Composition and Physiological Function of the Wax Layers Coating Arabidopsis Leaves: β-Amyrin Negatively Affects the Intracuticular Water Barrier

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Plants prevent dehydration by producing a cuticle, a lipophilic layer coating all aerial, primary organs. As the cuticle also forms the plant-environment interface, this primary function of blocking nonstomatal water loss (Kerstiens, 1996; Riederer and Schreiber, 2001) must be balanced against other functions including deterring insects and pathogens, obstructing UV penetration, and maintaining surfaces clean of spores and other particulate (Eigenbrode and Espelie, 1995; Barthlott and Neinhuis, 1997; Krauss et al., 1997; Müller, 2006). The balance of all functions directly results from the composition of the cuticle. Cuticles are mixed structures consisting of an insoluble and mechanically robust matrix (cutin plus possibly cutan and/or polysaccharides) and organic-soluble compounds, termed wax (Walton, 1990; Nawrath, 2006; Pollard et al., 2008). Wax ubiquitously comprises linear very-long-chain (VLC) compounds, including varying ratios of acids, primary and secondary alcohols, esters, aldehydes, alkanes, and ketones (Walton, 1990; Jetter et al., 2006). In addition, cyclic compounds such as pentacyclic triterpenoids occur in the wax of many species (Jetter et al., 2006).

Wax composition varies between species but also between locations within a single species down to the subcuticular level. At the smallest currently attainable scale, two layers of wax within the cuticle have been distinguished (Jeffree, 2006). The outer layer, termed epicuticular wax, can be physically stripped from the surfaces of many robust leaves and fruit using aqueous glue (Jetter and Schäffer, 2001; Buschhaus and Jetter, 2011). Consecutive adhesive applications reach a physical limit (presumably cutin) at which no additional wax can be removed. Subsequent solvent extraction releases additional wax that presumably resided within the cutin and is called intracuticular wax. Several studies have revealed that wax composition typically is not uniform between the two wax layers (Gniwotta et al., 2005; Guhling et al., 2005; Buschhaus et al., 2007a; Buschhaus et al., 2007b; Ji and Jetter, 2008; van Maarssen and Jetter, 2009). Most notably, cyclic compounds regularly accumulate almost exclusively in the intracuticular wax layer. The mechanism governing such compartmentalization is not known.

The contribution of various cuticle constituents to each cuticle function is currently unclear. Similarly, the contribution of the different substructures to the different roles the cuticle plays remains uncertain. Our
understanding is mainly hampered by the fact that the previous investigations aimed at chemically and biologically characterizing the cuticles from different species, and then searching for structure-function correlations based on species comparisons (Schreiber and Riederer, 1996). However, such descriptive comparisons were necessarily confounded by the multitude of cuticle differences found between species, rather than just the one factor being assessed. Using this approach, no structure-function relationships have been revealed to date. To overcome this fundamental limitation, analyses of single species prior to and after modification of the wax composition are required.

The quantitative chemical and functional characterization of genetically manipulated cuticles was attempted in a single study to date, in that case using a tomato (Solanum lycopersicum) fruit mutant with reduced VLC aliphatics (Vogg et al., 2004). In this species, the water barrier resulting mainly from VLC compounds was found to be split evenly between the epicuticular and intracuticular layers. However, the lack of easily available genetic tools has hampered further studies on other species.

Although tomato has the advantage of producing robust, astomatous fruit cuticles, Arabidopsis (Arabidopsis thaliana) already has a suite of characterized cuticular wax mutants (for review, see Samuels et al., 2008). Moreover, unlike tomato fruit and Arabidopsis stems, Arabidopsis leaves do not naturally produce detectable quantities of cuticular pentacyclic triterpenoids and can serve as a clean background for studying the effect of these compounds (Shan et al., 2008; Bessire et al., 2011). Thus, this study aimed to compare transpiration barrier properties with and without added cuticular triterpenoids in Arabidopsis leaf waxes. To this end, we genetically manipulated the triterpenoid level by ectopically expressing the triterpenoid synthase gene AtLUP4 (for lupeol synthase4 or β-aminor synthase) in (normally triterpenoid-deficient) Arabidopsis leaves. However, in order to correlate triterpenoid accumulation with barrier properties, we first analyzed Arabidopsis leaf waxes with the necessary spatial resolution. The Arabidopsis gl1 mutant was used for the experiments, because it is devoid of trichomes that interfere with the chemical and physiological experiments. Thus, experiments were carried out to determine (1) the exact composition of the leaf wax of gl1, (2) the composition of the waxes on both sides of the gl1 leaf, (3) the localization of compounds within the epicuticular and intracuticular wax layers of gl1, (4) the composition of the wax mixtures on both sides and in both layers of leaves overexpressing AtLUP4, and (5) the water barrier properties of Arabidopsis leaf cuticles on gl1 and AtLUP4 overexpressors.

