Chapter 12
Plant Surface Lipids and Epidermis Development

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Abstract The epidermis has a strategic position at the interface between the plant and the environment. In order to control exchanges with the environment as well as to protect the plant from external threats, the epidermis synthesises and secretes surface lipids to form a continuous, transparent and hydrophobic layer known as the cuticle. Cuticle formation is a strictly epidermal property in plants and all aerial epidermal cells produce some sort of cuticle on their surface. Conversely, all cuticularized plant surfaces are of epidermal origin. This seemingly anodyne observation has surprisingly profound implications in terms of understanding the function of the plant cuticle, since it underlies in part, the difficultly of functionally separating epidermal cell fate specification from cuticle biogenesis.

Keywords Cuticle • Epidermis • Regulation • Waxes • Development • Arabidopsis

Introduction

The lipid metabolism of the epidermis is highly specialized because this cell monolayer is located at the interface between the plant and its surrounding environment. Due to this highly strategic positioning, one important function of the epidermis is to form a hydrophobic barrier over aerial surfaces to permit the regulation of water and gas exchange as well as protecting the plant from both biotic and abiotic stresses. In order to achieve this role, the epidermis synthesises and secretes surface lipids to form a continuous transparent and hydrophobic layer on the outside of leaves, stems and flowers. In contrast to the lipid metabolism of most cortical cells,

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which is mainly channelled towards the generation of thylakoid membranes for photosynthesis, that of epidermal cells is therefore principally devoted to the biosynthesis of surface lipids.

All aerial parts of the plants as well as parts of the root system are delimitated by a layer of epidermal cells. Above ground epidermal cells include a "basal" cell type, known as pavement cells, which are abundant and show a highly organ dependent morphology, as well as trichomes, also known as leaf hairs, and the guard cells of stomata. Underground, young primary roots possess an epidermis layer, but this tissue, together with the endodermis and the cortex, disappears during secondary development, when a periderm originating from the pericycle develops. Aerial and mature root surfaces are covered by lipid layers known as the cuticle and suberin, respectively. Over the last 20 years, the biosynthetic pathways producing the lipids of these apoplastic diffusion barriers have been well described, especially in the plant model *Arabidopsis thaliana*, where many mutants have been isolated and characterised (Nawrath et al. 2013). None of the suberin mutants identified to date have been associated with root epidermal or peridermal defects, but this may be due to technical difficulties in studying the precise morphology of these underground tissues. In contrast, the fact that several mutants affected in their cuticle composition and/or content also display defects in epidermal development, such as organ fusions, abnormal trichome morphology or decreased stomatal index, suggests a link between surface lipids and the differentiation of epidermal cells (Bird and Gray 2003). Furthermore, two studies have highlighted a potential role of the epidermis in restricting plant growth (Savaldi-Goldstein et al. 2007; Nobusawa et al. 2013a). Interestingly, cutinase treatments in rapidly growing internodes of deepwater rice suggest that the cuticle acts as a growth-limiting structure, and could thus be a key epidermal element in keeping the underlying parenchyma cells under compression (Hoffmann-Benning and Kende 1994).

In this chapter, we will present our current knowledge of the biosynthesis of surface lipids and describe in more detail how certain mutants with defects in their surface lipid content and/or composition are also affected in their epidermal development. We will then discuss the complex transcriptional regulation of plant surface lipid biosynthesis and epidermis development. Finally, we will take advantage of recent advances in our understanding of the establishment of the protocuticule in Arabidopsis embryos to present the inter-cellular signaling pathway monitoring the formation of a functional embryonic cuticle and enabling the physical separation of the embryo from the endosperm.

Biosynthesis of the Cuticular Barrier by Epidermal Cells

The plant cuticle is a continuous lipophilic layer, of which the main roles are to limit non-stomatal water loss, control gaseous exchanges, protect the plant from both biotic and abiotic environmental stresses, provide mechanical strength, and prevent organ fusion during development. The cuticle is composed of a fatty acid derived

polymer, known as cutin, which is impregnated with, and covered by, a mixture of very long-chain (VLC) aliphatic compounds, called cuticular waxes (Yeats and Rose 2013).

Cuticular waxes consist of a mixture of very-long chain aliphatic compounds, containing from 22 to 48 carbon atoms, which are produced in the endoplasmic reticulum (ER) (Bernard and Joubès 2013). Their biosynthesis relies on the elongase complex, which produces VLC-acyl-CoAs (and free VLC fatty acids; VLCFAs), the alcohol-forming pathway, which converts these into even-numbered primary alcohols and alkyl esters, and the alkane-forming pathway, which yields aldehydes and odd-numbered alkanes, secondary alcohols and ketones (Fig. 12.1). The cutin polymer is mainly made of interesterified hydroxy fatty acids, but also contains some glycerol and dicarboxilic acids, as well as phenolic compounds. Cutin biosynthesis starts in the ER where hydroxyl fatty acids are produced and esterified to mono-acyl glycerol (MAG), and ends in the apoplasm where polymerization takes place (Fig. 12.1). A key step in surface lipid biosynthesis is therefore the transport of the cutin monomers and cuticular waxes across both the plasma membrane and the hydrophilic cell wall (Fig. 12.1).

The Fatty Acid Elongase Complex Produces Very Long Chain Fatty Acids

Free C_{16} and C_{18} fatty acids, issuing from *de novo* fatty acid synthesis in the plastids, are used as precursors for fatty acid elongation (Haslam and Kunst 2013). Recent reports revealed the important role of at least three Long-Chain-Acyl-CoA Synthetase (LACS) iso-enzymes, which activate free fatty acids into CoA thioesters, in Arabidopsis wax synthesis (Lü et al. 2009; Weng et al. 2010; Jessen et al. 2011). Each cycle of the ER-bound multi-enzymatic fatty acid elongase (FAE) consists of four successive reactions that generate an acyl-chain extended by two carbons. The reactions involve (1) formation of β-ketoacyl-CoA by condensation of malonyl-CoA with an a C_n-acyl-CoA catalysed by a β-ketoacyl-CoA synthase (KCS), (2) reduction to β-hydroxyacyl-CoA by a β-ketoacyl-CoA reductase (KCR), (3) dehydration to enoyl-CoA by a β-hydroxyacyl-CoA dehydratase (HCD) and (4) a final reduction by a enoyl-CoA reductase (ECR) yielding a C_{n+2}-acyl-CoA. Biochemical studies and the co-existence of 21 putative KCS-encoding genes annotated in the Arabidopsis genome (Joubès et al. 2008), led to the idea that multiple elongase complexes with distinct chain-length specificities perform sequential and parallel reactions to produce the wide range of chain-lengths found in plant VLCFAs (von Wettstein-Knowles 1982).

