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DARWIN REVIEW

Composition differences between epicuticular and intracuticular wax substructures: How do plants seal their epidermal surfaces?

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Abstract

The protective wax coating on plant surfaces has long been considered to be non-uniform in composition at a subcellular scale. In recent years, direct evidence has started to accumulate showing quantitative compositional differences between the epicuticular wax (i.e. wax exterior to cutin that can be mechanically peeled off) and intracuticular wax (i.e. wax residing within the mechanically resistant layer of cutin) layers in particular. This review provides a first synthesis of the results acquired for all the species investigated to date in order to assign chemical information directly to cuticle substructures, together with an overview of the methods used and a discussion of possible mechanisms and biological functions. The development of methods to probe the wax for *z*-direction heterogeneity began with differential solvent extractions. Further research employing mechanical wax removal by adhesives permitted the separation and analysis of the epicuticular and intracuticular wax. In wild-type plants, the intracuticular (1–30 μg cm^{-2}) plus the epicuticular wax (5–30 μg cm^{-2}) combined to a total of 8–40 μg cm^{-2}. Cyclic wax constituents, such as triterpenoids and alkylresorcinols, preferentially or entirely accumulate within the intracuticular layer. Within the very-long-chain aliphatic wax components, primary alcohols tend to accumulate to higher percentages in the intracuticular wax layer, while free fatty acids and alkanes in many cases accumulate in the epicuticular layer. Compounds with different chain lengths are typically distributed evenly between the layers. The mechanism causing the fractionation remains to be elucidated but it seems plausible that it involves, at least in part, spontaneous partitioning due to the physico-chemical properties of the wax compounds and interactions with the intracuticular polymers. The arrangement of compounds probably directly influences cuticular functions.

Key words: Chain lengths, cuticular transpiration, cutin, fatty acids, plant–insect interactions, quantitative analysis, review, triterpenoids, wax.

Introduction

Land plants must cope with adverse conditions including high doses of ultraviolet light, prolonged exposure to a dry atmosphere, leaching by heavy rains, harmful concentrations of air-borne pollutants, contamination by shading surface particulates, and attack by pathogens and herbivores. These abiotic and biotic stresses initially affect the plant surface and therefore may be countered effectively by protective mechanisms located in an outer skin. Over non-woody, above-ground plant parts, these protective functions are performed by a lipid coating called the cuticle.

Plant cuticles are lipophilic structures deposited onto the outer side of epidermal cell walls. Two major components of plant cuticles are typically distinguished based on their solubility in organic solvents: the lipophilic compounds released by solvent extraction are collectively designated as 'cuticular wax', whereas the second lipophilic component

Abbreviations: AFM, atomic force microscopy; GC, gas chromatography; MS, mass spectrometry; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

that cannot be extracted due to its polymer structure is called 'cutin'. Cutin is a polyester of hydroxylated C_{16} and C_{18} fatty acids and glycerol, although dicarboxylic acids may also be prominent compounds (Walton, 1990; Nawrath, 2006; Pollard *et al.*, 2008). Cuticular wax, on the other hand, is typically a complex mixture of dozens of compounds with diverse hydrocarbon chain or ring structures (Walton, 1990; Jetter *et al.*, 2007). The most ubiquitous, and frequently most prominent, group of compounds are aliphatics with fully saturated (no C=C double bonds), unbranched hydrocarbon chains containing at least 20 carbons. These 'very-long-chain' (VLC) compounds are biosynthesized by elongation of fatty acids beyond chain lengths of C_{18}, and by further modification into corresponding alkanes, aldehydes, and ketones, primary and secondary alcohols, as well as the esters formed by combining fatty acids and alcohols (Samuels *et al.*, 2008). A second group of compounds accumulating in the cuticular wax of many plant species are the pentacyclic triterpenoids. These alicyclic constituents also have largely saturated aliphatic structures, but contain condensed hydrocarbon rings rather than chains (Jetter *et al.*, 2007). Aromatic compounds can also be found in low quantities in cuticular wax mixtures from certain plant species (Jetter *et al.*, 2007).

Based on microscopic evidence, it has long been recognized that the cuticular wax is dispersed across the entire depth of the cuticle, with some of the wax embedded within the cutin polymer matrix and some of it deposited on the outer surface of the polymer (Jeffree, 1996). The former has been designated as 'intracuticular wax' while the latter is the 'epicuticular wax'. The two layers of wax thus defined are the major substructures occurring within the cuticular wax of all vascular plants. It must be noted, however, that many reports erroneously use the term 'epicuticular wax' to refer to the bulk soluble cuticular waxes, such as are extracted by organic solvents. Although various attempts have been made over the years, reliable information on the composition of these two layers has been scarce until recently. It was not clear whether compositional differences, and thus gradients in one or more of the wax constituents, existed between the two wax compartments. It was, therefore, also not clear how much each of the wax compounds and layers contributes to the overall biological functions of the cuticular wax.

Beyond the distinction between epi- and intracuticular wax layers, two further small-scale features may be observed within cuticles in certain cases. The first of these are lamellae that may be seen within the intracuticular layer using TEM, but whose chemical composition and mode of formation remain elusive (Jeffree, 2006). The second nanoscale structural element are wax crystals protruding from the epicuticular layer into the atmosphere, present in many plant species and varying dramatically between them, as documented by SEM (Barthlott *et al.*, 1998). Indirect evidence mainly acquired from comparisons across species or between mutant lines within species showed correlations between crystal shapes and corresponding bulk wax compositions (Baker, 1982; Jeffree, 2006). This led to the conclusion that certain crystal types are formed by specific compounds. However, since these conclusions were based on total cuticular wax composition, the contribution of specific compounds could only be inferred indirectly. In order to quantify the exact crystal composition, selective sampling and analysis of only the epicuticular wax crystals is required.

Over the past decade, new methods have been devised that allow the selective removal of epicuticular waxes, finally enabling the composition of the epicuticular layer to be quantified directly. Where it could be shown that the methods were able to remove the epicuticular material exhaustively, the remaining intracuticular wax could also be analysed selectively. Even though a number of species have been investigated with these methods over the past decade, the accumulated evidence has not been reviewed so far. Therefore, the current review will summarize our current knowledge on the composition of intra- and epicuticular wax layers in an attempt to assign chemical information directly to cuticle substructures.

Previous reviews on cuticle structure rarely connected it to chemical composition of the waxes. Conversely, most reviews on the chemical composition of plant cuticular waxes did not correlate it with cuticle substructures. However, two noteworthy exceptions are a review on the chemical composition of epicuticular wax crystals (Baker, 1982) and a recent, very comprehensive book chapter on cuticle structures (Jeffree, 2006) that also summarizes the mainly indirect, correlative evidence on the formation and composition of epicuticular crystals. To complement these reviews, the present paper will focus more on the composition of the intracuticular wax layer and summarize our knowledge on the epicuticular composition, mainly to contrast it against the adjacent intracuticular wax.