**RESULTS**

This work had the two overarching objectives of providing detailed information on the spatial arrangement of cuticular waxes on Arabidopsis leaf surfaces, and of testing whether the addition of triterpenoids to this arrangement would change the transpiration barrier properties of the waxes. Both goals required experiments in which trichomes would have interfered, and thus our analyses were performed in the gl1 mutant completely devoid of leaf hairs. We performed detailed analyses of the gl1 leaf wax composition and water barrier properties at enhanced spatial resolutions, distinguishing between the adaxial and abaxial surfaces and further resolving between the epicuticular and intracuticular wax layers. Finally, we engineered plants to produce β-aminor in their leaves, located this triterpenoid within the cuticular wax, and assessed its effect on the water barrier properties of Arabidopsis leaf cuticles by comparing gl1 with AtLUP4 overexpressors.

**Total Leaf Wax of gl1**

The extractable wax from the leaves of the gl1 mutant totaled 0.9 ± 0.1 μg cm⁻². The largest portion of the identified wax was alkanes (0.32 ± 0.05 μg cm⁻²; Fig. 1). Lesser quantities of free acids (0.22 ± 0.04 μg cm⁻²) and primary alcohols (0.19 ± 0.01 μg cm⁻²), and very minor amounts of aldehydes (0.016 ± 0.002 μg cm⁻²) were also present. The remainder of the wax

![Figure 1](https://www.plantphysiol.org/)

**Figure 1.** Relative quantities of compound classes in Arabidopsis leaf waxes. The percentages of all compound classes in the total, adaxial, and abaxial leaf waxes from gl1 and the AtLUP4 overexpressor are shown (% ± SD; n = 3).
(0.13 ± 0.04 μg cm⁻²) could not be identified. No pentacyclic triterpenoids were detected.

The distribution of compound chain lengths in each compound class paralleled previous findings (Fig. 2). Alkanes ranged from 27 to 35 carbons in length, with most containing 31 carbons (49 ± 1% of the alkane fraction). Alkanes with odd carbon numbers predominated, although minor quantities with even numbers of carbons were also detected (Supplemental Table S1). The free fatty acids contained 24 to 34 carbons, with C₃₄ acid being the most abundant. Primary alcohols were present both as straight-chain (40%) and branched compounds (60%). The n-alcohol chain length range was nearly as broad as that of the acids, with compounds ranging from 26 to 34 carbons in length. The branched-chain primary alcohols displayed a more restricted range from 30 to 32 carbons (including carbons in branches), with branched C₃₂ alcohol constituting 40% of the total alcohols. The chain length distribution of aldehydes paralleled that of branched alcohols; C₃₀ and C₃₂ aldehydes were present in almost equal relative quantities. These final three classes of compounds were all dominated by homologs with even numbers of carbons.

Adaxial versus Abaxial Leaf Wax of gl1

Leaf wax compositions were further resolved by separately analyzing the top and bottom leaf surfaces of the gl1 mutant. The wax load on the adaxial surface totaled 0.8 ± 0.1 μg cm⁻². The same four compound classes were detected on the adaxial surface as for the total leaf wax samples (Fig. 1). Alkanes (0.29 ± 0.4 μg cm⁻²) contributed the largest portion (40% of identified wax) to the adaxial wax. Free acids (0.22 ± 0.05 μg cm⁻²) and primary alcohols (0.12 ± 0.02 μg cm⁻²) formed 30% and 20% of the wax, respectively. Aldehydes were present at very low levels (0.07 ± 0.03 μg cm⁻²). A total of 0.11 ± 0.03 μg cm⁻² could not be identified, and pentacyclic triterpenoids were not detected.

The abaxial surface of gl1 was covered in much less wax (0.4 ± 0.1 μg cm⁻²) than the adaxial side of the leaf. Moreover, the relative and absolute abundances of classes differed from the adaxial surface (Fig. 1). The proportions between alcohols and alkanes were reversed from the adaxial layer. Primary alcohols and alkanes were present at one-half of the quantity (0.04 ± 0.02 μg cm⁻²). Similar to the adaxial surface, one-third of the identified wax was free acids (0.08 ± 0.02 μg cm⁻²), whereas aldehydes constituted only 0.01 ± 0.005 μg cm⁻². No pentacyclic triterpenoids were detected, and 0.13 ± 0.02 μg cm⁻² remained unidentified. Although the quantities of the compound classes differed from both the adaxial surface and the total leaf wax, the distribution of chain lengths within each class did not differ from that of the adaxial surface.

Intracuticular versus Epicuticular Adaxial Leaf Wax of gl1

After determining the specific wax composition of the upper leaf surface in comparison with the lower surface, the adaxial surface was selected to further examine possible partitioning of classes between the epicuticular and intracuticular wax layers (Fig. 3). A single application of gum arabic removed 0.8 ± 0.1 μg cm⁻² of wax from gl1. The majority of wax identified in this layer (60%) consisted of alkanes (0.4 ± 0.1 μg cm⁻²). Primary alcohols (0.09 ± 0.01 μg cm⁻²) and free acids (0.11 ± 0.04 μg cm⁻²) contributed equal amounts (approximately 15% each). Aldehydes were the least abundant at 0.05 ± 0.01 μg cm⁻². Only 15% of the wax sampled by gum arabic (0.12 ± 0.2 μg cm⁻²) was not identified. The epicuticular wax layer was found to have the same chain length distribution within classes as the overall adaxial wax.