Over the last decade, major advances in understanding VLCFA synthesis in yeast have permitted the identification of the ECR, KCR and HCD enzymes of Arabidopsis. Sequence similarity searches with ECR from yeast revealed one single locus in Arabidopsis. Complementation of the Arabidopsis *cer10* (*cer* standing for *eceriferum*, i.e. wax-less) mutant by expression of *AtECR* demonstrated that *CER10*

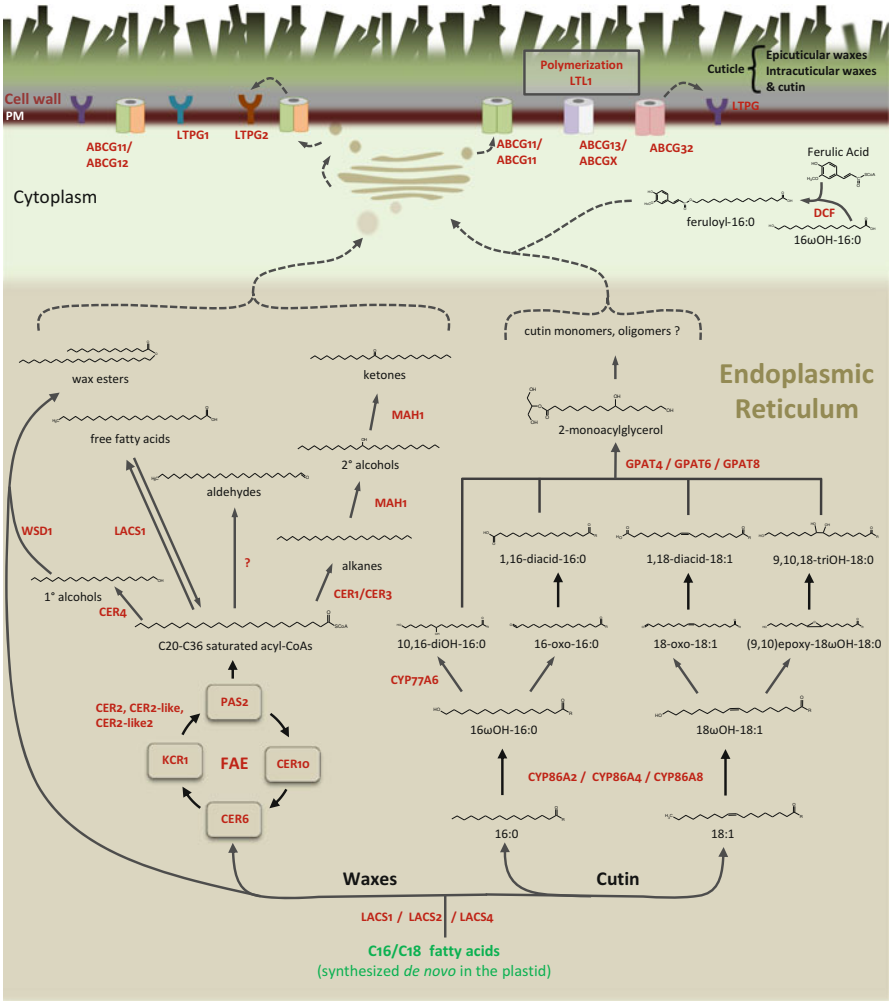

Fig. 12.1 Cuticle biosynthetic pathways. The genes in *red* are those discovered in *Arabidopsis thaliana*. Wax biosynthetic pathways: Very long chain fatty acyl-CoAs are synthesized by the Fatty Acid Elongase (FAE) multienzymatic complex from C16-C18 fatty acids. VLCFAs are then modified via two distinct biosynthetic pathways to generate the aliphatic compounds of waxes; the alcohol forming pathway resulting in the formation of primary alcohols (1° alcohols) and wax esters, and the alkane forming pathway which leads to the production of aldehydes, alkanes, secondary alcohols (2° alcohols) and ketones. Cutin biosynthetic pathways: Addition of a hydroxyl group at the terminal or mid-chain position of C16 and C18 is catalyzed by members of the cytochrome P450 family (CYPs). Formation of dicarboxylic acids (DCA) from ω-hydroxyacids (ω-OH) may involve oxydoreductases. Intermediates are annotated "COOR", where R could be H, CoA or glycerol, since the extact substrate of P450 and order of reaction remains unclear. Esterification of ω-OH and DCA to glycerol-3-phosphate by glycerol-3-phosphate acyl-CoA transferase (GPATs) produces *sn*-2-monoacylglycerol. Incorporation of phenolic components into the cutin polymer requires BAHD-type acyltransferases. Extracellular polymerisation are performed by cutin synthases. Export of precursors to the cuticular matrix: Cuticle precursors produced in the endoplasmic reticulum (ER) reach the plasma membrane (PM) via the Golgi apparatus through the secretory pathway. Export of cuticle compounds from the plasma membrane is carried out by ATP-Binding Cassette (ABCs) transporters and glycosylphosphatidylinositol-anchored Lipid Transfer Proteins (LTPGs)

encodes a functional ECR activity (Zheng et al. 2005). Likewise, two Arabidopsis loci were identified as encoding potential HCDs and two loci as encoding potential KCRs (Bach et al. 2008; Beaudoin et al. 2009). Surprisingly, complementation assays in corresponding yeast mutants revealed that only *PASTICCINO2* encodes a functional HCD and only *KCR1* has KCR activity. Although several *KCS* mutants show defects in their cuticular waxes, *CER6* is the only one considered to be strictly wax-specific (Millar et al. 1999; Hooker et al. 2002), indicating a preponderant role in wax synthesis. The recent functional characterization of CER2 and related proteins suggests that these proteins are necessary for elongation up to C_{34}, even though the precise function of these proteins remains to be elucidated (Haslam et al. 2012, 2015; Pascal et al. 2013).

The VLC-acyl-CoAs produced by the FAEs are either used as precursors in the alcohol- and alkane-forming pathways (below), or simply converted by thioesterase(s) to free VLCFAs, a significant quantity of which are detected in surface lipids.

The Alcohol Forming Pathway Produces Primary Alcohols and Alkyl Esters

The first biochemical studies of primary alcohol formation suggested a two-step reaction in which fatty acyl reductase (FAR) reduces VLCFAs to aldehydes, which are further reduced to primary alcohol by an aldehyde reductase (Kolattukudy 1971). However, biochemical studies on jojoba seeds and pea leaves, as well as expression of genes encoding FAR alcohol-forming activities in heterologous systems, revealed that a single enzyme produced fatty alcohols, with the potential intermediate aldehyde remaining bound to the enzyme (Rowland and Domergue 2012). In Arabidopsis, the *cer4* mutant shows a severe reduction of primary alcohols and wax esters, suggesting that *CER4* could play a role in this biosynthetic pathway (Jenks et al. 1995). Expression of *CER4* in yeast resulted in the production of VLC-primary alcohols with C_{24} and C_{26} chain lengths, confirming the FAR activity of CER4 (Rowland et al. 2006). However, the activity of CER4 on C_{28} and C_{30} fatty acids has never been demonstrated, indicating that another FAR or functionally related enzyme, is likely responsible for C_{30} primary alcohol formation (Rowland and Domergue 2012). Detailed analysis of wax ester chain-lengths from the stems of Arabidopsis *cer4* mutants indicated that primary alcohols formed by CER4 act as substrates for subsequent alkyl ester formation (Lai et al. 2007). Wax Synthase (WS) enzymes catalyze the esterification of primary alcohols with acyl-CoAs in higher plants, mammals and bacteria (Lardizabal et al. 2000; Cheng and Russell 2004; Stoveken et al. 2005). In Arabidopsis, sequence similarity with jojoba seed WS and bi-functional WS/Diacylglycerol acyltransferases (DGATs) from *Acinetobacter calcoaceticus*, revealed 12 and 11 similar sequences respectively. Analysis of a putative WS/DGAT encoding gene (*WSD1*), highly expressed in the epidermis, subsequently confirmed its involvement as the major WS in cuticular wax synthesis (Li et al. 2008).

