

Cuticle-like layer covering velamen realizes functional zoning of aerial roots in epiphytic orchids

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Abstract

Aerial roots of epiphytic orchids possess a velamen structure that is assumed to assist water uptake and reduce water loss. However, there is still debate over how this dual function is achieved. The discovery of a water-repelling layer that covers the velamen may provide answers. To determine what role this layer plays in velamen function, we examined the structure, chemical composition, gene expression, wettability and water loss prevention of epiphytic orchid roots. Results of our analyses indicate this water-repelling layer is similar to the plant cuticle. Therefore, we have named it the “cuticle-like layer”. Further analysis of epiphytic roots showed that when the velamen was in contact with bark, genes related to cuticle biosynthesis were down-regulated and root hairs developed. Furthermore, in root tissues close to bark, aquaporin gene expression responded positively to water-supply. The functional paradox of the velamen can be explained by a “functional zoning” hypothesis: epiphytic orchid roots are partitioned into spatially-separated regions that prevent water loss and increase water absorption. At different regions of the velamen, water loss is prevented by the development of a cuticle-like layer, and water absorption is increased by the development of root hairs.

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Abstract

Aerial roots of epiphytic orchids possess a velamen structure that is assumed to assist water uptake and reduce water loss. However, there is still debate over how this dual function is achieved. The discovery of a water-repelling layer that covers the velamen may provide answers. To determine what role this layer plays in velamen function, we examined the structure, chemical composition, gene expression, wettability and water loss prevention of epiphytic orchid roots. Results of our analyses indicate this water-repelling layer is similar to the plant cuticle. Therefore, we have named it the “cuticle-like layer”. Further analysis of epiphytic roots showed that when the velamen was in contact with bark, genes related to cuticle biosynthesis were down-regulated and root hairs developed. Furthermore, in root tissues close to bark, aquaporin gene expression responded positively to water-supply. The functional paradox of the velamen can be explained by a “functional zoning” hypothesis: epiphytic orchid roots are partitioned into spatially-separated regions that prevent water loss and increase water absorption. At different regions of the velamen, water loss is prevented by the development of a cuticle-like layer, and water absorption is increased by the development of root hairs.

Key words : cuticle-like layer, epiphytic orchids, functional zoning, root hair, velamen.

Introduction

The orchid family (Orchidaceae) initially consisted of terrestrial species, but after expansion and adaptation to forest canopy environments in the Eocene, is today characterized by numerous epiphytic species (Benzing, Ott, & Friedman, 1982; Chomicki et al., 2015; Givnish et al., 2016; Givnish et al., 2015). In contrast to terrestrial habitats, epiphytic habitats have limited water and nutrient supplies, and light intensity fluctuates greatly. These conditions present a great challenge to the roots of epiphytic orchids, because frequent exposure to dry air is completely different from their ancestral terrestrial habitats. Water has been suggested to be the most important limiting factor for growth and survival of vascular epiphytes (Zotz & Hietz, 2001). The limited water supply that aerial roots faced when orchids transitioned from terrestrial to epiphytic habitats is analogous to the transition of plants from the sea to the land—the first colonizer faced devastating desiccation (Leliaert, Verbruggen, & Zechman, 2011; Waters, 2003). Arguably, the most critical evolutionary innovation during the plant colonization of land was the development of the cuticle, a hydrophobic skin covering epidermal cell walls that limits water loss. Today, cuticles are present on the outer surfaces of almost all aerial organs of land plants (Fich, Segerson, & Rose, 2016; Kolattukudy, 1980), such as leaves, flower petals, primary stems, and fruits (Bessire et al., 2007; Buschhaus & Jetter, 2012; Kosma, Nemacheck, Jenks, & Williams, 2010; Li-Beisson et al., 2009; Panikashvili, Shi, Schreiber, & Aharoni, 2011). Surprisingly, the possibility that a cuticle covers the surface of aerial roots of epiphytic orchids has never been investigated.

The outer surface of aerial roots of epiphytic orchids and aroids (also of numerous terrestrial plants) is covered by a sponge-like tissue usually composed of multiple layers of dead cells called the velamen (Zotz, Schickenberg, & Albach, 2017). The velamen is thought to both reduce water loss and facilitate water absorption (Zotz et al., 2017; Zotz & Winkler, 2013). How can the velamen simultaneously prevent desiccation and assist water absorption? Previous studies have suggested that the function of the velamen is related to the structure and chemical composition of its cells (Benzing et al., 1982; Joca, de Oliveira, Zotz, Cardoso, & Moreira, 2020). The velamen traps air in different types of dead cells, which act as a buffer zone that reduces water loss from living root tissue to the atmosphere (Benzing et al., 1982). Efficient water retention by the velamen relies on the number of cell layers, the stratification of layers, and shape and size of individual cells (Joca et al., 2020). Chemical components in velamen cell walls (e.g., pectins, cellulose, and lignin) have been suggested to play a role in water absorption by facilitating apoplastic flow (Joca et al., 2020). One potential

structural adaptation that would prevent water loss would be the development of a cuticle covering over the velamen surface; however, a cuticle would also impede water absorption. Thus, the existence of cuticle may aggravate the contradiction on how multifunction of velamen is achieved.

After observing that the aerial roots of some epiphytic orchids exhibit water repellency when transferred from moist conditions to an environment with fluctuating water supply, and that genes mapped to cutin biosynthesis are upregulated during velamen development (Li, Zhang, Xi, Bradshaw, & Zhang, 2020), we hypothesized that a waterproof layer similar to a cuticle covers the velamen of some epiphytic orchids. The structure, regulation of synthesis, chemical constitution, and ecological function of plant cuticles have been studied extensively for decades, in different organs and different species. Most cuticles are composed of cutin, which is an insoluble polyester of long-chain hydroxy fatty acids that is impregnated by and covered with waxes. Cutin monomers are synthesized by the modification of plastid-generated C16 and C18 fatty acids, producing many kinds of monoacylglycerols (Dominguez, Heredia-Guerrero, & Heredia, 2011; Zhang et al.). Biosynthesis of cuticular wax starts with saturated very long chain fatty acids (VLCFAs) with 24 – 36 carbon atoms, and a fatty acid elongase (FAE) complex determines the length of final product (Denic & Weissman, 2007; Kunst & Samuels, 2009; Li et al., 2019; Millar & Kunst, 1997; Yu, Liu, & Wang, 2019). The basic chemical constitution of cuticles is similar in most plant species, and includes wax esters, aldehydes, alcohols and fatty acids, although plant species and organs differ in the total content and the proportions of the different components (Buschhaus & Jetter, 2012; Guo & Jetter, 2017; Jetter, Kunst, & Samuels, 2006; Leide, Hildebrandt, Reussing, Riederer, & Vogg, 2007; Zhao et al., 2019). Plant cuticles form a physical barrier that protects aerial parts from diverse biotic stresses, such as pests and pathogens, and abiotic stresses, such as excessive UV light and excessive water loss caused by transpiration (Lee & Suh, 2013). Insights gained from research on plant cuticle structure and function provide the basis for investigating the unexpected waterproof layer on the orchid velamen.

In nature, the roots of many epiphytic orchids grow on trees, with the ventral part of the velamen coming into close contact with the bark. Previous studies have found that root hairs develop on the surface of velamen of epiphytic orchids when they grow in soil or when attached to bark (Groom, 1893; Muthukumar & Ayyasamy, 2017; Ponert, Travnicek, Truong Ba, Rybkova, & Suda, 2016; Pridgeon, 1987; Stern & Judd, 1999). The development of root hairs in aerial roots, taken together with their water repellency, have led us to propose a “functional zoning” hypothesis: the velamen of aerial roots reduce water loss and assist water absorption by spatially-separated differences in morphological structure.