Subsequent treatments of Arabidopsis leaves with gum arabic caused significant damage and prevented further testing. In three cases (once for gl1 and twice for the AtLUP4 overexpressor, see below) gum arabic application was applied successfully a second time, to release 0.12 ± 0.01 μg cm⁻² of wax of which only 33% could be identified. With respect to absolute quantities of identifiable wax, the second application of gum arabic thus released less than 6% of the first. Because

**Figure 2.** Relative quantities of individual compounds in Arabidopsis leaf waxes. The percentages of each chain length within the respective compound class in total, adaxial, and abaxial leaf waxes of gl1 and the AtLUP4 overexpressor are shown (% ± se; n = 3).
single applications of gum arabic removed most of the wax accessible to adhesive treatment, it was decided to perform the extraction of the intracuticular wax in the following experiment immediately after the first gum arabic application.

The intracuticular wax totaled a mere $0.16 \pm 0.03 \mu g \ cm^{-2}$, slightly more than the quantity removed by the second application of gum arabic. This low quantity of total intracuticular wax was reflected in the absolute quantity of each compound class. In addition to this, the relative distribution of compound classes differed between the epicuticular and intracuticular wax layers (Fig. 3). Out of the identifiable intracuticular wax, only one-third was alkanes ($0.025 \pm 0.02 \mu g \ cm^{-2}$), as compared with two-thirds in the epicuticular wax layer. Conversely, primary alcohols increased from 14% in the epicuticular to 40% in the intracuticular wax layer ($0.035 \pm 0.003 \mu g \ cm^{-2}$). Equal percentages of free acids ($0.020 \pm 0.005 \mu g \ cm^{-2}$) and aldehydes ($0.004 \pm 0.001 \mu g \ cm^{-2}$) were found in the two layers. Approximately one-half of the intracuticular wax sample ($0.08 \pm 0.2 \mu g \ cm^{-2}$) could not be identified.

Small differences in chain length distributions were observed between the intracuticular and epicuticular layers (Fig. 4). Within the alkanes, $C_{30}$ alkane accounted for approximately 10% more of the alkane fraction in the inner layer than the outer layer, whereas the inverse was found for $C_{31}$ alkane. The intracuticular layer also contained higher relative amounts of branched $C_{32}$ alcohol and $C_{32}$ aldehyde, whereas the epicuticular layer had slightly higher percentages of $C_{32}$ and $C_{34}$ $n$-alcohols.

**Ectopic Expression of AtLUP4 in Leaves**

After characterizing the wax distribution in the $gl1$ mutant, we tested the partitioning of an artificial cuticle component into specific wax layers and its contribution to the water barrier. We engineered plants to produce pentacyclic triterpenoids—compounds found in the stem but not leaf wax of Arabidopsis—in the leaf wax of $gl1$ by ectopically overexpressing a triterpenoid synthase gene. The Arabidopsis gene AtLUP4 was chosen, as a previous study in yeast (*Saccharomyces cerevisiae*; gene characterization; Shibuya et al., 2009) had demonstrated that it encodes an enzyme responsible for the single product $\beta$-amyrin. Leaves of 16 Arabidopsis lines overexpressing AtLUP4 were screened for the presence of $\beta$-amyrin. All lines accumulated low quantities of $\beta$-amyrin (approximately 1%) within the leaf wax, but otherwise did not display any phenotypic differences (data not shown). The line with the highest amount of $\beta$-amyrin was selected for detailed wax and transpiration analyses.
The total wax load found for the AtLUP4 overexpressor (0.8 ± 0.1 μg cm⁻²) was equivalent to that of gl1. Similarly, the absolute and relative quantities of compound classes were identical for both lines with one exception (Fig. 1). Leaves overexpressing AtLUP4 contained 0.005 ± 0.001 μg cm⁻² of the triterpenoid β-amyrin, corresponding to 0.8 ± 0.2% of the identified wax. Chain length distributions within classes did not vary between the two lines (Fig. 2).

