a role for the cuticle in the interaction with bacterial pathogens is less obvious because bacteria enter plants through stomata. Interestingly, an increased susceptibility to virulent and avirulent *Pseudomonas syringae* DC3000 has been observed in a number of mutants with defects in cuticle formation, including cutin-deficient mutants (i.e., *att1*, *acp4, lacs2/sma4, lacs9, gl1, gl3* and *ttg1*), wax-deficient mutants (i.e., *cer1, cer3,* and *cer4*) and *CER1*-overexpressing transgenic plants (Xia et al., 2009; Bourdenx et al., 2011). Mutants with defects in the cuticle, including *lacs2*, *lacs9*, *cer1*, *cer3* and *cer4,* were also able to mount a systemic acquired resistance (SAR) response. SAR is a specific type of induced resistance in which exposure to a pathogen, chemical or physical stress generates an endogenous mobile signal that activates resistance mechanisms in distal non-exposed parts of a plant (Metraux et al., 2002). Unlike the typical hormones involved in plant defense, such as salicylic acid and jasmonic acid, GA was able to partially restore the defective SAR in *gl1* plants (Xia et al., 2010). These results point toward a complex interaction of pathways that lead to cuticle formation and pathogen defense. Some of the interactions between plant defense systems and cuticle formation may be indirect, because both pathways are dependent on acyl lipid molecules or on the increased movement of signal molecules of unknown nature across a permeable cuticle.

### *Concluding remarks*

A number of previous studies demonstrated that the cuticle is an important interface for interactions of plants with their biotic environment. Interactions between plants and microbes and plants and insects have coevolved for millions of years. Thus, general conclusions about the significance of the cuticle in a particular interaction cannot be made. For example, the infestation of wheat by the Hessian fly demonstrates that the cuticle may be modified in response to the invading organism (Kosma et al., 2010). Thus, the cuticle must be viewed as a dynamic interface between plants and invading pests and pathogens.

## ***The role of the cuticle in plant development***

### *The role of the cuticle in post-embryonic development*

Organs of aerial parts of the plant are generated from the shoot apical meristem and the floral apical meristem. Although adjacent organs maintain tight contact with each other, they remain separate and do not fuse. The *fiddlehead* (*fdh*) mutant and a number of other mutants have been identified because of striking organ fusions that occur among the rosette leaves or the floral organs (Lolle et al., 1992; Lolle et al., 1998; Wellesen et al., 2001). Organ fusions and the rapid leaching of chlorophyll were well phenocopied by transgenic Arabidopsis plants expressing a fungal cutinase, supporting the hypothesis that the fusion phenotypes are associated with a defective cuticle (Figure 10) (Sieber et al., 2000). Although FDH has been identified as member of the KCS family based on sequence similarity, the biochemical role of this protein in cutin formation has not yet been elucidated (Yephremov et al., 1999; Pruitt et al., 2000; Efremova et al., 2004). Similar to the *fdh* mutant, a link between cutin deposition and organ fusions has been identified in a number of other mutants that display a similar phenotype, including the *lcr, bdg, cer10, ace/hth,* and *abgc11* mutants (Yephremov et al., 1999; Pruitt et al., 2000; Wellesen et al., 2001; Kurdyukov et al., 2006a; Kurdyukov et al., 2006b; Bird et al., 2007; Panikashvili et al., 2007; Panikashvili et al., 2009; Voisin et al., 2009; Panikashvili et al., 2010; Panikashvili et al., 2011; Shi et al., 2011). Based on observed alterations in cutin amount and composition, a role for the mutated genes in cutin biosynthesis was established (see above). Because organ fusions have been described in mutants with a block in different steps of the cutin biosynthetic pathway, namely in cutin monomer synthesis (i.e., *lacs2*, *ace/hth*, and *lcr*) (Wellesen et al., 2001; Kurdyukov et al., 2006b; Bessire et al., 2007), transport (i.e., *pec1*, *abcg11* and *abcg13*) (Bird et al., 2007; Panikashvili et al., 2007; Bessire et al., 2011; Panikashvili et al., 2011), and extracellular polyester formation (i.e., *bdg*) (Kurdyukov et al., 2006a), as well as in CUTE plants that degrade their cutin (Sieber et al., 2000), these developmental changes may be triggered by an increased permeability of the cuticle during the early development of the organ undergoing fusion. However, no direct correlation between cuticle permeability and aberrations in epidermal cell development could be detected in mature plants (Yephremov and Schreiber, 2005).

In most organ fusion mutants, ultrastructural changes in the cuticle could be identified in zones that were not fused (see above). In the zones of organ fusion, the cell wall polysaccharides of epidermal cells were joined, as the cuticle was partially disrupted or entirely missing, such as in the *bdg* and *lacs2* mutants (Figure 11A, 11B)(Sieber et al., 2000; Kurdyukov et al.,

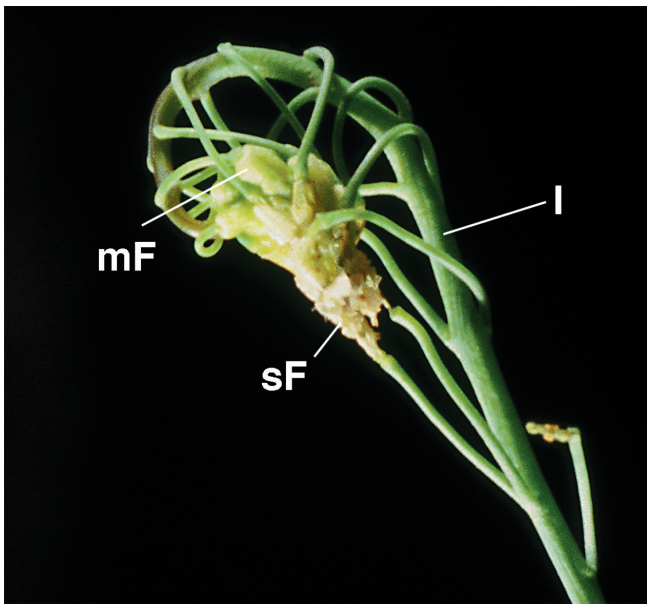


**Figure 10.** Visual phenotype of a cutinase-expressing transgenic Arabidopsis plant.

Inflorescence of a cutinase-expressing transgenic Arabidopsis plant with multiple organ fusion events between a series of flowers of different ages and between flower organs. I: inflorescence, mF: mature flower, sF: senescing flower. The points of cell contact are hidden.

2006a; Bessire et al., 2007; Voisin et al., 2009; Bessire et al., 2011). Only the cuticle of *fdh* was not visibly altered, although the cuticle permeability of this mutant was even higher than that of other cuticle mutants displaying organ fusions (Figure 11C) (Voisin et al., 2009).

The cells in a fusion zone retain their epidermal identity as they continue the differentiation process and form guard cells in the fusion zone. Although stomata are present, they are usually closed and are not likely to be physiologically functional (Sieber et al., 2000) (Figure 11D). However, a number of changes in epidermal patterning were observed, such as an altered shape of the epidermal pavement cells of the rosette leaves, as in the *dcr* mutant, or alterations in the number and shape of trichomes, as in the *fdh*, *lcr, yore-yore/wax2/cer3* and *dcr* mutants (Wellesen et al., 2001; Kurata et al., 2003; Yephremov and Schreiber, 2005; Kurdyukov et al., 2006a; Panikashvili et al., 2009). Thus, feedback regulation loops may control both the formation of a functional cuticle and epidermal differentiation (Yephremov et al., 1999; Wellesen et al., 2001; Kurdyukov et al., 2006a; Panikashvili et al., 2009; Panikashvili et al., 2010; Shi et al., 2011). The Arabidopsis cuticle mutants with the most pronounced phenotypes, such as CUTE-expressing plants, AtMYB41-overexpressing plants, and *abcg11* and *bgd* mutants, also exhibit dwarfism, indicating a pleiotropic influence of impaired cuticle formation on plant development (Sieber et al., 2000; Kurdyukov et al., 2006a; Bird et al., 2007; Panikashvili et al., 2007; Cominelli et al., 2008).

A comparison of the transcriptomes of expanding rosette leaves in the *bdg*, *lcr* and *fdh* mutants (Voisin et al., 2009) identified a set of approximately 90 genes that are upregulated in all three genotypes. Among these genes, a number of genes are involved in cutin and wax biosynthesis, indicating that cell walls and cuticles are remodeled and defenses against abiotic stresses and pathogens are activated in organ fusion mutants. A regulator of organ fusions has been identified from *in silico* studies of the transcriptomes of organ fusion mutants (Voisin et al., 2009). A mutation in *SERRATE* (*SE*) (At2g27100) suppresses the formation of organ fusions in *se lcr* and *se bdg* double mutants. *SERRATE* (*SE*) (At2g27100) encodes a zinc finger protein that functions in RNA splicing and the processing of pre-miRNAs to mature miRNAs, linking the regulation of RNA processing to cuticle formation (Prigge and Wagner, 2001; Grigg et al., 2005; Lobbes et al., 2006; Yang et al., 2006). However, the exact function of SE in cuticle formation has not been established (Figure 9).

Organ fusion always occurs during organ formation, when the epidermis of a newly developing organ is in tight contact with the epidermis of other organs (Sieber et al., 2000). Once the leaves or flowers have grown out, they are no longer fusion competent. However, if organs that are tightly fused are exposed to mechanical stress during development, the point of fusion may break, forming a wound site (Sieber et al., 2000; Wellesen et al., 2001). The formation of cutin is not only under developmental control but also under environmental control. Therefore, the type and severity of organ fusions may depend on the plant environment, which explains why the extent of organ fusion often varies when a single genotype (e.g. the *lacs-1lacs2* double mutant) is grown under different conditions (Lu et al., 2009; Weng et al., 2010).