To test this hypothesis, we compared six epiphytic orchids with velamina of different thickness and cell wall thickening patterns which are classified into three types: *Cymbidium*, *Vanda* and *Dendrobium* type. The velamen of the *Vanda* type is usually composed of three to five cell layers, and has massive helical thickenings. Velamina of the *Cymbidium* type is usually composed of about ten layers of cells, with long-sinuate thickenings. The velamen of the *Dendrobium* type is characterized by about eight cell layers, and its cells show helical thickenings of different diameter (Porembski & Barthlott, 1988). To study the spatially-separated development of a hydrophobic layer and a root hair zone in these epiphytic orchids, and to examine differential functions of spatially-separated parts, we combined several approaches including scanning electron microscopy, x-ray microtomography, gas chromatography-mass spectrometry, and transcriptome analysis. We are convinced that our work can provide a new entry point for the study of adaptive strategies of epiphytic orchids, and expand our understanding of the function of plant cuticles.

Material and Methods

Plant materials

We compared the roots of six epiphytic (*Cymbidium tracyanum*, *Acampe rigida*, *Papilionanthe teres*, *Rhynchostylis retusa*, *Dendrobium chrysotoxum*, and *D. thyrsiflorum*) and one terrestrial orchid species (*C. goeringii*). The root morphology and structure of these species differ; specifically, the number of cell layers and degree of cell wall thickening of the velamen vary. Their velamen has been classified into three types (Porembski & Barthlott, 1988): *Cymbidium* type, *Vanda* type and *Dendrobium* type. The details of

the experimental materials are shown in Table S1.

To minimize the potential effect of developmental differences on our experiments, we selected mature individuals of fairly uniform size for each species. Prior to experiments, 20 – 50 individuals of each epiphytic orchid species were cultivated hanging on shelves with wires, which allowed their roots to grow in the air without attaching to support materials. The plants were watered with about 1 L tap water per individual at 8:00 am every day. Thirty individuals of the terrestrial orchid *C. goeringii* were planted in plastic pots with a bark mixture watered to maintain a substrate water content of 65 – 75%. The substrate water content was calculated by weighing individual pots, and was calculated as (wet weight - dry weight)/dry weight \times 100. All plants were grown in a greenhouse at 22 – 35 °C under 30% full sunlight, and a relative humidity of 35 – 90%. Daily fluctuations of VPD are shown in Figure S1.

Bark attaching treatment of aerial roots

To simulate the natural growth conditions of epiphytic orchids, we brought aerial roots of *C. tracyanum* and *A. rigida* into close contact with pieces of bark (Figure S2). The area of every piece of bark was about 40 cm², and the thickness of the bark was about 1 cm. We watered the combination of roots and bark every morning, and recorded changes in bark water content by using redundant bark pieces of similar quality to those with orchid roots attached. Bark water content was calculated as (wet weight – dry weight)/dry weight \times 100.

Aerial roots were allowed to grow along and attach to the bark, which exposed the dorsal part of the root to air, while the ventral part of the root was tightly attached to bark. We separated root tips from the vascular bundle. For all experiments, root tips were collected once roots were tightly attached to bark and had elongated 5 cm in length (after about 20 days). Samples collected from the dorsal part of the root tip are referred to as the “aerial portion”; samples from the ventral part of the root tip, which was almost entirely attached to bark, leaving only a small portion to interact with air, are referred to as the “bark portion”.

Sudan red staining and microscopy observation of cuticle-like layer

Sudan red was prepared as a 0.05% (w/v) solution in 95% ethyl alcohol. Free-hand transverse sections of roots from six epiphytic orchids (*C. tracyanum*, *A. rigida*, *P. teres*, *R. retusa*, *D. chrysotoxum*, and *D. thyrsiflorum*) and one terrestrial orchid (*C. goeringii*) were made using razor blades, then the sections were stained in Sudan red solution for 1 h at room temperature. Before microscopic examination, samples were briefly cleaned in distilled water. Images were taken with a digital camera mounted on a Leica DM2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany). Observation of the position of the cuticle-like layer and measurement of velamen thickness were carried out 10 \times and 20 \times magnification. Observation and measurement of the thickness of the cuticle-like layer was carried out at 40 \times magnification.

Scanning electron microscopy (SEM) of the cuticle-like layer

Scanning electron microscopy was performed to observe possible wax crystals on the surface of the cuticle-like layer. Fresh fully developed parts of roots from six epiphytic species (*C. tracyanum*, *A. rigida*, *P. teres*, *R. retusa*, *D. chrysotoxum*, and *D. thyrsiflorum*) were sliced into a maximum of 10 mm \times 5 mm sections, and fixed in 4% glutaraldehyde fixative for 20 h. Gradient ethanol solutions (30 %, 50 %, 70 %, 80 %, 90 % and 100 %) were prepared for dehydration. After root fragments were washed with PBS buffer solution, they were successively dehydrated in the gradient ethanol solution for 1 h each concentration. Root fragments were then dried using the supercritical fluid drying technique (Leica EM CPD300, Wetzlar, Germany). Samples were then coated with platinum particles and observed with a Zeiss Sigma scanning electron microscope equipped with Oxford instruments HKL advanced electron backscatter diffraction system 140, EHT was set at 7.0 kV, and Signal A was set at InLens.

Analysis of chemical composition of cuticle-like layer using gas chromatography-mass spectrometry

The chemical mixtures of aerial root surfaces were extracted from four epiphytic orchids (*C. tracyanum*, *A.*

rigida, *P. teres* and *D. chrysotoxum*). To identify differences in chemical composition between aerial roots and leaves, we also obtained samples of mixtures of leaf surfaces from *C. tracyanum* and *A. rigida*. Each sample was immersed in 30 mL of chloroform for 30 s at room temperature, and n-heptadecane was added as an internal standard.

Dried chemical complexes were added into the dichloromethane. Sample solutions of 1 μL were injected into the temperature controlled capillary gas chromatography (Agilent 5973N, Palo Alto, USA) with HP-5MS quartz capillary column to separate the mixtures. The following procedure were applied in order: initial temperature of 40°C for 2 min, raised by 3 °C min⁻¹ to 80 °C, and then raised by 5 °C min⁻¹ to 280 °C and held 280 °C for 30 min. The qualitative composition was identified using a mass spectrometric detector (70 eV, m/z 35 to 500).

Water loss rate and water retention of aerial and terrestrial roots

We determined the rate of root water loss for five epiphytic orchids (*C. tracyanum*, *A. rigida*, *P. teres*, *R. retusa*, and *D. thyrsiflorum*) and one terrestrial orchid (*C. goeringii*). We collected 4 cm long root samples at 8:00 – 8:30 am after watering. After excision, fresh weight (FW) was determined and samples were immediately sealed with parafilm. The roots were then placed on a lab bench at a temperature of 21 °C and a relative humidity of 30 %. At set intervals (1 to 6 h), FW was recorded for a total of about 260 h for each individual. At the end of the observation, the roots were dried at 70 °C for 48 h to obtain dry weight (DW). At each recording time, water content was calculated as $(FW - DW)/FW \times 100$. After curve fitting for water content versus time using Sigmaplot 10.0, we estimated the water content at 100h dehydration (WC_{100}) for all species, and named the initial water content as WC_0 . In order to compare the rate of root water loss among species more directly, the proportional water loss after 100h dehydration was calculated as $(WC_0 - WC_{100})/WC_0 \times 100$.

Water repellency of aerial root surface of five epiphytic orchids (*C. tracyanum*, *A. rigida*, *P. teres*, *R. retusa*, and *D. thyrsiflorum*) and the wettability of the root surface of one terrestrial orchid (*C. goeringii*) were visually illustrated by taking images using digital camera. After applying 10 μL 0.1% (w/v) safranin solution to the surface of each root, we immediately took a picture, with additional pictures from the same angle taken after 5, 10 and 30 min for aerial roots, and 30 s and 1 min for the terrestrial *C. goeringii* root.