The adaxial leaf surface of the AtLUP4 overexpressor contained identical compound classes in the same abundance as found for gl1 (Fig. 1). In addition, 0.015 ± 0.006 μg cm⁻² of β-amyrin (2 ± 1%) was also present. The distribution of chain lengths within the classes for the adaxial sides of both gl1 and the AtLUP4 overexpressor matched those found in the total leaf wax, except for the aldehydes, where the chain length range extended up to 34 carbons and the C₃₂ homolog was dominant (Fig. 2). The abaxial leaf surface of the AtLUP4 overexpressor contained less wax (0.2 ± 0.1 μg cm⁻²) than the same surface of the gl1 mutant. Similar percentages were found for all compound classes except acids, where the absolute (and relative) quantity decreased to 0.03 ± 0.02 μg cm⁻² (Fig. 1). In addition, 4% (0.005 ± 0.001 μg cm⁻²) of the wax from the abaxial leaf surface of the AtLUP4 overexpressor was β-amyrin. No differences were found in the relative distribution of individual compounds within their respective classes as compared with the abaxial surface of gl1 and the adaxial surfaces of gl1 and the AtLUP4 overexpressor (Fig. 2).

The total quantities as well as the absolute and relative amounts of the various classes were nearly identical between wax samples prepared by gum arabic from the adaxial surfaces of gl1 and the AtLUP4 overexpressor (Fig. 3). The only difference between both samples was an increase in free acids from 17 ± 7% in gl1 to 28 ± 2% in the overexpressor. This difference was also reflected in the absolute quantities of the free acids. Notably absent from the epicuticular wax of the AtLUP4 overexpressor was β-amyrin. Relative percentages of compounds within classes were identical to those in the gl1 mutant.

The intracuticular wax layer of the AtLUP4 overexpressor had a composition matching that of gl1 in quantity, relative class composition, and relative chain length distributions (Figs. 3 and 4). The single exception was the presence of β-amyrin (0.004 ± 0.001 μg cm⁻²), present at 4% of the identifiable wax and with equal coverage (in μg cm⁻²) to that found in the total adaxial wax. The very low levels of typical cell membrane lipids detected in the same sample (Supplemental Table S1) shows that the detected β-amyrin had been extracted from the cuticle as opposed to accidental extraction of the cell membrane.

**Water Barrier Properties**

After determining the specific wax composition of the adaxial cuticle and further refining the differences in composition between the epicuticular and intracuticular wax layers in gl1 and the AtLUP4 overexpressor, the role of waxes in the water barrier was examined. Minimum water conductances (MWCs), and inversely the resistances, were determined for the adaxial surface of leaf discs with intact wax, with the epicuticular layer removed, and the entire wax mixture removed.

Water resistances decreased as consecutive wax layers were removed (Fig. 5). For the gl1 mutant, the resistance for leaves with intact wax equaled 1.1 ± 0.1 × 10⁴ s m⁻¹ (n = 43). Removal of the epicuticular wax layer reduced the resistance by 60% to 4.0 ± 0.5 × 10³ s m⁻¹ (n = 38). Solvent extraction of all wax decreased the water barrier to 6.0 ± 0.3 × 10² s m⁻¹ (n = 39), approximately 5% of the original value. The latter finding implies that, conversely, all the waxes taken together were responsible for 95% of the transpiration barrier of the intact cuticle (Fig. 5, right). Because water must necessarily move first through the intracuticular wax (and cutin/cutan) and then through the epicuticular wax, both layers impose separate barriers that are arranged in series (rather than in parallel), and their resistances behave additively. As a consequence, the epicuticular wax resistance (Fig. 5, right center) was calculated as the difference between the resistances of intact cuticles and cuticles from which the epicuticular layer had been removed. The intracuticular wax resistance (Fig. 5, far right) was calculated accordingly, as differences between resistances of cuticles from which either only the epicuticular wax or the entire wax had been removed. The epicuticular wax

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**Figure 5.** Transpiration barrier analysis of Arabidopsis gl1 and the AtLUP4 overexpressor leaf surfaces. Water resistances were determined for the intact adaxial leaf cuticle, for the adaxial cuticle remaining after the epicuticular wax had been removed, and for the adaxial cuticle remaining after the entire wax had been removed. From those three values, the resistances for the total wax, the epicuticular wax layer, and the intracuticular wax layer were calculated (by appropriate subtractions; s m⁻¹ ± s; n ≥ 9). Pairs marked with an asterisk indicate significant differences (P = 0.1).
contributed 66% of the total wax barrier, and the intracuticular wax contributed 34%.

Resistances for the AtLUP4 overexpressor followed a similar pattern as found for gl1 leaves (Fig. 5). The resistance for leaf discs with intact wax (1.0 ± 0.1 × 10^4 s m^-1) did not differ from that of gl1. However, removal of the epicuticular wax layer lowered the resistance (2.9 ± 0.2 × 10^4 s m^-1) by 70%, more than the decrease observed for the same treatment in gl1. Removal of all wax decreased the water resistance (4.76 ± 0.03 × 10^4 s m^-1) to 5% of the original, a percentage identical to that found for gl1.

