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APOPLASTIC BARRIERS OF ARABIDOPSIS THALIANA SEEDS:

THE CUTICLE

par

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1. Abstract

This monograph is a review concerning different aspects of an apoplastic barrier present in plants, the cuticle. The introduction treats of the evolution of land plants together with the presentation of *Arabidopsis* seeds morphology and their apoplastic barriers: cuticles and suberin. The first part of this work reviews the methods usually employed to observe or characterize the cuticle in the seed, from microscopy to biochemistry. Then, the localisations of the different seed cuticles – testa, endosperm and embryo cuticles – are examined. The cuticle synthesis is further described, and mutants with defective cuticle are reported. Finally, the general functions of cuticles are discussed, as they are linked to many essential functions of the whole plant, especially in the seed. Along this work, it is attempted to evaluate the conformity between different studies and to reveal some new perspectives on cuticle comprehension. Although some mentions of experiments about other plant species are pertinent in this work, this monograph principally deals with the model species *Arabidopsis thaliana*.

2. Introduction

The land colonisation by plants during the Silurian, which took place approximately 450 million years ago, forced plants to develop new structures adapted to the non-aquatic environment (Ducreux, 2002). Seeds and diffusion barriers are the kind of innovations the plant had to develop to support desiccation and other environmental stresses.

The seed, composed of the embryo covered by a nourishing and regulatory tissue called endosperm, itself surrounded by a protective layer, is one of the major innovations of vegetal evolution. The first vascular seed plants (spermatophytes) date from 360 million of years ago, in the late Devonian (Campbell *et al.*, 2012).

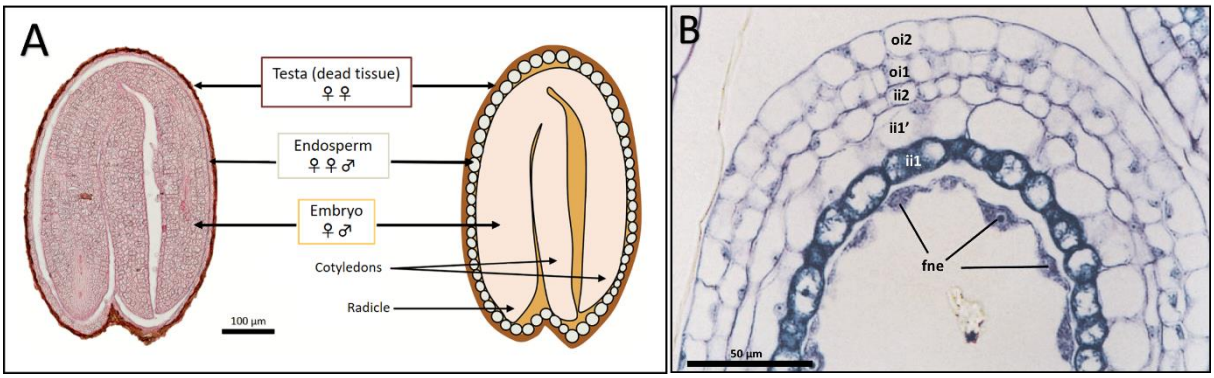

Figure 1. *Arabidopsis* seed morphology. (A) Anatomy of the mature dry seed. On the left, a histological section of a seed stained with the Sudan Red dye. Origin (maternal or paternal) and ploidy of tissues are indicated. Adapted from Chahtane *et al.*, 2017. **(B)** Seed coat of a two-cell embryo stage seed stained with toluidine blue. Adapted from Beeckman *et al.*, 2000. Abbreviations: fne, free nuclear endosperm; ii1, innermost layer of the inner integument or pigment layer or endothelial cell layer; ii1', median layer of inner integument; ii2, outer layer of inner integument; oi1, inner epidermis of outer integument; oi2, outer epidermis of outer integument.

Embryogenesis begins with a double fertilization between male gametes and cells contained in the female gametophyte: one male gamete fuses with the female egg cell, giving rise to the zygote (2n), while the second male gamete fuses with the central diploid cell of the female embryo sac, triggering the development of the triploid endosperm (Fig. 1A). The endosperm will first develop as a coenocytic tissue, then undergo cellularisation and partial degeneration, to finally display a one-cell-layer endosperm surrounding the embryo (Bowman, 2012; Windsor *et al.*, 2000).

During early embryogenic stages, the seed coat is formed by three layers of inner integuments (ii1, ii1' and ii2) and two layers of outer integuments (oi1 and oi2), both being entirely of maternal epidermal origin (Fig. 1B). In the literature, the ii1 layer is also called endothelial layer, endothelium or pigment layer, due to the accumulation in these cells of flavonoid polymers called tannins, which confer a brown colour to mature seeds after oxidation (Debeaujon *et al.*, 2003). At the embryo maturity stage, the inner integuments crush into a

single-cell-thick-walled layer called the brown pigment layer (bpl), responsible for the seed colour. Cells of outer integuments accumulate starch-containing amyloplasts; at the mature stage oi1 is mostly collapsed and oi2 synthetizes and secretes mucilage, a pectinaceous carbohydrate which provides protection and a moist environment for seed germination (Taiz, 2015; Windsor *et al.*, 2000). During the later stages of seed development, the cells of all seed coat layers finally die and form the testa (Beeckman *et al.*, 2000; Haughn and Chaudhury, 2005).

Regarding internal seed non-cellular structures, cuticle and suberin have been defined as apoplasmic specialized diffusion barrier. Both structures share some chemical characters and biosynthesis pathways, which results in similarities in their functions (Nawrath *et al.*, 2013). Suberin is a glycerolipid-phenolic heteropolymer occurring in the cell wall of external and internal plant tissues, including the seed coat; its ubiquitous presence in specific root tissues that control water and ion uptake suggests a role in the adaptation of vascular plants to terrestrial life (Pollard *et al.*, 2008; Vishwanath *et al.*, 2015). The cuticle is a lipophilic structure covering aerial plant surfaces (shoots and leaves) and seeds, but absent in regions of the roots that take up water and minerals (Russell, 2013). This impermeable layer is formed by waxes, cutin and cutan, the latter two being polymeric networks formed by fatty acids.

The best-known functions of the cuticle are to control the plant non-stomatal exchanges of water and gas. But recent studies revealed that mutations in cuticle biosynthesis genes induced phenotypes in seed development, thus pointing to specific and new roles of the cuticle in seeds (see below).