Specifically, this review will evaluate the various methods that are now available for selective sampling of epi- and intracuticular waxes (see 'Review of method developments' section), summarize our current knowledge on the compositions of both layers and on possible differences between them (see 'Differences in wax composition between layers' section), and finally address the biological implications of these results (see sections on 'Possible mechanisms causing compositional differences between intra- and epicuticular wax layers' and 'Implications of wax depth partitioning on cuticle functions').

Review of method developments: selectivity of wax sampling procedures

It had long been surmised that the intracuticular and epicuticular wax layers might differ in composition for a given plant species and organ. Accordingly, over the past three to four decades methods have been devised that aimed to sample both wax compartments selectively. These methods will be briefly summarized here, approximately following the order of their first descriptions in the literature.

Brief versus extended extraction with organic solvents

Originally, all methods for sampling cuticular waxes involved dipping intact plant organs into organic solvents. It seemed possible to preferentially probe the epicuticular waxes by using extremely short dipping times. By contrast, exhaustive extraction using extended time, hot organic solvent, or even overnight Soxhlet reflux should yield all of the cuticular wax. The intracuticular wax composition was inferred either by applying both methods in parallel to two samples and then subtracting the compositions or, alternatively, by employing both methods consecutively on the same sample in order first to extract the epicuticular wax preferentially and then extract the remaining (mainly) intracuticular material.

The discriminating extraction protocols were employed in some studies conducted in the 1970s in the laboratory of EA Baker (Silva Fernandes *et al.*, 1964; Baker and Procopiou, 1975; Baker *et al.*, 1982). The authors found differences between the waxes obtained by short and long extractions, thereby indicating that different depths within the cuticle exhibited compositional gradients as previously hypothesized. They also showed that the extraction protocols were indeed able to enrich portions of cuticular wax in the different extracts. However, this approach had clear limitations: first, even though this method showed qualitative gradients within the cuticular wax, they could not be quantified. Second, while these methods tended to sample parts of the wax that were located more towards the exterior or more towards the interior parts of the cuticle, they could not precisely differentiate between distinct, predefined layers within the cuticle. Third, solvents may preferentially extract certain compound classes due to differing solubilities. Thus, the results could not be interpreted strictly in terms of cuticle substructures, such as the epi- and intracuticular wax layers.

Collodion silver

Haas and Rentschler (1984) noticed discrepancies between widely ranging thicknesses of intracuticular compartments as judged by microscopy on cross-sections and the apparent intracuticular wax yields determined by differential extraction for various plant species. The authors argued that, at least for species with thin epicuticular and large intracuticular wax layers, superficial extraction was not sufficiently selective and another, non-extractive method was required. They adapted a protocol involving the mechanical removal of surface material for microscopy samples in order to allow chemical analyses: collodion, a nitrocellulose-based polymer, was applied to the plant surface in liquid solution and, after drying, was peeled off, concomitantly stripping the surface wax. Analysis of the wax attached to the collodion film yielded quantitative data on both the relative composition of the mixture and its coverage on the plant surface in μg cm^{-2}. The collodion samples, generated by mechanical wax removal rather than by chemical extraction, were interpreted to reflect the entire epicuticular wax layer. Conversely, the intracuticular wax was assumed to remain intact, accessible by superficial extraction of the (previously collodion-treated) specimen.

The collodion method was an important step towards selective sampling of wax layers, since it introduced the idea of mechanical wax stripping and further improved the approach of removing wax layers consecutively. However, the method had limitations regarding the accurate determination of both epi- and intracuticular wax compositions. First, it was not tested whether the epicuticular wax had been completely removed prior to the extraction of the remaining material. Thus, the latter samples might have contained intracuticular wax together with unknown quantities of epicuticular wax. Second, it must be noted that collodion is typically applied in the presence of an organic solvent. In the initial study the polymer was dissolved in amyl acetate (6% w/v; Haas and Rentschler, 1984), and commercial sources offer solutions in ether:ethanol (3:1 v/v, Merck Darmstadt). Organic solvent molecules can enter deep into the cuticle where they mobilize and mix intra- and epicuticular wax molecules (Jetter *et al.*, 2000). Consequently, the collodion samples will contain not only surface compounds but also some intracuticular material, and the following extraction step will yield intracuticular wax together with some epicuticular material. Thus, even though the collodion stripping can be assumed to remove mainly material located near the tissue surface, the overall method has limited selectivity for the wax layers.

Surface swiping with dry glass fabric

In one study focusing on plant epicuticular wax crystals and their ecological role in surface interactions with protective ants, dry glass fabric was employed to sample waxes from the epidermal surfaces of plant stems mechanically (Markstädter *et al.*, 2000). Glass fabric was repeatedly swiped over the plant surface and then exhaustively extracted. Relatively high wax quantities could be obtained after swiping small surface areas, showing that the dry swipes captured substantial amounts of material. However, the wax yield per surface area was not determined, presumably because of difficulties in uniformly swiping a set area. It also remains unknown whether the swiping removed only a part of the epicuticular crystals, the entire crystal layer, crystals and the epicuticular wax film, or even parts of the intracuticular wax. While the selectivity of the method can thus not be assessed quantitatively, it seems very likely that it did achieve a strong enrichment of epicuticular crystals in the samples, allowing their relative composition to be determined fairly accurately. The dry swiping method should therefore be noted as a method with (probably) relatively high selectivity for analysing epicuticular crystals, but not the overall epicuticular layer and/or the intracuticular wax.

Peeling with cryo-adhesives

The idea of mechanical wax sampling was further developed by Jeffree (1996) who introduced a method using frozen

glycerol to transfer surface wax onto artificial substrates for electron microscopy. Experimental details were not described at the time, and it took several years before the method was further developed by Ensikat *et al.* (2000). The authors showed by SEM, TEM, and AFM that the epicuticular crystals could be pulled off with the help of frozen droplets of polar solvents. The crystal shapes and arrangements were perfectly preserved in the process, indicating that the ice coating acted as a glue that exerted enough force to break the crystals off the plant surface, but not enough to alter them. Presumably, the liquid wetted the surface crystals just enough to embed their tips, but did not act as a solvent that would cause (partial) disassembly into molecular components.

Jetter *et al.* (2000) adapted this method for chemical analysis of epicuticular material. The authors first tested it on a plant surface covered by a smooth epicuticular wax film devoid of micro-crystal protrusions. They showed that both glycerol and water could be used as cryo-adhesives. Consecutive adhesive applications yielded wax amounts rapidly declining to zero, whereas much greater quantities of wax could be released by consecutive solvent extraction of the same surface. This revealed a sharp boundary between two wax layers defined by the mechanical accessibility of the outer compartment. It was concluded that, since the polymer cutin is the only cuticle component resistant to mechanical stress, it was responsible for blocking mechanical wax removal beyond a certain point. Hence, the waxes sampled by cryo-stripping came from the exterior layer deposited outside the cutin matrix, i.e. the epicuticular wax. By repeating the mechanical removal of epicuticular wax, it was made exhaustive and the following solvent extraction consequently released exclusively intracuticular wax. The two sampling methods together for the first time allowed the quantitative analysis of both layers with high selectivity.