RNA sequencing, data processing, and gene annotation related to cuticle biosynthesis, root hair development and aquaporin

To understand the genetic mechanisms that regulate spatially-separated “functional zoning” in epiphytic roots, we compared gene expression in the “aerial portion” and “bark portion” of roots. We detected differentially expressed genes related to cutin, wax biosynthesis, and root hair development. To further test for the presence of cutin and wax biosynthesis on the surface of aerial roots, we compared gene expression in root tips of the epiphytic *C. tracyanum* and of the terrestrial *C. goeringii*. Because bark water content changed with VPD, we compared the expression of aquaporin-related genes in the “bark portion” of roots with high water content (after watering 2 h) and low water content (at the peak of VPD).

Total RNA from each sample was extracted using the TIANGENRNA prep Pure kit (catalogue DP441, Beijing, China). A total of 1.5 μg RNA per sample was used as input material for RNA-Seq library construction. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), to which we added index codes. After cluster generation, we sequenced the library preparations with an Illumina HiSeq 2000 platform.

Transcriptomes were assembled using the Trinity method (Grabherr et al., 2011). Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (KEGG Orthology database) and GO (Gene Ontology). We used KOBAS software to test the enrichment of differential expression genes in KEGG pathways (Mao, Cai, Olyarchuk, & Wei, 2005). The sequencing data were uploaded

to the Sequence Read Archive (SRA) of the NCBI under accession number: SRR11440571, SRR11440590, SRR11442834, SRR11442916, SRR11443148, SRR11443976, SRR11665924.

X-ray micro-computerized tomography scanning

We scanned roots of *C. tracyanum* and *A. rigida* after bark attachment using x-ray micro-computerized tomography (CT) (Zeiss Xradia 410 Versa, Jena, German). To produce panoramic three-dimensional (3-D) CT images of *C. tracyanum*, the x-ray source was set at 30 kV and 200 μ A, camera binning at 2, pixel size at 25.2 μ m, and exposure time at 6 seconds. We also imaged the interaction site between root and bark in *C. tracyanum*, setting x-ray source at 60 kV and 133 μ A, camera binning at 2, pixel size at 2.0 μ m, and exposure time at 7 seconds. To produce panoramic 3-D CT images of *A. rigida*, the x-ray source was set at 40 kV and 200 μ A, camera binning at 2, pixel size at 34.4 μ m, and exposure time at 4 seconds. To image the interaction site between *A. rigida* root and bark, the x-ray source was 70 kV and 114 μ A, camera binning at 2, pixel size at 2.4 μ m, and exposure time at 7 seconds.

Statistical analysis

Gene expression was analyzed with three biological replicates using the DESeq package (1.10.1) in R (Love, Huber, & Anders, 2014). We identified differentially expressed genes based on the negative binomial distribution model. To correct for false discovery rate, we adjusted the resulting Type I error probabilities (P) using the Benjamini and Hochberg approach. We interpreted genes as differentially expressed when DESeq determined adjusted $P_{adj} < 0.05$.

Differences in the thickness of cuticle-like layers and chemical constitution were assessed with one-way analysis of variance (ANOVA), and Tukey's multiple comparison tests were used at the $\alpha = 0.05$ level to determine whether significant differences existed between species. Intraspecific differences in chemical constitution between leaves and roots were assessed with independent-sample t tests. All these analyses were performed with SPSS 20.0 software.

Results

Morphological and anatomical structure of the cuticle-like layer

Microscopy of aerial roots consistently showed that Sudan red dye stained a layer covering the velamen of epiphytic orchids (Figure 1 b, d, f, h, j, l); in contrast, we did not find such layer in terrestrial *C. goeringii* (Figure S3). Hereafter, we refer to this covering on velamen as the "cuticle-like layer".

The thickness of the cuticle-like layer and the velamen were inversely related (Figure S4). The cuticle-like layer was significantly thicker in species with thin velamen (e.g., species with *Vanda* type velamen) than in species with thicker types of velamen. The cuticle-like layer of *C. tracyanum*, which has the thickest velamen of all species, was thinnest of all species measured (Figure 1 a, c, e, g, i, k, Table 1). The thicknesses of the cuticle-like layer did not differ significantly between *A. rigida*, *P. teres*, and *R. retusa*, which have *Vanda* type velamen. Similarly, the thickness of the cuticle-like layer did not differ significantly between *D. chrysotoxum* and *D. thyrsiflorum*, which have *Dendrobium* type velamen. These results suggest that species with the same velamen type tend to possess cuticle-like layers of similar thickness.

The surface of aerial roots was examined using scanning electron microscopy to detect possible wax accumulation. In *A. rigida*, *P. teres* and *R. retusa*, which have *Vanda* type velamen, wax crystals were plate-shaped (Figure 2 d-l), whereas the wax crystals of *D. chrysotoxum*, *D. thyrsiflorum*, and *C. tracyanum* were granular (Figure 2 a-c, m-r). These results suggest that species with the same velamen type possibly tend to have similar patterns of wax accumulation.

Chemical constitution of cuticle-like layer

The cuticle-like layers of aerial roots and the cuticles of leaves in the tested orchids had similar chemical compositions with known chemical structures, including fatty acids, aldehydes, alcohols, alkenes, and alkanes. The carbon atom numbers of these components varied from C5 to C32. The contribution of chemical

components with less than 20 carbon atoms to cuticle-like layers differed among the four studied species (Figure 3). However, the composition of chemical components with more than 20 carbon atoms did not differ significantly in the cuticle-like layers of orchid species. In *A. rigida* and *P. teres*, which have the same velamen type, the cuticle-like layer consisted of similar numbers of chemical components regardless of carbon atom number. This may indicate that the chemical constitution of cuticle-like layers is similar within species with same velamen type. The chemical constitution of cuticle-like layers of aerial roots differed from that of the cuticle of leaves in some species. For example, in *C. tracyanum* chemical components with less and more than 20 carbon atoms were more prevalent in cuticle-like layers of aerial roots than in the cuticle of leaves. However, in *A. rigida* the chemical constitution of cuticle-like layers of aerial roots and the cuticle of leaves showed no statistical differences (Figure 3).

Regulatory genes related to cutin biosynthesis

Among differentially expressed genes involved in cutin and wax biosynthesis, we found five genes that encode subunits of long chain acyl-CoA synthetase. In *C. tracyanum*, all five of these genes were up-regulated in the “aerial portion” compared to the “bark portion” of the epiphytic root (Figure 4). Three of the genes encoding the subunits of long chain acyl-CoA synthetase were expressed at higher levels in the epiphytic *C. tracyanum* than in the terrestrial *C. goeringii* (Table S2). Cutin monomer oxidation reactions involve members of the CYP86A family; two genes encoding CYP86A were detected, and were up-regulated in the “aerial portion” compared with the “bark portion” of epiphytic roots (Figure 4), while one gene was expressed at higher levels in the epiphytic *C. tracyanum* than in the terrestrial *C. goeringii* (Table S2). We found four genes that encode subunits of glycerol-3-phosphate acyltransferase, which transfers acyl groups from acyl-CoA to glycerol-3-phosphate to generate cutin monomers. Three of these genes were up-regulated in the “aerial portion” (Figure 4). Three of these genes were up-regulated in epiphytic *C. tracyanum* (Table S2). The GDSL-motif lipase/esterase (GDSL) superfamily may function as a cutin synthase that polymerizes cutin monomers. Thirteen genes related to subunits of GDSL-motif lipase/esterase (GDSL) superfamily were differentially expressed in our study. Eight of these GDSL genes were up-regulated in the “aerial portion” of root tips (Figure 4). In addition, eight of these subunit genes were expressed at higher levels in the epiphytic orchid than the terrestrial orchid (Table S2).