3. Cuticle detection methods

The literature presents a few contradictory results concerning the presence or the localisation of a cuticle in the seed; in some cases, this may be due to the use of different methods involving delicate interpretations. The processes available to study the cuticles and their functions are listed below.

3.1 Optical microscopy

To observe the cuticle by optical microscopy, dyes with affinity for lipids are used. Several are commercially available and have been tested for their intensity, contrast, and specificity. The Sudan Red 7B stain has been revealed as one of the best, it is very efficient and is therefore generally employed, although other types of lipid stains are also used (Brundrett *et al.*, 1991).

Sudan Red allows to distinguish the lipid structures by staining them in a reddish colour (Fig. 2A). This dye can be used to observe the cuticle, but also suberin; however it displays some non-specific signal in the cell wall (Beisson *et al.*, 2007; Gou *et al.*, 2009). In some less frequent cases, the stain auramine O is used to visualize a fluorescent signal from cutin (Szczuka and Szczuka, 2003) (Nadiminti *et al.*, 2015). Auramine O also gives a non-specific and weaker signal in the cytoplasm, as visible in Figure 2B. Furthermore, this dye allows to see the cuticle with the advantage of not seeing tannins, which in brightfield microscopy may hamper the observation (Fig. 2A).

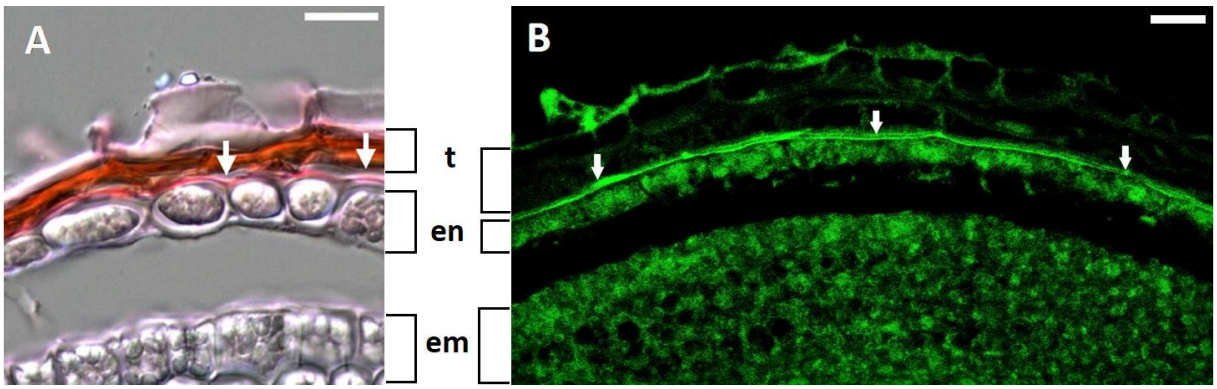

Figure 2. *Arabidopsis* endosperm cuticle observed by optical microscopy. (A) Close-up of a germinating seed section stained with Sudan Red 7B. Adapted from De Giorgi *et al.*, 2015. **(B)** Close-up of a seed at the maturation stage stained with auramine O (S. Loubéry, personal communication). Arrows point to the cuticle present between endosperm cells and the testa, visible as a pink or a green line in **(A)** and **(B)**, respectively. Bars: 15µm. Abbreviations: em, embryo; en, endosperm; t, testa.

3.2 Electron microscopy

Cuticle visualisation can be made by transmission electron microscopy (TEM), where it appears as a dark electron-dense structure that reveals some striations when observed at high magnification (Shumborski *et al.*, 2016). However, the way to fix the sample has an impact, as the fixatives influence cuticle morphology. It has been reported that the conventional chemical fixation of cuticular samples displays less details and not as suitable visual access as the preparation by high-pressure freezing/freeze-substitution (HPF/FS), which is known to preserve samples better. Compared to the conventional method, the HPF/FS preparation evinces more striations (and with a different morphology) than the conventional method, and reveals a thicker cuticle (Fig. 3A-B) (Shumborski *et al.*, 2016).

The external part of the cuticle, the epicuticular waxes, can be visualized with scanning electron microscopy (SEM). The epicuticular wax film is covered by epicuticular wax crystals, whose elaborate and variably shaped structures are nicely visible by SEM on *Arabidopsis* stems (Fig. 3C, D) and cabbage leaves (Fig. 3E). The epicuticular wax crystals are responsible for the glaucous appearance of these tissues in a macroscopic view, while the epicuticular wax film with less or no crystals display a glossy appearance (Lee and Suh, 2015; Yeats and Rose, 2013). SEM of the cuticle surrounding *Arabidopsis* seeds has not yet been performed, probably because of the testa cuticle instability upon mucilage extrusion provoked by the fixation process.