Peeling with carbohydrate polymer films

After the first method for the consecutive mechanical epicuticular and extractive intracuticular sampling had been established, it could serve as a point of reference to judge the layer-selectivity for other methods. Jetter and Schäffer (2001) demonstrated that aqueous solutions of gum arabic, an adhesive consisting of polysaccharides and arabinogalactan-proteins prepared from *Acacia senegal* or *Acacia seyal* trees, could be painted onto leaf surfaces and, after drying, formed a glue that could lift off epicuticular wax as effectively as the cryo-adhesives. SEM observations provided additional visual support for the effectiveness of this method. Coward (2007) then showed that other carbohydrates can also be used to remove and transfer epicuticular wax crystals. However, in this study the effectiveness of the sampling method was confirmed only by SEM and not also by chemical analysis.

In a series of studies it was shown that the gum arabic method is fairly versatile, successfully removing the epicuticular wax layer (both smooth films and films with crystals) from leaves and fruit of diverse plant species (see below). However, cellular protrusions limit the effectiveness. For example, trichomes contaminate the stripped epicuticular wax while papillose cells prevent exhaustive extraction of the epicuticular wax from surface regions above the cell margins. On the other hand it should be noted that, unlike all previous methods, this method can be performed *in vivo* and thus is useful for epicuticular wax regeneration studies.

Differences in wax composition between layers

The different methods described above have been used to study various plant species over the past three decades. Specifically, the gum arabic and cryo-adhesive methods have been applied to over 20 plant surfaces (Table 1), including the adaxial and abaxial leaf surfaces of *Kalanchoë daigremontiana* Hamet et Perr. de la Bathie (van Maarseveen and Jetter, 2009), *Macaranga tanarius* L. (Muell. Arg.) (Guhling *et al.*, 2005), *Pisum sativum* L. cv. Avanta (Gniwotta *et al.*, 2005), and *Taxus baccata* L. (Wen *et al.*, 2006); the adaxial leaf surfaces of *Ligustrum vulgare* L. (Buschhaus *et al.*, 2007*b*), *Prunus laurocerasus* L. (Jetter and Schäffer, 2001), and *Rosa canina* L. (Buschhaus *et al.*, 2007*a*); the abaxial leaf surface of *Secale cereale* L. (Ji and Jetter, 2008); the inner, slippery surfaces of pitchers from *Nepenthes alata* Blanco (Riedel *et al.*, 2003), *N. albomarginata* Lobb ex Lindl. (Riedel *et al.*, 2007), *N. khasiana* (Riedel *et al.*, 2007), *N.*×*henriana* (Riedel *et al.*, 2007), *N.*×*intermedia* (Riedel *et al.*, 2007), and *N.*×*superba* (Riedel *et al.*, 2007); and the fruit of both wild-type *Solanum esculentum* and the *lecer6* mutant (Vogg *et al.*, 2004). The results from these studies will be summarized below, first addressing the overall coverages and thicknesses of epicuticular and intracuticular wax layers, and then the distribution of individual compound classes.

Wax quantities in epi- and intracuticular layers

As the gum arabic and cryo-adhesive methods have been used most frequently and provide reliable data, the results from all studies using them and reporting quantitative data have been compiled and, as necessary, standardized to μg cm^{-2} ±SD (see Table 1 for a complete list of references). Absolute quantities of waxes in both intracuticular and epicuticular layers showed a wide range, similar to the great variability of total extractable wax reported across the species analysed. Total wax loads ranged from 8 μg cm^{-2} to over 40 μg cm^{-2}. Within these overall wax coverages, intracuticular wax amounts ranged from 1 μg cm^{-2} to 30 μg cm^{-2} (10–80% of the total wax) with a median of 7 μg cm^{-2}. For the epicuticular wax layer, quantities varied from 5 μg cm^{-2} to nearly 30 μg cm^{-2} (20–90% of the total wax). Assuming a density of 0.8–1.0×10^6 g m^{-3} (Le Roux, 1969), this equates to thicknesses of 10–375 nm for the intracuticular layer (excluding cutin) and 50–375 nm for the epicuticular wax. This, in turn, corresponds to an approximate range of 14–100 molecules stacked head to tail for the

Table 1. List of plant species and organs for which the intra- and epicuticular waxes have been selectively and quantitatively measured along with various parameters

Species	Organ	Surface	Method (times applied; replicates)[a]	Crystals	SEM confirmation[a]	Reference
Kalanchoë daigremontiana	Leaf	Adaxial	G.A. (4×; *n*=5)	Twisted ribbons	Before	van Maarseveen and Jetter, 2009
	Leaf	Abaxial	G.A. (4×; *n*=5)	Twisted ribbons	Before	van Maarseveen and Jetter, 2009
Ligustrum vulgare	Leaf	Adaxial	G.A. (3×; *n*=5)	Film	Before; After 1× (line); After 3×	Buschhaus *et al.*, 2007
Macaranga tanarius	Leaf	Adaxial	G.A. (2×; *n*=n.r.)	Film with granules	Before; After 1× (line); G.A.	Guhling *et al.*, 2005
	Leaf	Abaxial	G.A. (2×; *n*=n.r.)	Platelets	Before; After 1× (line); G.A.	Guhling *et al.*, 2005
Pisum sativum cv. Avanta	Leaf	Adaxial	G.A. (4×; *n*=5)	Platelets	Before	Gniwotta *et al.*, 2005
	Leaf	Abaxial	G.A. (4×; *n*=5)	Ribbons	Before	Gniwotta *et al.*, 2005
Prunus laurocerasus	Leaf	Adaxial	G.A. (3×; *n*=6)/Cryo	Film with granules	Before; After 1× (line); G.A.	Jetter and Schäffer, 2001
Rosa canina	Leaf	Adaxial	G.A. (3×; *n*=4)	Film	Before; After 1× (line)	Buschhaus *et al.*, 2007
Secale cereale	Leaf	Abaxial	G.A. (4×; *n*=6)	n.r.	n.r.	Ji and Jetter, 2008
Taxus baccata	Needle	Adaxial	G.A. (3×; *n*=5)	Tubules	Before; After 1× (line); After 3×; After $CHCl_3$; G.A.	Wen *et al.*, 2006
	Needle	Abaxial	G.A. (3×; *n*=5)	Tubules	Before; After 1× (line); After 3×; After $CHCl_3$; G.A.	Wen *et al.*, 2006
Nepenthes alata	Pitcher	Slippery zone	Cryo (4×; *n*=5); G.A.	Platelets	Before; After 1× (line); G.A.	Riedel *et al.*, 2003
Nepenthes albomarginata	Pitcher	Slippery zone	Cryo (5×; *n*=4)	Platelets	Before; After 1× (line); After $CHCl_3$	Riedel *et al.*, 2007
Nepenthes×henriana	Pitcher	Slippery zone	Cryo (3×; *n*=3)	Platelets	Before; After 1× (line); After $CHCl_3$	Riedel *et al.*, 2007
Nepenthes×intermedia	Pitcher	Slippery zone	Cryo (5×; *n*=4)	Platelets	Before; After 1× (line); After $CHCl_3$	Riedel *et al.*, 2007
Nepenthes khasiana	Pitcher	Slippery zone	Cryo (5×; *n*=3)	Platelets	Before	Riedel *et al.*, 2007
Nepenthes×superba	Pitcher	Slippery zone	Cryo (4×; *n*=2)	Platelets	Before; After 1× (line); After $CHCl_3$	Riedel *et al.*, 2007
Solanum lycopersicum	Fruit	WT	G.A. (2×; *n*=5)	Film	n.r.	Vogg *et al.*, 2004
	Fruit	*lecer6*	G.A. (2×; *n*=5)	Film	n.r.	Vogg *et al.*, 2004