The Alkane Forming Pathway Produces Aldehydes and Odd-Numbered Alkanes, Secondary Alcohols and Ketones

Analyses of *cer* mutants and biochemical experiments established a putative alkane forming pathway in which VLCFAs are used as precursors to form alkanes via a potential aldehyde intermediate (Bernard and Joubès 2013). Several *cer* mutants with a decreased alkane load have been biochemically characterized (Jenks et al. 1995). The *cer3* mutant showed a dramatic reduction in aldehydes, alkanes, secondary alcohols and ketones, while the *cer1* mutant exhibits a drastic decrease in alkanes and a near abolition of secondary alcohol and ketone production, accompanied by a slight increase in aldehyde content (Aarts et al. 1995; Chen et al. 2003; Kurata et al. 2003; Bourdenx et al. 2011). It has been proposed from these phenotypes that *CER3* could encode a potential VLCFA reductase producing aldehydes, whereas *CER1* could encode the alkane-forming enzyme, catalyzing the presumed decarbonylation of aldehydes to alkanes. Consistent with a role in wax associated processes, the expression of *CER1* and *CER3* was found to be restricted to the aerial epidermis, and to be up-regulated in drought conditions when wax synthesis is particularly active (Kurata et al. 2003; Bourdenx et al. 2011). Additionally, wax analyses of *CER1* overexpressors revealed a specific increase in alkanes with chain-lengths between 27 and 33 carbon atoms, consistent with *CER1* encoding an alkane-forming activity with strict substrate specificity for compounds containing more than 27 carbon atoms (Bourdenx et al. 2011). Recently, the proof that CER1 and CER3 act as an enzymatic complex catalyzing the synthesis of VLC-alkanes was provided by co-expression of the two proteins in yeast (Bernard et al. 2012).

Whereas alkanes are the end products of the alkane-forming pathway in Arabidopsis leaves, they can also be further modified by consecutive oxidation to produce secondary alcohols, and subsequently ketones, in stems. By looking for genes up-regulated in the epidermis and encoding proteins potentially involved in lipid oxidation, a cytochrome P_{450} encoding gene, *CYP96A15*, was pinpointed as a candidate for a catalytic role in secondary alcohol and ketone formation (Greer et al. 2007). Its ectopic expression resulted in the production of secondary alcohols and ketones in leaves, suggesting that CYP96A15 functions as a mid-chain alkane hydroxylase (MAH1).

Acyl-Oxydation, Esterification to Glycerol and Extracellular Polymerization Generate the Insoluble Cutin Polymer

Although the major steps involved in the biosynthesis of cutin have been elucidated over the last 10 years, its exact three-dimensional structure remains unknown. Cutin isolation followed by depolymerization reactions have shown that it is a polyester mostly composed of C16 and C18 ω-hydroxyacid, polyhydroxyacid and α,ω-dicarboxylic acid (DCA), as well as of glycerol and small amounts of

phenylpropanoids (Beisson et al. 2012). In Arabidopsis, the cutin of leaves and stems in enriched in DCA, while that of flowers is dominated by polyhydroxyacids, suggesting that the composition of cutin varies between different organs of the same plant species (Bonaventure et al. 2004; Franke et al. 2005). The oxidative reactions converting fatty acids to ω-hydroxyacids are catalyzed by members of the CYP86 cytochrome P450 family. The characterization of the *lcr* and *att1* mutants showed that LCR (CYP86A8) and ATT1 (CYP86A2) are the two major cytochrome P450s involved in the acyl-oxydation of cutin precursors in Arabidopsis leaves and stems (Wellesen et al. 2001; Xiao et al. 2004). The mechanism underlying further conversion to DCA is still unclear but could involve oxydoreductases such as HOTHEAD (HTH), even though HTH is not specifically expressed in the epidermis (Krolikowski et al. 2003; Kurdyukov et al. 2006a). In Arabidopsis flowers, CYP86A4 and CYPA77 respectively are the ω-hydroxylase and in-chain hydroxylase responsible for the formation of 10,16-dihydroxypalmitate, the main floral cutin precursor (Li-Beisson et al. 2009). Although the sequential order of reactions is still a matter of debate, substrate specificities tested in vitro suggest that fatty acyl oxidation is most probably followed by acyl activation before transfer to glycerol.

Two long chain acyl-CoA acyltransferases, LACS1 and LACS2 have been shown to be important for cutin synthesis in Arabidopsis (Lü et al. 2009; Schnurr et al. 2004). Once activated as acyl-CoAs, hydroxylated fatty acyls are most probably esterified to the *sn-2* position of glycerol by glycerol-3-phosphate:acyl-CoA acyltransferases such as GPAT4 and GPAT8 in leaves and stems (Li et al. 2007) and GPAT6 in flowers (Li-Beisson et al. 2009). These enzymes also possess phosphatase activity so that end products are 2-monoacylglycerol instead of 2-acyl-lysophos-phatidic acids. This additional activity could be instrumental in the separation of glycerolipid precursors for cutin and membrane lipid biosynthesis (Yang et al. 2012; Rautengarten et al. 2012). Incorporation of phenylpropanoids into the cutin polyester requires BAHD acyltransferases such as Deficient in Cutin Ferulate (DCF) and possibly Defective in Cuticular Ridges (DCR) which transfer ferulic acid to ω-hydroxyacids (Rautengarten et al. 2012; Panikashvili et al. 2009).

Using fluorescent fusion proteins, all the reactions described above, with the exception of those catalyzed by BAHD-type proteins which could be cytosolic, were shown to take place in the endoplasmic reticulum. Thus precursors must be transported across the plasma membrane and through the cell wall (see below) before polymerization occurs. Although this process is the least well understood part of cutin biosynthesis, the recent identification of a extracellular tomato cutin synthase (GDSL1/CD1), has provided a major step forward (Yeats et al. 2012; Girard et al. 2012). The *cd1* mutant shows a dramatic reduction in polymerized cutin, and in vitro assays showed that CD1 catalyzes the formation of polyester oligomers from 2-monoacylglycerol (Girard et al. 2012). Loss of function of Li-tolerant lipase 1 (LTL1), which encodes an Arabidopsis ortholog of CD1, causes typical characteristics of cutin mutants, while LTL1 can also catalyze cutin polymerization in vitro (Yeats et al. 2014).

ABC Transporters and LTPs Are Involved in the Export of Precursors to the Cuticular Matrix

Although most cuticle biosynthetic enzymes have been shown to be localized in the ER, little is known about the intracellular trafficking of the cuticular precursors before their export out of epidermal cells. Nevertheless, the recent use of mutants defective in vesicle trafficking and protein secretion suggests that the transfer of these hydrophobic molecules through the hydrophilic cytoplasm involves vesicles, and that they transit through the ER-Golgi interface and the trans-Golgi network to deliver cargo to the plasma membrane (PM) (McFarlane et al. 2014). Once the cuticle components have reached the PM, their export is carried out by ABC transporters. The gene encoding the first ABC transporter identified as required for wax transport was found to be allelic to *CER5*, and was named *ABCG12* (Pighin et al. 2004). A search for ABC protein-encoding genes with an expression pattern similar to *ABCG12* revealed *ABCG11* as a potential candidate for a role in wax export (Bird et al. 2007; Panikashvili et al. 2007). The *abcg11* mutant and the double mutant *abcg11 cer5* show similar wax composition, suggesting that ABCG11 and ABCG12 act in the same pathway or ABC transporter unit. Furthermore, *abcg11* showed organ fusions, defects in cuticle permeability and reduced cutin load, indicating that ABCG11 is also involved in cutin monomer export in vegetative organs (Panikashvili et al. 2007) as well as in flowers, siliques and seedcoats (Panikashvili et al. 2010). However, residual export of waxes and cutin monomers onto the plant surface in the absence of ABCG11 and ABCG12 indicates that other ABC transporters might also export these compounds. Interestingly, ABCG11 and ABCG12 are half transporters, and ABCG11/ABCG12 heterodimers have a function in wax export, while ABCG11 can also homodimerize or heterodimerize with other unknown component(s) to transport cutin monomers (McFarlane et al. 2010). Recently, two other ABCG transporters have been characterized. ABCG13, which is closely related to ABCG11 and ABCG12, contributes to cutin formation in flowers (Panikashvili et al. 2011). In contrast, ABCG32/PEC1, which is a full length transporter, is required for hydroxylated fatty acid transport in leaves and flowers (Bessire et al. 2011).

