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Physiological roles of Casparian strips and suberin in the transport of water and solutes

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Summary

- The formation of Casparian strips (CS) and the deposition of suberin at the endodermis of plant roots are thought to limit the apoplastic transport of water and ions. We investigated the specific role of each of these apoplastic barriers in the control of hydro-mineral transport by roots and the consequences on shoot growth.
- A collection of Arabidopsis thaliana mutants defective in suberin deposition and/or CS development was characterized under standard conditions using a hydroponic system and the Phenopsis platform.
- Mutants altered in suberin deposition had enhanced root hydraulic conductivity, indicating a restrictive role for this compound in water transport. In contrast, defective CS directly increased solute leakage and indirectly reduced root hydraulic conductivity. Defective CS also led to a reduction in rosette growth, which was partly dependent on the hydro-mineral status of the plant. Ectopic suberin was shown to partially compensate for defective CS phenotypes.
- Altogether, our work shows that the functionality of the root apoplastic diffusion barriers greatly influences the plant physiology, and that their integrity is tightly surveyed.

Introduction

As sessile organisms, plants strongly depend on the ability of their root system to cope with variable and possibly stressing soil conditions. Roots have evolved as plastic organs (Gruber et al., 2013), able to operate water and nutrient uptake in a wide spectrum of conditions going from deficiency to excess or toxicity. This capacity depends on multiple mechanisms, including the tuning of root system architecture, the regulation of membrane transporters and channels (Maurel et al., 2015), as well as alterations in root anatomical structures, such as the exodermis and endodermis (Liska et al., 2016). The radial transport of water and solutes from the soil to the root xylem vessels is considered as a major site of control (Steudle & Peterson, 1998). Radial transport can occur through the nonselective apoplastic pathway, or from cell-to-cell whereby membrane transporters and channels exert variable resistance and selectivity (Maurel et al., 2015). In contrast to the exodermis, which is lacking in certain species such as Arabidopsis thaliana (Arabidopsis), the endodermis is ubiquitous among the angiosperms and acts as the main apoplastic barrier. Two types of structures can be found in the fully developed endodermis: Casparian strips (CS) and suberin lamellae. The CS form a longitudinal belt encircling all endodermal cells, while suberin lamellae covers them, except for passage cells (Andersen et al., 2018). The CS result from a coordinated and localized impregnation of the primary cell wall by lignin (Naseet et al., 2012). This process is regulated by the MYB36 transcription factor (Kamiya et al., 2015), and its integrity surveyed by the SCHENGEN3/CIF1&2 receptor/ligand complex (Doblas et al., 2017b; Nakayama et al., 2017). Suberin is a heterogeneous biopolymer primarily composed of aliphatic monomers and some minor aromatic moieties. It is deposited around endodermal cells, eventually coating the entire endodermal cell surface (Haas & Carothers, 1975) and progressing into a suberized periderm from the pericycle (Campilho et al., 2020). Suberin is thought to create a diffusion barrier for water, gases and solutes (Enstone et al., 2002; Franke et al., 2012).

The function of endodermal CS and suberin as barriers to the radial transfer of water and solutes has been extensively questioned, notably over the last 25 yr (Peterson et al., 1993; Steudle et al., 1993; Frensch et al., 1996, and references cited therein; for recent reviews see: Geldner, 2013; Nawrath et al., 2013; Doblas et al., 2017a). A brief historical overview shows, however, that this issue is not settled yet. In 1993, mechanical disruption of maize endodermis led authors to conclude that this cell layer is not a barrier to water but to solutes, although roots were not...
Research et al. In 1996, Frensch et al. concluded, also from studies in maize roots, that the suberized endodermis impedes both solute and water flow (Frensch et al., 1996). However, no clear distinction between the role of suberin and CS was made until 2000, when it was shown that suberin is a barrier to water while CS influence solute transport in maize roots (Zimmermann et al., 2000). But this conclusion was restricted to root exodermis while no role of the endodermis was found in this study. The role of suberin as a barrier to root water transport was assessed later through genetic alteration of its deposition or composition in the horst-1 and esb1 mutants (Ranathunge & Schreiber, 2011). At this time though, the esb1 mutant was characterized for an enhanced suberin accumulation at the endodermis (Baxter et al., 2009), and it was only later that its primary defect in CS formation was established (Hosmani et al., 2013; Pfister et al., 2014; Li et al., 2017). Thorough phenotypic characterization of sgn3 mutants also pointed to the specific role of CS in altering water and solute relations (Pfister et al., 2014). However, we added to this representation that hydraulic alterations can be induced through a signaling process induced by damaged CS towards aquaporin activity (Wang et al., 2019). Therefore, a comprehensive study addressing the impact of CS and suberin on both water and solute transports at the root level would help integrate, and possibly reconcile, current knowledge. Such a study has become possible thanks to the large collection of CS and suberin-defective mutants which is now available.