[a] n.r., not reported; Before, before treatment; 1×, 2×, 3× etc., after one, two, three etc. gum arabic or cryo adhesive treatment(s); line, dividing line between treated and untreated surface; $CHCl_3$, after chloroform treatment; G.A., wax embedded in gum arabic.

epicuticular layer, assuming an all-*trans*-conformation for the compounds. Since the presence of great quantities of epicuticular wax did not in all cases coincide with the occurrence of epicuticular wax crystals on the surface, other factors also possibly contribute to the formation of crystals, such as the relative concentration of individual compounds or the chemical structure of surrounding non-wax compounds (e.g. cutin). The ratios of intracuticular wax to epicuticular wax also showed great variability, ranging from as little as 1:9 for *P. sativum* (Gniwotta *et al.*, 2005) to as high as 4:1 for *L. vulgare* (Buschhaus *et al.*, 2007*b*). The ratios do not correlate with the absolute quantities of extractable wax.

The compounds identified within the extracted wax mixture can be grouped into two large categories, namely cyclic and straight-chain compounds (Fig. 1). Excluding the fruit of the *S. lycopersicon* mutant *lecer6*, cyclic compounds constituted 0–75% of the total, identifiable wax while, conversely, straight-chain compounds formed 25–100%. These numbers changed within the individual wax layers. In the intracuticular layer, cyclic compounds accounted for as low as 0% and as high as 95% of the identified wax while straight-chain compounds formed the balance. Smaller percentages of cyclic compounds (0–35%) and correspondingly greater percentages of straight-chain compounds (65–100%) were found for the identifiable wax in the epicuticular layer. The ratios between cyclic and straight-chain compounds did not correlate with the total quantity of wax within the respective layer. For example, *M. tanarius* leaves were found to contain 1.5 μg cm^{-2} and 1.3 μg cm^{-2} of intracuticular wax on their adaxial and abaxial surfaces, respectively (Guhling *et al.*, 2005). However, on the top surface, the ratio of straight-chain:cyclic compounds was approximately 4:1 while on the lower surface it was 1:3. On the other hand, although the *L. vulgare* leaf and *N. alata* pitcher both had over 25 μg cm^{-2} of intracuticular wax, cyclic compounds greatly dominated in *L. vulgare* (Buschhaus *et al.*, 2007*b*) while straight-chain compounds were greatly in excess for the pitcher surface (Riedel *et al.*, 2003).

In most cases, differences in the relative compositions of the epi- and intracuticular wax layers were found. In some instances, these dissimilarities between the exterior and interior wax compartments were rather subtle, manifested only as small percentage variations that affected only minor compounds or only chain length distributions within compound classes. In many other cases, however, drastic differences were reported, including cases where a compound was absent from one layer and enriched at high concentration in the other. General trends suggesting which compounds tend to accumulate in which layer are slowly emerging and these trends will be summarized in the following sections.

Cyclic wax constituents

In the original studies establishing both the cryo-adhesive and the gum arabic methods on *P. laurocerasus* leaf waxes,

it was shown that triterpenoid acids were entirely restricted to the intracuticular wax layer in this species, while various VLC aliphatics were found distributed between both wax compartments (Jetter *et al.*, 2000; Jetter and Schäffer, 2001). Subsequent examinations on several species showed a similar trend with the vast majority (or frequently all) of the triterpenoids located within the intracuticular layer (Fig. 1). This pattern does not appear to depend on absolute or on relative wax quantities, as they range from 0.3 μg cm^{-2} in *M. tanarius* (Guhling *et al.*, 2005) to over 20 μg cm^{-2} in *L. vulgare* intracuticular wax (Buschhaus *et al.*, 2007*b*), and from 20% in *M. tanarius* to over 80% in *L. vulgare* intracuticular wax, respectively, nor does it matter which triterpenoid derivatives are involved (e.g. triterpenoid alcohols versus triterpenoid acids).

Kalanchoë daigremontiana leaf wax follows the same trend, albeit only weakly, with nearly equal absolute amounts of triterpenoids in the epi- and intracuticular layers but higher relative proportions in the inner layer (van Maarseveen and Jetter, 2009). It was speculated that, in this case, surface crystals might be formed out of the triterpenoids. It must yet be determined whether or not the epicuticular film beneath these crystals is composed of a triterpenoid-containing wax mixture. It is possible that the film may entirely lack triterpenoids (except for those being transported towards the crystals) although being