Based on transcriptome analysis of Arabidopsis stems and stem epidermal cells, seven candidate LTPs, which could play a role in wax and/or cutin monomer transport were isolated (Suh et al. 2005). The analysis of the cuticle phenotype of *ltpg1* and *ltpg2* mutants suggests that both of these glycosylphosphatidylinositol-anchored LTPs (LTPGs) could be involved in cuticle formation (DeBono et al. 2009; Lee et al. 2009; Kim et al. 2012). However, the very specific transport shown to be affected in *ltpg1* or *ltpg2* mutants has given rise to the proposition that multiple specialized LTPs, possibly with overlapping functions, are required to deliver the whole diversity of cuticle compounds to the epidermal surface.

Cuticle Mutants with Epidermal Defects

As illustrated above, numerous Arabidopsis lines with mutations in genes involved in cuticle metabolism have been characterized by reverse and forward genetics. Besides their defects in surface lipid composition and/or content, several also present abnormalities in the development of their epidermis, the most common being post-genital organ fusions. The severity of these phenotypes seems to vary dramatically depending on the enzymatic step affected, as summarized in Table 12.1.

Mutations in Genes of the Fatty Elongation Complex Affect Epidermal Development to Various Degrees

Loss of KCR or HCD, two of the three activities of the elongation complex that are encoded by a single functional gene, causes embryo lethality. Corresponding knockdown mutants (*AtKCR-RNAi* lines or *pas2*-1, a weak allele of *HCD*) display strong developmental defects, such as dwarfism, spontaneous organ fusions and abnormal epidermal cell morphology. For example, *pas2-1* lines show ectopic cell divisions and loss of cell adhesion (Faure et al. 1998), while *AtKCR-RNAi* lines have abnormally short trichomes with fused branches, and produce swollen pavement cells with adhesion defects (Beaudoin et al. 2009). Knock-out mutants in the other activity of the elongation complex encoded by a single functional gene (*ECR*) are viable because of a partial functional complementation by a yet unidentified enzyme. Nevertheless, *ecr* mutants exhibit severe morphological abnormalities such as size reduction in aerial organs, and flower buds with fused tissues (Zheng et al. 2005). However, because the elongation complex is also necessary for the production of the VLCFAs present in sphingolipids, it is not possible to distinguish whether epidermal defects are linked to abnormal surface lipid composition or to impaired sphingolipid metabolism and/or plasma membrane abnormality.

Among the *KCS* multigene family, loss of function alleles of four genes show epidermal abnormalities, amongst which *kcs10*/*fiddlehead*/*fdh* is the most strongly affected. *fdh* mutant lines are dwarf, show fusions in both floral organs and leaves, have an increased cuticular permeability, allow pollen to germinate on leaves, and show a 50 % reduction in trichome density (Lolle et al. 1997; Yephremov et al. 1999.). Although *FDH* is specifically expressed in the epidermis and appears to affect epidermal cell interactions (Pruitt et al. 2000), no strong alterations in the lipid composition of *fdh* has been reported, even though mutant cell walls are enriched in one particular C24-VLCFA (Lolle et al. 1997). Similarly, the *kcs13*/*hic* mutant shows no major modification in its lipid profiles, but exhibits a 20–25 % increase in stomatal index at elevated atmospheric CO_2 concentrations (Gray et al. 2000). Consistent with this phenotype, *KCS13* is specifically expressed in guard cells. KCS6/CER6/CUT1 is the most important condensing enzyme for cuticular wax biosynthesis since *cer6* mutants show a 48 and 81 % decreases in leaf and stem

Table 12.1 Epidermal defects observed in Arabidopsis cuticular mutants

Mutant	Locus	Pathway affected	General features				Organ fusion		Epidermal cell defects			Defective cuticular permeability[b]	References
			Dwarfism	Male Sterility	Glossy stems	Curly leaves	Flowers	Leaf	Pavement cells morphology	Trichome morphology	Stomatal density		
kcr[a]	At1g67730	VLCFA biosynthesis	+	+	+	+	+	+	+	+		+	Beaudoin et al. (2009)
hcd	At5g10480		+	+			+	+					Bach et al. (2008) and Faure et al. (1998)
cer10[a]	At3g55360		+	+	+	+	+		+	+			Zheng et al. (2005)
cer6	At1g68530			+	+						+		Bird and Gray (2003), Millar et al. (1999), and Hooker et al. (2002)
fdh	At2g26250		+			+	+	+		+		+	Bird and Gray (2003), Lolle et al. (1997), Yephremov et al. (1999), and Pruitt et al. (2000)
hic	At2g46720										+		Bird and Gray (2003) and Gray et al. (2000)
kcs1	At1g01120		+	+									Bird and Gray (2003) and Todd et al. (1999)
cer1	At1g02205	Wax biosynthesis		+	+						±		Aarts et al. (1995), Bourdenx et al. (2011), and Gray et al. (2000)
cer3	At5g57800			+	+		+	+		+	+	+	Chen et al. (2003), Kurata et al. (2003), Ariizumi et al. (2003), and Rowland et al. (2007)

Mutant	Locus	Pathway affected	General features				Organ fusion		Epidermal cell defects			Defective cuticular permeability[b]	References
			Dwarfism	Male Sterility	Glossy stems	Curly leaves	Flowers	Leaf	Pavement cells morphology	Trichome morphology	Stomatal density		
wbc11	At1g17840	Cutin biosynthesis	+	+		+	+	+	+	+	+	+	Bird et al. (2007) and Panikashvili et al. (2007)
abcg13	At1g51460						+					+	Panikashvili et al. (2011)
lcr	At2g45970		+			+	+	+		+			Wellesen et al. (2001)
lacs2	At1g49430		+	+		+			+	+	+	+	Lü et al. (2009), Weng et al. (2010), and Schnurr et al. (2004)
hth	At1g72970						+						Krolikowski et al. (2003) and Kurdyukov et al. (2006a)
gpat4 gpat8	At1g01610/ At4g00400		+						+		+	+	Li et al. (2007) and Yang et al. (2010, 2012)
dcr	At3g48720						+		+	+			Panikashvili et al. (2009)
gpat6	At2g38110						+		+			+	Li-Beisson et al. (2009) and Yang et al. (2010, 2012)
bdg	At1g64670		+			+		+		+		+	Kurdyukov et al. (2006b)

[a]Embryo lethal knock-out
[b]Assayed using toluidine blue, chlorophyll leaching, weight loss or pollen germination on leaf test

wax loads, respectively (Millar et al. 1999; Fiebig et al. 2000). Despite these dramatic reductions in wax load, *cer6* mutants do not display any major epidermal defect, with the exception of a 30 % increase in stomatal index (Gray et al. 2000). The defects observed in *kcs1* mutants depend on the relative humidity (RH) used for growth. At high RH, *kcs1-1* plants resemble wild-type segregants but have 25 % fewer flowers, a 35 % reduction in silique length and very thin stems. In contrast, at low RH, 2 week old seedlings are very small and have a mortality rate of over 90 % (Todd et al. 1999). Finally, no epidermal defects were reported in plants with a mutation in *KCS9* or any CER2-like protein, although the corresponding mutants are affected in fatty acid elongation and/or the chain length of epicuticular wax compounds (Haslam et al. 2012; Pascal et al. 2013; Kim et al. 2013).