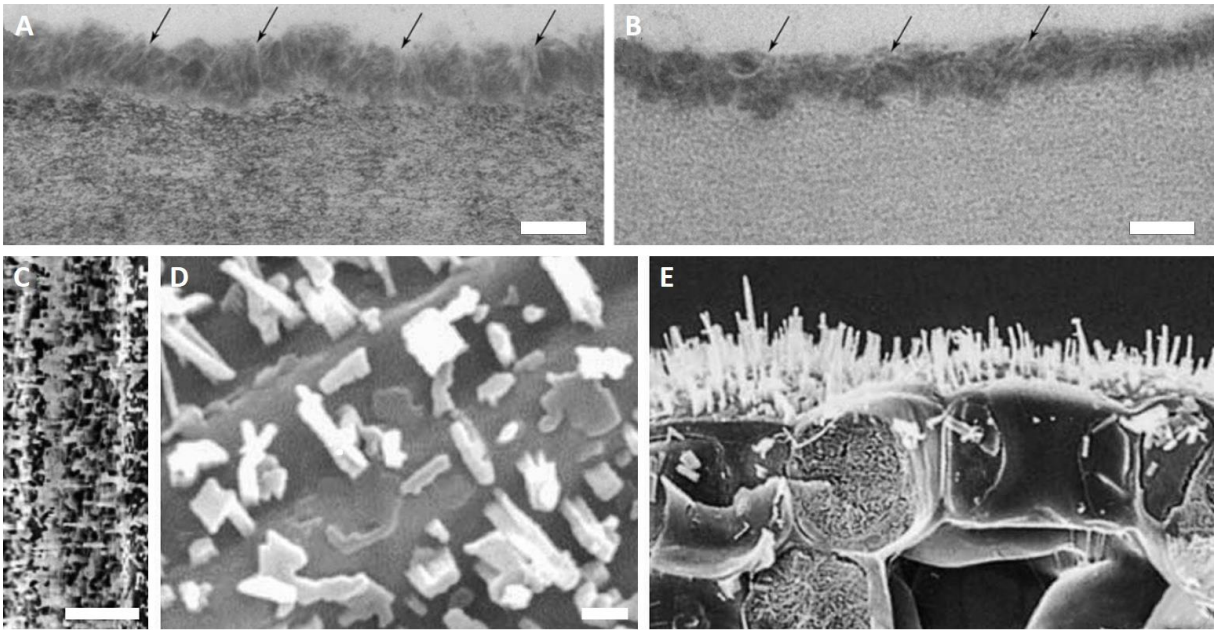

Figure 3. Cuticular structures by electron microscopy. (A,B) Ultrastructure by TEM of wild-type *Arabidopsis* stem cuticles. Arrows indicate electron-translucent striations. Bars: 100 nm. Adapted from Shumborski *et al.*, 2016. **(A)** Preparations by HPF/FS and **(B)** conventional chemical fixation. **(C-E)** Epicuticular waxes observed by SEM. **(C)** *Arabidopsis* stem. Bar: 10µm. Adapted from Luo *et al.*, 2007. **(D)** Close-up of *Arabidopsis* stem surface. Bar: 1µm. Adapted from Jenks *et al.*, 2002. **(E)** Leaf cross-section of *Brassica oleracea*. Scale bar size was missing in the reference paper. Adapted from Shepherd and Wynne Griffiths, 2006.

3.3 Permeability tests

Then, there is another way to control the proper function of a cuticle: tests of permeability. Tetrazolium penetration assay is a common permeability test based on the reduction of tetrazolium salts by NADH-dependent reductases of the endoplasmic reticulum. This reaction gives a coloured product called formazan, which dyes the sample proportionally to the degree of permeability (Berridge *et al.*, 1996; Debeaujon *et al.*, 2000). Because of the reaction depending on NADH, this method rests on the hypothesis that the sample provides elements necessary for the reduction. It can be imagined that cells in a state of dormancy or inactivity, inducing a lack of metabolic activity, would not be able to reduce the dye, therefore giving falsely negative results and biasing the conclusions. Effects of metabolism may also mislead the permeability interpretation as evaluated by the staining intensity: a weak staining signal may indicate a decreased metabolic activity instead of a weak permeability.

For these reasons, some research groups turned to another test of permeability using toluidine blue. This rapid and inexpensive method is generally used to test the permeability of the cuticle of the leaves (Tanaka *et al.*, 2004). A functional cuticle of a wild-type plant does not allow the dye to penetrate and stain the tissues, whereas a discontinuous or altered cuticle will display a clearly visible blue coloration. The toluidine blue test was developed with cuticle mutants in the leaves (Fig. 4A), and later adapted for seeds (Fig. 4B,C) (De Giorgi *et al.*, 2015; Watanabe *et al.*, 2004).

Figure 4. Examples of tetrazolium salt and toluidine blue permeability tests on *Arabidopsis thaliana*. (A) Test on leaves with toluidine blue. Wild-type (left) and *acp4* mutant (right) leaves. Adapted from Xia *et al.*, 2009. **(B)** Test on seeds with tetrazolium salt. Wild-type (left) and *gpat5* mutant (right) seeds. Adapted from Fedi *et al.*, 2017. **(C)** Test on seeds with toluidine blue. Wild-type (left) and *bdg1* mutant (right) seeds (embryos have been extracted from the seeds and subsequently imaged). Adapted from De Giorgi *et al.*, 2015.

3.4 Biochemistry

Finally, biochemical methods can be useful to study the cuticle, allowing to quantify its contents. The composition of the aliphatic monomers mixture derived from lipid polyesters contained in the cuticle can be revealed by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS). It is first necessary to proceed to depolymerization, by cleaving the ester bonds to isolate the fatty acid monomers, which are next sorted and quantified (Nawrath, 2006).

Practicing GC or GC-MS allows to compare different cuticles contents within a species (between the leaves and the seeds for example), but also between different plant species. GC-MS analyses have revealed that the predominant aliphatic monomers of *Arabidopsis* cutin are C16 and C18 ω-hydroxylated fatty acids, which is unusual compared to the typical cutin composition in other species (Beisson *et al.*, 2012). Furthermore, analyses of cuticle composition are of great help for studies on new genes involved in cuticle biosynthesis, by displaying impacts of mutations on monomers loads (Molina *et al.*, 2007).

However, this kind of analysis does not give any indication about the geographical localisation of different cuticles in an organ. This is an issue in particular with *Arabidopsis* seeds, because the small size of samples (seed size is approximatively 0.4 x 0.6 mm^2 (Jiang *et al.*, 2013)) makes it very difficult to separate neighbouring tissues. Nevertheless, chemical analyses enable to know whether specific cuticle compounds are present in the sample, and so to confirm the presence of a putative apoplastic barrier.

4. Cuticle localisation in *Arabidopsis* seeds

Studies report different putative localisations of the cuticle in the seed of *Arabidopsis thaliana*. Most of them are in agreement with the presence of a cuticle covering the endosperm cell wall (Fig. 5D-F), facing the testa, or more exactly the ii1 layer (Andeme Ondzighi *et al.*, 2008; Beeckman *et al.*, 2000; De Giorgi *et al.*, 2015). Because of its thickness which is about 10 times superior to the cuticle of the leaves (>300 nm) (De Giorgi *et al.*, 2015), this cuticle is more readily observable by TEM or by optical microscopy than other thinner cuticles. This endosperm cuticle has been already detected from the pre-fertilization stage and persists during seed development until maturity (Beeckman *et al.*, 2000; Bowman, 2012). Imaging the seed coat development allows to notice that this cuticle is first localized bordering the inner cell wall of the ii1 layer, facing the embryo sac. During the different seed development steps, endosperm cellularization takes place between the embryo and the cuticle, this latter becoming the endosperm cuticle (Beeckman *et al.*, 2000). Consequently, this could support the hypothesis that the endosperm cuticle is synthetized and released by cells belonging to the ii1 layer (which are of epidermal origin), and not by the endosperm cells.

Another cuticle, called here embryo cuticle, has been identified by TEM, at the boundary between the embryo and the endosperm (Fig. 5G-I). It is thinner than the endosperm cuticle, and it has been observed surrounding the embryo from the globular heart transition stage (De Giorgi *et al.*, 2015; Moussu *et al.*, 2017; Szczuka and Szczuka, 2003; Tanaka *et al.*, 2001; Yang *et al.*, 2008). This cuticle becomes thicker and denser while the endosperm is cellularized, degenerates and becomes detached from the embryo (Szczuka and Szczuka, 2003; Tanaka *et al.*, 2001).