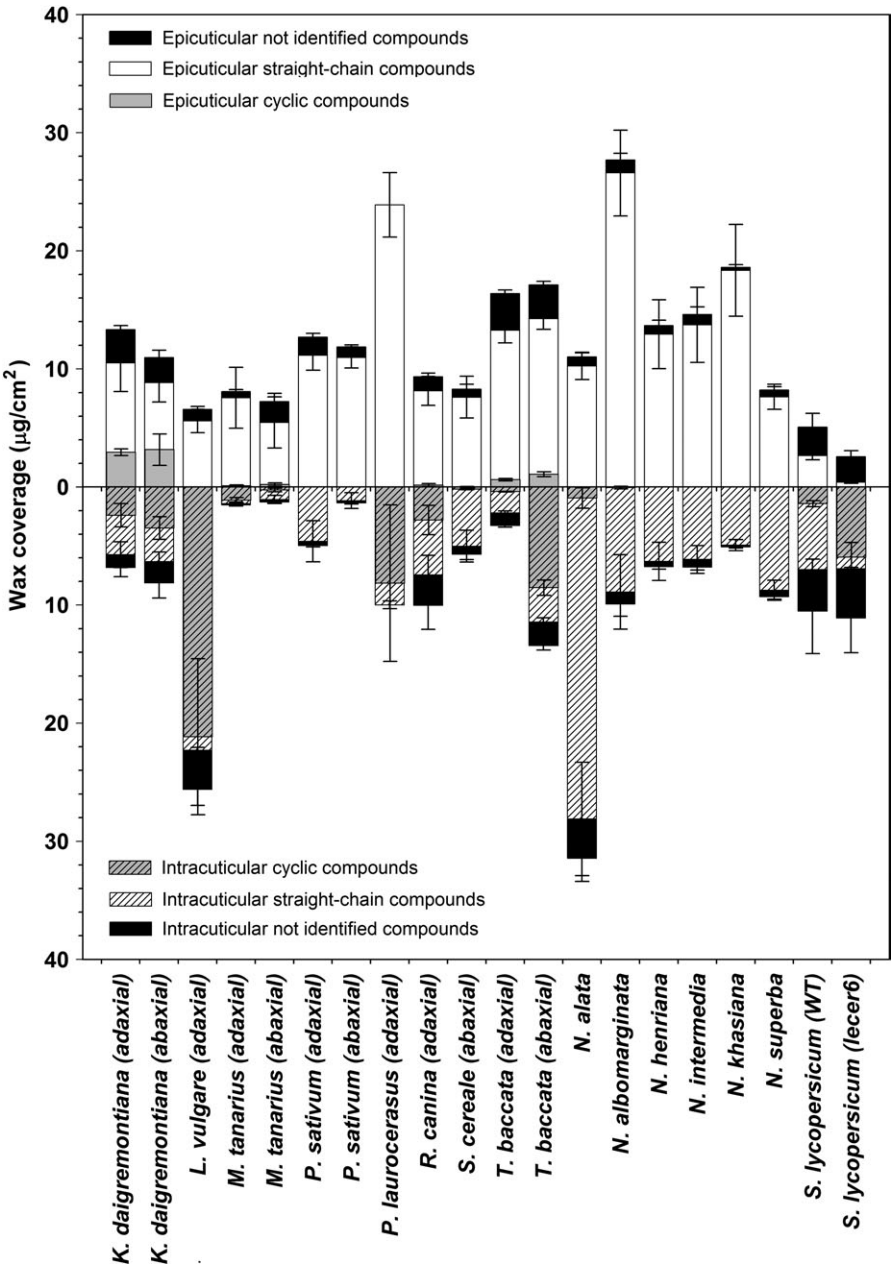

Fig. 1. Absolute quantities of straight-chain, cyclic, and not identified compounds in epicuticular and intracuticular wax. The total epicuticular and intracuticular wax (μg cm^2 ±SD) is represented by the bar height above and below zero, respectively. Together these sum to the total extractable wax. Samples were from adaxial or abaxial leaf surfaces, the slippery zones of pitchers, or fruit.

surrounded by triterpenoid-containing intracuticular wax and epicuticular crystals. It has been reported that epicuticular crystals of various other plant species also consist of triterpenoids, for example, various ant-plants in the genus *Macaranga* (Markstädter *et al.*, 2000). However, the distribution of triterpenoids between the epicuticular and the intracuticular wax layers has not been determined in any of these species, and thus it can not be concluded whether gradients between both layers exist in these cases. Consequently, it also remains unknown why the triterpenoids partition into the epicuticular wax (and crystals) of these species, but not in other species like *L. vulgare* (Buschhaus *et al.*, 2007*b*) and *P. laurocerasus* (Jetter and Schäffer, 2001) that contain both higher relative and absolute quantities of triterpenoids yet do not have triterpenoids in the epicuticular wax.

As for most pentacyclic triterpenoids, steroids in *S. cereale* leaves were also restricted to the intracuticular wax layer (Ji and Jetter, 2008). Other terpenoids seem to follow the same trend, as tocopherols have been found in minor quantities exclusively in the intracuticular layer on yew needles (Wen *et al.*, 2006). Overall, terpenoids displace the aliphatic constituents in intracuticular wax to varying degrees.

Aromatic compounds have sporadically been described in plant waxes and, similar to other cyclic compounds, they have been found to accumulate mainly within the intracuticular wax. For example, the phenylethyl esters were located exclusively in the intracuticular wax of *L. vulgare* (0.6 μg cm^{-2}; 2%) (Buschhaus *et al.*, 2007*b*). In *T. baccata*, the absolute quantities of phenylpropanoid and phenylbutanoid esters were similar to aromatic esters in *L. vulgare*, ranging from 0.7 μg cm^{-2} to 1.6 μg cm^{-2} (Wen *et al.*, 2006). However, as a percentage of the total wax within the layer, the intracuticular wax layer as compared to the epicuticular layers contained nearly 8-fold and 2-fold higher levels on the adaxial and abaxial surfaces, respectively. Leaves of *R. canina* contained minor quantities of phenylethyl esters (0.1 μg cm^{-2}) in each layer; they contributed 1.1% to the intracuticular wax versus 0.9% to the epicuticular wax layer (Buschhaus *et al.*, 2007*a*). The single study reporting the localization of benzyl esters indicated that they occur in the intracuticular wax layer (0.3 μg cm^{-2}; 3%) of the leaves of *R. canina* at nearly ten times the absolute and relative quantity found in the epicuticular wax layer (0.04 μg cm^{-2}; 0.4%; Buschhaus *et al.*, 2007*a*).

A single report to date describes the localization of cuticular alkylresorcinols (Ji and Jetter, 2008). In leaves of *S. cereale*, alkylresorcinols occur exclusively in the intracuticular layer (0.2 μg cm^{-2}; 2%). Although the universality of this finding remains to be confirmed, it does follow the trend set by other cyclic wax compounds. Moreover, the intracuticular localization of alkylresorcinols suggests that the presence of the aromatic group outweighs the presence of an alkyl group in determining the partitioning of these compounds. It must yet be determined whether an increase in the concentrations of cyclic compounds would partially shift alkylresorcinols to the epicuticular wax and, if so, whether these would amalgamate into the film or form crystals.

Very-long-chain aliphatic classes

Various classes of straight VLC aliphatics are ubiquitous in plant waxes; frequently, they contribute the majority or sometimes the totality of the wax. The absolute quantities of any given VLC compound or compound class can vary dramatically between the intra- and epicuticular wax layers. Moreover, plants contain various combinations of VLC compound classes and unique distributions of homologous series of chain lengths within each class. Considering such variability, do gradients exist within this category of straight, very-long-chain compounds?

Partitioning occurs in various VLC compound classes (Fig. 2). By comparing the percentage (within the total VLC compounds) of a specific compound class in the epicuticular layer to the percentage in the intracuticular layer, partitioning can be assessed. If the percentages are equal between the two layers for every compound class, then the wax can be assumed to be homogeneous between the two layers. Conversely, differences in percentages imply preferential partitioning of a compound class into a respective layer.

Primary alcohols occurred at higher percentages within the intracuticular wax layer in one-third of the species where leaf waxes were analysed, namely the adaxial surfaces of *L. vulgare*, *P. laurocerasus*, *R. canina*, and the abaxial surface of *P. sativum* (Jetter *et al.*, 2000; Gniwotta *et al.*, 2005; Buschhaus *et al.*, 2007*a*, *b*). Higher percentages of primary alcohols were also observed in the intracuticular layer of pitchers of all six *Nepenthes* species tested (Riedel *et al.*, 2003, 2007). For the remaining species investigated, the percentages of primary alcohols were approximately equal between the intra- and epicuticular layers; in no cases have the percentages of this constituent class been shown to be higher in the epicuticular wax.