To test whether the observed differences between cuticular resistances might have been affected by mechanical damage during wax removal steps, the water barrier properties of cuticles after puncturing with a fine needle were also determined (Supplemental Figure S1). The MWC increased linearly with increasing numbers of holes by approximately 6 × 10^3 m s^-1 per hole for the intact leaf disc. A similar linear increase was observed for holes in leaf discs with the epicuticular layer removed (data not shown). The differences of cuticular resistances before and after adhesive removal of epicuticular waxes thus corresponded to those expected from three punctures. It was therefore unlikely that the increased permeability found after removal of epicuticular waxes was an artifact due to local cuticle damage by the adhesive.

**DISCUSSION**

Although wax biosynthesis in the model plant Arabidopsis has been extensively studied, little is known about the distribution of wax compounds on scales smaller than the organ level in this species. This has hampered studies seeking to correlate wax composition with function, specifically the primary physiological function of blocking uncontrolled water loss. To remedy this, the wax compositions on the adaxial and abaxial leaf surfaces along with the epicuticular versus intracuticular wax layers were individually assessed here in the gl1 mutant. Mutant alleles of this gene, including the allele used in this study, have been shown to prevent trichome formation (Koomneef et al., 1982; Oppenheimer et al., 1991; Wang et al., 2004). A trichome-less mutant was essential for the current study because preliminary tests had shown that the crucial gum arabic treatments tore trichomes off the surface of Arabidopsis wild-type leaves. This mechanical damage in turn caused contamination of wax extracts from internal lipids, and the resulting holes in the cuticle hampered water barrier studies. Both artifacts could be avoided using the gl1 mutant. Moreover, the use of a trichome-less mutant allowed for accurate measuring of the surface area. The total wax load and composition per surface area described in this study for this mutant closely matched published values for wild-type leaves (Lu et al., 2009; Bourdenx et al., 2011) and supports previous findings where gl1 was shown to have relatively small differences in wax composition per fresh weight as compared with the wild type (Xia et al., 2010). Moreover, the leaf wax composition in this study parallels that found by Reisberg et al. (2012), assuming they grouped straight and branched alcohols together.

**Distinct Wax Compositions on Both Sides of the Leaf**

Waxes from the adaxial and abaxial leaf surfaces showed distinct total loads and compositions. The large reduction of alkanes on the abaxial surface might be due to differential posttranslational modification, but is much more likely caused by transcriptional control. Thus we hypothesize a lower expression level of genes coding for the enzymes involved in alkane formation. Data on the relative expression levels of candidate genes for alkane biosynthesis in epidermal cells of both leaf sides are not available at present, and it will be interesting to test this experimentally.

In sharp contrast to the compound class composition, the chain length distributions did not differ between both sides of the leaf. This suggests that neither the enzymes responsible for fatty acyl chain elongation nor the substrate-specificity of the enzymes modifying the fatty acyl precursors into wax end products differ between the upper and lower epidermal layers.

**Distinct Wax Compositions of the Epicuticular and Intracuticular Wax Layers**

The wax amounts and composition determined by total wax extraction from the adaxial side was used as a reference value for the subsequent investigations, which employed adhesive stripping to sample the epicuticular wax followed by chloroform extraction of the intracuticular wax. The amounts of waxes found by the separate sampling steps added up to the total adaxial wax quantity. Approximately 6 times more wax was released by the adhesive than by the consecutive solvent extraction, although further tests showed that a second gum arabic application, prior to chloroform extraction, would have released an additional 5% to 10% of the adhesive-accessible material (15% to 20% of the quantity from the first application, of which one-quarter was typical wax VLC aliphatics). Thus, the single application of gum arabic used in this study was likely not quite exhaustive for sampling the epicuticular wax layer. All the results taken together, the epicuticular wax layer accounted for approximately 90% of the wax on the adaxial side of the leaf, with the remaining 10% in the intracuticular layer. It should be noted that this intracuticular wax load may be smaller than that normally found in wild-type leaves as the total quantity of cutin and some of the cutin monomers are reduced in the gl1 mutant (Xia et al., 2010). Unfortunately, no methods currently exist to quantify intracuticular wax loads for trichome-studded wild-type leaves. It has been reported that...
other species have similarly higher proportions of wax in the epicuticular layer (Gniwotta et al., 2005; Guhling et al., 2005; Ji and Jetter, 2008; Buschhaus and Jetter, 2011).

Differences in the compound class distributions were observed between the epicuticular and intracuticular wax layers. The outer layer contained mainly nonpolar alkanes, following a trend observed across several species tested to date, where in all cases the outer wax layer had equal or higher percentages of alkanes as compared with the inner layer. Conversely, the intracuticular wax layer on the adaxial side of Arabidopsis leaves contained a higher proportion of alcohols. A similar partitioning has also been observed in several species, and it has been speculated that hydrogen bonding between alcohols and cutin restricts diffusion of wax alcohols into the epicuticular wax layer (Buschhaus and Jetter, 2011).

In addition to differences in compound classes, small differences in chain length distributions were found between the epicuticular and intracuticular layers. However, as no general trends were observed, this chain length partitioning cannot be interpreted at this point. It is unlikely that the differences in chain lengths between both wax layers resulted from differing diffusion rates.