Knock-Out Mutations in Cuticular Wax Biosynthesis Have no Major Epidermal Defects

The *cer4* and *wsd1* mutants, which are affected in the reduction pathway producing fatty alcohols and wax esters, are phenotypically similar to wild-type plants, and show normal epidermis development (Rowland et al. 2006; Li et al. 2008). A mutation in *CER1*, which is responsible for the synthesis of the major wax components in Arabidopsis (alkanes), results in conditional male sterility (at low RH) despite the fact that the pollen coat (tryphine) of the mutant looks like that of wild-type (Aarts et al. 1995). Contradictory results have been reported concerning the stomatal index of *cer1* plants: Gray et al. (2000) report an increase, but in contrast Bourdenx et al. (2011) report no difference compared to wild-type, suggesting that in a *cer1* background, growth conditions could affect the development of stomata. Conversely, mutations in *CER3*, which is also involved in the biosynthesis of alkanes, cause a reduced stomatal index but also result in male sterility at low RH (Chen et al. 2003). Depending on the study, *cer3* mutants either have no other epidermal defect (Ariizumi et al. 2003; Rowland et al. 2007), show smaller trichomes and aerial organ adhesions (Kurata et al. 2003), or can even display postgenital organ fusions in leaves and flower buds (Chen et al. 2003). Although this last study attributed fusions to a putative role for CER3 in cutin formation, lipid analysis later showed that CER3 has no role in the biosynthesis of cutin, at least in leaves (Rowland et al. 2007).

Cutin Mutants Often Present Pleiotropic Defects in Epidermal Development

In contrast to cuticular wax mutants in which development and morphology is often indistinguishable from that of wild-type plants, many cutin mutants present extremely severe phenotypes with multiple epidermal defects, especially in reproductive organs. Mutations in the ABC transporters responsible for the export of

cuticle precursors best exemplify this discrepancy. The *cer5* mutant, which is affected in wax load (54 % less wax on stems) but not in cutin content, does not present any visible phenotype (Pighin et al. 2004; Bird et al. 2007). In contrast, the *wbc11* mutant, which is affected in both wax and cutin loads, shows many surface defects which are typical of an abnormal epidermal development (Bird et al. 2007; Panikashvili et al. 2007). Mutant plants present a strong growth retardation at the vegetative stage, and multiple thin-stemmed short inflorescences at the reproductive stage (loss of apical dominance). Numerous fusion events between leaves or between leaves and flower buds are also observed in this background, as are the production of misshapen rosette leaves, an increase in permeability to solutes and the appearance of pavement cell abnormalities. In addition the production of asymmetric stomata, shorter trichomes with irregular branching and frequent trichome collapse has been reported. Finally flower morphology is affected in the *wbc11* mutant where the fertility of pollen and the functionality of stigmatic papillae are reduced, and siliques are shorter and contain fewer seeds than those of wild-type plants. Consistent with the strong expression of *ABCG13* in flowers, the *abcg13* mutant also shows inter-organ post-genital fusions in inflorescences, but shows no defects during the vegetative phase (Panikashvili et al. 2011). The analysis of *abcg13* flower surface lipids showed that although wax load is normal, cutin load is reduced by 50 %. Petals are most strongly affected, with a loss of the typical conical shape of the epidermal cells and an absence of cuticular ridges (Panikashvili et al. 2011). An identical floral phenotype was observed in the *gpat6*, *cyp77a6*, and *dcr* mutants, which all show a reduction in 10,16-dihydroxypalmitate, the main floral cutin precursor, of over 90 % (Li-Beisson et al. 2009; Panikashvili et al. 2009).

Although mutants with reduced floral organ cutin always show strong epidermal defects such as post genital organ fusions, phenotypes associated with a reduction in leaf cutin content can vary dramatically. For example, the *lcr* mutant has multiple developmental abnormalities whereas the *att1* mutant looks morphologically normal, although both genes are thought to code for P450 hydroxylases involved in fatty acid oxidation (Wellesen et al. 2001; Xiao et al. 2004). Unfortunately, lipid analyses were only conducted for *att1*, where the authors did not report any epidermal defects although the stem cutin load was decreased by 70 % (Xiao et al. 2004). Similar to the situation for *lcr*, the effect of a mutation in *LACS2* on cutin content and/or composition has not been reported, even though epidermis development is strongly affected in *lacs2* mutants (Schnurr et al. 2004). In particular leaf pavement cells, which are shaped like jigsaw puzzle pieces in wild-type, have fewer lobes or even collapse in *lacs2* mutants. This phenotype is reminiscent to that of *dcr* mutants, in which the pavement cell patterning is disrupted and trichomes often collapse (Panikashvili et al. 2009). In contrast, mutation in the *DCF* gene, encoding another BAHD, does not result in any epidermal defect even though the leaf cutin of *dcf* is devoid of ferulate. Finally, *gapt4gpat8* double mutants, containing 65 % less cutin in their stems, have deformed pavement cells and stomata lacking cuticular edges, while *bdg* mutants, in which cutin load is 1.2–3 times higher than that of wild type, have deformed leaves with flattened trichomes that often collapse and adhere to neighboring pavement cells (Kurdyukov et al. 2006b). It should nevertheless be

noted that growing conditions can apparently strongly modulate the consequences of cutin defects on epidermis development, since *lacs1lacs2* double mutants were described as unaffected in size, growth and development by Lü and coworkers (Lü et al. 2009), but severely disrupted with temporary organ fusion in flowers, by Weng and coworkers (Weng et al. 2010).

Together, these results suggest that epidermal developmental defects are related to modifications in the composition and/or content of cutin, rather than waxes. This idea is further supported by the fact that similar epidermal defects have been observed in Arabidopsis transgenic plants expressing a fungal cutinase (Sieber et al. 2000) or an Arabidopsis putative cutinase (Takahashi et al. 2010). In addition, the primary role of cuticular waxes is widely considered to be related to the waterproof function of the cuticle, consistent with their having a much stronger hydrophobic character than cutin monomers. In contrast, cutin monomers or derivatives have been shown to function as elicitors of plant defense suggesting that plants actively monitor cutin integrity (Schweizer et al. 1996; Fauth et al. 1998). Intriguingly, an increased permeability of the cuticle to pathogen-derived lipid-signals eliciting plant defenses was proposed as an explanation for the enhanced biotic resistance observed in certain cutin-deficient mutants (Reina-Pinto and Yephremov 2009). Similarly, Bird and Grey (2003) suggested that a lipid molecule, most probably a VLCFA (or derivative) produced in guard cells, could diffuse in the cutin matrix and prevent surrounding cells from differentiating in guard cells, thereby participating in the fine-tuning of stomatal patterning. The control of epidermal cell fate by non-autonomous VLCFA-derivative signals is further supported by the *fdh* mutant which presents higher levels of a cell-wall localized C24:0 fatty acid derivative and strong developmental defects (Lolle et al. 1997), as well as by the recent demonstration that synthesis of VLCFAs in the epidermis can restrict cell proliferation in the vasculature (Nobusawa et al. 2013b). The cutin polymer could therefore function to restrict the mobility of lipid signaling molecules within the cuticle, thus fine tuning the development of the epidermis.