Diols also appear to parallel the same trend as primary alcohols with a 2-fold higher percentage in the intracuticular layer on the adaxial surface of *T. baccata* than in the epicuticular wax (Wen *et al.*, 2006). Approximately equal percentages of diols were found between the two layers for *P. sativum* (Gniwotta *et al.*, 2005) and for the abaxial surface of *T. baccata* (Wen *et al.*, 2006). Analyses of more species containing cuticular diols are needed to see how far this trend can be generalized.

In contrast to the primary alcohols and diols, secondary alcohols tend to accumulate to higher percentages in the epicuticular layer then in the intracuticular, as was observed in the adaxial leaves of *R. canina* and *T. baccata* (Wen *et al.*, 2006; Buschhaus *et al.*, 2007*a*). The other four leaf surfaces that contain secondary alcohols (*P. sativum*—adaxial and abaxial, *S. cereale*—adaxial, and *T. baccata*—abaxial) showed similar percentages between the two layers (Gniwotta *et al.*, 2005; Wen *et al.*, 2006; Ji and Jetter, 2008).

Alkanes and free fatty acids also existed at equal or higher percentages in epicuticular wax as compared to intracuticular wax; the adaxial surface of *R. canina* (Buschhaus *et al.*, 2007*a*), the abaxial surface of *P. sativum* (Gniwotta *et al.*, 2005), and both upper and lower surfaces of *T. baccata* (Wen

et al., 2006) had higher percentages of alkanes in their epicuticular layers than in the intracuticular. The same pattern was observed for free fatty acids on the adaxial surfaces of *P. laurocerasus* and *T. baccata* (Jetter *et al.*, 2000; Wen *et al.*, 2006). For all of the other surfaces tested, not a single one had significantly higher percentages of alkanes or free fatty acids in the intracuticular layer.

Aldehydes and alkyl esters did not display consistent trends of partitioning between both wax layers. Aldehydes were present at higher percentages in the intracuticular wax as compared to the epicuticular wax on the abaxial surface of *M. tanarius* leaves (Markstädter *et al.*, 2000) but, conversely, at lower percentages on the adaxial surfaces of *M. tanarius*, *P. laurocerasus*, and *T. baccata* leaves (Markstädter *et al.*, 2000; Jetter *et al.*, 2000; Wen *et al.*, 2006). For the leaves of other species, no differences in percentages were found. Moreover, aldehydes also formed a higher percentage of the epicuticular wax layer in all of the pitchers of the *Nepenthes* species as compared to the intracuticular wax layer (Riedel *et al.*, 2003, 2007). Esters constituted a greater portion of the very-long-chain compounds in epicuticular wax than in the intracuticular layer on the abaxial side of *M. tanarius* (Markstädter *et al.*, 2000) and the adaxial side of *P. laurocerasus* leaves (Jetter *et al.*, 2000). The opposite trend

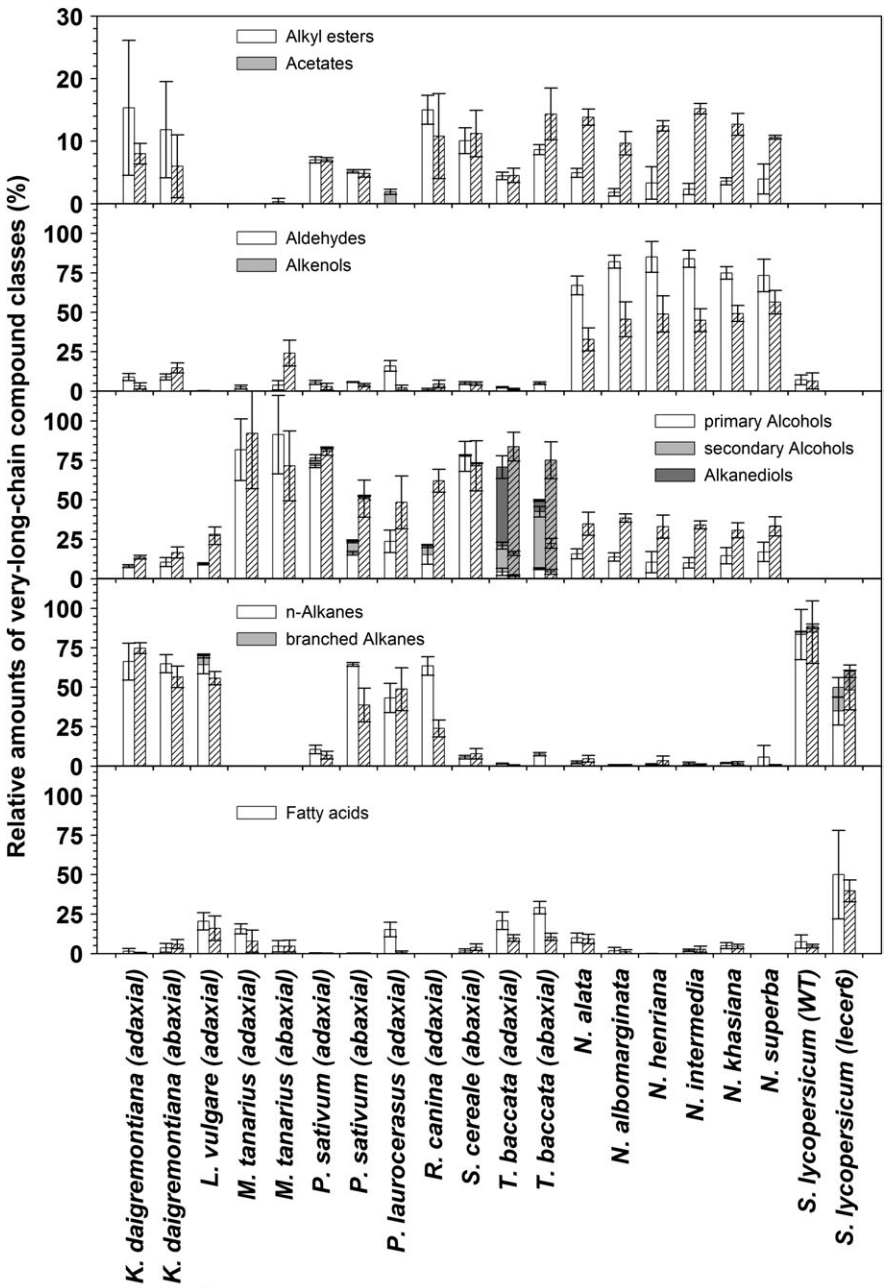

Fig. 2. Relative quantities of very-long-chain compound classes. Relative quantities (% ±SD) were determined as the quantity per total straight chain compounds with the respective layer. The left and right bars for each species are the epicuticular and intracuticular layers, respectively.

was observed for the pitchers of all of the *Nepenthes* species analysed (Riedel *et al.*, 2003, 2007).