The last cuticle reported has been observed in the external face of the seed coat, surrounding the testa (Fig. 5A-C). It has been found in mature seeds and at torpedo stage (DeBolt *et al.*, 2009; Panikashvili *et al.*, 2009; Watanabe *et al.*, 2004). During development, the seed surface undergoes alterations due to the differentiation of mucilage secreting cells, which could maybe involve suppression or modifications of this cuticle, and so explain why it has been reported only recently. Consistent with this hypothesis, Aharoni and coll. have reported that this testa cuticle can been observed covering the *dcr* mutant seeds, which lack a BAHD acyltransferase involved in cutin formation and presents defaults in mucilage extrusion, while it is not visible in wild-type imbibed seeds (Panikashvili *et al.*, 2009).

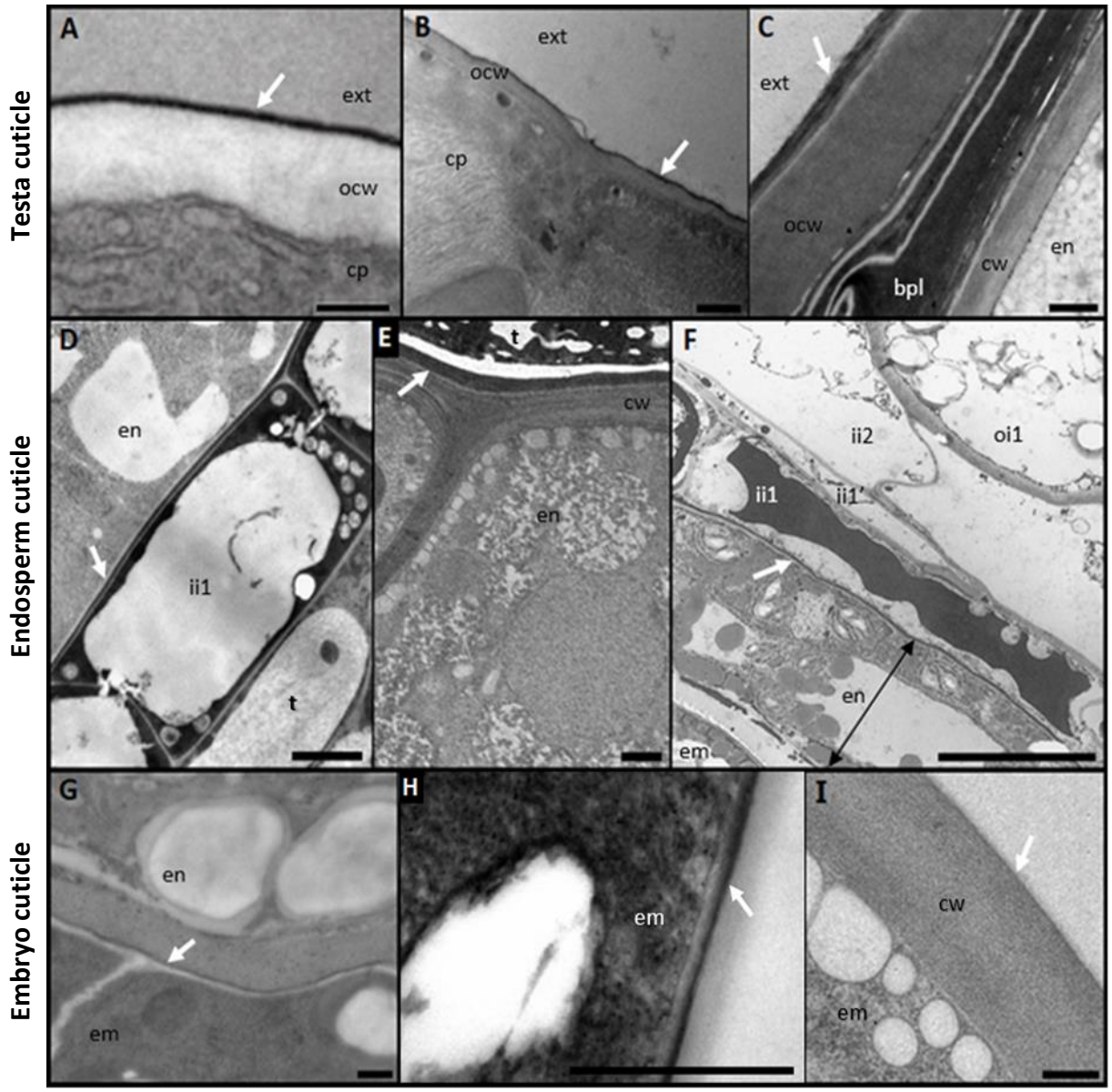

Figure 5. Cuticles localisation in *Arabidopsis* seed observed by TEM. (A-C) Testa cuticle. **(D-F)** Endosperm cuticle. **(G-I)** Embryo cuticle. **(A)** Seed coat epidermal cell of a wild-type seed at torpedo stage. Bar: 0.2µm. Adapted from Watanabe *et al.*, 2004. **(B)** Seed coat epidermis of a *dcr-1* mutant mature seed. Mucilage is retained between epidermal columella cells and the outer cell, which allows to observe the cuticle. Bar: 1µm. Adapted from Panikashvili *et al.*, 2009. **(C)** Outer layer of a mature wild-type seed. Bar: 1µm. Adapted from DeBolt *et al.*, 2009. **(D)** Endosperm and ii1 layer of a pre-embryo stage wild-type seed. Bar: 3µm. Adapted from Andeme Ondzighi *et al.*, 2008. **(E)** Endosperm cells of an imbibed arrested wild-type seed. Bar: 1µm. Adapted from De Giorgi *et al.*, 2015. **(F)** Endosperm and epidermal layers of a wild-type seed at torpedo stage. Bar: 10µm. Adapted from Beeckman *et al.*, 2000. **(G)** Embryo-endosperm interface of a wild-type seed at torpedo stage. Bar: 0.1µm. Adapted from Moussu *et al.*, 2017. **(H)** Embryonic cell of a wild-type seed at torpedo stage. Bar: 0.5µm. Adapted from Yang *et al.*, 2008. **(I)** Embryonic cell of an imbibed arrested wild-type seed. Bar: 0.3µm. Adapted from De Giorgi *et al.*, 2015. The white arrows indicate **(A-C)** the testa cuticle, **(D-F)** the endosperm cuticle, and **(G-I)** the embryo cuticle. Abbreviations: bpl, brown pigment layer; cp, cytoplasm; cw, cell wall; em, embryo; en, endosperm; ext, exterior; ii1, innermost layer of the inner integument or pigment layer or endothelial cell layer; ii1', median layer of inner integument; ii2, outer layer of inner integument; oi1, inner epidermis of outer integument; t, testa.