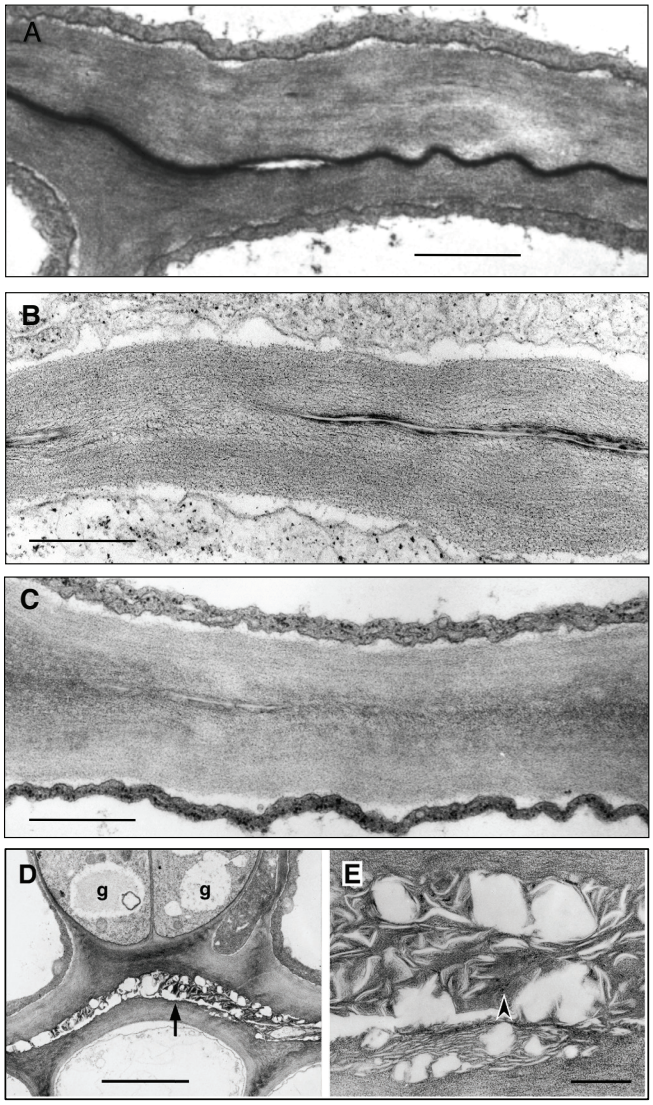


**Figure 11.** Organ fusions between epidermal cells of different organs in cutin mutants.

Organ fusions between epidermal cell layers of the leaves **(A-C)** and between inflorescence stems **(D-E)**. Visual appearance of the organ fusion zone in the Arabidopsis mutants *fdh* **(A)** (picture by Jean Mac Donald-Petetot, University Lausanne ), *bdg* **(B)** (from (Kurdyukov et al., 2006a) and *lacs2-3* **(C)** (from Bessire et al., 2007), as well as in transgenic plants expressing a fungal cutinase **(D, E)** (from Sieber et al. 2000). Scale bars (A-E): 800 nm.

*The role of the cuticle during embryo development*

While the formation of organ fusions during post-embryonic development has been instrumental in the identification of mutants with defects in cutin biosynthesis, no cutin biosynthetic genes with a role during embryogenesis have been characterized. Instead, several genes have been isolated that are involved in regulating the establishment of the epidermal surface and the cuticle. *ABNORMAL LEAF SHAPE 1* (*ALE1*) (At1g62340) encodes

an extracellular subtilisin-like serine protease that is required for the formation of the epidermal surface in embryos and juvenile plants. ALE1 is expressed in the endosperm but not in the epidermis of the embryo (Tanaka et al., 2001). However, mutations in *ALE1* enhance the mutant phenotypes of *crinkly4*. *CRINKLY4* (*ACR4*) (At3g59420) encodes a receptor-like kinase involved in epidermal cell differentiation. Mutations in this gene lead to organ fusions and a permeable cuticle in juvenile leaves, suggesting that ALE1 may also act in the formation of the cuticle of the embryo (Watanabe et al., 2003; Watanabe et al., 2004). The receptor kinases GASSHO1 and GASSHO2 act redundantly in the establishment of the epidermis and the cuticle during embryo development (Tsuwamoto et al., 2008). Mutation of both *GSO* genes leads to the fusion of cotyledons, a permeable cuticle and an altered pattern of stomata on cotyledons. The phenotypes of this double mutant are similar to those of the *ale1* mutant, although *GSO* genes are expressed only in the embryo and not in the endosperm. Genetic analysis demonstrated that ALE1 and GSO1/GSO2 act in the same signaling pathway as ZHOUPI (ZOU) (At1g49770), an endosperm-specific transcription factor of the basic helix loop helix family, which regulates the expression of ALE1, corroborating the significance of the endosperm for the formation of the embryo surface (Xing et al., 2013). Interestingly, a number of mutants with organ fusion phenotypes at maturity do not form organ fusions during embryogenesis and *vice versa,* indicating that organ fusions may be prevented in different ways over the course of plant development.

## Physiological Functions of Diffusion Barriers in Roots

### *The role of root diffusion barriers in water and nutrient uptake*

The organization of the apoplastic barrier in the shoots of Arabidopsis and other herbaceous plants without extensive secondary growth is fairly simple, with the cuticle covering the surface of all organs and forming ledges surrounding the stomata. In contrast, the structure of the apoplastic transport barriers in the roots is more complex. Roots are designed for taking up water and dissolved nutrients, but unfavorable or toxic compounds must be filtered and restricted from entering the plant (Steudle and Peterson, 1998). In addition, harmful microorganisms, especially pathogens, which occur in the soil at a much higher density than in air, must be prevented from colonizing the living plant tissues. To solve this problem, Casparian strips and suberin lamellae are deposited in the apoplast of the root in its primary developmental state, chemically modifying the primary cell wall (Schreiber, 2010). In Arabidopsis, these structures are found in the endodermis (Franke et al., 2005), while in other species, Casparian strips and suberin lamellae are also found in the hypodermis (Wilson and Peterson, 1983). In the secondary developmental state of the Arabidopsis root, one to two suberized cells cover the outer root surface (Franke et al., 2005).

Water and nutrient uptake are thought to be highest in younger root sections of Arabidopsis. Thus, the endodermis, with its cell wall modifications, should be a major barrier that allows the plant to separate interior and exterior compartments. Asymmetric localization of membrane bound transporters (influx and efflux transporters) in the endodermis supports this idea (Alassimone et al., 2010). Dyes that act as apoplastic tracers have been used for microscopic investigations of sites of water and solute uptake and for localization of apoplastic transport barriers (Bayliss et al., 1996; Aloni et al., 1998). In Arabidopsis, it was demonstrated that the radial movement of the dye in the cell wall was restricted at the outer side of the endodermis (Alassimone et al., 2010). This finding provides evidence that water and ions cannot diffuse radially in the cell wall space across the endodermis. However, it must be noted that tracer dyes are fairly large molecules with molecular weights of several hundred Daltons (e.g., propidium iodine has a molecular weight of 668 Da). Water and dissolved nutrients are much smaller (10-100 Da); thus, an endodermal barrier may not efficiently inhibit radial movement of these molecules.

The radial flow of water and solutes can be measured in roots using the pressure probe technique (Steudle et al., 1993). Three pathways represent routes of radial water and nutrient uptake into roots: (i) the apoplastic pathway, (ii) the symplastic pathway, and (iii) the transcellular pathway, which represents a mixture of the former 2 pathways (Steudle, 1994). Whole root systems of Arabidopsis have been mounted onto the pressure probe, and overall water flow (i.e., water uptake via all three pathways) was measured by applying a hydrostatic pressure gradient between the surrounding medium and the root xylem vessels (Ranathunge and Schreiber, 2011). The determined hydraulic hydrostatic conductivity was 3.7 x 10-8 m $s^{-1}$ $MPa^{-1}$. This value is not significantly different from those measured for rice (Ranathunge et al., 2003). Two to ten times higher values were reported for maize (Zimmermann and Steudle, 1998). In parallel, osmotic pressure gradients can be used for measuring the symplastic flow of water. Thus, from hydraulic conductivities, which are obtained with hydrostatic and osmotic pressure gradients, the extent to which the apoplastic pathway contributes to the overall water uptake of the root can be estimated. Because the hydraulic conductivity of the Arabidopsis root did not differ with the application of different pressure gradients (i.e., hydraulic vs. osmotic), water uptake in Arabidopsis primarily occurs via the symplastic pathway (Ranathunge and Schreiber, 2011).

Mutants characterized by a 60% decrease (e.g., *cyp86a1/horst*) (Höfer et al., 2008) or a 100% increase (e.g., *esb1*) in the amount of root suberin (Baxter et al., 2009) were also investigated using the pressure probe. Hydraulic conductivities in the presence of a hydrostatic pressure gradient significantly increased (by a factor of three) in roots with reduced suberin amounts, while they were unchanged in the presence of an osmotic pressure gradient. Thus, the apoplastic barrier was less effective towards radial water flow in the mutant with reduced suberization. However, in comparison to wt, no differences in hydraulic conductivities were observed with *esb1* mutants, which have increased root suberization. Interestingly, *esb1* was previously characterized as having a significant reduction in calcium, nickel and iron content in the shoot. This shoot phenotype might represent an indirect measure of reduced ion translocation through the root, but it may also be a secondary effect of physiological adaptations that affect ion homeostasis. A recently identified mutant in sphingolipid metabolism, which also shows suberin phenotypes and changes in the leaf ionome, supports the former hypothesis (Chao et al., 2011). In conclusion, the relationship between the amount of the aliphatic biopolymer

suberin in roots and radial hydraulic conductivity is not simple. The molecular arrangement of suberin in the cell wall and the amount of other cell wall polymers, such as lignin, should also be considered as potential factors affecting radial hydraulic conductivity in the Arabidopsis root.

## CONCLUSIONS

Recent advances in our understanding of the composition and biosynthesis of apoplastic diffusion barriers in Arabidopsis, combined with the establishment of accurate methods for the assessment of the physical properties of diffusion barriers, provide the opportunity to address challenging questions in the future. The regulation of the deposition of diffusion barriers during development and under different environmental conditions necessits further investigation. More sophisticated methods, particularly non-destructive methods or partially destructive methods, for analysis of the chemical structure of these barriers must be developed to elucidate the relationship between different lipid and aromatic compounds and cell wall structure. An improved understanding of diffusion barriers as dynamic cell wall modifications that contribute the survival of the plant under changing environmental conditions will be the next challenge. Studies with Arabidopsis have significantly advanced our knowledge of the structure and function of diffusion barriers. In the future, studies in other plants (i.e., tomato and potato) may provide complementary information that will further advance our knowledge of diffusion barriers in Arabidopsis.

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## APPENDIX

## METHODS FOR THE INVESTIGATION OF DIFFUSION BARRIERS

### Histochemical Stains for the Detection of Polyesters

#### *Sudan*

Sudan dyes (i.e., Sudan III, Sudan IV and Sudan 7B) can be used for staining cutin and suberin polymers in plants (Brundrett et al., 1991). To date, these dyes have been exploited in Arabidopsis for staining root suberin. Cross-sections of primary roots with an endodermis or secondary roots with a periderm are stained with Sudan 7B (0.1% w/v) in PEG-400:90% glycerol (1:1, v:v) at room temperature for 1 h. After staining, sections are washed with water, embedded in glycerol:water (1:1, v:v) and examined using light microscopy.

#### *Fluorol Yellow*

Fluorol Yellow can be used for the detection of polyesters in Arabidopsis roots (Franke et al., 2005). 5-day-old seedlings are incubated in a 0.01% solution of Fluorol Yellow 088 (w/v, in lactic acid) at 70°C for 30 min, rinsed with water, counterstained with aniline blue (0.5% w/v, in water) at room temperature for 30 min, washed thoroughly with water and mounted in 50% glycerol before microscopic examination.