More definitive trends are likely to be obscured by the combination of dramatic inter-species wax variability coupled with class partitioning possibly depending in part on the presence and abundance of other classes. Overall, however, primary alcohols and diols tended to accumulate to higher concentrations in the intracuticular wax layer while alkanes, free fatty acids, and secondary alcohols tended towards the epicuticular layer. No consistent trends were observed for aldehydes and esters.

Chain lengths of very-long-chain aliphatics

No consistent trends in chain length partitioning occurred across the species tested (see species list in Table 1). Most species showed no differences in the relative chain length distribution for any compound class, with exceptions in the fatty acids and alcohols in *L. vulgare* and *T. baccata* (Wen *et al.*, 2006; Buschhaus *et al.*, 2007*b*), and fatty acids in *S. cereale* (Ji and Jetter, 2008). In those three cases where chain length differences were observed, the intracuticular wax layer contained a higher percentage of the shorter compounds within the homologous series. No examples are known where longer chain lengths of a homologous series dominate the intracuticular wax layer while shorter chain lengths accumulate in the epicuticular wax.

Possible mechanisms causing compositional differences between intra- and epicuticular wax layers

With the evidence accumulating that compositional gradients exist between the intra- and epicuticular wax layers on organs of many plant species, the question arises: what causes the observed gradients between the intracuticular and epicuticular wax layers?

In general terms, the spatial segregation of different wax compounds into the two sub-compartments within the cuticle can be explained as a phase separation of constituents. Numerous experiments using mostly artificial binary mixtures have shown that (very-) long-chain aliphatic lipids may, to varying degrees, spontaneously separate into two solid and/or liquid phases, depending on the physical conditions and the differences in molecular geometry between compounds (Small, 1984). For example, pairwise combinations of n-alkanes are immiscible at room temperature if their chain lengths differ by more than six methylene units. Similarly, it can be expected that more complex natural mixtures of wax aliphatics would also undergo phase separation, albeit only partially due to the presence of many different homologues with a contiguous chain length distribution. Further segregation is known to occur between compound classes where differences between the shape and polarities of molecules are too big to allow mixed packing in a condensed phase.

In the plant cuticular wax mixtures, such differences in molecule geometry are most pronounced between the VLC aliphatics and cyclic compounds such as aromatics and triterpenoids. While the former are thought to exhibit largely one-dimensional molecule geometries, due to the long and narrow all-*trans* conformation of the hydrocarbon chains (Kreger, 1948; Small, 1984), the pentacyclic triterpenoids are relatively compact molecules extending in two dimensions. Both types of molecules cannot be packed effectively together in the condensed state and, therefore, must be expected to form separate phases. It has been hypothesized that the VLC aliphatics form crystalline domains within the wax, while triterpenoids together with other constituents segregate into amorphous regions surrounding the crystals (Riederer and Schreiber, 1995).

However, while the phase separation model described above seems to be in accordance with the (at least partial) segregation of triterpenoids and VLC aliphatics into intra- and epicuticular wax layers, it must be interpreted with caution. On the one hand, the length scales differ widely between the predicted crystalline/amorphous domains (Riederer and Schreiber, 1995) and the observed intra- and epicuticular layers (Jeffree, 2006). On the other hand, even though the model may explain segregation of VLC aliphatics and triterpenoids, it does not predict a preference for either of the compounds and phases to be associated with the intracuticular wax. Nevertheless, the triterpenoids were found to accumulate primarily in the intracuticular layer (see above), an effect that must be explained by mechanisms beyond simple phase separation. Three such mechanisms seem feasible and will be outlined below.

First, proteins or other molecules could chaperone the wax compounds to their respective locations. Since this mechanism would essentially be reversible, it could also explain the decline in specific wax compounds observed over time in *P. laurocerasus* (Jetter and Schäffer, 2001). However, the paucity of detected proteins within the cuticle argues against this option (Martin and Juniper, 1970; Pyee *et al.*, 1994; Yeats *et al.*, 2010), along with the fact that the diffusive movement of bulky protein molecules, or of smaller chaperones with cargo, through a partially crystalline lipid layer would probably be very slow.

Second, layered partitioning could result from the differential biosynthesis of compound classes. If wax is simply extruded, with newer compounds being accrued to the inner parts of the growing cuticle and displacing older wax layers towards the atmosphere, then gradients could be achieved by simply regulating wax biosynthesis to stagger the production of specific compound classes. While developmental changes in compound class quantity do occur, such as have been seen in time-course experiments of *Prunus laurocerasus* and other species (Hauke and Schreiber, 1998; Jetter and Schäffer, 2001; Bringe *et al.*, 2006), these changes do not follow a sequential epicuticular to intracuticular order where those layers have been investigated. Thus, partitioning due to ontogenetically regulated waves of wax biosynthesis appears unlikely. Further, the high reproducibility of the gradients within a species across sample batches, whether they are harvested over the course of a growing season or across years, counters this suggestion.

Third, the differences in composition between the intra- and epicuticular wax layers may be due to differential interaction of the wax constituents with the polymers present only in the intracuticular space. The cutin matrix (and also cell wall fibrils that possibly extend into the intracuticular compartment) contains more oxygen functionalities and, hence, is more polar than the wax compounds. Consequently, the more polar of the wax constituents will interact more strongly with the matrix, and accumulate preferentially in the intracuticular compartment. This effect may enhance the phase separation between wax compound classes, further separating the phases of VLC aliphatics and of cyclic compounds, expanding them to larger scales and orienting them to the epi- and intracuticular layers, respectively.

Furthermore, fractionation based on polarity differences within the waxes may also account for the gradients observed between various classes of VLC aliphatics, as the least polar constituents, alkanes, tend to accumulate in the epicuticular wax layer while the most polar compounds, alcohols and diols, tend to remain in the intracuticular layer. Long-chain aliphatic acids are known to form hydrogen-bonded dimers in the condensed state, rendering them relatively unpolar (Leiserowitz, 1976; Bond, 2004; Moreno *et al.*, 2006). If such dimer structures exist between free fatty acids within waxes as well, then they should be expected to partition into the epicuticular layer due to their low polarity. Finally, it also seems plausible that functional groups of the cutin polymer may interact specifically with some of the functional groups on wax compounds, for example, in hydrogen bonds between cutin hydroxyacids and wax alcohols or triterpenoids, thereby preferentially retaining certain compound classes.