Transcriptional Regulation of Cuticular Lipid Biosynthesis and Epidermis Specification

The epidermis-specific expression of many genes involved in the biosynthesis and transport of cuticle components suggests that these processes must be under strict transcription regulation. Large-scale screens of Arabidopsis overexpressor/activation tagged lines identified the AP2/EREBP transcription factor SHN1/WIN1 as a major positive regulator of cuticle production (Broun et al. 2004; Aharoni et al. 2004). Plants over expressing SHN1/WIN1, or one the two other members of this small AP2/EREBP clade (SHN2 and SHN3), display clear visual phenotypes including dwarfism, shiny leaves that curl, smaller and more shiny siliques, and flowers with affected morphology, especially in petals. In addition, the differentiation of their leaf epidermal cells was clearly altered as trichomes were less abundant and often single branched, pavement cells were enlarged, and stomatal density was

decreased by two thirds (Aharoni et al. 2004). The first published lipid analyses conducted on these plants indicated that their wax load was several fold increased, consistent with their shiny appearance (Broun et al. 2004; Aharoni et al. 2004). However, further analyses showed that cutin production was the primary target of regulation (resulting in a 2–3 times higher cutin load in leaves), with *LACS2* identified as a direct target of WIN1, while wax deposition was only up-regulated in a second later phase (Kannangara et al. 2007).

The redundant functions of SHN1, 2 and 3 in shaping the surface and morphology of Arabidopsis flowers was shown using microRNA technology. Plants silenced simultaneously for all three genes displayed flowers with organs fusions and lacking the typical nanoridges on the adaxial epidermis of petals (Shi et al. 2011). Transcriptomic analyses further showed that downstream targets are either related to cutin biosynthesis or to cell wall metabolism, suggesting that SHN transcription factors not only regulate the deposition of cuticular lipids, but also modulate cell wall pectins and epidermal cell elongation, adhesion and separation, to prevent organ fusion. The relation between cutin metabolism and epidermal development is further supported by a study conducted on *SlSHN3* in tomato. In fruits from *SlSHN3*-silenced lines, a 60 % decrease in cutin load was correlated with strong modification of the morphology and patterning of the fruit epidermal cells. Most interestingly, among the putative SlSHN3 target genes were several encoding transcription factors associated with epidermal cell patterning. These included *SlSHN2*, the R2R3-MYB-encoding *SlMIXTA*, and three HDZIPIV genes (*SlGL2*, *SlHDG11a* and *SlANL2c*). In Arabidopsis, the MIXTA-like MYB transcription factor MYB106, which was known to regulate the formation and branching of trichomes, was recently shown to act as a positive regulator of WIN1/SHNs, supporting the link between cuticle and epidermal cell differentiation (Oshima et al. 2013). Similarly, Arabidopsis HDG1, a member of the HDZIP IV family of homeodomain transcription factors, was shown to bind the promoter of the cutin-related genes *BDG* and *FDH*, and HDG1 chimeric repressor plants were shown to have a defective cuticle resulting in leaf fusion events (Wu et al. 2011).

In Arabidopsis, the *HDZIP IV* gene family comprises 16 members, of which several have been shown to be involved in both epidermal specification and differentiation (Abe et al. 2003; Nakamura et al. 2006; Marks et al. 2009; Vernoud et al. 2009; Depege-Fargeix et al. 2011; Javelle et al. 2011a; Nadakuduti et al. 2012; Roeder et al. 2012; Takada et al. 2013; San-Bento et al. 2014). This gene family encodes plant-specific transcription factors which play a key role in regulating the intimately intertwined processes of epidermal fate specification and cuticle biogenesis. Loss of function of two closely related family members, ATML1 and PDF2, leads to a complete loss of epidermal cell fate specification during early embryogenesis (Abe et al. 2003; San-Bento et al. 2014). Conversely, the ectopic expression of *ATML1*, and/or of the closely related *HDG2* gene leads to the ectopic formation of stomata in internal positions within developing organs, suggesting that these proteins are not only necessary, but also sufficient for the specification of some epidermal cell types (Takada et al. 2013; Peterson et al. 2013). Transcriptional analyses of *ATML1* and *HDG2* ectopic expression lines show an over-expression (probably also ectopic) of other members of the *HDZIPIV* gene family, such as PDF2.

Interestingly however, over-expression of either *ATML1* or *PDF2* specifically within the epidermis leads to a repression of the endogenous *ATML1* and *PDF2* genes during post-embryonic development, suggesting that the level of expression of these two genes, and probably of other family members, is tightly regulated by complex feedback mechanisms. In support of this hypothesis, it has been shown that ATML1 and PDF2 are capable of binding to their own, and each other's, promoters. Furthermore, the normal expression of the ATML1 gene is at least in part dependent upon the presence of an L1 box, the cognate binding site for HDZIPIV proteins (Sessions et al. 1999; Abe et al. 2001; Nakamura et al. 2006; Takada and Jurgens 2007). Interestingly, ATML1 and PDF2 are capable of both homodimerization and heterodimerization *in planta*, the latter both with each other and with other HDZIPIV proteins (our unpublished results) and indeed with other transcription factor families (San-Bento et al. 2014; Rombola-Caldentey et al. 2014). Taken together, these observations suggest a complex picture in which the production of multiple different heterodimeric complexes could fine-tune both feedback regulation and target gene activation by this protein family.

Because of their important role in specifying epidermal cell fate it could be argued that the effects of altering HDZIPIV activity on cuticle composition are largely indirect. Nonetheless, several pieces of evidence support a more direct role for members of the HDZIPIV family in the control of cuticle production. Ectopic overexpression of the maize HDZIPIV protein OCL1 leads to changes in cuticle composition associated with regulation of several genes involved in cuticle biogenesis. In the case of at least one gene, *ZmWBC11a*, this regulation requires an L1 box (Javelle et al. 2010). More evidence for regulation of cuticle biogenesis by HDZIP proteins, albeit potentially indirect, comes from *Arabidopsis* lines ectopically overexpressing *ATML1* and *HDG2*, which show strong up-regulation of both *FDH* and *WBC12*/*CER5* (Takada et al. 2013). Furthermore the defective trichomes of *hdg2* mutants show reduced cuticle load and reduced expression of *CYP94C*, a gene required for the production of dicarboxylic acids potentially involved in cutin biosynthesis (Marks et al. 2009). Finally, defects in both the tomato HDZIPIV encoding *CUTIN DEFICIENT2* gene, and its *Arabidopsis* orthologue *ANTHOCYANINLESS2* (*ANL2*) reduce cuticle loads (Nadakuduti et al. 2012). At a more global level, both co-expression data and the presence of L1 boxes in the promoters of a subset of genes with important roles in cuticle production support a link between HDZIPIV expression and cuticle biogenesis (unpublished results), although how this is integrated with the activity of other key cuticle-regulating transcription factors, such as members of the WIN/SHINE clade, remains rather unclear.