**β-Amyrin in Cuticular Wax**

Previous studies had shown a tendency for cyclic compounds to accumulate within the intracuticular wax layer of diverse species (Buschhaus and Jetter, 2011). To test whether artificially added triterpenoids would also accumulate in the intracuticular wax of cuticles normally lacking such pentacyclic compounds, the triterpenoid synthase gene AflLUP4 was ectopically expressed in Arabidopsis leaves. This in planta experiment resulted in the presence of cuticular β-amyrin, thus also confirming previous yeast expression studies (Shibuya et al., 2009). Our results showed that β-amyrin accumulated solely in the intracuticular wax layer. As Arabidopsis leaf wax is normally devoid of cuticular pentacyclic triterpenoids, the localization of the newly accumulating β-amyrin points to a general mechanism likely directing their distribution. It seems plausible that triterpenoid partitioning is based on the physicochemical properties of common cuticular components.

How does β-amyrin travel from the site of synthesis inside epidermal cells to the cuticle? Because it is unlikely that triterpenoids diffuse from the plasma membrane into the aqueous cell wall (en route to the cuticle), export probably is facilitated by proteins. Because pentacyclic triterpenoids are naturally absent from the leaf cuticle of Arabidopsis, leaf epidermal cells likely do not express transporters specific to these compounds. Instead, it is possible that steroid or lipid transporters may be present that have a broad enough substrate spectrum to also handle pentacyclic triterpenoids. Sterols have been shown to be transported by some ABCG transporters (for review, see Tarr et al., 2009), which have homology to the cutin and wax exporters ABCG11 and ABCG12/CER5 in Arabidopsis (Bird et al., 2007; Pighin et al., 2004). As well, a lipid transfer protein has been shown to bind steroids (Cheng et al., 2004). All taken together, it seems plausible that proteins capable of transporting diverse triterpenoids from the cell exist in Arabidopsis leaves. Conversely, the same transporters might also be involved in exporting other steroids including sitosterol (see Supplemental Table S1) found in wax samples (and notably in Arabidopsis leaf wax, but not in stem wax, which contains pentacyclic triterpenoids instead). Thus, the repeatedly reported presence of steroids in wax samples from Arabidopsis (and other species) may not simply be contamination from damaged epidermal cells, but instead might reflect bona fide wax constituents.

**Water Barrier Properties**

Methods capable of providing reproducible, quantitative values of water barrier resistances have been applied to many species, but not to Arabidopsis. In contrast, studies examining the cuticular water barrier properties in Arabidopsis have relied upon comparative methods assessing differential rates of dehydration (Goodwin and Jenks, 2005) or else the rates of surrogate compound uptake or exit (i.e. toluidine blue; Tanaka et al., 2004; Xia et al., 2010; benzoic acid; Kerstiens et al., 2006; Ballmann et al., 2011; and chlorophyll; Xia et al., 2010). Here we have achieved the combination, applying the resistance measurements now to Arabidopsis. Thus, comparisons of cuticle permeabilities can now be made not only between Arabidopsis lines, but also between Arabidopsis and other species. The MWC for an intact g/l leaf disc 

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(1.0 \times 10^{-4} \text{ m s}^{-1})
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fell between the 25th and 50th percentiles (median = 1.5 \times 10^{-4} \text{ m s}^{-1}) of 80 stomatal tissues of diverse species examined using similar methods (Kerstiens, 1996). As our transpiration measurements were likewise carried out on surfaces with stomates (albeit present only in small numbers in the adaxial epidermis of Arabidopsis) incubated in the dark, the values reflect cuticular resistances only under the assumption of complete stomatal closure.

The total wax resistance of Arabidopsis leaves was found here to be 10.0 \pm 0.8 \times 10^{3} \text{ s m}^{-1}, showing that the wax provided nearly 95% of the water barrier effectiveness of the entire cuticle. The location and structural basis of the water barrier were further probed by examining the contribution of individual layers of wax. For barriers in series, such as the epicuticular wax layer versus the underlying parts of the cuticle, individual resistances may be summed or subtracted (Riederer and Schreiber, 2001). For example, by subtracting the resistance of the cuticle without epicuticular wax from the resistance of the intact leaf, the resistance of the epicuticular wax may be determined.
For the adaxial side of Arabidopsis leaves, the epicuticular wax resistance (6.6 ± 0.9 × 10^3 s m^-1) was twice as large as the intracuticular resistance (3.4 ± 0.5 × 10^3 s m^-1; Fig. 5). In comparison, the epicuticular wax layer had approximately 9 times more wax than the intracuticular layer, suggesting that the relative composition and/or alignment of compounds contributes significantly to the barrier properties. Overall, these data provide a useful baseline for further studies: Modifying wax composition in this control background followed by water resistance analyses using the methods established here may elucidate the specific contributions of wax compounds to the water barrier.