Due to the importance of CS and suberin in the control of plant water transport and mineral nutrition, variations in these structures are expected to impact shoot development. Accordingly, Baxter et al. (2009); Hosmani et al. (2013); Pfister et al. (2014); Kamiya et al. (2015); Barberon et al. (2016) and Reyt et al. (2020) showed that genotypes with CS and/or suberin disorders have both alterations in shoot ionome and shoot development. However, the causal links between modifications of the endodermal barriers and changes in shoot growth still hold many questions. In particular, are growth alterations due to disorders in water supply and/or ion provision?

The objectives of the present study were (1) to clarify the specific roles of the CS and endodermal suberin in water and/or solute transport in roots, and (2) to assess their long-term effects on shoot growth and development. For this purpose, we used Arabidopsis wild-type (WT) and a group of 17 Arabidopsis mutants that we classified into five distinctive groups based on standard CS and suberin characterizations.

Materials and Methods

Hydroponic experiments

Plant materials and growth conditions Arabidopsis accession Col-0 and 17 mutants with defects in CS and/or suberin (Supporting Information Table S1), were surface sterilized and sowed into clear polystyrene culture plates containing a 1/2 Murashige and Skoog (MS) medium (Sigma-Aldrich, St Louis, MO, USA). Plates were kept for 2 d at 4°C, then incubated vertically for 10 d under environmentally-controlled conditions: 60% relative humidity, 16 h d⁻¹ of 250 µmol photons m⁻² s⁻¹, 20°C. Plants were then transferred on 35 cm × 35 cm plastic plates floating over a basins filled with 81 of hydroponic solution (1.25 mM KNO₃, 0.75 mM MgSO₄, 1.5 mM, Ca(NO₃)₂, 0.5 mM K₂HPO₄, 50 µM FeEDTA, 50 µM H₂BO₃, 12 µM MnSO₄, 0.70 µM CuSO₄, 1 µM ZnSO₄, 0.24 µM MoO₃N₂H₂, 100 µM Na₂SiO₃). Physiological and molecular determinations were done after 10–11 d of hydroponic culture (i.e. on 20–21 d-old plants). Previously unpublished mutants are T-DNA insertions provided by the Nottingham Arabidopsis Stock Centre (NASC): gelp51-2, GK_016A11; anac038-1, SALK_103716; anac038-2, WiscDsLoxH5007-11H (Fig. S1).

Casparian strip permeability and suberin quantification Propidium iodide (PI) staining was performed as previously described (Allassime et al., 2010) on 21 d-old plants with a 1 h incubation period. Root suberin was extracted and quantified using gas chromatography following the procedures explained in Franke et al. (2005). Despite Fluorol Yellow being the standard dye for suberin staining, we faced issues when working with more mature and bigger root systems. Auramine O staining was therefore performed instead, as described in Ursache et al. (2018), on 21 d-old plants.