### Histochemical Methods for the Detection of Polyphenolics

A "clearing" of the root preceding the evaluation of autofluorescence leads to good visualization of the localization of polyphenolics in roots (Naseer et al., 2012). This procedure is suitable for the visualization of lignin in Casparian strips and xylem vessels. Seedlings are incubated in 0.24N HCl in 20% methanol at 57°C for 15 min. This solution is replaced with 7% NaOH in 60% ethanol for 15 min at room temperature. Roots are then rehydrated for 5 min each in 40%, 20% and 10% ethanol and infiltrated for 15 min in a solution of 5% ethanol and 25% glycerol. Roots are mounted in 50% glycerol for microscopic analysis.

### Staining Methods for the Assessment of the Permeability of Diffusion Barriers

#### *Toluidine blue*

Toluidine blue (TB) staining is a versatile method that can be easily adapted for the analysis of the permeability of the cuticle of different organs, plants with different genotypes, and plants grown under different environmental conditions (Tanaka et al., 2004). For the evaluation of highly permeable cuticles, entire organs may be submerged for 5 min in a solution of 0.01% TB and 0.01% Triton and washed with water (Li et al., 2007b). To assess the permeability of less permeable cuticles, 5-µl droplets of a solution of 0.01% TB and 0.01% Triton are applied to detached leaves or

whole plants maintained under 100% humidity for different time periods. Since TB can enter the plant via excision sites experiments with whole plants are to be favored. Incubation times depend on the phenotype being investigated and are usually longer (15 min to overnight) (Bessire et al., 2007; Bessire et al., 2011).

### *Propidium iodide*

To determine the functionality of the apoplastic barrier of roots, seedlings are incubated in the dark for 10 min in a fresh solution of 15 µM (10 µg/mL) propidium iodide and rinsed two times in water (Alassimone et al., 2010).

## Quantitative Methods for Assessing Functionality of Diffusion Barriers

### *Radoactive tracer uptake*

A 50-ml donor volume of the radioactive tracer (e.g., $^{14}$C-epoxiconazole) is dissolved in water. The radioactive concentration should be in the range of 1012 dpm ml$^{-1}$. Three leaves from entirely expanded rosettes are excised under water and fully immersed in the donor solution. The large external volume and the high radioactive concentration ensure that (i) the external donor concentration does not drop due to the uptake of radioactivity into the leaf and (ii) detectable amounts of radioactivity are taken up into the leaf. After 5, 10 and 15 minutes, leaves are removed from the donor solution, superficially adhering donor solution is washed away with water, and leaves are carefully blotted dry using filter paper. Leaf disks of a defined surface area are punched out using a cork borer (diameter: 5 mm). This procedure ensures that the amount of radioactive uptake can be directly related to a defined leaf surface, which is given as the sum of the upper and lower leaf sides. For each sampling time, approximately 20 leaf disks are harvested, and the amount of radioactivity in each leaf disk is determined. The leaf disks are digested with a tissue solubilizer for 3 hours before adding a liquid scintillation cocktail. The amount of radioactive uptake into each individual leaf disk is plotted as a function of the sampling time. With 3 sampling times (5, 10 and 15 min) and 20 leaf disks per sampling time, a total of 60 leaf disks are investigated for uptake kinetics measurements. A linear regression is fitted to the uptake kinetics, and from the slope of the regression line, permeance, in m s$^{-1}$, can be calculated by dividing the slope, dpm s$^{-1}$, by the donor concentration, dpm m-$^{3}$, and the leaf surface area, m$^2$. Further details can be found in Ballmann *et al.* (2011) (Ballmann et al., 2011) and Schreiber and Schönherr (2009) (Schreiber and Schönherr, 2009).

### *Measurements of hydraulic conductivity*

Arabidopsis plants are grown hydroponically for 4 weeks, allowing easy access to the root system. Aerial parts of the plants are excised under water close to the base of the root system. Roots are directly mounted to the pressure probe, with each root system sitting in an aerated nutrient solution. After equilibrium pressure has been established (in Arabidossis, this occurs at approximately 0.05 MPa), positive and negative hydrostatic pressure gradients are induced by mechanically moving the rod in the cylinder of the pressure probe, which initiates water flow in the central cylinder of the root. Pressure relaxations are recorded, and hydraulic conductivities (Lpr) are calculated from the rate constants of water exchange between the root system and the surrounding medium. For measurements of root hydraulic conductivity under an osmotic pressure gradient, the external nutrient solution is replaced by an osmotically active medium (e.g., 50 mOsmol NaCl solution), inducing an osmotically driven water flow out of the root system. Further details are provided in Ranathunge and Schreiber (2011) and Steudle (1993).

## REFERENCES

**Agrawal, V.P., and Kolattukudy, P.E.** (1978). Purification and characterization of a wound-induced ω-hydroxyfatty acid:NADP oxidoreductase from potato tuber disks (*Solanum tuberosum* L.). Arch. Biochem. Biophys. **191,** 452-465.

**Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G., and Pereira, A.** (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell **16,** 2463-2480.

**Alassimone, J., Naseer, S., and Geldner, N.** (2010). A developmental framework for endodermal differentiation and polarity. Proc. Natl. Acad. Sci. USA **107,** 5214-5219.

**Alejandro, S., Lee, Y., Tohge, T., Sudre, D., Osorio, S., Park, J., Bovet, L., Lee, Y., Geldner, N., Fernie, A.R., and Martinoia, E.** (2012). AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr. Biol. **22,** 1207-1212.

**Aloni, R., Enstone, D.E., and Peterson, C.A.** (1998). Indirect evidence for bulk water flow in root cortical cell walls of three dicotyledonous species. Planta **207,** 1-7.

**Ariizumi, T., and Toriyama, K.** (2011). Genetic regulation of sporopollenin synthesis and pollen exine development. In Annual Review of Plant Biology, **62**, S.S. Merchant, W.R. Briggs, and D. Ort, eds, pp. 437-460.

**Asselbergh, B., Curvers, K., Franca, S.C., Audenaert, K., Vuylsteke, M., Van Breusegem, F., and Hofte, M.** (2007). Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol. **144,** 1863-1877.

**Balazs, E., and Lebeurier, G.** (1981). Arabidopsis is a host of cauliflower mosaic virus. Arabidopsis Inf. Serv. **18,** 130-134.

**Ballmann, C., De Oliveira, S., Gutenberger, A., Wassman, F., and Schreiber, L.** (2011). A radioactive assay allowing the quantitative measurement of cuticular permeability of intact *Arabidopsis thaliana* leaves. Planta **234,** 9-20.

**Baxter, I., Hosmani, P.S., Rus, A., Lahner, B., Borevitz, J.O., Muthukumar, B., Mickelbart, M.V., Schreiber, L., Franke, R.B., and Salt, D.E.** (2009). Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in Arabidopsis. PloS Genet. **5,** e1000492.

**Bayliss, C., vanderWeele, C., and Canny, M.J.** (1996). Determinations of dye diffusivities in the cell-wall apoplast of roots by a rapid method. New Phytol. **134,** 1-4.

**Behrisch, R.** (1926). Zur Kenntnis der Endodermiszelle. Ber. Deut. Bot. Ges. **44,** 162-164.

**Beisson, F., Li-Beisson, Y., and Pollard, M.** (2012). Solving the puzzles of cutin and suberin polymer biosynthesis. Curr. Opin. Plant Biol. **15,** 329-337.

**Beisson, F., Li, Y.H., Bonaventure, G., Pollard, M., and Ohlrogge, J.B.**

(2007). The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of Arabidopsis. Plant Cell **19,** 351-368.

**Benveniste, I., Tijet, N., Adas, F., Philipps, G., Salaün, J.P., and Durst, F.** (1998). *CYP86A1* from *Arabidopsis thaliana* encodes a cytochrome P450-dependent fatty acid ω-hydroxylase. Biochem. Biophys. Res. Co. **243,** 688-693.

**Bernard, A., Domergue, F., Pascal, S., Jetter, R., Renne, C., Faure, J.-D., Haslam, R.P., Napier, J.A., Lessire, R., and Joubes, J.** (2012). Reconstitution of plant alkane biosynthesis in yeast demonstrates that Arabidopsis ECERIFERUM1 and ECERIFERUM3 are core components of a very-long-chain alkane synthesis complex. Plant Cell **24,** 3106-3118.

**Bernards, M.A.** (2002). Demystifying suberin. Can. J. Bot. **80,** 227-240.

**Bessire, M., Chassot, C., Jacquat, A.C., Humphry, M., Borel, S., Petetot, J.M.C., Metraux, J.P., and Nawrath, C.** (2007). A permeable cuticle in Arabidopsis leads to a strong resistance to *Botrytis cinerea*. EMBO J. **26,** 2158-2168.

**Bessire, M., Borel, S., Fabre, G., Carraca, L., Efremova, N., Yephremov, A., Cao, Y., Jetter, R., Jacquat, A.C., Metraux, J.P., and Nawrath, C.** (2011). A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in Arabidopsis. Plant Cell **23,** 1958-1970.

**Bird, D., Beisson, F., Brigham, A., Shin, J., Greer, S., Jetter, R., Kunst, L., Wu, X.W., Yephremov, A., and Samuels, L.** (2007). Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J. **52,** 485-498.

**Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N.** (2003). A gene expression map of the Arabidopsis root. Science **302,** 1956-1960.

**Blacklock, B.J., and Jaworski, J.G.** (2006). Substrate specificity of Arabidopsis 3-ketoacyl-CoA synthases. Biochem. Biophys. Res. Co. **346,** 583-590.

**Boerjan, W., Ralph, J., and Baucher, M.** (2003). Lignin biosynthesis. Ann. Rev. Plant Biol. **54,** 519-546.

**Boller, T.** (1995). Chemoperception of microbial signals in plant cells. Ann. Rev. Plant Biol. **46,** 189-214.

**Bonaventure, G., Beisson, F., Ohlrogge, J., and Pollard, M.** (2004). Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-cis-6, cis-9-diene-1,18-dioate as the major component. Plant J. **40,** 920-930.

**Bornette, G., and Puijalon, S.** (2011). Response of aquatic plants to abiotic factors: a review. Aquat. Sci. **73,** 1-14.

**Borodich, F., Gorb, E., and Gorb, S.** (2010). Fracture behaviour of plant epicuticular wax crystals and its role in preventing insect attachment: a theoretical approach. Appl. Physics A: Materials Science & Processing, 1-9.

**Bourdenx, B., Bernard, A., Domergue, F., Pascal, S., Leger, A., Roby, D., Pervent, M., Vile, D., Haslam, R.P., Napier, J.A., Lessire, R., and Joubes, J.** (2011). Overexpression of Arabidopsis ECERIFERUM1 promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic atresses. Plant Physiol. **156,** 29-45.

**Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., and Benfey, P.N.** (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. Science **318,** 801-806.