Regulatory genes related to wax biosynthesis

For wax biosynthesis, we found that three genes encoding the subunits of fatty acyl-ACP thioesterase were up-regulated in the “aerial portion” of root tips (Figure 4), and four genes encoding subunits of this thioesterase were expressed at higher levels in the epiphytic *C. tracyanum* than in the terrestrial *C. goeringii* (Table S2). In the endoplasmic reticulum, the fatty acid elongase complex (comprised of four enzymes a b-ketoacyl-CoA synthase, a b-ketoacyl-CoA reductase, a b-hydroxyacyl-CoA dehydratase and an enoyl-CoA reductase) synthesizes very long chain fatty acids (VLCFs) which are precursors for wax production. We detected eight genes related to the subunits of a b-ketoacyl-CoA synthase, one gene encoding subunits of a b-hydroxyacyl-CoA dehydratase and one gene encoding subunits of enoyl-CoA reductase. All of these genes were up-regulated in the “aerial portion” of epiphytic roots (Figure 4). Four out of six genes related to subunits of a b-ketoacyl-CoA synthase were expressed at higher levels in *C. tracyanum* than in *C. goeringii*, while four genes encoding the subunits of enoyl-CoA reductase were up-regulated in *C. goeringii* (Table S2).

Regulatory genes related to cutin and wax transportation

The ABCG subgroup of the ABC-type transporters family is involved in cutin deposition. We found 12 of 13 genes related to the subunit of ABCG members were up-regulated in the “aerial portion” of root tips (Figure 4), and 12 of 31 genes encoding ABCG subunits were up-regulated in epiphytic *C. tracyanum* (Table S2). Glycosylphosphatidylinositol-anchored lipid transfer protein (LTPG) is confirmed to be involved in lipid export. One gene related to the subunit of LTPG was found in our study, but it was down-regulated in the “aerial portion” of root tips (Figure 4), and one gene encoding its subunit was up-regulated in epiphytic *C. tracyanum* compared with terrestrial *C. goeringii* (Table S2).

Water loss and water repellency

Proportional water loss from aerial roots after 100 h dehydration differed among species by more than an order of magnitude from 0.8% in *R. retusa* to 9% in *C. tracyanum* (Figure 5 a-f). Water loss in the three species with *Vanda* type velamen was consistently smaller than that of *D. thyrsiflorum* or *C. tracyanum*. In the terrestrial *C. goeringii*, the proportion of water loss was about twice as large as in the epiphytic *C. tracyanum*, which has the same velamen type (Figure 5 e, f).

We found that all the roots of epiphytic orchids (Figure 6 a, b, c, d, e) showed strong water repellency: the droplet remained on the surface with little change after 30 min. In contrast, the root of terrestrial *C. goeringii* was easily wettable: a 10 μ L water drop was almost completely absorbed by the root after 30 s (Figure 6 f). When the droplet on the root surface disappeared after 1 – 2 h, the residual stains were different between epiphytic species and *C. goeringii*. This indicates that the aerial roots covered by a cuticle-like layer have a very slow rate of water absorption (Figure S5).

Root hair development and aquaporin gene expression

We investigated the development of root hair after bark attachment through three-dimensional observation of root structure and by analyzing the differential expression of related genes. Using X-ray micro-computerized tomography scanning, we found that the epi-velamen cells of velamen in both *C. tracyanum* and *A. rigida* developed root hairs when they attached to bark (Figure 7 a-f). By comparing gene expression between the “aerial portion” and the “bark portion” of roots, we found that genes related to the bHLH transcription factor and EXPANSIN, which are essential to root hair development, were up-regulated in the “bark portion” of *C. tracyanum* root tips (Figure 8 a, c).

In the bark attaching experiment, we also examined gene expression of aquaporin which facilitates water flow through membranes. By comparing the “bark portion” of roots with high water content (after watering 2 h) and low water content (at the peak of VPD), we found that the expression of genes related to aquaporins were significantly up-regulated after the roots attached to bark with high water content (Figure 9).

Discussion

Almost 200 years after its original description by Link (1824) our understanding of the function of velamen is still incomplete. In the present study, we found aerial roots of epiphytic orchids growing in air were strongly water repellent due to the existence of a cuticle-like layer covering the velamen, while root hairs were produced on the contact zone of aerial roots with bark as an alternative way to promote water absorption (Figure 10). Thus, the aerial roots of these epiphytic orchids can resolve the conflict of functional requirements for water absorption and reduction of water loss through “functional zoning”. We further suggest to name the part of the aerial root that grows in air causing the development of the cuticle-like layer as the “control zone”, and the part which attaches to bark causing the development of root hair as the “passable zone”.

Development of the cuticle-like layer in the “control zone” of aerial roots

Plant cuticles form a physical barrier that protects against biotic stressors (e.g., pathogens and pests), and abiotic stress (e.g., excessive UV irradiation), but its most prominent role is related to preventing water loss (Fich et al., 2016; Lee & Suh, 2013). Our results establish a link between a thicker cuticle-like layer and lower water loss from the aerial roots. However, species with thicker cuticle-like layers tend to have thinner velamina. This suggests that there may be a trade-off between the two “barriers” to reduce water loss – a thinner velamen needs support from the cuticle-like layer, whereas thick velamen is equivalent to a thick boundary layer with little demand for additional reduction of diffusion by a cuticle. However, a thicker cuticle-like layer seems more effective than a thicker velamen in preventing water loss from aerial roots of epiphytic orchids (Table 1, Figure 5 a-f). The existence of two distinct barriers to water loss in aerial roots could explain the discrepancies in reports on the variation in root traits, especially for velamen thickness, of epiphytic orchids along gradients of water availability. Two studies along rainfall gradients in, respectively, West Africa and Puerto Rico (Sanford, & Adanlawo, 1973; Díaz, & Ackerman, 1990) show that species in drier habitats tend to develop thicker velamina. In contrast, a recent study in lowland Panama reports substantial variation in velamen thickness which is, however, unrelated to a substantial moisture gradient

(Einzmann, Schickenberg, & Zotz, 2020). None of these studies assessed the presence of the cuticle-like layer. Future studies should include the thickness of the cuticle-like layer as a potentially important trait.

Studies on foliar cuticles suggest that there is no correlation between cuticle thickness and water permeability (Riederer & Schreiber, 2001). The amount of cutin is not necessarily an indication of cuticular water permeability (Isaacson et al., 2009), while cuticular waxes contribute largely to the cuticle-mediated resistance to water diffusion, for instance c. 95% in the case of tomato fruit (Leide et al., 2007). Alkanes and other more nonpolar components tend to be associated with decreased cuticular water permeability, while nonaliphatic wax compounds, such as triterpenoids, are probably a less effective water barrier (Buschhaus & Jetter, 2012; Leide et al., 2007). We found that species with a thicker cuticle-like layer had more chemical components with less than 20 carbon atoms, while there was no significant difference in possibly waxy composition (constitution with more than 20 carbon atoms) among the four tested species (Figure 3). Since water loss rate tends to decrease with increased thickness of the cuticle-like layer (Figure S6), both composition and thickness influenced the function of the cuticle-like layer in preventing water loss in aerial roots, suggesting the rate of water loss possibly depends more upon the thickness of the layer. This would make our results about aerial roots surprisingly different from previous leaf- and fruit-based studies.