5. Cuticle biosynthesis

The cuticle is formed by a cutin layer, embedded with intracuticular waxes and covered by epicuticular waxes, and cutan. The latter has been first discovered owing to its resistance to depolymerisation by saponification, one of the typical methods to achieve cutin depolymerisation (Nip *et al.*, 1986). Cutan, less known and studied than cutin, is rich in ether and C-C bonds; it has been demonstrated that its preferred precursor was linoleic acid, while the preferred precursors for cutin are palmitic or oleic acids (Heredia, 2003; Villena *et al.*, 1999).

Cutin is a polymer formed by long fatty acid chains connected by ester bounds and glycerol (Fig. 6). The composition of cutin monomers can be identified using GC-MS, which revealed that C16 and C18 ω-hydroxylated fatty acids are predominant. Those usually carry in the mid-chain position hydroxy or epoxy groups (Nawrath, 2006).

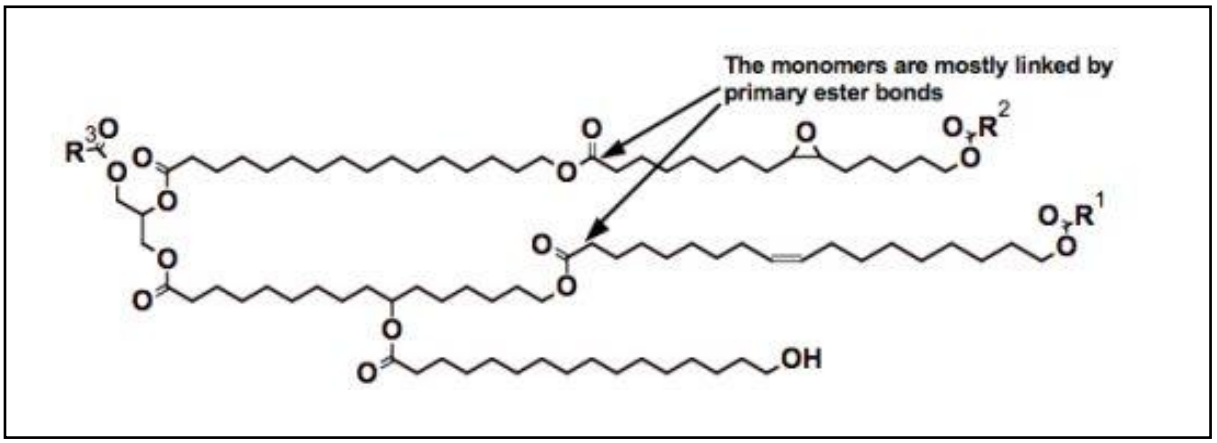

Figure 6. Hypothetical cutin monomers and polymeric structure. Arrangement of monomers that could be found in an ω-hydroxy fatty acid-rich cutin. Picture from Nawrath, 2002.

Cutin biosynthesis begins by the *de novo* synthesis of fatty acids in cells plastids; then they must be converted (activated) to fatty acyl-CoAs thioesters by long-chain acyl-CoA synthetase (LACS) acyltransferase activity to be exported to the endoplasmic reticulum (Schnurr, 2004). Nine members of the LACS family are known to activate acyl chains and allow them to be translocated into subcellular compartments (Shockey *et al.*, 2002), and three of them have been shown to be involved in cuticle functions. It has been reported that *LACS1* (*CER8*) and *LACS2* have overlapping functions which modulate the relative load of cutin monomers (Lü *et al.*, 2009). *LACS9* is highly expressed in plastid envelopes of developing seed cells and a mutation in this gene induces a phenotype of elevated permeability of the cuticle (Schnurr *et al.*, 2002; Xia *et al.*, 2009).

The hydroxylation of the fatty acids terminal methyl (position ω) is catalysed by hydroxylases encoded by members of the CYP86 subfamily of cytochromes P450s. In *Arabidopsis*, two

members of the CYP family with a major importance in seed cuticle formation have been reported: *CYP86A2* and *CYP86A8*. *att1* mutants (*aberrant induction of type three genes*), which have a defective CYP86A2 protein, display alterations in the cuticle ultrastructure and a higher water loss rate in the leaves, which suggests an increased permeability of the cuticle (Xiao *et al.*, 2004). Mutations in the CYP86A8 protein (*LACERATA* gene, *lcr* mutants) induce an altered cuticle (TEM), postgenital organ fusions (Wellesen *et al.*, 2001) and a lesser dormancy of the seeds (De Giorgi *et al.*, 2015).

Other intracellular acyltransferases, members of the glycerol-3-phosphate O-acyltransferases (GPAT) family, catalyse the formation of acylglycerols. It has been shown that GPAT4, GPAT6 and GPAT8 are necessary for cutin formation in the aerial parts of the plant (Li *et al.*, 2007). Concerning *Arabidopsis* seeds, it has been shown that the rate of *GPAT4* expression during germination depends on environmental conditions (De Giorgi *et al.*, 2015).

Biosynthesis of cuticular waxes shares steps and enzymes with cutin biosynthesis; the composition differs in that the major lipids are the very-long-chain fatty acids (VLCFA, C20-C34), but these are derived from the same C16-C18 supplied by the plastids. C16-C18 fatty acids are substrates for the fatty acid elongase (FAE) complex, which successively adds carbons to form VLCFAs (Lee and Suh, 2015).

The transport of the cutin and waxes precursors, from the endoplasmic reticulum to the site of deposition is provided by transporters of the G-family of ATP-binding cassette proteins (ABCG), localised in the plasma membrane (Nawrath *et al.*, 2013). DSO/ABCG11 is such a protein involved in cutin translocation in *Arabidopsis* seeds, polarly localised in embryonic epidermal cells and endosperm (Panikashvili *et al.*, 2010).