**Broun, P., Poindexter, P., Osborne, E., Jiang, C.Z., and Riechmann, J.L.** (2004). WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. Proc. Natl. Acad. Sci. USA **101,** 4706-4711.

**Brundrett, M.C., Kendrick, B., and Peterson, C.A.** (1991). Efficient lipid staining in plant-material with Sudan Red-7B or Fluoral Yellow-088 in polyethylene glycol-glycerol. Biotech. Histochem. **66,** 111-116.

**Buda, G.J., Isaacson, T., Matas, A.J., Paolillo, D.J., and Rose, J.K.C.** (2009). Three-dimensional imaging of plant cuticle architecture using confocal scanning laser microscopy. Plant J. **60,** 378-385.

**Buschhaus, C., and Jetter, R.** (2011). Composition differences between epicuticular and intracuticular wax substructures: How do plants seal their epidermal surfaces? J. Exp. Bot. **62,** 841-853.

**Buschhaus, C., and Jetter, R.** (2012). Composition and physiological function of the wax layers coating Arabidopsis leaves: beta-amyrin negatively affects the intracuticular water barrier. Plant Physiol. **160,** 1120-1129.

**Calo, L., Garcia, I., Gotor, C., and Romero, L.C.** (2006). Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a *Trichoderma* alpha-1,3-glucanase. J. Exp. Bot. **57,** 3911-3920.

**Cantu, D., Vicente, A.R., Labavitch, J.M., Bennett, A.B., and Powell, A.L.** (2008). Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends Plant Sci. **13,** 610-617.

**Caspary, R.** (1865). Bemerkungen über die Schutzscheide und die Bildung des Stammes und der Wurzel. Jahrb. wissenschaft. Bot. **4,** 101-124.

**Cereijido, M., Contreras, R.G., and Shoshani, L.** (2004). Cell adhesion, polarity, and epithelia in the dawn of metazoans. Physiol. Rev. **84,** 1229-1262.

**Chao, D.-Y., Gable, K., Chen, M., Baxter, I., Dietrich, C.R., Cahoon, E.B., Guerinot, M.L., Lahner, B., Lue, S., Markham, J.E., Morrissey, J., Han, G., Gupta, S.D., Harmon, J.M., Jaworski, J.G., Dunn, T.M., and Salt, D.E.** (2011). Sphingolipids in the root play an important role in regulating the leaf ionome in *Arabidopsis thaliana*. Plant Cell **23,** 1061-1081.

**Chassot, C., and Métraux, J.P.** (2005). The cuticle as source of signals for plant defense. Plant Biosyst. **139,** 28-31.

**Chassot, C., Nawrath, C., and Metraux, J.P.** (2007). Cuticular defects lead to full immunity to a major plant pathogen. Plant J. **49,** 972-980.

**Chassot, C., Nawrath, C., and Metraux, J.P.** (2008). The cuticle: Not only a barrier for plant defence: A novel defence syndrome in plants with cuticular defects. Plant Cell Behav. **3,** 142-144.

**Chen, G.X., Komatsuda, T., Ma, J.F., Nawrath, C., Pourkheirandish, M., Tagiri, A., Hu, Y.G., Sameri, M., Li, X.R., Zhao, X., Liu, Y.B., Li, C., Ma, X.Y., Wang, A.D., Nair, S., Wang, N., Miyao, A., Sakuma, S., Yamaji, N., Zheng, X.T., and Nevo, E.** (2011a). An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proc. Natl. Acad. Sci. USA **108,** 12354-12359.

**Chen, W., Yu, X.-H., Zhang, K., Shi, J., De Oliveira, S., Schreiber, L., Shanklin, J., and Zhang, D.** (2011b). *MALE STERILE2* encodes a plastid-localized fatty acyl carrier protein reductase required for pollen exine development in Arabidopsis. Plant Physiol. **157,** 842-853.

**Chen, X.B., Goodwin, S.M., Boroff, V.L., Liu, X.L., and Jenks, M.A.** (2003). Cloning and characterization of the *WAX2* gene of Arabidopsis involved in cuticle membrane and wax production. Plant Cell **15,** 1170-1185.

**Clement, J., Porter, R., Butt, T., and Beckett, A.** (1994). The role of hydrophobicity in attachment of urediniospores and sporelings of *Uromyces viciae-fabae*. Mycol. Res. **98,** 1217-1228.

**Cominelli, E., Sala, T., Calvi, D., Gusmaroli, G., and Tonelli, C.** (2008). Over-expression of the Arabidopsis *AtMYB41* gene alters cell expansion and leaf surface permeability. Plant J. **53,** 53-64.

**Compagnon, V., Diehl, P., Benveniste, I., Meyer, D., Schaller, H., Schreiber, L., Franke, R., and Pinot, F.** (2009). *CYP86B1* is required for very long chain ω-hydroxyacids and α, ω-dicarboxylic acids synthesis in root and seed suberin polyester. Plant Physiol. **150,** 1831-1843.

**DeBolt, S., Scheible, W.R., Schrick, K., Auer, M., Beisson, F., Bischoff,**

**V., Bouvier-Nave, P., Carroll, A., Hematy, K., Li, Y.H., Milne, J., Nair, M., Schaller, H., Zemla, M., and Somerville, C.** (2009). Mutations in UDP-glucose:sterol glucosyltransferase in Arabidopsis cause transparent testa phenotype and suberization defect in seeds. Plant Physiol. **151,** 78-87.

**DeBono, A., Yeats, T.H., Rose, J.K.C., Bird, D., Jetter, R., Kunst, L., and Samuels, L.** (2009). Arabidopsis LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. Plant Cell **21,** 1230-1238.

**Dolan, L., Linstead, P., Poethig, R.S., and Roberts, K.** (1994). The contribution of chimeras to the understanding of root meristem organization. Linnean Soc**.** 195-208.

**Domergue, F., Vishwanath, S.J., Joubès, J., Ono, J., Lee, J.A., Bourdon, M., Alhattab, R., Lowe, C., Pascal, S., Lessire, R., and Rowland, O.** (2010). Three *Arabidopsis* fatty acyl-CoA reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. Plant Physiol. **153,** 1539–1554.

**Dunn, T.M., Lynch, D.V., Michaelson, L.V., and Napier, J.A.** (2004). A post-genomic approach to understanding sphingolipid metabolism in *Arabidopsis thaliana*. Ann. Bot. **93,** 483-497.

**Efremova, N., Schreiber, L., Bar, S., Heidmann, I., Huijser, P., Wellesen, K., Schwarz-Sommer, Z., Saedler, H., and Yephremov, A.** (2004). Functional conservation and maintenance of expression pattern of FIDDLEHEAD-like genes in Arabidopsis and Antirrhinum. Plant Mol. Biol. **56,** 821-837.

**Eigenbrode, S.D.** (2004). The effects of plant epicuticular waxy blooms on attachment and effectiveness of predatory insects. Arthropod Struct. Dev. **33,** 91-102.

**Esau, K.** (1977). Anatomy of seed plants. (New York: Wiley).

**Fauth, M., Schweizer, P., Buchala, A., Markstadter, C., Riederer, M., Kato, T., and Kauss, H.** (1998). Cutin monomers and surface wax constituents elicit H2O2 in conditioned cucumber hypocotyl segments and enhance the activity of other H2O2 elicitors. Plant Physiol. **117,** 1373-1380.

**Fiebig, A., Mayfield, J.A., Miley, N.L., Chau, S., Fischer, R.L., and Preuss, D.** (2000). Alterations in *CER6*, a gene identical to *CUT1*, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell **12,** 2001-2008.

**Franke, R., and Schreiber, L.** (2007). Suberin - a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. **10,** 252-259.

**Franke, R., Briesen, I., Wojciechowski, T., Faust, A., Yephremov, A., Nawrath, C., and Schreiber, L.** (2005). Apoplastic polyesters in Arabidopsis surface tissues - A typical suberin and a particular cutin. Phytochemistry **66,** 2643-2658.

**Franke, R., Hofer, R., Briesen, I., Emsermann, M., Efremova, N., Yephremov, A., and Schreiber, L.** (2009). The *DAISY* gene from Arabidopsis encodes a fatty acid elongase condensing enzyme involved in the biosynthesis of aliphatic suberin in roots and the chalaza-micropyle region of seeds. Plant J. **57,** 80-95.

**Franke, R.B., Dombrink, I., and Schreiber, L.** (2012). Suberin goes genomics: Use of a short living plant to investigate a long lasting polymer. In Front. Plant Sci.

**Fraser, C.M., and Chapple, C.** (2011). The phenylpropanoid pathway in Arabidopsis. In The Arabidopsis Book, **9,** e0152.

**Girard, A.-L., Mounet, F., Lemaire-Chamley, M., Gaillard, C., Elmorjani, K., Vivancos, J., Runavot, J.-L., Quemener, B., Petit, J., Germain, V., Rothan, C., Marion, D., and Bakan, B.** (2012). Tomato GDSL1 Is required for cutin deposition in the fruit cuticle. Plant Cell **24,** 3119-3134.

**Gniwotta, F., Vogg, G., Gartmann, V., Carver, T., Riederer, M., and Jetter, R.** (2005). What do microbes encounter at the plant surface? Chemical composition of pea leaf cuticular waxes. Plant Physiol. **139,** 519-530.

**Gou, J.Y., Yu, X.H., and Liu, C.J.** (2009). A hydroxycinnamoyltransferase responsible for synthesizing suberin aromatics in Arabidopsis. Proc. Natl. Acad. Sci. USA **106,** 18855-18860.

**Graça, J., and Santos, S.** (2006). Glycerol-derived ester oligomers from cork suberin. Chem. Phys. Lipids **144,** 96-107.

**Graça, J., and Santos, S.** (2007). Suberin: A biopolyester of plants' skin. Macromol. Biosci. **7,** 128-135.

**Graça, J., and Lamosa, P.** (2010). Linear and branched poly(omega-hydroxyacid) esters in plant cutins. J. Agr. Food Chem. **58,** 9666-9674.

**Graça, J., Schreiber, L., Rodrigues, J., and Pereira, H.** (2002). Glycerol and glyceryl esters of ω-hydroxyacids in cutins. Phytochemistry **61,** 205-215.

**Greer, S., Wen, M., Bird, D., Wu, X.M., Samuels, L., Kunst, L., and Jetter, R.** (2007). The cytochrome P450 enzyme CYP96A15 is the mid-chain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax of arabidopsis. Plant Physiol. **145,** 653-667.

**Grigg, S.P., Canales, C., Hay, A., and Tsiantis, M.** (2005). SERRATE coordinates shoot meristem function and leaf axial patterning in Arabidopsis. Nature **437,** 1022-1026.

**Han, J., Clement, J.M., Li, J., King, A., Ng, S., and Jaworski, J.G.** (2010). The cytochrome P450 CYP86A22 is a fatty acyl-CoA omega-hydroxylase essential for estolide synthesis in the stigma of Petunia hybrida. J. Biol. Chem. **285,** 3986-3996.