Key role of root hair, aquaporin and cuticle-like layer peeling for “passable zone” of aerial root

Most roots of bark epiphytes are tightly attached to the bark of a host tree. Parts of these roots are exposed to air, other parts are in contact with bark and develop root hairs (Groom, 1893; Muthukumar & Ayyasamy, 2017; Ponert et al., 2016; Pridgeon, 1987; Stern & Judd, 1999). Although the function of root hairs has been little studied in epiphytes, candidate roles include effective attachment to the host tree, nutrient acquisition, and water uptake (Pridgeon, 1987; Zotz, 2016). We found that the roots of epiphytic orchids developed root hairs on the outermost layer cells of velamen when they attached to bark (Figure 7 a-f, Figure 8 b). At the same time, gene expression related to cutin and wax biosynthesis were down-regulated in the root part close to bark (Figure 4). These results suggest that the development of root hairs may compensate for the part of the root that is covered by the cuticle-like layer, which repels water, by increasing water absorption from bark.

After water is absorbed by root hairs, it is transported in roots through both cell-to-cell and apoplastic pathways. The cell-to-cell route is symplastic through plasmodesmata and via a transcellular path involving the crossing of membranes. Water movement through cell membranes is facilitated by aquaporins (Maurel, 2007), which belong to the family of major intrinsic proteins (MIPs) (Hachez, Moshelion, Zelazny, Cavez, & Chaumont, 2006; Hachez, Zelazny, & Chaumont, 2006; Katsuhara, Hanba, Shiratake, & Maeshima, 2008). When roots come into contact with the bark, genes related to aquaporin are significantly up-regulated after watering (Figure 9). This suggests that the entry of water into roots through root hair is facilitated by aquaporins.

Cuticle-like layer peeling—the shedding of the cuticle-like layer from the surface of aerial roots—was detected in *A. rigida* and *P. teres*, which both have *Vanda* type velamen (unpublished observation with reference to Figure S7 a-f). Thus, when the roots get longer, the older part of roots obtain the ability to absorb water as a result of cuticle-like layer peeling; this may change the part of roots from the “control zone” to the “passable zone”. It is currently unknown how widespread this phenomenon is among orchids, and we can only speculate about the underlying mechanism and functional consequences.

Discovery of the cuticle-like layer of aerial roots is an opportunity to understand the plant cuticle

Plant cuticles are not homogeneous, but typically divided into two main parts based on their chemical composition (Fich et al., 2016). The inner layer adjacent to the outer epidermal cell wall is mainly composed of cutin, although how cutin interacts with cell wall polysaccharides remains unresolved. Analyses of cuticles using chemical stains and enzyme treatments have identified a layer rich in pectins and cellulase-susceptible glycans at the interface between cutin and polysaccharides (Jeffrey, 2006; Lendzian, Nakajima, & Ziegler,

1986). Evidence from cuticles of fruit suggests that the linkage between cutin and polysaccharides is achieved via cutin acids and sugars (Fang et al., 2001; Tian et al., 2008). Several models have been developed to explain this tight association (Agullo, Collar, & Seoane, 1984; Tian et al., 2008). The “peeling phenomenon” of the cuticle-like layer observed in *A. rigida* and *P. teres* indicates that this layer does not tightly interact with cell walls or is linked to cell wall polysaccharide in a completely different way. However, such the “peeling phenomenon” was not found in the roots of species with *Cymbidium* and *Dendrobium* type velamen (Figure S7 a-f). One possible explanation for this is that clades differ in how the cuticle-like layer is connected to the cell wall. Thus, further investigations into the cuticle-like layer in aerial roots may offer an opportunity to advance our understanding of the connection between the cuticle and cell wall.

Waxes are abundant in the outer layer of cuticles, and are either deposited within a cutin matrix as intra-cuticular wax or accumulated on the outermost surface as epi-cuticular wax (Lee & Suh, 2013). Epi-cuticular waxes exhibit great micromorphological diversity, (e.g., arranged in continuous layers or as solitary crystalloids). Crystalloids vary characteristically in size and shape (Porembski & Barthlott, 1988). This diversity contributes to different wettability. For example, when drop contact angle is used to quantify wettability, surfaces with crystalloid tubules shapes have larger angles than those with platelets and crust shapes, i.e. water repellency is highest in the presence of tubule-shaped crystalloids (Muhammad, Wuyts, Nuyts, de Wael, & Samson, 2020). The papillae-shaped crystalloid has a strong hydrophobic enhancement, and sheet structure helps achieve super hydrophobicity and self-cleaning activities (Guan, Feng, Zhang, Niu, & Han, 2019). We found wax crystals on the outermost surface of the cuticle-like layer of all epiphytic orchids, and the shape of wax crystals varied from plate-like in species with a relatively thick cuticle-like layer to granular in species with a thin cuticle-like layer (Figure 2 a-r). However, it is currently not possible to establish an unambiguous link between the limited observations of our study to an ecologically relevant function, (i.e., ability of wettability).

The cuticle-like layer was not found on the root surface of terrestrial *C. goeringii* (Figure S3). Previous studies have reported a reduction of the velamen thickness in terrestrial *Cymbidium* (Li, Chen, Hu, Ma, & Zhang, 2018; Stern & Judd, 2002). In *Cymbidium*, the epiphytic or lithophytic habitat is the ancestral state, whereas the terrestrial habit is derived. The loss of the cuticle-like layer and thinning of velamen may have contributed to more efficient water utilization and successfully adaptation to terrestrial habitats. When we think about the cuticle-like layer from an evolutionary perspective, many questions are need to be answered, such as whether terrestrial roots in *Cymbidium* have totally lost the ability to produce cuticle-like layers, and if so, whether the loss coincides with the return to terrestrial habitats. In the future, the cuticle-like layer should be considered when studying life form evolution in Orchidaceae.

Conclusions

Anatomical observations, determination of chemical composition and expression of genes involved in cuticle biosynthesis consistently suggest that the outermost layer covering velamina of epiphytic orchids is comparable to a cuticle. The thickness of this layer is species-specific, and aerial roots with thinner velamen tended to have a thicker cuticle-like layer. We also observed wax crystals on the outer surface of this layer. The chemical composition of aerial root surfaces was quite similar to that of foliar cuticles, while there were no significant species differences in waxy components. Although a cuticle-like layer dramatically slowed water absorption, development of root hairs on the ventral parts of the velamen in contact with bark helped to absorb water. Thus, the velamen of aerial roots in epiphytic orchids can solve the conflicting functional requirements — assisting water absorption and reducing water loss — through “functional zoning”. More than 20,000 epiphytic species of orchids (Zotz, 2013) are evidence of their outstanding ability to thrive in water-limited condition in tree crowns, and “functional zoning” in velamentous roots is yet another piece of the puzzle in the understanding of the actual mechanisms.

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Author Contributions

J-W L, H-P X, S-B Z and J-L Z designed research; J-WL and H-P X conducted experiments; J-WL, G Z, S-B Z and J-L Z analyzed data; J-WL, G Z, S-B Z and J-L Z wrote the manuscript; all authors read and approved the manuscript.

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Figures and Tables

Figure 1 Cross sections of aerial roots (a, c, e, g, i, k) and details of the cuticle-like layer (b, d, f, h, j, l) for six epiphytic orchid species with three types of velamen. Blue arrows indicate the cuticle-like layer.

Figure 2 Scanning electronic microscopy (SEM) images at three different magnifications of the surface of aerial roots of *Cymbidium tracyanum* (a, b, c), *Acampe rigida* (d, e, f), *Papilionanthe teres* (g, h, i), *Rhynchostylis retusa* (j, k, l); *Dendrobium chrysotoxum* (m, n, o) and *D. thyrsiflorum* (p, q, r). A scale is given for each column in the uppermost image.