Root hydraulics and root balancing pressure Root hydraulic conductance (K) was determined in de-topped plants using a set of pressure chambers filled with hydroponic solution (Boursiac et al., 2005). Excised roots were subjected to 350 kPa for 10 min, followed by successive measurements at 320, 160, and 240 kPa. The value of Kᵣ was calculated as the slope of the flow to pressure relationship. The hydrostatic hydraulic conductivity (Lpᵣ,₀) was calculated by dividing Kᵣ by the root dry weight. Osmotic hydraulic conductivity (Lpᵣ,₀) was determined using the free-exudation method. The plants were de-topped with a razor blade and the sectioned hypocotyl immediately introduced into a 100 µl micro capillary. Dental paste (Colïène/Whaledent s.a.r.l., Lezennes, France) was used to ensure a proper seal between the hypocotyl and the capillary. The sap exuded for the first 10 min was discarded, and the sap exuded over the next 45 min was collected and analyzed. Its osmolality (as well as osmolality of the bath medium) was measured using a Vapro 5520 osmometer (Wescor, Logan, UT, USA). The value of Lpᵣ,₀ was obtained by dividing the exudation rate by the root dry weight and osmotic potential gradient between the exuded sap and the bath. The contribution of the aquaporin-related pathway to Lpᵣ was tested by the application of 1 mM sodium azide (NaN₃), a plant respiration inhibitor known to induce the gating of aquaporins (Tournaire-Roux et al., 2003).

The passive leakage of solutes into the root was approximated by determining the root balancing pressure (Pᵣ₀) after 1 h treatment with 100 mM sodium chloride (NaCl). The value of Pᵣ₀ is the extrapolated intercept with the pressure axis (Pᵣ = 0) of the flow/pressure relationship obtained from pressure chamber measurements. It is related to the selectivity of the root to solutes, or reflection coefficient σᵣ, as explained in Boursiac et al. (2005).
Active solutes pumping by root cells, which is responsible for the free exudation of the root, may impair this measurement by mimicking solutes leakage (Knipfer & Fricke, 2010). However, we usually observed a free exudation in the range of $1/20^{th}$ to $1/30^{th}$ of the flow obtained under pressurization, making its influence negligible.

**Total RNA isolation and aquaporin expression** RNA was isolated from 30 to 50 mg frozen roots using the RNA Isolation Kit Z3100 and DNase kit from Promega (Promega Corp., Madison, WI, USA). Total RNA was quantified by optical density measurements at 280/260 nm and stored at $-80^\circ$C until use. Transcript abundance was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR), using the sequences of primer pairs corresponding to the 13 Arabidopsis PIPs described in Sutka et al. (2011). For each gene, relative quantification was made by the Delta cycle threshold method with correction for PCR efficiency. The references genes tested were those described in Sutka et al. (2011), of which TIP41-like, PP2A3, and SFP were selected as the most stable ones among the different mutants.

**Phenopsis experiment conditions**

**Plant material and growth conditions** Plant phenotyping was realized using the Phenopsis platform (Granier et al., 2006). Seeds were surface sterilized and sown in pots as prescribed. The Plant material and growth conditions used in the Phenopsis experiment are described in Table S1. The 13 Arabidopsis PIPs described in Sutka et al. (2011). For each gene, relative quantification was made by the Delta cycle threshold method with correction for PCR efficiency. The references genes tested were those described in Sutka et al. (2011), of which TIP41-like, PP2A3, and SFP were selected as the most stable ones among the different mutants.

**Physiological determinations** Five weeks after sowing, the rosettes (which were close to bolting stage, $n=7$) were detached from the root and were weighted prior to and after oven-drying at $65^\circ$C for 72 h, to determine their fresh weight (FW) and dry weight (DW).

Leaves were frozen at $-20^\circ$C for 2 d, pending extraction of the cellular medium with a centrifuge (8 min $\times$ 1000 g). Next, 10 µl of the extract was transferred to an absorbent paper disc and measured using a vapor pressure osmometer (Vapro 5520; Wescor).

Whole plant transpiration was determined in seven plants with the soil covered by a plastic sheet to prevent evaporation (Granier et al., 2006). Pot weights were monitored six times a day during 2 d. Day/night transpiration per unit rosette area was determined as the slope of the pot weight loss over time (g cm$^{-2}$ h$^{-1}$). The rosette area was calculated based on the photographs taken at the end of the transpiration period.

**Shoot elemental analysis** Briefly, 30 mg of ground dried tissue from young and old leaves of five plants per treatment and genotype ($n=5$), were digested in 1 ml of 48.75% nitric acid (HNO$_3$) and 7.5% hydrogen peroxide (H$_2$O$_2$) in a quartz tube at $110^\circ$C for 2 h. Cations were determined with an atomic absorption spectrophotometer (SpectrAA 220; Varian, Palo Alto, CA, USA). Results were expressed in mg g$^{-1}$ DW.