**Haslam, T.M., Manas-Fernandez, A., Zhao, L., and Kunst, L.** (2012). Arabidopsis ECERIFERUM2 is a component of the fatty acid elongation machinery required for fatty acid extension to exceptional lengths. Plant Physiol. **160,** 1164-1174.

**Hauke, V., and Schreiber, L.** (1998). Ontogenetic and seasonal development of wax composition and cuticular transpiration of ivy (*Hedera helix* L.) sun and shade leaves. Planta **207,** 67-75.

**Hawes, M.C., Gunawardena, U., Miyasaka, S., and Zhao, X.W.** (2000). The role of root border cells in plant defense. Trends Plant Sci. **5,** 128-133.

**Heide-Jorgensen.** (1991). Cuticle development and ultrastructure: evidence for a procuticle of high osmium affinity. Planta **183,** 511-519.

**Höfer, R., Briesen, I., Beck, M., Pinot, F., Schreiber, L., and Franke, R.** (2008). The *Arabidopsis* cytochrome P450 *CYP86A1* encodes a fatty acid ω-hydroxylase involved in suberin monomer biosynthesis. J. Exp. Bot. **59,** 2347-2360.

**Holloway, P.J.** (1982a). The chemical constitution of plant cutins. In The Plant Cuticle, D.F. Cutler, A.K. I., and C.E. Price, eds (London: Academic Press), pp. 45-86.

**Holloway, P.J.** (1982b). Structure and histochemistry of plant cuticular membranes: An overview. In The Plant Cuticle, D.F. Cutler, K.L. Alvin, and C.E. Price, eds (London: Academic Press), pp. 1-32.

**Hooker, T.S., Lam, P., Zheng, H.Q., and Kunst, L.** (2007). A core subunit of the RNA-processing/degrading exosome specifically influences cuticular wax biosynthesis in Arabidopsis. Plant Cell **19,** 904-913.

**Huckelhoven, R.** (2007). Cell wall-associated mechanisms of disease resistance and susceptibility. Ann. Rev. Phytopathol. **45,** 101-127.

**Inada, N., and Savory, E.A.** (2011). Inhibition of prepenetration processes of the powdery mildew *Golovinomyces orontii* on host inflorescence stems is reduced in the Arabidopsis cuticular mutant *cer3* but not in *cer1*. J. Gen. Plant Pathol. **77,** 273-281.

**Isaacson, T., Kosma, D.K., Matas, A.J., Buda, G.J., He, Y., Yu, B., Pravitasari, A., Batteas, J.D., Stark, R.E., Jenks, M.A., and Rose, J.K.C.** (2009). Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties,

but not transpirational water loss. Plant J. **60,** 363-377.

**James, D.W., Lim, E., Keller, J., Plooy, I., Ralston, E., and Dooner, H.K.** (1995). Directed tagging of the Aradidopsis *FATTY ACID ELONGATION1* (*FAE1*) gene with the maize transposon activator. Plant Cell **7,** 309-319.

**Jeffree, C.E.** (2006). The fine structure of the plant cuticle. In Biology of the Plant Cuticle, M. Riederer and C. Müller, eds (Oxford: Blackwell Publisheing), pp. 11-125.

**Jenks, M.A., Eigenbrode Sanford, D., and Lemieux, B.** (2002). Cuticular waxes of Arabidopsis. In The Arabidopsis Book, **1**, e0016.

**Jessen, D., Olbrich, A., Knuefer, J., Krueger, A., Hoppert, M., Polle, A., and Fulda, M.** (2011). Combined activity of LACS1 and LACS4 is required for proper pollen coat formation in Arabidopsis. Plant J. **68,** 715-726.

**Jetter, R., and Schaffer, S.** (2001). Chemical composition of the *Prunus laurocerasus* leaf surface. Dynamic changes of the epicuticular wax film during leaf development. Plant Physiol. **126,** 1725-1737.

**Jetter, R., Schaffer, S., and Riederer, M.** (2000). Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. Plant Cell Environ. **23,** 619-628.

**Jetter, R., Kunst, L., and Samuels, A.L.** (2006). Composition of plant cuticular waxes. In Biology of the Plant Cuticle, M. Riederer and C. Müller, eds (Oxford: Blackwell Publishing), pp. 145-181.

**Joubes, J., Raffaele, S., Bourdenx, B., Garcia, C., Laroche-Traineau, J., Moreau, P., Domergue, F., and Lessire, R.** (2008). The VLCFA elongase gene family in *Arabidopsis thaliana*: phylogenetic analysis, 3D modelling and expression profiling. Plant Mol. Biol. **67,** 547-566.

**Jun, J.H., Song, Z.H., Liu, Z.J., Nikolau, B.J., Yeung, E.S., and Lee, Y.J.** (2010). High-spatial and high-mass resolution imaging of surface metabolites of *Arabidopsis thaliana* by laser desorption-ionization mass spectrometry using colloidal silver. Analyt. Chem. **82,** 3255-3265.

**Kader, J.C. (1996).** Lipid-transfer proteins in plants. Ann. Rev. Plant Physio. **47**, 627-654.

**Kandel, S., Sauveplane, V., Compagnon, V., Franke, R., Millet, Y., Schreiber, L., Werck-Reichhart, D., and Pinot, F. (**2007). Characterization of a methyl jasmonate and wounding-responsive cytochrome P450 of *Arabidopsis thaliana* catalyzing dicarboxylic fatty acid formation *in vitro.* FEBS J. **274**, 5116-5127.

**Kannangara, R., Branigan, C., Liu, Y., Penfield, T., Rao, V., Mouille, G., Höfte, H., Pauly, M., Riechmann, J.L., and Broun, P.** (2007). The transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis thaliana*. Plant Cell **19**, 1278-1294.

**Karahara, I., and Shibaoka, H.** (1992). Isolation of Casparian strips from pea roots. Plant Cell Physiol. **33,** 555-561.

**Katagiri, F., Thilmony, R., and He Sheng, Y.** (2002). The *Arabidopsis thaliana-Pseudomonas syringae* interaction. In The Arabidopsis Book, **1**, e0039.

**Kerstiens, G.** (1996). Diffusion of water vapour and gases across cuticles and through stomatal pores presumed closed. In Plant Cuticles: An integrated functional approach, G. Kerstiens, ed (Oxford: BIOS Scientific Publishers), pp. 121-134.

**Kirsch, T., Kaffarnik, F., Riederer, M., and Schreiber, L.** (1997). Cuticular permeability of the three tree species *Prunus laurocerasus* L, *Ginkgo biloba* L and *Juglans regia* L: Comparative investigation of the transport properties of intact leaves, isolated cuticles and reconstituted cuticular waxes. J. Exp. Bot. **48,** 1035-1045.

**Knepper, C., and Day, B.** (2010). From perception to activation: The molecular-genetic and biochemical landscape of disease resistance signaling in plants. In The Arabidopsis Book, **8**, e0012.

**Kolattukudy, P.E.** (2001a). Polyesters in higher plants. Adv. Biochem. Eng. Biot. **71,** 1-49.

**Kolattukudy, P.E.** (2001b). Suberin from plants. In Biopolymers, Y. Doi and A. Steinbüchel, eds (Weinheim: Wiley-VCH), pp. 41-68.

**Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A.** (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. USA **92,** 4080-4087.

**Kosma, D.K., Nemacheck, J.A., Jenks, M.A., and Williams, C.E.** (2010). Changes in properties of wheat leaf cuticle during interactions with Hessian fly. Plant J. **63,** 31-43.

**Kosma, D.K., Molina, I., Ohlrogge, J.B., and Pollard, M.** (2012). Identification of an Arabidopsis fatty alcohol: caffeoyl-Coenzyme A acyltransferase required for the synthesis of alkyl hydroxycinnamates in root waxes. Plant Physiol. **160,** 237-248.

**Kosma, D.K., Bourdenx, B., Bernard, A., Parsons, E.P., Lu, S., Joubes, J., and Jenks, M.A.** (2009). The impact of water deficiency on leaf cuticle lipids of Arabidopsis. Plant Physiol. **151,** 1918-1929.

**Krolikowski, K.A., Victor, J.L., Wagler, T.N., Lolle, S.J., and Pruitt, R.E.** (2003). Isolation and characterization of the Arabidopsis organ fusion gene HOTHEAD. Plant J. **35,** 501-511.

**Krömer, K.** (1903). Wurzelhaut, Hypodermis und Endodermis der Angiospermwurzel. Bibl. Bot. **59,** 156.

**Kunst, L., and Samuels, A.L.** (2003). Biosynthesis and secretion of plant cuticular wax. Prog. Lipid Res. **42,** 51-80.

**Kunst, L., and Samuels, L.** (2009). Plant cuticles shine: Advances in wax biosynthesis and export. Curr. Opin. Plant Biol. **12,** 721-727.

**Kunst, L., Taylor, D.C., and Underhill, E.W.** (1992). Fatty-acid elongation in developing seeds of *Arabidopsis thaliana*. Plant Physiol. Biochem. **30,** 425-434.

**Kurata, T., Kawabata-Awai, C., Sakuradani, E., Shimizu, S., Okada, K., and Wada, T.** (2003). The *YORE-YORE* gene regulates multiple aspects of epidermal cell differentiation in Arabidopsis. Plant J. **36,** 55-66.

**Kurdyukov, S., Faust, A., Nawrath, C., Bär, S., Voisin, D., Franke, R., Schreiber, L., Saedler, H., Métraux, J.-P., and Yephremov, A.** (2006a). The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in Arabidopsis. Plant Cell **18,** 321-339.

**Kurdyukov, S., Faust, A., Trenkamp, S., Bar, S., Franke, R., Efremova, N., Tietjen, K., Schreiber, L., Saedler, H., and Yephremov, A.** (2006b). Genetic and biochemical evidence for involvement of HOTHEAD in the biosynthesis of long-chain alpha-omega-dicarboxylic fatty acids and formation of extracellular matrix. Planta **224,** 315-329.

**L'Haridon, F., Besson-Bard, A., Binda, M., Serrano, M., Abou-Mansour, E., Balet, F., Schoonbeek, H.J., Hess, S., Mir, R., Leon, J., Lamotte, O., and Metraux, J.P.** (2011). A permeable cuticle is associated with the release of reactive oxygen species and induction of innate immunity. PLoS Pathog. **7,** e1002148.

**Lai, C., Kunst, L., and Jetter, R.** (2007). Composition of alkyl esters in the cuticular wax on inflorescence stems of *Arabidopsis thaliana cer* mutants. Plant J. **50,** 189-196.

**Lam, P., Zhao, L., McFarlane, H.E., Aiga, M., Lam, V., Hooker, T.S., and Kunst, L.** (2012). RDR1 and SGS3, components of RNA-mediated gene silencing, are required for the regulation of cuticular wax biosynthesis in developing inflorescence stems of Arabidopsis. Plant Physiol. **159,** 1385-1395.