Figure 3 Chemical constitution of cuticle-like layer from aerial roots of *Cymbidium tracyanum* (Ct), *Acampe rigida* (Ar), *Papilionanthe teres* (Pt) and *Dendrobium chrysotoxum* (Dc) and of foliar cuticles of *C. tracyanum* and *A. rigida* . “< C20” represents the total chemical components with less than 20 carbon atoms. “[?]C20” represents total chemical components with equal to or more than 20 carbon atoms. Data are means +- SD for three measurements. Different letters above bars indicate significant differences in “< C20” or “[?]C20” of aerial roots between species (p < 0.05, based on one-way ANOVA, followed by Tukey’s post hoc tests for comparison). Intraspecific differences in chemical coverage between leaves and roots were assessed with independent-sample t tests (n.s. p > 0.05; *p < 0.05; **p < 0.01).

Figure 4 Differentially expressed genes related to cutin and wax biosynthesis between the “aerial portion” and the “bark portion” of *Cymbidium tracyanum* root tips after bark attaching treatment. All of the “aerial portion” of root tips interact with air; most of the “bark portion” is attached to bark.

Figure 5 Water loss curve for roots from five epiphytic species, *Acampe rigida* (a), *Rhynchostylis retusa* (b), and *Papilionanthe teres* (c), *Dendrobium thyrsiflorum* (d), and *Cymbidium tracyanum* (e); one terrestrial *C. goeringii* (f). Data are means +- SD for 6 – 10 individuals.

Figure 6 Water repellency behavior on velamen from epiphytic orchids *Cymbidium tracyanum* (a), *Acampe rigida* (b), *Papilionanthe teres* (c), *Rhynchostylis retusa* (d), and *Dendrobium thyrsiflorum* (e); three enlarged images from left to right indicate the droplet standing time was 5, 10, and 30 min. Strong wettability of velamen from terrestrial *C. goeringii* (f); two enlarged images from left to right indicate that standing time was 30s and 1 min.

Figure 7 The plasticity of velamen interacting with air or in contact with bark from x-ray computerized tomography scans. General 3D image (a, d); 3D image of velamen and velamen attached to bark (b, e); and reconstructed 2D transverse section of the velamen and velamen attached to bark (c, f). Solid blue arrows

indicate velamen interacting with air. Hollow blue arrows indicate velamen in contact with bark and the development of root hairs.

Figure 8 Expression heat maps for bHLH transcription factor (a) and EXPANSIN (c) involved in development of root hair. Yellow or blue represents up-regulation or down-regulation. The scale bar represents the \log_2 FoldChange in differentially expressed genes. Anatomical observations of root hair development as velamen contacts bark (b).

Figure 9 Differentially expressed aquaporin-related genes in *Cymbidium tracyanum*. Differentially expression was detected by comparing gene expression in the “bark portion” of roots tip contacting bark with high water content (after watering 2 h) and low water content (at the peak of vapor pressure deficit).

Figure 10 Diagrammatic representation of the proposed “functional zoning” of velamen in epiphytic orchids.

Table 1 Anatomical traits of velamen and cuticle layer of *Cymbidium tracyanum* with *Cymbidium* type velamen; *Acampe rigida*, *Papilionanthe teres*, and *Rhynchostylis retusa* with *Vanda* type velamen; *Dendrobium chrysotoxum* and *D. thyrsiflorum* with *Dendrobium* type velamen. Different letters indicate significant differences in parameters of aerial roots among species using one-way ANOVA, followed by Tukey’s post hoc tests for comparison ($p < 0.05$).

Complementary information

Figure S1 Diurnal changes in vapor pressure deficit and bark water content during experiment.

Figure S2 Bark attaching treatment on *Cymbidium tracyanum* and *Acampe rigida*.

Figure S3 Cross sections of roots and root surface for terrestrial *Cymbidium goeringii*.

Figure S4 Correlation between thickness of cuticle-like layer and velamen.

Figure S5 Residual stains of water drop on the root surface on *Acampe rigida*, *Dendrobium thyrsiflorum*, *Cymbidium tracyanum*, *Papilionanthe teres*, *Rhynchostylis retusa* and *C. goeringii*.

Figure S6 Correlation between thickness of cuticle-like layer and proportion of water loss after 100 h.

Figure S7 Cross sectional observations of velamen with cuticle-like layer “peeling phenomenon” (a, b, c, d); direct observation of cuticle-like layer peeling (e, f).

Table S1 Experiments in selected species.

Table S2 Differentially expressed genes related to cutin and wax biosynthesis between root tip of *Cymbidium tracyanum* and *C. goeringii*.

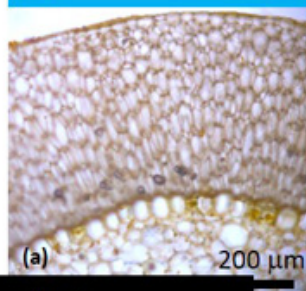
Table 1 Anatomical traits of velamen and cuticle layer of *Cymbidium tracyanum* with *Cymbidium* type velamen; *Acampe rigida*, *Papilionanthe teres*, and *Rhynchostylis retusa* with *Vanda* type velamen; *Dendrobium chrysotoxum* and *D. thyrsiflorum* with *Dendrobium* type velamen. Different letters indicate significant differences in parameters of aerial roots among species using one-way ANOVA, followed by Tukey’s post hoc tests for comparison ($p < 0.05$).

Species	ὕψος-λίκε λαψερ τηςκνεσς (μμ)	ἐλαμεν τηςκνεσς (μμ)	Cuticle-like layer thickn
<i>Cymbidium</i> type			
<i>C. tracyanum</i>	7.5±2.7 ^a	696.2±86.4 ^a	1.1±0.4 ^a
<i>Vanda</i> type			
<i>A. rigida</i>	24.4±6.7 ^{bc}	119.4±12.2 ^{bc}	20.4±5.6 ^b
<i>P. teres</i>	13.1±5.6 ^{ac}	88.1±7.7 ^b	14.9±6.4 ^b
<i>R. retusa</i>	25.3±10.9 ^{bc}	150.0±30.7 ^b	16.8±7.2 ^b
<i>Dendrobium</i> type			
<i>D. chrysotoxum</i>	17.4±3.9 ^c	291.1±38.8 ^d	6.0±1.3 ^a

Species	ὀτιςλε-λικε λαψερ τηικνεος (μμ)	ἐλαμεν τηικνεος (μμ)	Cuticle-like layer thickness
<i>D. thyrsoflorum</i>	18.6±8.0 ^{bc}	689.0±17.0 ^a	2.7±1.2 ^a

Cymbidium type

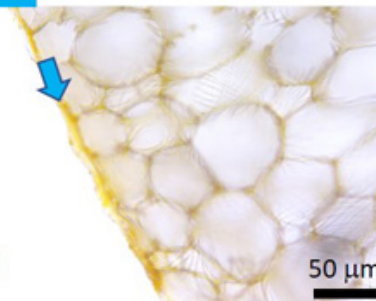
Cymbidium tracyanum



(a)

200 μm

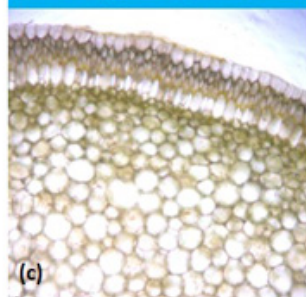
(b)



50 μm

Vanda type

Acampe rigida



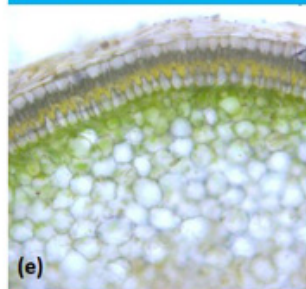
(c)

(d)