Implications of wax depth partitioning on cuticle functions

Finally, the implications that distinct layer compositions within the cuticle may have for its biological functions can be considered. Plant cuticular waxes perform a variety of functions, the most important of them being the protection of the tissue against non-stomatal water loss (Riederer and Schreiber, 1995). For the tomato fruit wax, it has been shown that the water barrier is formed mainly by the VLC aliphatics in the wax mixture, and that the cuticular triterpenoids make little direct contribution to the physiological function (Vogg *et al.*, 2004). While the barrier function necessitates a continuous, hydrophobic zone coating the apoplast, it could be equally effective if present in either the intra- or epicuticular (or both) layers. To date, the location of the water barrier has been determined only for one case, the tomato fruit, where Vogg *et al.* (2004) found that approximately equal parts of the transpiration resistance were located in the intracuticular and the epicuticular wax layers. However, it is possible that the barrier location within the two wax layers may vary between species and/or organs, and more species have to be studied before general conclusions can be drawn. With the methods summarized here for the stepwise removal of epi- and intracuticular waxes, it is possible, in principle, to generate samples that will allow the permeances (and transpiration resistances) of both layers to be determined independently.

Because the water barrier function is crucial for plant survival, it cannot be compromised and will take priority over other, secondary functions. Consequently, these functions must either be performed by the same compounds and are then likely to be centred in the same layer as the transpiration barrier, or else the additional functions must be performed in a separate layer so as not to hamper the physiological function. The additional cuticle functions can be divided into two broad categories based on the location of the required compounds – those functions that must be exerted at the very surface of the plant and those independent of the surface.

First, several cuticle functions are achieved by compounds that must be located external to the epidermal cells to be effective, but not necessarily at the plant–atmosphere interface. A prominent example for one such function is the protection of underlying tissues against UV damage. Aromatic compounds are known to absorb UV-B and UV-C radiation, and the cuticular concentrations of these wax constituents were shown to be high enough to provide moderate UV protection at least in some of the plant species investigated (Krauss *et al.*, 1997). It should be noted that the aromatics and triterpenoids may also function as anti-feedants to smaller organisms or as chemical signalling compounds for those herbivores that probe into the plant surface (Eigenbrode and Espelie, 1995). All these functions depend on the molecular properties of the compounds, and not on their physical organization within the cuticle. For these compounds, therefore, the exact location within the cuticle is not essential, and they can be located in either the intracuticular or the epicuticular wax, or in both. However, taken together with the general finding that the same compound classes tend to accumulate preferentially in the intracuticular compartment, the major part of the cuticular UV screening and the anti-feeding function may be assigned to this inner layer of the waxes. These functions, then, reside in the same layer as the transpiration barrier or underneath it.

On the other hand, some functions can only be performed at the plant–atmosphere interface and must therefore be performed by the epicuticular wax layer. For example, the self-cleaning surface properties (i.e. the Lotus effect) for the removal of dust, spores, and other foreign matter require a hydrophobic micro-relief on the surface (Barthlott and Neinhuis, 1997). Certain cell–cell interactions between plant tissues and the signalling between plants and small herbivores probably also occur on the outer surface of the cuticle, even though the mechanisms and, thus, the compounds involved remain unknown (Müller, 2006).

Finally, plant organs may also be protected against walking insect herbivores, or serve to catch insect prey in some carnivorous plants, through the action of epicuticular wax (Müller, 2006). It has been shown that the presence of epicuticular wax crystals renders the cuticle surface slippery for insect feet, and the resulting non-adhesive surfaces of

vertical plant parts can be insurmountable mechanical barriers for walking insect herbivores (Knoll, 1914; Harley, 1991; Federle *et al.*, 1997). In many plant species, the slippery epicuticular wax crystals are formed by VLC aliphatics, for example, aldehydes and their polymer derivatives on the inner surfaces of *Nepenthes* pitchers (Riedel *et al.*, 2003, 2007). Because, in these cases, the plant–insect interaction is mediated through crystals composed of similar compound types to those of the transpiration barrier, both functions may (or may not) coincide in the epicuticular layer. By contrast, the slippery stem surfaces of *Macaranga* species mediating interactions with various ant species rely on the presence of triterpenoid crystals on the plant surface (Markstädter *et al.*, 2000). In these cases, the water barrier must be located in a layer below the surface where VLC aliphatics are sufficiently concentrated and triterpenoid concentrations are low.

In order to understand the various functions performed by cuticular waxes, the distinct compositions of the intra- and epicuticular wax layers must be considered in the context of the possible mechanisms causing the partitioning into wax layers. If plants could precisely position each compound type irrespective of physico-chemical properties, through chaperone guidance or differential regulation of biosynthesis generating ontogenetic waves of compounds, then multiple, optimized functions could be achieved in each of the discrete layers.

However, it appears likely that the depth partitioning of the intra- and epicuticular layers is largely driven by the physico-chemical properties of waxes in combination with the cutin/polysaccharide matrices (see above). Thus, the scope of molecular properties given by the available VLC aliphatics and cyclic wax components will define the possibilities for partitioning, and it probably sets strict limits to the layered structures that can be realized. Plant cuticles might be fundamentally restricted to developing only two layers, with gradients in only some wax constituents, with sometimes only shallow concentration differences and only one possible direction of the gradient. It might also be difficult to maintain a contiguous layer close to the apoplast, and/or to minimize the thickness of the functional layer. In this case, the barrier function would dictate a certain chemical composition and this, in turn, would restrict the realization of layered arrangements. Only those secondary functions that are compatible with the barrier composition and structure could be exerted, and a balance between fulfilling the primary physiological function and certain secondary ecological functions would be imposed. Although such trade-offs between one major and multiple minor functions may be hard to quantify, more detailed investigations into the layered structure of the cuticle and the biological functions associated with the layers will certainly shed some light on these questions.

Future perspectives

Great gains in our knowledge of cuticle composition have been made over the last decade: two discrete, compositionally distinct layers of wax can now be quantified. However, the vast majority of studies conducted so far focused on wild types of diverse species, rather than on closely related taxa and/or even near isogenic lines of one species. It would therefore now be of particular interest to advance to mutant and transgenic plant lines, exploiting the many recently identified genes involved in cuticle formation to elucidate further the observed partitioning patterns and/or the mechanisms controlling partitioning. However, to this end, it will be essential to adapt the methods described here to plant species for which the necessary genetic tools are available. It will be especially important to investigate the intracuticular and epicuticular wax layers of *Arabidopsis thaliana* leaves, a very important species for which the composition of both wax layers has not been reported to date. If the methods for probing both wax layers can be used on this model system, then it will become possible to address the following questions: if cyclic compounds are synthesized in plants/organs where they do not naturally occur, do they still preferentially accumulate in the intracuticular layer? Which compounds (or compound classes) constitute the intracuticular wax, if alcohols are omitted? Do the same partitioning patterns occur if the cutin composition and/or structure is modified? Does a set intracuticular (cutin) layer have a maximum wax capacity?

The progress made on defining the intracuticular and epicuticular wax layers also opens up larger issues that will require additional tool development: It remains possible that additional, chemically distinct sub-layers exist within these two larger layers. Moreover, the lateral distribution of wax components will have to be taken into consideration, together with the properties of these compounds. The precise (absolute and relative) wax composition at a particular location on different epidermal cell types and on different parts of each cell will need to be examined to develop a clear understanding of how cuticles perform their many functions.

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