**Le Bouquin, R., Skrabs, M., Kahn, R., Benveniste, I., Salaun, J.P., Schreiber, L., Durst, F., and Pinot, F.** (2001). CYP94A5, a new cytochrome P450 from *Nicotiana tabacum* is able to catalyze the oxidation of fatty acids to the omega-alcohol and to the corresponding diacid. Eur. J. Biochem. **268,** 3083-3090.

**Lee, S.B., Jung, S.J., Go, Y.S., Kim, H.U., Kim, J.K., Cho, H.J., Park, O.K., and Suh, M.C.** (2009). Two Arabidopsis 3-ketoacyl CoA synthase genes, *KCS20* and *KCS2/DAISY*, are functionally redundant in cuticu-

lar wax and root suberin biosynthesis, but differentially controlled by osmotic stress. Plant J. **60,** 462-475.

**Lee, Y., Rubio, M.C., Alassimone, J., and Geldner, N.** (2013). A mechanism for localized lignin deposition in the endodermis. Cell **153,** 402-412.

**Li, H., Pinot, F., Sauveplane, V., Werck-Reichhart, D., Diehl, P., Schreiber, L., Franke, R., Zhang, P., Chen, L., Gao, Y.W., Liang, W.Q., and Zhang, D.B.** (2010). Cytochrome P450 family member CYP704B2 catalyzes the omega-hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell **22,** 173-190.

**Li, Y.H., and Beisson, F.** (2009). The biosynthesis of cutin and suberin as an alternative source of enzymes for the production of bio-based chemicals and materials. Biochimie **91,** 685-691.

**Li, Y.H., Beisson, F., Ohlrogge, J., and Pollard, M.** (2007a). Monoacylglycerols are components of root waxes and can be produced in the aerial cuticle by ectopic expression of a suberin-associated acyltransferase. Plant Physiol. **144,** 1267-1277.

**Li, Y.H., Beisson, F., Koo, A.J.K., Molina, I., Pollard, M., and Ohlrogge, J.** (2007b). Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. Proc. Natl. Acad. Sci. USA **104,** 18339-18344.

**Li-Beisson, Y., Pollard, M., Sauveplane, V., Pinot, F., Ohlrogge, J., and Beisson, F.** (2009). Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. Proc. Natl. Acad. Sci. USA **106,** 22008-22013.

**Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S., Bird, D., Debono, A., Durrett, T.P., Franke, R.B., Graham, I.A., Katayama, K., Kelly, A.A., Larson, T., Markham, J.E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K.M., Wada, H., Welti, R., Xu, C., Zallot, R., and Ohlrogge, J.** (2013). Acyl-lipid metabolism. In The Arabidopsis Book, **11,** e0161.

**Lippold, F., Sanchez, D.H., Musialak, M., Schlereth, A., Scheible, W.-R., Hincha, D.K., and Udvardi, M.K.** (2009). AtMYB41 regulates transcriptional and metabolic responses to osmotic stress in Arabidopsis. Plant Physiol. **149,** 1761-1772.

**Liu, C.-J.** (2012). Deciphering the enigma of lignification: precursor transport, oxidation, and the topochemistry of lignin assembly. Mol. Plant **5,** 304-317.

**Liu, C.-J., Miao, Y.-C., and Zhang, K.-W.** (2011). Sequestration and transport of lignin monomeric precursors. Molecules **16,** 710-727.

**Liu, L.S., Chen, G.Y., Fishman, M.L., and Hicks, K.B.** (2005). Pectin gel vehicles for controlled fragrance delivery. Drug Deliv. **12,** 149-157.

**Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C., and Clarke, J.** (2006). SERRATE: a new player on the plant microRNA scene. EMBO Rep. **7,** 1052-1058.

**Lolle, S.J., Cheung, A.Y., and Sussex, I.M.** (1992). *Fiddlehead* - an Arabidopsis mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells. Dev. Biol. **152,** 383-392.

**Lolle, S.J., Hsu, W., and Pruitt, R.E.** (1998). Genetic analysis of organ fusion in *Arabidopsis thaliana*. Genetics **149,** 607-619.

**Lu, S., Zhao, H., Parsons, E.P., Xu, C., Kosma, D.K., Xu, X., Chao, D., Lohrey, G., Bangarusamy, D.K., Wang, G., Bressan, R.A., and Jenks, M.A.** (2011). The *glossyhead1* allele of ACC1 reveals a principal role for multidomain acetyl-Coenzyme A carboxylase in the biosynthesis of cuticular waxes by Arabidopsis. Plant Physiol. **157,** 1079-1092.

**Lu, S., Zhao, H., Des Marais, D.L., Parsons, E.P., Wen, X., Xu, X., Bangarusamy, D.K., Wang, G., Rowland, O., Juenger, T., Bressan, R.A., and Jenks, M.A.** (2012). Arabidopsis ECERIFERUM9 involvement in cuticle formation and maintenance of plant water status. Plant Physiol. **159,** 930-944.

**Lu, S.Y., Song, T., Kosma, D.K., Parsons, E.P., Rowland, O., and Jenks, M.A.** (2009). Arabidopsis *CER8* encodes LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. Plant J. **59,** 553-564.

**Luo, B., Xue, X.Y., Hu, W.L., Wang, L.J., and Chen, X.Y.** (2007). An ABC transporter gene of *Arabidopsis thaliana*, *AtWBC11*, is involved in cuticle development and prevention of organ fusion. Plant Cell Physiol. **48,** 1790-1802.

**Macgregor, D.R., Deak, K.I., Ingram, P.A., and Malamy, J.E.** (2008). Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. Plant Cell **20,** 2643-2660.

**Mazurek, S., Mucciolo, A., Humbel, B.M., and Nawrath, C.** (2013). Transmission Fourier transform infrared microspectroscopy allows simultaneous assessment of cutin and cell-wall polysaccharides of Arabidopsis petals. Plant J. **74,** 880-891.

**Mc Farlane, H.E., Shin, J.H., Bird, D.A., and Samuels, A.L.** (2010). Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. Plant Cell **22,** 3066-3075.

**Metraux, J., Nawrath, C., and Genoud, T.** (2002). Systemic acquired resistance. Euphytica **124,** 237-243.

**Millar, A.A., and Kunst, L.** (1997). Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J. **12,** 121-131.

**Millar, A.A., Clemens, S., Zachgo, S., Giblin, E.M., Taylor, D.C., and Kunst, L.** (1999). *CUT1*, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell **11,** 825-838.

**Molina, I., Ohlrogge, J.B., and Pollard, M.** (2008). Deposition and localization of lipid polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. Plant J. **55,** 437-449.

**Molina, I., Bonaventure, G., Ohlrogge, J., and Pollard, M.** (2006). The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds. Phytochemistry **67,** 2597-2610.

**Molina, I., Li-Beisson, Y., Beisson, F., Ohlrogge, J.B., and Pollard, M.** (2009). Identification of an Arabidopsis feruloyl-Coenzyme A transferase required for suberin synthesis. Plant Physiol. **151,** 1317-1328.

**Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C., and Geldner, N.** (2012). Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. Proc. Natl. Acad. Sci. USA **109,** 10101-10106.

**Niederl, S., Kirsch, T., Riederer, M., and Schreiber, L.** (1998). Co-permeability of H-3-labeled water and C-14-labeled organic acids across isolated plant cuticles - Investigating cuticular paths of diffusion and predicting cuticular transpiration. Plant Physiol. **116,** 117-123.

**Nishimura, M.T., and Dangl, J.L.** (2010). Arabidopsis and the plant immune system. Plant J. **61,** 1053-1066.

**Olson, D.A., and Sheares, V.V.** (2006). Preparation of unsaturated linear aliphatic polyesters using condensation polymerization. Macromolecules **39,** 2808-2814.

**Olsson, A., Lindstrîm, M., and Iversen, T.** (2007). Lipase-catalyzed synthesis of an epoxy-functionalized polyester from the suberin monomer cis -9,10-epoxy-18-hydroxyoctadecanoic acid. Biomacromolecules **8,** 757-760.

**Panikashvili, D., Shi, J.X., Schreiber, L., and Aharoni, A.** (2009). The Arabidopsis *DCR* encoding a soluble BAHD acyltransferase is required for cutin polyester formation and seed hydration properties. Plant Physiol. **151,** 1773-1789.

**Panikashvili, D., Shi, J.X., Schreiber, L., and Aharoni, A.** (2011). The Arabidopsis ABCG13 transporter is required for flower cuticle secretion and patterning of the petal epidermis. New Phytol. **190,** 113-124.

**Panikashvili, D., Shi, J.X., Bocobza, S., Franke, R.B., Schreiber, L.,**

**and Aharoni, A.** (2010). The Arabidopsis DSO/ABCG11 transporter affects cutin metabolism in reproductive organs and suberin in roots. Mol. Plant **3,** 563-575.

**Panikashvili, D., Savaldi-Goldstein, S., Mandel, T., Yifhar, T., Franke, R.B., Hofer, R., Schreiber, L., Chory, J., and Aharoni, A.** (2007). The Arabidopsis DESPERADO/AtWBC11 transporter is required for cutin and wax secretion. Plant Physiol. **145,** 1345-1360.

**Pascal, S., Bernard, A., Sorel, M., Pervent, M., Vile, D., Haslam, R.P., Napier, J.A., Lessire, R., Domergue, F., and Joubes, J.** (2013). The Arabidopsis *cer26* mutant, like the cer2 mutant, is specifically affected in the very long chain fatty acid elongation process. Plant J. **73,** 733-746.

**Paul, S., Gable, K., Beaudoin, F., Cahoon, E., Jaworski, J., Napier, J.A., and Dunn, T.M.** (2006). Members of the Arabidopsis FAE1-like3-ketoacyl-CoA synthase gene family substitute for the Elop proteins of *Saccharomyces cerevisiae*. J. Biol. Chem. **281,** 9018-9029.

**Peterson, C.A., Dewildt, P.P.Q., and Edgington, L.V.** (1978). Rationale for ambimobile translocation of nematicide oxamyl in plants. Pestic. Biochem. Phys. **8,** 1-9.

**Pighin, J.A., Zheng, H.Q., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L., and Samuels, A.L.** (2004). Plant cuticular lipid export requires an ABC transporter. Science **306,** 702-704.

**Pinot, F., and Beisson, F.** (2011). Cytochrome P450 metabolizing fatty acids in plants: characterization and physiological roles. FEBS J. **278,** 195-205.

**Podila, G.K., Rogers, L.M., and Kolattukudy, P.E.** (1993). Chemical signals from Avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol. **103,** 267-272.

**Pollard, M., Beisson, F., Li, Y.H., and Ohlrogge, J.B.** (2008). Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci. **13,** 236-246.