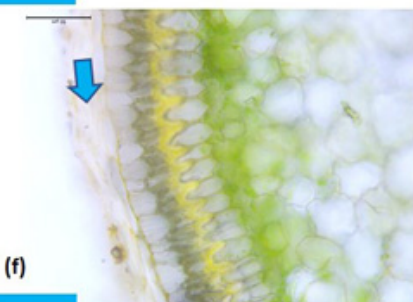
50 μm

Papilionanthe teres



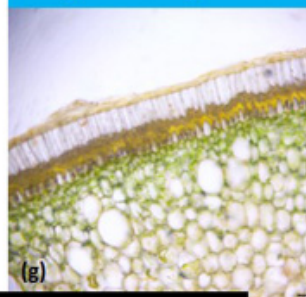
(e)

(f)



50 μm

Rhynchostylis retusa



(g)

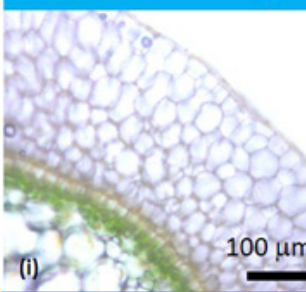
(h)



50 μm

Dendrobium type

Dendrobium chrysotoxum

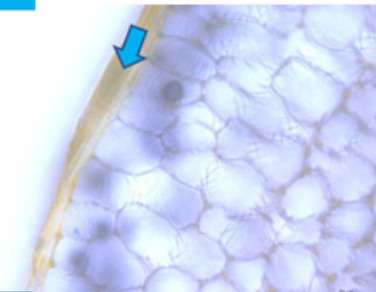


(i)