An acyltransferase of the BAHD family encoded by *DEFECTIVE IN CUTICULAR RIDGES* (*DCR*) is necessary for the incorporation of cutin monomers into the cutin polymeric structure. Seeds cuticle is affected by a mutation of this gene, which induces an altered morphology of seeds and a defective mucilage (Panikashvili *et al.*, 2009). Moreover, it has been reported that cutin monomers could form nanoparticles, lipid vesicles named cutinsomes, formed by the self-assembly of endogenous hydroxylated fatty acids. It is suggested that cutinsomes facilitate the transport of monomers across the plasma membrane to reach the site of cutin deposition, participating to the early cuticle formation (Domínguez *et al.*, 2015; Heredia-Guerrero *et al.*, 2008). Interestingly in the case of seed cuticles, these specific vesicles have been observed in *Olea europaea* seeds. It has been hypothesised that cutinsomes participate to the extrusion of cutin monomers from the seed coat, where they are produced, towards the endosperm, where they are deposited to form the endosperm cuticle (D'Angeli *et al.*, 2013).

Other genes have functions not totally understood yet in the finalisation of the cuticle formation process. For example, *BODYGUARD1* (*BDG1*), whose inactivation by mutation leads to phenotypes of defective cuticles, assumes the regulation of cutin C18 monomers load. It is

suggested that *BDG* controls an essential step in cutin synthesis or incorporation into polyesters, making it a limiting factor in cutin biosynthesis. Furthermore, this gene could potentially be an interesting new tool to modify cutin polymers, since its overexpression results in higher cutin load, without altering the water barrier activity (De Giorgi *et al.*, 2015; Jakobson *et al.*, 2016; Kurdyukov *et al.*, 2006).

Finally, several genes have functions in the upstream regulation of cutin biosynthesis. The first reported one was SHINE/WAX INDUCER1 (SHN/WIN1), co-discovered in 2004 by two research groups who reported different functions (Aharoni *et al.*, 2004; Broun *et al.*, 2004). In *Arabidopsis*, this transcription factor has two important roles that influence cuticle properties: activation of cuticular waxes biosynthesis and deposition; and control of cutin biosynthesis, by binding to the *LACS2* promoter (Kannangara *et al.*, 2007). Another example of a gene regulating cutin biosynthesis is *KNOX4*. One of its downstream targets is *CYP86A* and *knox4* mutants display defective cuticles and altered amounts of cutin monomers, consistent with the functions of *CYP86A* (Chai *et al.*, 2016).

6. Cuticle functions

Today, little is known about cuticle function in seeds. There are three cuticles in *Arabidopsis* seeds, and it is not known if each one has a main specific role, or if they share the same roles. Another fundamental dimension to consider is time. A same cuticle could first have a function during seed development, for example preventing organ fusion, and then at the mature stage perform a different task, as the control of water movement. Consequently, the following chapter treats of properties of cuticles in general, including the three cuticles at different possible moments of the life of the seed, in order to discuss (and extrapolate) what the seed cuticles can be important for.

6.1 The cuticle as a barrier for abiotic molecules

6.1.1 Water

In the 18th century, the British physiologist Stephen Hales calculated that a sunflower plant absorbs and transpires seventeen times more water in 24 hours than a human being, normalized to mass (Hales, 1727). These fast dynamics of water are linked to the way plants draw energy, photosynthesis. The consequence is that almost 99% of the water absorbed is rejected in the air by vaporous transpiration; 90-95 % of this large quantity passes through stomata on the leaves, the rest (5-10 %) is dispelled through the cuticle, which is therefore not totally impermeable (Raven *et al.*, 2014; Taiz and Zeiger, 2010). Thus, it is clear that the cuticle on the aerial parts of plants allows to reduce water evaporation through the epidermis, which could lead to desiccation if not controlled.

As previously mentioned, different cuticles from distinct organs can have variable thicknesses. It has been demonstrated that there is no correlation between the thickness of a cuticle and its permeability (Riederer and Schreiber, 2001); in particular, some mutants possessing a thicker cuticle display increased permeability, in the leaves (Kurdyokov *et al.*, 2006; Voisin *et al.*, 2009; Xiao *et al.*, 2004) or in the seeds (De Giorgi *et al.*, 2015). While the cuticle thickness cannot be considered as a standard of water permeability, other features could help to estimate it. The structural organization of the cuticle, visible by TEM as variations in density and compaction, can bring more information. TEM examination of cuticle biosynthesis mutants has revealed that their defects in cuticle permeability was correlated with a disorganised and discontinuous cuticle (Voisin *et al.*, 2009). For example, the cuticles in *bdg1* display spaces with low optical density, a phenotype associated with defects in seed permeability (Kurdyokov *et al.*, 2006; De Giorgi *et al.*, 2015). Thus, seed water permeability may be affected by three physical characteristics perceivable by TEM: thickness, structure and density. Those are relatively interdependent and may allow to predict the resulting permeability: a structurally disorganised cuticle is generally correlated to a superior thickness

with high density variations, which leads to an increased permeability. Furthermore, it has been reported that in general the cuticular water permeability is not linked to the amount of cutin or waxes (Isaacson *et al.*, 2009; Jakobson *et al.*, 2016; Riederer and Schreiber, 2001). This supports the hypothesis that defects in the cuticular organization can lead to an altered permeability, but that abnormal quantities of cuticular components, which induce variations in cuticle thickness, do not.

Recent studies have used cutin biosynthesis mutant to test the role of the cuticle in hydric stress. *lacs1/2* mutants displayed necrosis symptoms upon drought more pronounced than wild-type plants and only a little proportion could recover after rewatering, while all the wild-type plants could (Weng *et al.*, 2010). A similar experiment has demonstrated that *CED1*, a gene allelic to *BDG*, is necessary for the increase of the levels of ABA during an osmotic stress. *ced1* mutants displayed drought-stressed phenotypes and their rate of viability was low (Wang *et al.*, 2011). Thus, defects in cuticle impermeability induce a decreased resistance of plants against drought stress. Seeds are generally submitted to high drought stresses, sometimes for years, and they must be able to withstand it to assure the survival of the species they belong to. At least for this reason, cuticles may be as indispensable in the seeds as they are on the leaves epidermis.

Conversely, seed cuticles could play a role in the regulation of the inward movement of water, to control imbibition. Because water diffusion is mostly unidirectional in the aerial parts of plants, less is known about the cuticle function concerning the penetration of water. Experiments on various species showed that removal or disruption of the testa cuticle can induce water imbibition (Taylorson, 2012). This suggests that the testa cuticle, in combination with other deeper barriers (probably suberin and more internal cuticles) participates to imbibition prevention. It has been discovered that small cracks on the soybean testa cuticle (visible by SEM) are responsible for seed coat permeability and are sites of initial water penetration during imbibition (Ma *et al.*, 2004). The features of this cracked cuticle reside in a particular chemical composition, inducing a different and probably less solid organisation of the cuticular network (Shao *et al.*, 2007).