**Prigge, M.J., and Wagner, D.R.** (2001). The Arabidopsis SERRATE gene encodes a zinc-finger protein required for normal shoot development. Plant Cell **13,** 1263-1279.

**Pruitt, R.E., Vielle-Calzada, J.P., Ploense, S.E., Grossniklaus, U., and Lolle, S.J.** (2000). *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in Arabidopsis, encodes a putative lipid biosynthetic enzyme. Proc. Natl. Acad. Sci. USA **97,** 1311-1316.

**Quist, T.M., Sokolchik, I., Shi, H., Joly, R.J., Bressan, R.A., Maggio, A., Narsimhan, M., and Li, X.** (2009). *HOS3*, an *ELO*-like gene, inhibits effects of ABA and implicates a S-1-P/ceramide control system for abiotic stress responses in *Arabidopsis thaliana*. Mol. Plant **2,** 138-151.

**Raffaele, S., Vailleau, F., Leger, A., Joubes, J., Miersch, O., Huard, C., Blee, E., Mongrand, B., Domergue, F., and Roby, D.** (2008). A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in Arabidopsis. Plant Cell **20,** 752-767.

**Ranathunge, K., and Schreiber, L.** (2011). Water and solute permeabilities of Arabidopsis roots in relation to the amount and composition of aliphatic suberin. J. Exp. Bot. **62,** 1961-1974.

**Ranathunge, K., Steudle, E., and Lafitte, R.** (2003). Control of water uptake by rice (*Oryza sativa* L.): role of the outer part of the root. Planta **217,** 193-205.

**Rani, S.H., Krishna, T.H.A., Saha, S., Negi, A.S., and Rajasekharan, R.** (2010). *DEFECTIVE IN CUTICULAR RIDGES* (*DCR*) of *Arabidopsis thaliana*, a gene associated with surface cutin formation, encodes a soluble diacylglycerol acyltransferase. J. Biol. Chem. **285,** 38337-38347.

**Rashotte, A., and Feldmann, K.** (1996). Epicuticular waxes and aphid resistance in Arabidopsis *cer* mutants and ecotypes. Plant Physiol. **111,** 304-304.

**Rashotte, A.M.** (1999). Epicuticular wax in *Arabidopsis thaliana*: a study of the genetics, chemistry, structure, and interactions with insects. Ph.D. Dissertation. Plant Science. University of Arizona, Tucson.

**Rashotte, A.M., Jenks, M.A., and Feldmann, K.A.** (2001). Cuticular waxes on *eceriferum* mutants of *Arabidopsis thaliana*. Phytochemistry **57,** 115-123.

**Rautengarten, C., Ebert, B., Ouellet, M., Nafisi, M., Baidoo, E.E.K., Benke, P., Stranne, M., Mukhopadhyay, A., Keasling, J.D., Sakuragi, Y., and Scheller, H.V.** (2012). The Arabidopsis *DEFICIENT IN CUTIN FERULATE* (*DCF*) encodes a transferase required for feruloylation of ω-hydroxyfatty acids in cutin polyester. Plant Physiol. **158,** 654-665.

**Razem, F.A., and Bernards, M.A.** (2002). Hydrogen peroxide is required for poly(phenolic) domain formation during wound-induced suberization. J. Agr. Food Chem. **50,** 1009-1015.

**Razem, F.A., and Bernards, M.A.** (2003). Reactive oxygen species production in association with suberization: evidence for an NADPH-dependent oxidase. J. Exp. Bot. **54,** 935-941.

**Reina, J.J., Dominguez, E., and Heredia, A.** (2001). Water sorption-desorption in conifer cuticles: The role of lignin. Physiol. Plant. **112,** 372-378.

**Reina-Pinto, J.J., and Yephremov, A.** (2009). Surface lipids and plant defenses. Plant Physiol. Biochem. **47,** 540-549.

**Reisige, K., Gorzelanny, C., Daniels, U., and Moerschbacher, B.M.** (2006). The C28 aldehyde octacosanal is a morphogenetically active component involved in host plant recognition and infection structure differentiation in the wheat stem rust fungus. Physiol. Mol. Plant Pathol. **68,** 33-40.

**Richardson, A., Wojciechowski, T., Franke, R., Schreiber, L., Kerstiens, G., Jarvis, M., and Fricke, W.** (2007). Cuticular permeance in relation to wax and cutin development along the growing barley (*Hordeum vulgare*) leaf. Planta **225,** 1471-1481.

**Riederer, M., and Schonherr, J.** (1988). Development of plant cuticles - fine-structure and cutin composition of *Clivia miniata* Reg leaves. Planta **174,** 127-138.

**Riederer, M., and Schreiber, L.** (2001). Protecting against water loss: analysis of the barrier properties of plant cuticles. J. Exp. Bot. **52,** 2023-2032.

**Roppolo, D., De Rybel, B., Dénervaud Tendon, V., Pfister, A., Alassimone, J., Vermeer, J.E.M., Yamazaki, M., Stierhof, Y.-D., Beeckman, T., and Geldner, N.** (2011). A novel protein family mediates Casparian strip formation in the endodermis. Nature **473,** 380-383.

**Rowland, O., Lee, R., Franke, R., Schreiber, L., and Kunst, L.** (2007). The *CER3* wax biosynthetic gene from *Arabidopsis thaliana* is allelic to *WAX2/YRE/FLP1*. FEBS Lett. **581,** 3538-3544.

**Rowland, O., Zheng, H., Hepworth, S.R., Lam, P., Jetter, R., and Kunst, L.** (2006). *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. Plant Physiol. **142,** 866-877.

**Samuels, L., Kunst, L., and Jetter, R.** (2008). Sealing plant surfaces: Cuticular wax formation by epidermal cells. Ann. Rev. Plant Biol. **59,** 683-707.

**Sauveplane, V., Kandel, S., Kastner, P.-E., Ehlting, J., Compagnon, V., Werck-Reichhart, D., and Pinot, F.** (2009). *Arabidopsis thaliana* CYP77A4 is the first cytochrome P450 able to catalyze the epoxidation of free fatty acids in plants. FEBS J. **276,** 719-735.

**Schmutz, A., Buchala, A.J., and Ryser, U.** (1996). Changing the dimensions of suberin lamellae of green cotton fibers with a specific inhibitor of the endoplasmic reticulum-associated fatty acid elongases. Plant Physiol. **110,** 403-411.

**Schnurr, J., Shockey, J., and Browse, J.** (2004). The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in Arabidopsis. Plant Cell **16,** 629-642.

**Schönherr, J., and Riederer, M.** (1986). Plant cuticles sorb lipophilic compounds during enzymatic digestion. Plant Cell Environ. **6,** 459-466.

**Schreiber, L.** (1996). Chemical composition of Casparian strips isolated from *Clivia miniata* Reg roots: Evidence for lignin. Planta **199,** 596-601.

**Schreiber, L.** (2010). Transport barriers made of cutin, suberin and associated waxes. Trends Plant Sci. **15,** 546-553.

**Schreiber, L., and Schönherr, J.** (2009). Water and solute Permeability of Plant Cuticles. (Springer Berlin Heidelberg).

**Schreiber, L., Franke, R., and Lessire, R.** (2005a). Biochemical characterization of elongase activity in corn (*Zea mays* L.) roots. Phytochemistry **66,** 131-138.

**Schreiber, L., Franke, R., and Hartmann, K.** (2005b). Wax and suberin development of native and wound periderm of potato (*Solanum tuberosum* L.) and its relation to peridermal transpiration. Planta **220,** 520-530.

**Schreiber, L., Hartmann, K., Skrabs, M., and Zeier, J.** (1999). Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. J. Exp. Bot. **50,** 1267-1280.

**Schweizer, P., Felix, G., Buchala, A., Müller, C., and Métraux, J.** (1996). Perception of free cutin monomers by plant cells. Plant J. **10,** 331-341.

**Seo, P.J., Lee, S.B., Suh, M.C., Park, M.J., Go, Y.S., and Park, C.M.** (2011). The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in Arabidopsis. Plant Cell **23,** 1138-1152.

**Serra, O., Hohn, C., Franke, R., Prat, S., Molinas, M., and Figueras, M.** (2010). A feruloyl transferase involved in the biosynthesis of suberin and suberin-associated wax is required for maturation and sealing properties of potato periderm. Plant J. **62,** 277-290.

**Shi, J.X., Malitsky, S., De Oliveira, S., Branigan, C., Franke, R.B., Schreiber, L., and Aharoni, A.** (2011). *SHINE* transcription factors act redundantly to pattern the archetypal surface of Arabidopsis flower organs. PLoS Genet. **7,** e1001388.

**Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Metraux, J.P., and Nawrath, C.** (2000). Transgenic Arabidopsis plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721-738.

**Sitte, P., and Rennier, R.** (1963). Untersuchungen an cuticulären Zellwandschichten. Planta **60,** 19-40.

**Soliday, C.L., and Kolattukudy, P.E.** (1977). Biosynthesis of cutin - omega-hydroxylation by a microsomal preparation from germinating *Vicia faba* Plant Physiol. **59,** 1116-1121.

**Soliday, C.L., and Kolattukudy, P.E.** (1978). Midchain hydroxylation of 16-hydroxypalmitic acid by endoplasmic reticulum fraction from germinating *Vicia faba*. Arch. Biochem. Biophys. **188,** 338-347.

**Stark, R.E., and Tian, S.** (2006). The cutin biopolymer matrix. In Biology of the Plant Cuticle, M. Riederer and C. Müller, eds (Oxford: Blackwell Publishing), pp. 126-144.

**Steudle, E.** (1994). Water transport across roots. Plant Soil **167,** 79-90.

**Steudle, E., and Peterson, C.A.** (1998). How does water get through roots? J. Exp. Bot. **49,** 775-788.

**Steudle, E., Murrmann, M., and Peterson, C.A.** (1993). Transport of water and solutes across maize roots modified by punctuating the endodermis - Further evidence for the composite transport model of the root. Plant Physiol. **103,** 335-349.

**Suh, M.C., Samuels, A.L., Jetter, R., Kunst, L., Pollard, M., Ohlrogge, J., and Beisson, F.** (2005). Cuticular lipid composition, surface structure, and gene expression in Arabidopsis stem epidermis. Plant Physiol. **139,** 1649 - 1665.

**Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L., and Bennett, M.J.** (2008). The auxin influx carrier LAX3 promotes lateral root emergence. Nat. Cell Biol. **10,** 946-954.

**Szczuka, E., and Szczuka, A.** (2003). Cuticle fluorescence during embryogenesis of *Arabidopsis thaliana* (L.) Heynh. Acta Biol. Cracov. Ser. Bot. **45,** 63-67.

**Takahashi, K., Shimada, T., Kondo, M., Tamai, A., Mori, M., Nishimura, M., and Hara-Nishimura, I.** (2010). Ectopic expression of an esterase, which is a candidate for the unidentified plant cutinase, causes cuticular defects in *Arabidopsis thaliana*. Plant Cell Physiol. **51,** 123-131.