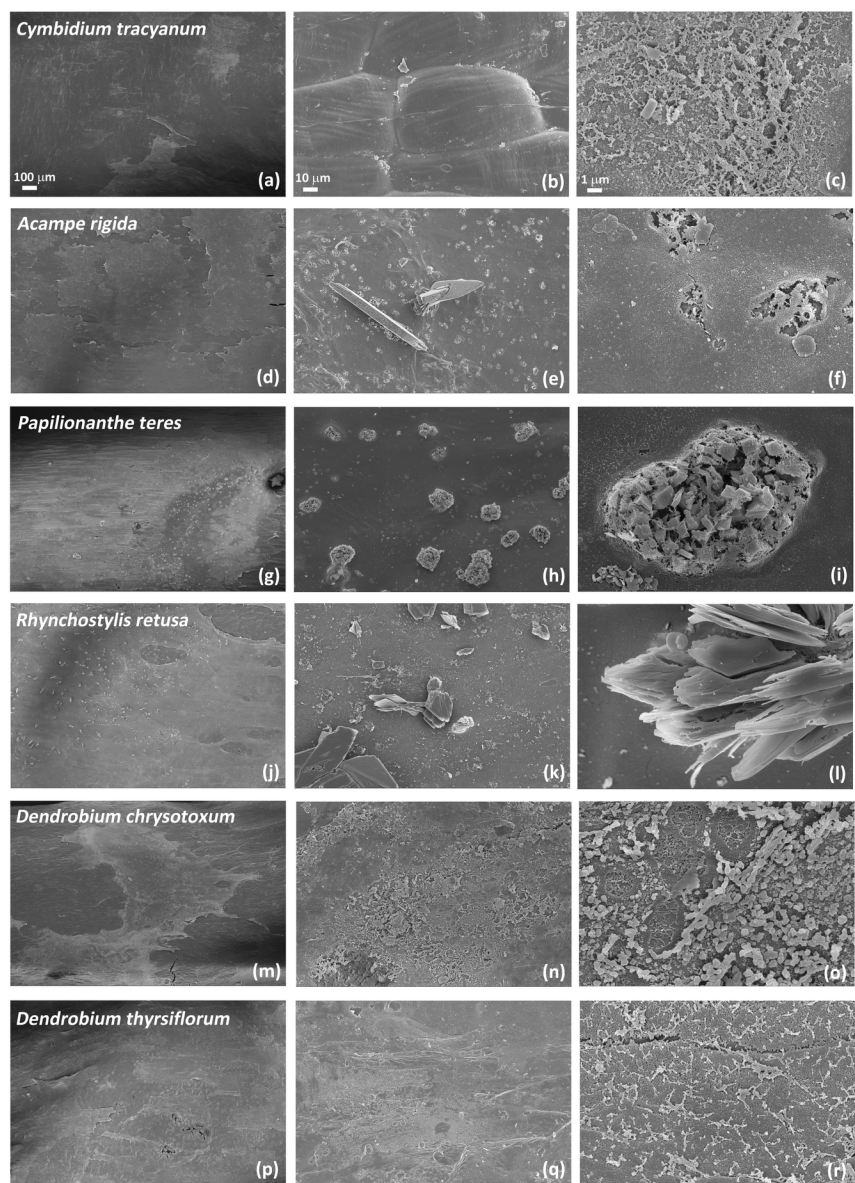
100 μm

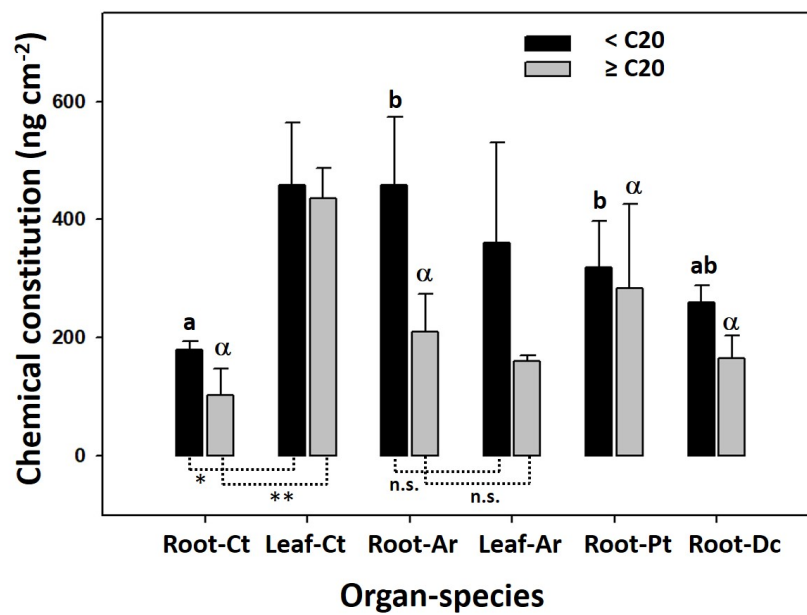
17

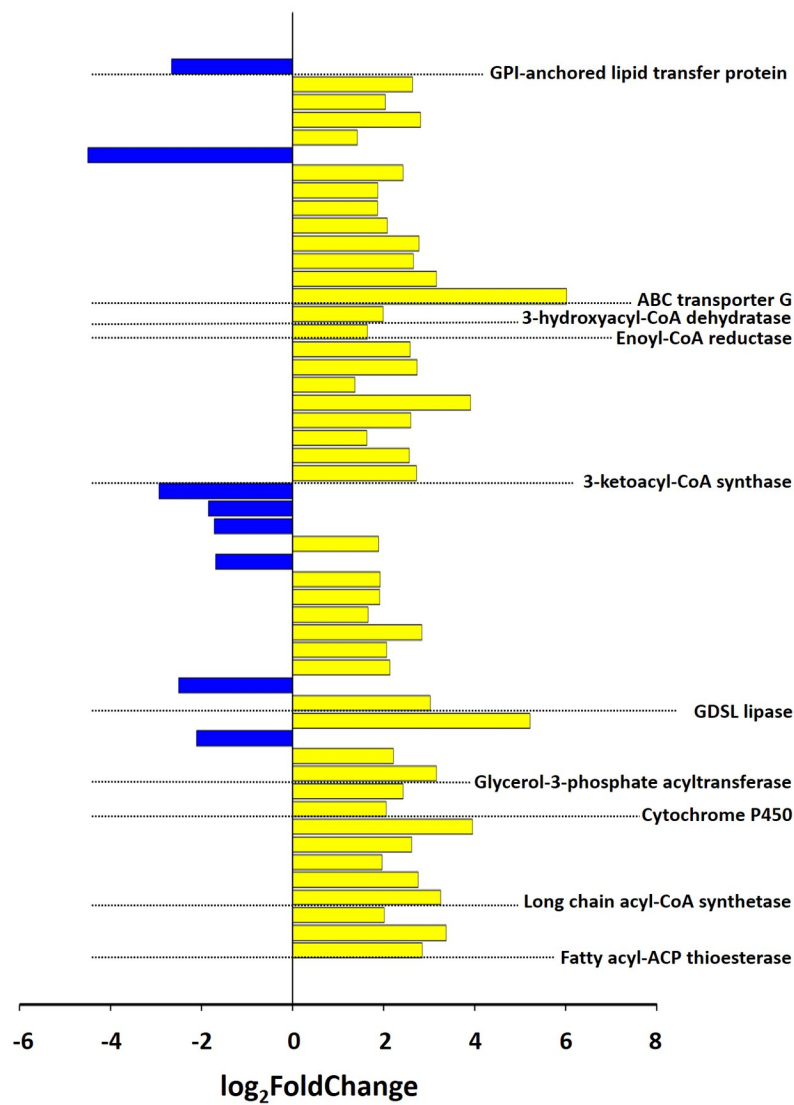
(j)

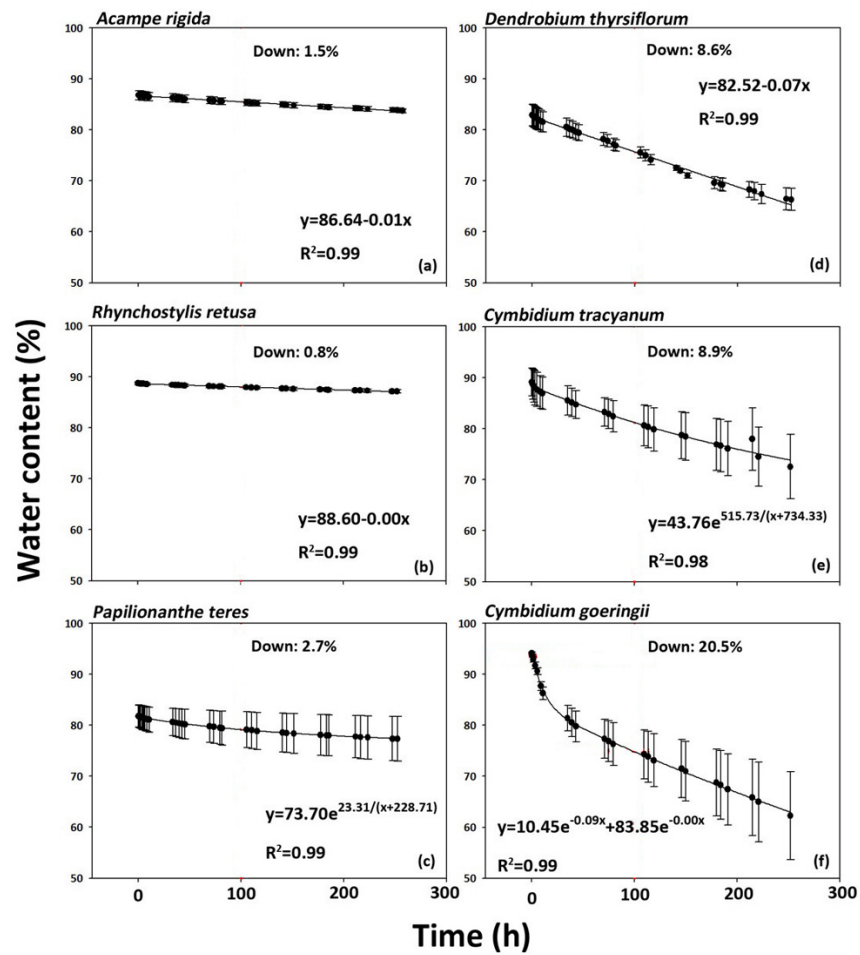


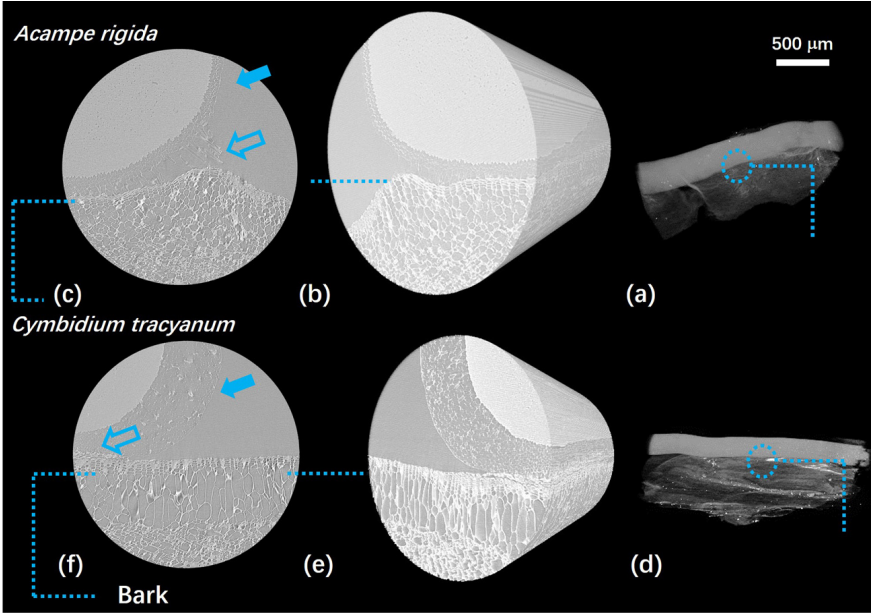
50 μm









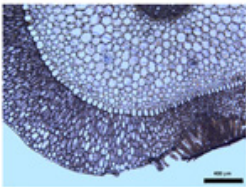


bHLH transcription factor

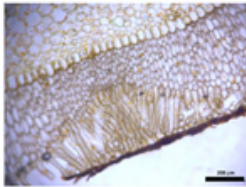
- Cluster-41504.0
Transcription factor bHLH87-like isoform X1
- Cluster-37657.46025
Transcription factor bHLH19-like
- Cluster-37657.17685
Putative transcription factor bHLH086
- Cluster-37657.120858
Transcription factor bHLH110
- Cluster-37657.15282
Transcription factor bHLH30
- Cluster-37657.17901
Transcription factor bHLH147-like
- Cluster-37657.31036
Transcription factor bHLH149-like
- Cluster-37657.23893
Transcription factor bHLH53-like
- Cluster-37657.22585
Transcription factor bHLH113
- Cluster-37657.28934
Putative transcription factor bHLH041
- Cluster-37657.21071
Transcription factor bHLH96
- Cluster-37657.19179
Transcription factor bHLH128-like
- Cluster-37657.57767
Transcription factor ILR3-like
- Cluster-37657.39416
Transcription factor bHLH155-like isoform X1
- Cluster-37657.73681
Transcription factor bHLH19-like

Aerial portion vs. Bark portion

(a)



(b)



Expansin

(c)

- Cluster-37657.137449
Expansin-A16
- Cluster-37657.67453
Expansin-A10-like
- Cluster-37657.82415
Expansin-B16-like
- Cluster-37657.70342
Expansin-A4
- Cluster-37657.51545
Expansin-A10