Protocuticle and Epidermal Cell Fate

Early plant embryogenesis involves the sequential specification of key cell types including the epidermis. In Arabidopsis, where this process has perhaps been most extensively studied, epidermal cell fate is generally considered to be "fixed" at the

dermatogen stage of embryogenesis. At this stage, the eight roughly geometrically equivalent cells derived from the original apical cell of the two celled embryo by two perpendicular longitudinal and one latitudinal rounds of division, each undergo a periclinal cell division to give 16 cells, of which 8 form the skin (protoderm) of the embryo. Interestingly, markers of epidermal cell fate, including the *ATML1* gene, are expressed as early as the two cell embryo stage suggesting that "epidermis" or at least "protoderm" may be a default state, at least of the embryo proper. Our unpublished results suggest that at this stage in embryo development, and indeed during early preglobular stages, there is no detectable trace of cuticle deposition within the cell wall separating the membrane of the embryo from that of the surrounding syncytial endosperm. However, in silico transcriptomic data issuing from painstaking laser microdissection and microarray analysis of Arabidopsis seed development (Le et al. 2010), shows that many key components of the cuticle biosynthesis pathway including *LACS2*, *FDH*, and *BDG1* are already strongly expressed in the pre-globular embryo proper, suggesting that cuticle biogenesis and deposition is likely initiated very early in the developing embryo. Both *FDH* and *BDG1* contain L1 boxes upstream their transcription start sites, consistent with a potential regulation by HDZIPIV proteins (Abe et al. 2001, 2003; Nakamura et al. 2006). However, although it is clear that well before embryo maturity cotyledons are covered in a layer of cuticular material (Tanaka et al. 2001), frustratingly little is known about the biogenesis of the embryonic cuticle.

In terms of cuticle biogenesis, plant embryos are unique from two important points of view. Firstly, because they arise from gametophyte cells, which in turn are derived from non-epidermal cells buried within the ovule nucellus, their cuticle, like the epidermal identity of the cells on which it appears, arises de novo. In contrast, clonal analyses in several species suggests that after early embryogenesis the overwhelming majority of, if not all plant epidermal cells arise from anticlinal divisions of existing epidermal cells, and are thus "pre-equipped" with a cuticle bearing surface (Javelle et al. 2011b). Secondly, not only does the embryonic epidermis and therefore its cuticle arise de novo, but it also arises in a position where it is not juxtaposed by another cuticularized surface. In angiosperms, the embryo develops in an invagination of the endosperm, which initially takes the form of a rapidly growing coenocytic single cell (commonly called the syncytial endosperm). This cell subsequently undergoes cellularization in a wave, starting in the zone surrounding the developing embryo. There is no evidence in the literature or from TEM data (our unpublished results) that the endosperm produces a cuticle at its junction with the developing embryo. The embryonic cuticle, and thus the apoplastic separation of the embryo from the endosperm, therefore arises in developmentally unique circumstances since in all other aerial plant organs the outer, cuticle bearing surfaces of all epidermal cells juxtapose the equivalent surfaces of other epidermal cells. The juxtaposed cuticles of these cells play a seminal role in preventing post genital fusion of epidermal surfaces, no matter how tightly they are packed together in developing buds. The developmental importance of this "separation" function is manifest in the many cuticle-related mutants where this function is impacted, and in which organs fuse, impacting growth, fertility and viability (see previous section).

The unique circumstances surrounding angiosperm embryonic cuticle formation might be predicted to demand the deployment of specific molecular and cellular mechanisms which permit the correct definition and positioning of the nascent cuticle, and the successful separation of the embryo from surrounding endosperm tissues. Consistent with this idea, recent research has uncovered a seed-specific signaling pathway, involving both endosperm and embryonically expressed components, which is necessary for the formation of a functional embryonic cuticle, and for physical separation of the embryo and endosperm, in *Arabidopsis*. In the interests of conciseness, we will refer to this pathway as the Embryo Cuticle Functionality (ECF) pathway. To date, five critical components of this pathway have been identified. The first two comprise two very distantly related bHLH transcription factors: ZHOUPI (ZOU)/RGE1 which is expressed specifically in the embryo surrounding endosperm (Kondou et al. 2008; Yang et al. 2008; Xing et al. 2013), and ICE1/SCREAM which acts as a heterodimer with ZOU (Denay et al. 2014), but is also involved in several other developmental and physiological processes (Shirakawa et al. 2014; Kanaoka et al. 2008; Chinnusamy et al. 2003; Lee et al. 2005; Miura et al. 2007). Both *zou* and *ice1* mutants fail to form a functional embryonic cuticle, and their cotyledons are highly permeable to toluidine blue (Denay et al. 2014). In addition mutant embryos fail to physically separate from the surrounding endosperm (Yang et al. 2008). Interestingly however, cuticle components appear to be present at the surface of *zou* mutant embryos, and, importantly, the expression of neither epidermal markers (Yang et al. 2008; Xing et al. 2013; Denay et al. 2014) nor genes involved in cuticle biosynthesis (our unpublished results) is strongly affected in either mutant background. The third gene involved in the ECF pathway is again expressed specifically in the embryo-surrounding endosperm, but in this case encodes a Subtilisin-like serine protease called ABNORMAL LEAF SHAPE1 (ALE1), which is necessary for normal embryonic surface formation and endosperm/embryo separation (Tanaka et al. 2001). ALE1 is predicted to act in the secretory pathway or apoplast, and therefore represents a non-cell autonomous component of the pathway. The expression of *ALE1* is almost completely abolished in *zou* and *ice1* mutants, and reintroduction of *ALE1* expression into *zou* mutants partially complements their cuticle defects (Yang et al. 2008; Xing et al. 2013; Denay et al. 2014). Consistent with the fact that *ZOU* and *ALE1* act in the same genetic pathway their mutant phenotypes show no additivity in double mutants (Yang et al. 2008). Finally, two genes encoding the closely related receptor kinases GASSHO1 (GSO1) and GSO2, and which show strong expression in developing embryos, act redundantly to promote the production of a functional embryonic cuticle and embryo/endosperm separation (Tsuwamoto et al. 2008). Double *gso1 gso2* mutant shows remarkably similar phenotypes to *ale1* mutants and non-additive genetic interactions with both *ale1* and *zou* mutants, confirming its likely participation in a signaling processes involving communication between the developing embryo and endosperm necessary for normal embryonic cuticle biogenesis (Xing et al. 2013; Waters et al. 2013).