Finally, cuticle water permeability can be discussed in the framework of germination. The phytohormone gibberellic acid (GA), which is repressed in an unfavourable environment, has a germination-promoting function. It acts in an antagonist manner with abscisic acid (ABA), whose accumulation in response to RGL2, a response factor repressed by GA, inhibits germination (Taiz, 2015). Testa rupture is a process which positively correlates with an expansion and an increased cellular transparency of the micropylar endosperm cells (De Giorgi *et al.*, 2015); the expansion of the cells would be driven by the uptake of water, while their transparency can be explained by the filling of intracellular vacuoles. It has been recently demonstrated that some cutin biosynthesis mutants were able to undergo testa rupture under low GA conditions (conditions that prevent this event in wild-type seeds); this suggests that a

function of the endosperm cuticle could be the prevention of water uptake in endosperm cells. Besides, cutin mutants are not able to block endosperm cellular expansion, which could highlight an additional mechanical function of this cuticle (De Giorgi *et al.*, 2015).

6.1.2 Gases

As water loss is controlled by the cuticle, exchanges of carbon dioxide (CO_2) and oxygen (O_2) are similarly regulated by the cuticle (Voesenek *et al.*, 2006). In the fifties, scientists confirmed that the cuticle on leaves is permeable to CO_2 (Dugger, 1952), the carbon atom of which is used to form carbohydrates during photosynthesis. It has been reported that the conductance of the cuticle is in ascending order for O_2, CO_2 and gaseous H_2O, whose diffusions are operated in different directions. This order may be explained by differences in the size of each molecule, or also by differences in the paths lengths of each molecule (Borisjuk and Rolletschek, 2009; Boyer *et al.*, 1997).

O_2 in plant tissues is either derived from photosynthesis or collected from the environment, and is then used for respiration during day and night (Stitt, 2013). In seeds, the cuticle permits to limit O_2 penetration, which ensures a low internal concentration of oxygen. This condition minimizes respiration and catabolism, in order to store nutrients and avoid the formation of high levels of reactive oxygen species (ROS). For carbon dioxide, the cuticle acts as a barrier preventing its escape, which induces a high CO_2 seed concentration. Coupled to low O_2 levels, this helps to decrease respiration (Goffman *et al.*, 2004) and supplies carbon economy for storage (Borisjuk and Rolletschek, 2009).

Oxidation events lead to the accumulation of ROS, which presents correlations with loss of dormancy and affects seed viability (El-Maarouf-Bouteau *et al.*, 2013). It has been recently proven that mutants in cutin biosynthesis genes are more sensitive to oxidation: *bdg1*, *lacs2*, *gpat4/8* and *dcr* seeds display a lower seed viability when in contact with an accelerated aging treatment, and are less dormant than wild-type controls; this correlates with higher amounts of oxidized lipids in these mutant seeds (De Giorgi *et al.*, 2015). Therefore, this confirms that defects in the cuticle lead to increased amounts of ROS responsible for this seed behaviour.

6.2 Cuticle and pathogens

The cuticle represents a barrier against invading pests who cannot penetrate the plant by wound, vector, or through stomata in the case of the leaves. Regarding seeds, one should keep in mind the fact that different parts can be dead, dormant, or alive may influence both the attraction of the pathogens, and the immune response in case of invasion.

Cutin, the main component of the cuticle, seems to be the principal element preventing penetration; indeed it has been shown that some fungal pathogens produce a specific lipase, cutinase, able to hydrolyse cutin polyesters and release free hydroxylated fatty acids monomers (Ettinger *et al.*, 1987; Longhi and Cambillau, 1999). This enzymatical degradation can be complementary to a mechanical rupture of the cuticle accomplished by the formation of an appressorium structure, operating by turgor pressure. This latter physical mechanism of invasion may be sufficient to breach the cuticle when it is thin enough (Bechinger *et al.*, 1999). However, because seed cuticles are much thicker than the leaves cuticle, the chemical method with cutinase probably would be more likely to be successful in the case of a pathogen attacking a seed.

The cutin monomers released by the cutinase hydrolysis action can have multiple actions on both interacting organisms. They are recognized by the fungal pathogen, in which it induces a positive loop by stimulating cutinase expression and therefore strongly enhances cutinase activity (Lin and Kolattukudy, 1978). Furthermore, still for the pest advantage, they also could induce appressorium gene expression (Dickman *et al.*, 2003). In parallel, the breakdown products of cutin can act as elicitors of plant defence responses, named *damage-associated molecular patterns* (DAMPS) (Boller and Felix, 2009); they are therefore able to induce the production of reactive oxygen species (ROS), a signal under ABA control for innate immunity (Benikhlef *et al.*, 2013; L'Haridon *et al.*, 2011).

Interestingly, a paradoxical situation takes place when resistance to plant pathogens on cuticle biosynthesis mutants is tested. Studies have reported that some *Arabidopsis* mutants impaired in various aspects of cuticle biosynthesis have an altered permeability correlating with pathogen resistance (Serrano *et al.*, 2014). For example, mutants for *lacs2*, *bdg* and *lcr* which have more permeable cuticles, display an enhanced resistance to the fungus *Botrytis cinereal*; this is manifested by less and smaller lesions on the leaves than the wild-type (Chassot *et al.*, 2007; Tang *et al.*, 2007; Voisin *et al.*, 2009). This could be explained by the fact that a more permeable cuticle enhances the diffusion of elicitors in the plants, which then leads to a more efficient resistance. Alternatively, it could also enhance the export of antifungal molecules (Bessire *et al.*, 2007). On the other hand, *lacs2* mutant, which expresses a higher resistance to a fugal pathogen, shows a lesser resistance to a bacterial pathogen (*Pseudomonas syringae* pv *tomato*). When inoculated with these bacteria, *lacs2* plants present rather a hypersensitive response (HR, a type of programmed cell death) than true susceptibility. It seems that the higher permeability of the cuticle allows an enhanced and faster leaking of cell contents, leading to more severe symptoms of necrotic collapse (Tang *et al.*, 2007).

6.3 Seed development and prevention of organ fusion

The role of cuticles in *Arabidopsis* development is prominent in the definition of organ boundaries, and several cases of ectopic organs fusion have been observed in cuticle mutants.