**Tanaka, H., Onouchi, H., Kondo, M., Hara-Nishimura, I., Nishimura, M., Machida, C., and Machida, Y.** (2001). A subtilisin-like serine protease is required for epidermal surface formation in Arabidopsis embryos and juvenile plants. Development **128,** 4681-4689.

**Tanaka, T., Tanaka, H., Machida, C., Watanabe, M., and Machida, Y.** (2004). A new method for rapid visualization of defects in leaf cuticle reveals five intrinsic patterns of surface defects in Arabidopsis. Plant J. **37,** 139-146.

**Tang, D.Z., Simonich, M.T., and Innes, R.W.** (2007). Mutations in LACS2, a long-chain acyl-coenzyme a synthetase, enhance susceptibility to avirulent *Pseudomonas syringae* but confer resistance to *Botrytis cinerea* in Arabidopsis. Plant Physiol. **144,** 1093-1103.

**Thomas, R., Fang, X.X., Ranathunge, K., Anderson, T.R., Peterson, C.A., and Bernards, M.A.** (2007). Soybean root suberin: Anatomical distribution, chemical composition, and relationship to partial resistance to *Phytophthora sojae*. Plant Physiol. **144,** 299-311.

**Tian, S.Y., Fang, X.H., Wang, W.M., Yu, B.W., Cheng, X.F., Qiu, F., Mort, A.J., and Stark, R.E.** (2008). Isolation and identification of oligomers from partial degradation of lime fruit cutin. J. Agr. Food Chem. **56,** 10318-10325.

**Trenkamp, S., Martin, W., and Tietjen, K.** (2004). Specific and differential inhibition of very-long-chain fatty acid elongases from *Arabidopsis thaliana* by different herbicides. Proc. Natl. Acad. Sci. USA **101,** 11903-11908.

**Tresch, S., Heilmann, M., Christiansen, N., Looser, R., and Grossmann, K.** (2012). Inhibition of saturated very-long-chain fatty acid biosynthesis by mefluidide and perfluidone, selective inhibitors of 3-ketoacyl-CoA synthases. Phytochemistry **76,** 162-171.

**Tsuwamoto, R., Fukuoka, H., and Takahata, Y.** (2008). *GASSHO1* and *GASSHO2* encoding a putative leucine-rich repeat transmembrane-type receptor kinase are essential for the normal development of the epidermal surface in Arabidopsis embryos. Plant J. **54,** 30-42.

**Tucker, S., and Talbot, N.** (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. Ann. Rev. Phytopathol. **39,** 385-417.

**Tudzynski, P., and Kokkelink, L.** (2009). *Botrytis cinerea* : Molecular aspects of a necrotrophic life style plant relationships, H.B. Deising, ed (Springer Berlin Heidelberg), pp. 29-50.

**Uppalapati, S.R., Ishiga, Y., Doraiswamy, V., Bedair, M., Mittal, S., Chen, J., Nakashima, J., Tang, Y., Tadege, M., Ratet, P., Chen, R., Schultheiss, H., and Mysore, K.S.** (2012). Loss of abaxial leaf epicuticular wax in *Medicago truncatula irg1/palm1* mutants results in reduced spore differentiation of anthracnose and nonhost rust pathogens. Plant Cell **24,** 353-370.

**van den Brule, S., and Smart, C.C.** (2002). The plant PDR family of ABC transporters. Planta **216,** 95-106.

**Vanfleet, D.S.** (1961). Histochemistry and function of the endodermis. Bot. Rev. **27,** 165-220.

**Vogt, T.** (2010). Phenylpropanoid biosynthesis. Mol. Plant **3,** 2-20.

**Voisin, D., Nawrath, C., Kurdyukov, S., Franke, R.B., Reina-Pinto, J.J.,**

**Efremova, N., Will, I., Schreiber, L., and Yephremov, A.** (2009). Dissection of the complex phenotype in cuticular mutants of Arabidopsis reveals a role of SERRATE as a mediator. PLoS Genet. **5,** e1000703.

**Walton, J.D.** (1996). Host-selective toxins: agents of compatibility. Plant Cell **8,** 1723-1733.

**Walton, T.J., and Kolattukudy, P.** (1972). Determination of structures of cutin monomers by a novel depolymerization procedure and combined gas-chromatography and mass-spectrometry. Biochemistry **11,** 1885-1897.

**Wang, Z.H., Guhling, O., Yao, R.N., Li, F.L., Yeats, T.H., Rose, J.K.C., and Jetter, R.** (2011a). Two oxidosqualene cyclases responsible for biosynthesis of tomato fruit cuticular triterpenoids. Plant Physiol. **155,** 540-552.

**Wang, Z.Y., Xiong, L., Li, W., Zhu, J.K., and Zhu, J.** (2011b). The plant cuticle is required for osmotic stress regulation of abscisic acid biosynthesis and osmotic stress tolerance in Arabidopsis. Plant Cell **23,** 1971-1984.

**Watanabe, M., Tanaka, H., Machida, C., and Machida, Y.** (2003). ACR4, a putative receptor-like kinase gene involved in differentiation of the epidermis in *Arabidopsis thaliana*. Plant Cell Physiol. **44,** S171-S171.

**Watanabe, M., Tanaka, H., Watanabe, D., Machida, C., and Machida, Y.** (2004). The ACR4 receptor-like kinase is required for surface formation of epidermis-related tissues in *Arabidopsis thaliana*. Plant J. **39,** 298-308.

**Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., Steiner-Lange, S., Saedler, H., and Yephremov, A.** (2001). Functional analysis of the *LACERATA* gene of Arabidopsis provides evidence for different robes of fatty acid omega-hydroxylation in development. Proc. Natl. Acad. Sci. USA **98,** 9694-9699.

**Wen, M., and Jetter, R.** (2009). Composition of secondary alcohols, ketones, alkanediols, and ketols in *Arabidopsis thaliana* cuticular waxes. J. Exp. Bot. **60,** 1811-1821.

**Weng, H., Molina, I., Shockey, J., and Browse, J.** (2010). Organ fusion and defective cuticle function in a *lacs1 lacs2* double mutant of Arabidopsis. Planta **231,** 1089-1100.

**Wilson, C.A., and Peterson, C.A.** (1983). Chemical-composition of the epidermal, hypodermal, endodermal and intervening cortical cell-walls of various plant-roots. Ann. Bot. **51,** 759-769.

**Wu, R., Li, S., He, S., Waßmann, F., Yu, C., Qin, G., Schreiber, L., Qu, L.-J., and Gu, H.** (2011). CFL1, a WW domain protein, regulates cuticle development by modulating the function of HDG1, a Class IV homeodomain transcription factor, in rice and Arabidopsis. Plant Cell **23,** 3392-3411.

**Xia, Y., Yu, K.S., Navarre, D., Seebold, K., Kachroo, A., and Kachroo, P.** (2010). The *glabra1* mutation affects cuticle formation and plant responses to microbes. Plant Physiol. **154,** 833-846.

**Xia, Y., Yu, K., Gao, Q.-M., Wilson, E.V., Navarre, D., Kachroo, P., and Kachroo, A.** (2012). Acyl CoA binding proteins are required for cuticle formation and plant responses to microbes. Front. Plant Sci. **3,** 224-224.

**Xia, Y., Gao, Q.M., Yu, K.S., Lapchyk, L., Navarre, D., Hildebrand, D., Kachroo, A., and Kachroo, P.** (2009). An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. Cell Host Microbe **5,** 151-165.

**Xiao, F.M., Goodwin, S.M., Xiao, Y.M., Sun, Z.Y., Baker, D., Tang, X.Y., Jenks, M.A., and Zhou, J.M.** (2004). Arabidopsis CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J. **23,** 2903-2913.

**Xing, Q., Creff, A., Waters, A., Tanaka, H., Goodrich, J., and Ingram, G.C.** (2013). ZHOUPI controls embryonic cuticle formation via a signalling pathway involving the subtilisin protease ABNORMAL LEAF-SHAPE1 and the receptor kinases GASSHO1 and GASSHO2. Development **140,** 770-779.

**Yang, L., Liu, Z., Lu, F., Dong, A., and Huang, H.** (2006). SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. Plant J. **47,** 841-850.

**Yang, W., Pollard, M., Li-Beisson, Y., Beisson, F., Feig, M., and Ohlrogge, J.** (2010). A distinct type of glycerol-3-phosphate acyltransferase with *sn-2* preference and phosphatase activity producing 2-monoacylglycerol. Proc. Natl. Acad. Sci. USA **107,** 12040-12045.

**Yang, W.L., Simpson, J.P., Li-Beisson, Y., Beisson, F., Pollard, M., and Ohlrogge, J.B.** (2012). A land-plant-specific glycerol-3-phosphate acyltransferase family in Arabidopsis: substrate specificity, sn-2 preference, and evolution. Plant Physiol. **160,** 638-652.

**Yeats, T.H., Howe, K.J., Matas, A.J., Buda, G.J., Thannhauser, T.W., and Rose, J.K.C.** (2010). Mining the surface proteome of tomato (*Solanum lycopersicum*) fruit for proteins associated with cuticle biogenesis. J. Exp. Bot. **61,** 3759-3771.

**Yeats, T.H., Martin, L.B.B., Isaacson, T., He, Y., Zhao, L., Matas, A.J., Buda, G.J., Domozych, D.S., and Rose, J.K.C.** (2012a). An extracellular acyltransferase catalyses cutin polymerization. Pharm. Biol. **50,** 542-543.

**Yeats, T.H., Martin, L.B.B., Viart, H.M.F., Isaacson, T., He, Y.H., Zhao, L.X., Matas, A.J., Buda, G.J., Domozych, D.S., Clausen, M.H., and Rose, J.K.C.** (2012b). The identification of cutin synthase: Formation of the plant polyester cutin. Nat. Chem. Biol. **8,** 609-611.

**Yephremov, A., and Schreiber, L.** (2005). The dark side of the cell wall: Molecular genetics of plant cuticle. Plant Biosyst. **139,** 74-79.

**Yephremov, A., Wisman, E., Huijser, P., Huijser, C., Wellesen, K., and Saedler, H.** (1999). Characterization of the *FIDDLEHEAD* gene of Arabidopsis reveals a link between adhesion response and cell differentiation in the epidermis. Plant Cell **11,** 2187-2201.

**Zeier, J., Ruel, K., Ryser, U., and Schreiber, L.** (1999). Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. Planta **209,** 1-12.

**Zheng, Z.F., Xia, Q., Dauk, M., Shen, W.Y., Selvaraj, G., and Zou, J.T.** (2003). Arabidopsis AtGPAT1, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility. Plant Cell **15,** 1872-1887.

**Zimmermann, H.M., and Steudle, E.** (1998). Apoplastic transport across young maize roots: Effect of the exodermis. Planta **206,** 7-19.