Despite their defective cuticle phenotypes, like *zou* mutants, neither *gso1 gso2* double mutants nor *ale1* mutants show significant changes in the expression of epi-

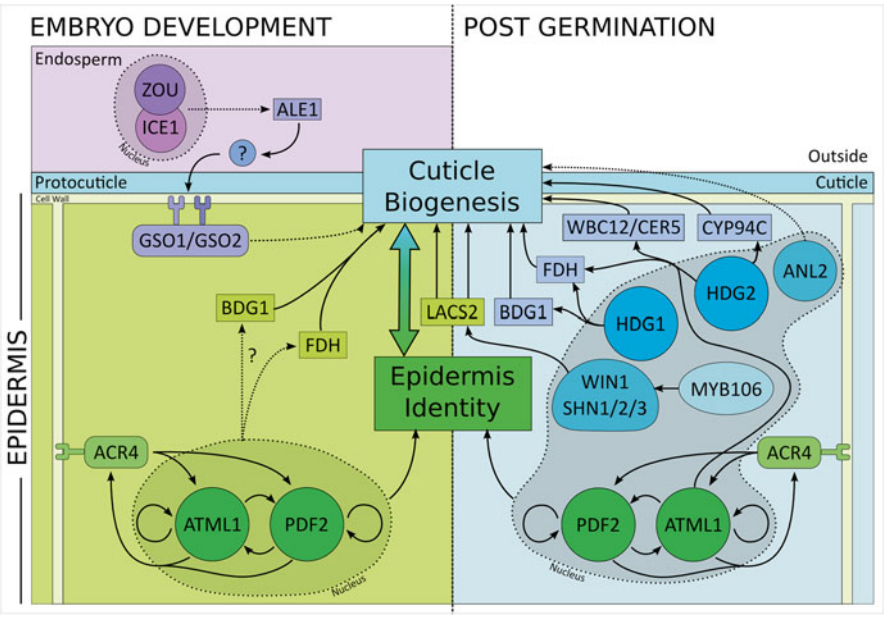

Fig. 12.2 Transcriptional control of cuticle biosynthesis and epidermis specification during development. During plant development, epidermis identity is thought to be specified by the HDZIPIV transcription factors ATML1 and PDF2. Cell-cell signaling, for example mediated by the ACR4 receptor, generates a feedback loop allowing identity maintenance. During zygotic embryogenesis, cuticle biogenesis is affected by two different genetic pathways. The first involves an intercompartmental signaling pathway between the endosperm and the embryo. The second is poorly understood but may be regulated by ATML1, PDF2 and other HDZIPIV transcription factors, and thus be intricately linked to epidermis identity. Later during development, many transcription factors from different classes, including HDZIPIV transcription factors have been shown to regulate the expression of different effectors involved in cuticle biosynthesis. Similar transcription factors could be involved in cuticle biogenesis during embryogenesis, although evidence to support this is currently lacking. Transcription factors are represented by circles, effectors by rectangles and receptor by round-cornered boxes. *Left* panel shows an epidermal cell and its environment during early embryogenesis. *Right* panel shows the same cell after germination

dermal marker genes such as *ATML1* and *PDF2* (San-Bento et al. 2014). Consistent with this observation, the formation of an epidermal cell layer does not appear to be significantly affected in these mutants suggesting that the defects in cuticle integrity which they display are not linked to a fundamental defect in epidermal cell fate specification. Interestingly however, mutants in the ECF pathway show very strong synergistic interactions with single mutants in either *ATML1*, *PDF2* and with mutant alleles of *ACR4*, a receptor-kinase encoding gene, homologues of which are required for normal epidermal development in several species (Roeder et al. 2012; Pu et al. 2012; Becraft et al. 1996; Gifford et al. 2005; Tanaka et al. 2004, 2007) and which has recently been shown to be both directly regulated by, and necessary for the maintained expression of, ATML1 and PDF2 (San-Bento et al. 2014) (Fig. 12.2). Although *atml1 pdf2* mutants show early embryo lethality, single mutant *atml1*,

pdf2 or *acr4* seedlings show only very weak defects in cotyledon permeability, and no major morphological abnormalities (Abe et al. 2003; San-Bento et al. 2014; Tanaka et al. 2004). In addition genetic interactions between *acr4* mutants and either *atml1* or *pdf2* single mutants are consistent with the signaling feedback loop described above (San-Bento et al. 2014; Moussu et al. 2013). In contrast, mutants in the ECF pathway, when combined with either *atml1*, *pdf2* or *acr4* mutant alleles, lead at best to the production of extremely abnormal seedlings with highly abnormal epidermal surfaces, and in most cases to early embryo lethality and a loss of epidermal identity (San-Bento et al. 2014; Xing et al. 2013; Tanaka et al. 2007), effectively phenocopying *atml1 pdf2* double mutants.

The genetic interactions described in the above paragraph are complex and rather non-intuitive. In addition, the fact that they involve a pathways active in specific compartments of the developing seed makes them extremely difficult to dissect. However a possible explanation for the observed genetic synergism between the ECF pathway and the epidermal specification pathway described above, was provided by mathematical modeling of the predicted behavior of the feedback loop involved in epidermal cell fate specification and maintenance (San-Bento et al. 2014). Molecular and genetic analysis of the regulatory behavior of this feedback loop post germination suggested that although ACR4 mediated signaling was necessary for maintaining the expression levels of ATML1 and PDF2, these two proteins fed back negatively on the expression of the *ACR4*, *ATML1* and *PDF2* genes. In mathematical models, this scenario gives a robust maintenance of *ATML1* and *PDF2* expression (and thus epidermal identity) over a wide range of possible values for ACR4 signaling activity (which can be considered to be equivalent to the presence of the, as yet unidentified, ligand of ACR4). Interestingly however, data concerning the regulation of elements of the epidermal specification feedback loop in developing seeds suggested that during early embryogenesis the net regulation of *ACR4* expression, and likely the expression of *ATML1* and *PDF2* by HDZIP IV class proteins, is likely to be positive rather than negative (San-Bento et al. 2014; Takada et al. 2013; Takada and Jurgens 2007). Mathematical modeling of this scenario leads to an unstable scenario, in which modestly decreasing the level of ACR4 signaling activity can lead to a sudden and irreversible loss of expression of *ATML1* and *PDF2* (San-Bento et al. 2014). A plausible scenario then is that the production of a functional embryonic cuticle, mediated by the ECF pathway, is necessary for the maintenance of ACR4 signaling activity, possibly via the restriction of a diffusible apoplastically localized ligand to the embryo. In an otherwise wild-type background, defects in the ECF pathway are not sufficient to destabilize the epidermal specification feedback loop. However in backgrounds where this loop is defective, cuticle defects are able to "tip the balance" leading to a sudden and catastrophic loss of epidermal identity. Interestingly, and in accord with this hypothesis, loss of epidermal cell fate in ECF component mutant combinations with mutants in elements of the epidermal feedback loop, occurs at the early heart stage, at the time when endosperm dramatically increases the potential for apoplastic diffusion in the embryo-surrounding endosperm. However, until more is known about when and where the cuticle is formed during embryogenesis, it will be difficult to address its function as a developmentally important diffusion barrier in more detail.

Concluding Remarks

The differentiation of a distinct, continuous epidermal cell layer containing highly specialized cells such as stomata, and the appearance of a cuticle on the surface of epidermal cells, represent key adaptive responses developed by the green phyla to cope with dry conditions when plants colonized non-aqueous environments about 450 million years ago. Ever since, epidermal differentiation and the biosynthesis of surface lipids, and their respective regulation, have co-evolved to allow plants to successfully colonize an astounding range of terrestrial habitats. Recent studies focusing on the transcriptional regulation of epidermal characters have started to unravel a highly complex network and have highlighted interplay in the regulation of key epidermal traits. Major cuticle regulators interact with proteins necessary for epidermal cell differentiation, which in turn can control the expression of genes coding for proteins involved in surface lipid biosynthesis. Tantalizingly, HDZIPIV transcription factors, which play a central role in specifying epidermal cell fate and in the differentiation of various epidermal cell types, contain a START domain that has the potential to bind regulatory lipids, while VLCFA derivatives have been identified as candidate signaling lipids involved in fine-tuning the development of both the epidermis and the cuticle in response to environmental clues. Together, these data hint at mechanisms underlying a complex cross-talk between cuticle formation and epidermis differentiation both at the transcriptional and metabolic level.

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