The most internal seed cuticle, at the boundary between embryo and endosperm, prevents organs fusion in the seed, but also in aerial parts of the plant (Tanaka *et al.*, 2001). Genes specifically involved in embryo cuticle integrity have been identified. A basic helix-loop-helix (bHLH) transcription factor, *ZHOUPI* (*ZOU*), is expressed exclusively in the endosperm of developing seeds and is necessary for endosperm degradation, embryo growth, embryo-endosperm separation and normal embryo cuticle formation (Yang *et al.*, 2008). The genes *KERBEROS* and *ALE1* (*ABNORMAL LEAF SHAPE1*) are under control of ZOU; the embryo cuticle in mutants of these genes is altered, showing structural discontinuities (Moussu *et al.*, 2017; Tanaka *et al.*, 2001; Yang *et al.*, 2008). More precisely, it has been reported that the *ALE1* gene, coding for a protein probably member of the subtilisin-like serine protease family, is involved in embryo cuticle formation. *ALE1* is expressed in certain endosperm cells adjacent to the embryo and in the young embryo before germination. Phenotypes of *ale1* mutants highlight the importance of this cuticle for the whole plant and represent a genetic link between altered cuticle, organ fusion and a conditional plant lethality: a discontinuous cuticle on the developing embryos and seedlings, leading to adhesion of endosperm and embryo, seedlings showing conditional lethality under low humidity, altered morphology of epidermal cells, and fusions between lateral organs (Tanaka *et al.*, 2001).

Finally, it has been shown that the cuticle surrounding the testa controls the morphology of epidermal cells of the seed coat. Mutants for *ACR4*, a gene coding for a receptor-like protein kinase, display an altered cuticle and phenotypes typical of a disturbed epidermis: irregularities in the epidermal cells disposition, protrusions and cell fusions (Watanabe *et al.*, 2004). *ACR4*, involved in the differentiation and/or maintenance of epidermis-related tissues, may be involved in a signalling pathway controlling cuticle formation and epidermal cell divisions in the seed coat. It would be interesting to understand whether this epidermal disorder of the seed induces the cuticle alteration observed, or if conversely, an altered cuticle triggers this epidermal disorder, which would establish a new function for this external cuticle. Mutation in the *DCR* gene display a phenotype resembling to *acr4* in some points: irregularities of the epidermal cells and an abnormal seed morphology, *dcr* additionally showing a failure to release mucilage upon imbibition and a lower rate of germination (Panikashvili *et al.*, 2009). Knowing that *DCR* directly controls cutin composition, this strongly suggests that the other phenotypes of *dcr* are consequences of a cuticle malfunction, thus establishing new functions for the external cuticle in the morphogenesis and the homeostasis of the epidermis.

6.4 Protection against environmental stresses

Other roles in relation with environment perception and adaptation have been assigned to cuticles. In particular, they have been shown to play roles in adaptations against temperature variations and UVs stresses.

At high temperatures, external cuticles are able to reflect light, allowing to reduce heat load (Taiz, 2015; Yeats and Rose, 2013). Offering the possibility of adaptation in opposite situations, the endosperm cuticle has been reported to increase in thickness during seed exposure to low temperature in *Olea europaea* (D'Angeli *et al.*, 2013). Cold-acclimation activates the transcription of genes coding for FA-desaturases (FAD family), which are responsible for increased rates of specific fatty acids. These are mainly used to adjust plasma and chloroplast membranes fluidity, but are also involved in cutinsomes formation, hence participating to the cutinisation process (D'Angeli *et al.*, 2016). Still in *Olea europaea*, it has been shown that *FAD* genes expression strongly increase during development, probably contributing to the building up of endosperm cuticle (D'Angeli and Altamura, 2016). This leads to the conclusion that FAD genes may have temporally different functions: first during normal development for cuticle synthesis, then during cold-acclimation, allowing the plant to survive under extreme conditions.

In the framework of plant evolution, a significant advantage was conferred by protection against radiations. The cuticle allows to prevent damages caused by UV light (in the UV-B spectrum), which can affect DNA, the photosynthetic apparatus, and membrane lipids (Rozema *et al.*, 1997; Tepfer *et al.*, 2012; Yeats and Rose, 2013). Reflection of UV-B presumably depends on the abundance of epicuticular waxes; it has been demonstrated that if cuticular waxes are removed, UV-B are less reflected (Mulroy, 1979) and that irradiation with enhanced UV-B levels caused an increase of total wax (Steinmüller and Tevini, 1985), which provide a better reflection and an enhanced resistance. Measures of transmittance spectra through isolated plant cuticles confirmed that only a small fraction reach the sensitive tissues under the cuticle (Krauss *et al.*, 1997). This experiment was performed on different plants species, and it revealed that leaves from tropical species tend to display lower UV-B cuticular transmittance than species with deciduous leaves. This permits to class the cuticle among features having been selected during evolutive speciation, depending on environmental conditions.

7. Conclusion

The understanding of *Arabidopsis thaliana* seed cuticles is challenging, especially if one wants to specifically discriminate the role of each cuticle. The majority of this work treated of cuticles in general, because of the quasi impossibility to physically separate the three cuticles in *Arabidopsis* seeds; however, observations by microscopy allowed to precisely determine their localizations, which enables to extrapolate more specialized functions for each of them. Cuticle roles determination is delicate because of interdependence between its characteristics: water and gas impermeability, mechanical support and capacity of light reflexion are its primary roles that together underlie a plethora of global functions in homeostasis control. Maintaining an equilibrium in seed gas and water contents together allows the seed to survive in slow motion and to economize energy for germination and plant growth. Experiments with transgenic *Arabidopsis* plants expressing a fungal cutinase, degrading cutin from the whole plant, confirmed the eclectic functions of this structure. This study indeed highlighted its importance in environment interactions as well as in the development and differentiation of several organs (Sieber *et al.*, 2000). Regarding the cuticle role face to biotic threats such as pathogens, experiments have been accomplished principally on plants aerial parts. It would be interesting to carry on with this line of research by exposing mutant seeds to pathogens, to test a cuticle function in seed (embryo/endosperm)-pathogen interactions.

Up to now, most studies have been done in *Arabidopsis* because it is only in this model system that we have the genetic tools to dissect molecular mechanisms and to deeply understand the functions of our structures of interest. In the near future, the different putative functions of the cuticle will probably be tested and, for some of them, confirmed. The knowledge acquired in these different studies is likely to be useful in agriculture in different ways, in a world where ecological questions become increasingly dominant. For example, a better understanding of cuticles will help to optimize water use, and a better understanding of the physiology of seeds will help to better keep them, have higher germination/growth rates, superior yields, etc.

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