Finally, the water barrier effectiveness was also analyzed for the AtLUP4 overexpressor. The water resistance caused by the epicuticular wax layer (6.8 ± 0.6 × 10^3 s m^-1) equaled the corresponding value in the control (gl1; Fig. 5). Because the epicuticular wax compositions were identical in absolute and relative quantities between the two lines, this finding is expected and further supports the unbiased reproducibility of the method. The intracuticular wax resistance of the AtLUP4 overexpressor (2.4 ± 0.2 × 10^3 s m^-1), however, was only one-third of the epicuticular wax resistance, and less than the corresponding intracuticular wax resistance in gl1, despite the equal absolute and relative quantities of VLC constituents. Instead, the decrease in water resistance in the intracuticular wax layer corresponds with the presence of β-amyrin in the AtLUP4 overexpressor.

Generalizing the effect of β-amyrin on the transpiration barrier in gl1 leaves to other triterpenoids and Arabidopsis lines requires caution. β-amyrin behaves similarly to other triterpenoid alcohols investigated to date, accumulating preferentially in the intracuticular wax layer both in the Arabidopsis transgenic lines described here and in the wild types of other species. Due to similarities in chemical structures and physical properties, other triterpenoid alcohols will likely—but not necessarily—have similar effects on cuticular permeability as β-amyrin. Further caution is required when extrapolating the results for gl1 to other Arabidopsis lines and other plant species. gl1 mutants show increased chlorophyll leaching and water loss, possibly due to higher cuticle permeability (Xia et al., 2010). Although an effect by β-amyrin alone seems plausible, specific interactions between the triterpenoids and the gl1 cuticle leading to neomorphic phenotypes cannot be ruled out. Distinguishing between direct triterpenoid effects and synergistic interactions with the mutant cuticle structure will require repeating the experiments in other trichome-less lines that lack a cuticle (permeability) phenotype. However, no such lines are known at this point.

The decrease in water resistance co-occurring with the presence of β-amyrin supports a model proposed to explain the molecular mechanisms underlying cuticular wax water barriers (Riederer and Schreiber, 2001). According to this model, the parallel alignment of VLC aliphatic compounds in all-trans conformations leads to the formation of crystalline domains which, in turn, exclude water and force it to follow a tortuous path around the crystalline domains, through amorphous regions of functional groups and nonlinear molecules surrounding the crystals. Accordingly, additional triterpenoids such as β-amyrin were, by this model, predicted to increase the volume of the amorphous domain, either by decreasing the size of crystals by blocking aggregation or by increasing the distance between the crystals. Thus, the addition of triterpenoids to the cuticle should shorten the route and/or open more routes through which water might diffuse, thereby decreasing the resistance of the cuticle overall. This predicted decrease in resistance was now supported by our experiments.

Because triterpenoids hinder cuticular water barriers in the leaves of the Arabidopsis gl1 mutant, and maybe also in other species, why do some (organs of) plants produce them and export them to the cuticle? The results presented here lead to two alternative hypotheses: Either the cuticle fine structure and composition of other species allows them to accommodate the triterpenoids without compromising the transport barrier, or they accumulate triterpenoids to levels where their negative effect on the barrier is balanced against other, positive effects. It may be speculated that the cuticular triterpenoids participate in secondary functions, such as protection from herbivores or pathogen spores, or that they help maintain flexibility in the cuticle and avoid cuticular cracks.

CONCLUSION

Arabidopsis leaves are coated with waxes with distinct compositions on their upper and lower surfaces. Moreover, within the adaxial wax, the epicuticular layer contains more wax and a higher relative quantity of alkanes, whereas the intracuticular wax has a higher percentage of alcohols. The wax forms a transpiration barrier, with the outer layer contributing to the barrier twice as much as the inner layer. The overproduction of β-amyrin leads to accumulation of the triterpenoid solely in the intracuticular wax layer and causes a reduction in the water barrier effectiveness of this layer.

MATERIALS AND METHODS

Plant Material

Seeds from the trichome-less Arabidopsis (Arabidopsis thaliana) mutant gl1 (SALK_039478 Columbia-0 background; Koornneef et al., 1982; Herman and Marks, 1989; Alonso et al., 2003) and the AtLUP4 overexpression in the gl1 background were plated on Arabidopsis media agar (Somerville and Ogren, 1992), stratified for 2 to 3 d at 4°C, and then germinated under continuous light (approximately 150 μmol m^-2 s^-1 photosynthetically active radiation) for 7 to 10 d at 20°C. Seedlings were transplanted into soil (Sunshine mix 4), grown at 20°C under 12-h-days/night at the same photosynthetically active radiation as germination, and watered twice weekly with MiracleGro. Plants from the same batches were used for both wax composition and MWC analyses.

Construction of the AtLUP4 Overexpressor

AtLUP4 (At1g79950) was PCR-amplified from complementary DNA obtained from the stems of wild-type Arabidopsis (Col-0) using gene-specific primers with Gateway-compatible ends. The PCR product was transferred into pDONR-221 using a Gateway cloning kit (Invitrogen) and then sequenced. Subsequently, the gene of interest was recombined into pMDC32 (Curtis and Grossniklaus, 2003) behind double constitutive cauliflower mosaic virus 35S promoters, creating pMDC32-LUP4. The Arabidopsis mutant gl1 was transformed with the construct using the Agrobacterium tumefaciens-mediated floral-dip plant transformation method (Clough and Bent, 1998).

Wax Extraction and Derivatization

Mature leaves with a cumulative surface area exceeding 10 cm² were harvested from multiple plants for bulk wax extraction. Leaves were submersed for 30 s in chloroform containing a defined quantity of n-tetracosane as an internal standard. Samples were resubmerged for 30 s in fresh chloroform, and the two solutions were pooled. For side-specific wax extraction, a glass cylinder was pressed on to the leaf surface (Jetter et al., 2000). Chloroform with a defined quantity of n-tetracosane was added for 30 s with gentle agitation and then removed. A second wash with chloroform for 30 s was added, and the two solutions pooled. If leakage occurred, samples were discarded.

Layer-specific wax sampling was performed according to Jetter and Schäffer (2001): an aqueous solution of gum arabic (1 mg mL⁻¹) was applied to the entire adaxial leaf surface, allowed to dry, and then peeled off. The gum arabic was then extracted with chloroform to obtain epicuticular wax. Subsequent to gum arabic treatment, the cylinder method (see above) was applied to the leaf surface to extract the intracuticular wax. For each of the samples collected by the various above methods the solvent was evaporated under a gentle stream of N₂ gas while heating at 50°C before derivatizing with excess of bis-N,O-(trimethylsilyl)trifluoroacetamide in pyridine for 30 min at 70°C. The solvents were then evaporated as above before chloroform was added back to the derivatized wax. Three separate extractions (and subsequent individual analyses) were performed for each group (n = three per group), and thus the two values represent natural variability.

Wax Compound Identification and Quantification

Wax constituents were separated by capillary gas chromatography (GC: 6890N, Agilent, Avondale, PA; column 30 m HP-1, 0.32-mm inner diameter, film thickness = 0.1 μm) using the following temperature regime: on-column injection at 50°C, oven-held for 2 min at 50°C, raised by 40°C min⁻¹ to 200°C, held for 2 min at 200°C, raised by 3°C min⁻¹ to 320°C, and held for 30 min at 320°C. For compound identification, the GC was linked to a mass spectrometric detector (5973N, Agilent), and the inlet pressure programmed for a constant 1.4 mL min⁻¹ flow of helium carrier gas. For compound quantification, the GC with inlet pressure programmed for constant flow of 2.0 mL min⁻¹ of H₂ carrier gas was connected to a flame ionization detector. The quantity (μg) was established by comparison with a defined amount of n-tetracosane, the internal standard added into the wax extracts. The extracted surface areas were measured with ImageJ software (Abramoff et al., 2004) from digital photographs of the samples (and multiplied by 2 for the total leaf). Alternatively, for the cylinder method, the area of the cylinder opening was measured and used as the extracted surface area.

MWC Analyses

Water loss was measured to follow Knoche et al. (2001). Briefly, leaves were sealed across the opening of a cylindrical, water-filled chamber with silicon grease applied along the ring of leaf-chamber contact. The surfaces facing the water were scratched to ensure continuous access of water into the leaves. Chambers were placed over silica desiccant in order to create gradients approaching 100% internal water to 0% external water. Samples equilibrated overnight at 25°C in a light-excluding container before gravimetrically measuring water loss. Measurements were typically made every 2 to 4 h and usually included at least six measurements per sample; longer intervals or additional measurements altered neither the slope nor the r² value. The weight time series of each cuticle sample was individually subjected to correlation analysis, and those with r² > 0.99 were discarded; these discarded samples could nearly always be traced to sealing or cuticle failure. For the remaining curves, the water flux was divided by the water vapor concentration difference, thus yielding the MWC. MWCs are reported as geometric means in accordance with Baur (1997). Statistical differences were tested using Welch’s t-tests on log-normal values. Controls were created by puncturing submillimeter holes through the cuticle using a fine needle attached to a micromanipulator. Samples were also discarded if the measured fluxes were greater than the geometric means of fluxes from corresponding leaf discs punctured with a microscope.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transpiration analysis of punctured Arabidopsis leaf surfaces. MWC through the adaxial leaf surfaces (wax intact) from gl1 and the AtLUP4 overexpressor after puncturing with one, two, or four holes (m s⁻¹ ± SD; n = 4).

Supplemental Table S1. Absolute composition of various wax compartments on the gl1 mutant leaf. Comparison of coverages for all identified compounds on both leaf surfaces together, only on the abaxial or the adaxial surfaces, and in the intracuticular or epicuticular layers on the adaxial side of the leaf (μg cm⁻² ± SD; n = 3; n.d. = not detected).

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LITERATURE CITED