**Statistical analyses**

All data, except for the regression analyses, were analyzed using one-way ANOVA with the R software (R Core Team, 2020). Tukey’s post hoc adjustment was used to test mean differences between treatments at $x=0.05$. Spearman’s correlation analyses were performed in order to elucidate the relations between Lp, and aquaporin expression.

For the experiment at the Phenopsis platform, plants were set-up in a random block design that was analyzed using a two-way ANOVA together with Tukey’s adjustment at $x=0.05$ with R.

**Results**

**Presentation of the mutant collection**

In order to study the physiological role of CS and suberin while avoiding pitfalls related to single-mutant studies, we gathered a collection of Arabidopsis mutants that aimed at covering various combinations of alterations. The chosen genotypes were (Table S1): (1) mutants known for their alterations in CS and the formation of ectopic suberin: myb36-1 and myb36-2 (Kamiya et al., 2015), esb1-1 and esb1-2 (Baxter et al., 2009), casp3-1 casp3-1 (Roppolo et al., 2011; Pfister et al., 2014). (2) Mutants with altered CS and with unaffected suberin content: sgn3-3 esb1-1, sgn3-3 (Pfister et al., 2014; Wang et al., 2019), and sgn3-4 (Tsuwamoto et al., 2008). (3) Mutants with reduced or altered suberin but no information on the functionality of CS: horst-1 and horst-2 (Hofer et al., 2008), and the double mutant horst-1 ralph-1 (present work). We added to this group a plant line expressing the CDEF1 cutinase under the control of the CASP1 promoter (pCASP1::CDEF1), which has reduced content in suberin but no functional CS (Naseer et al., 2012). (4) Mutants with altered suberin composition and no differences in total suberin content: ralph-1 and ralph-2 (Compagnon et al., 2009). (5) A set of new, not yet described mutants, with potentially modified suberin content and unknown properties of the CS (present work). These included gelp51-2, an insertion mutant in a GDSL-type esterase/lipase family (GELP), and anac038-1 and anac038-2, insertion mutants in a NAC transcription factor gene family member. Genes encoding the latter mutants were identified based on strong in silico coexpression using suberin biosynthetic genes such as RALPH as a ‘bait’ in the ATTEDII analysis tool (Obayashi et al., 2007) and in silico expression data showing a expression in the root endodermis (Fig. S1).

**Casparian strip mutants maintain barrier defects in mature root systems**

The functionality of the CS was assessed through monitoring of PI penetration into the stele (Alassimone et al., 2010). PI diffuses through the apoplast where it binds to the carboxyl groups of the cell wall homogalacturonans (Rounds et al., 2011) and thereby

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stains the vessels if not blocked by the CS. Assays with PI on 7 d-old seedlings have previously revealed a defective CS in myb36-1 and myb36-2 (Kamiya et al., 2015), esb1-1 and casp1-1/casp3-1 (Baxter et al., 2009; Hosmani et al., 2013), and sgn3-3 (Pfister et al., 2014). We performed our experiment on more mature and complex root systems of 21 d-old plants grown for 10 d in vitro and 11 d in hydroponics. Observations were made at various zones along the root: zone of first root hairs formation, lateral root primordia (LRP) at stages I and II, first lateral root emergence, an intermediate zone, and a zone close to the base, on the primary root and lateral roots (Figs 1a, S2). While xylem or proto-xylem vessels were stained by PI in the root hairs zone in almost all genotypes, no more staining could be observed after proto-xylem vessels were stained by PI in the root hairs zone in zones along the root: zone of first root hairs formation, lateral (Baxter et al., 2009; Hosmani et al., 2013), and sgn3-3 (Pfister et al., 2014). We performed our experiment on more mature and complex root systems of 21 d-old plants grown for 10 d in vitro and 11 d in hydroponics. Observations were made at various zones along the root: zone of first root hairs formation, lateral root primordia (LRP) at stages I and II, first lateral root emergence, an intermediate zone, and a zone close to the base, on the primary root and lateral roots (Figs 1a, S2). While xylem or proto-xylem vessels were stained by PI in the root hairs zone in almost all genotypes, no more staining could be observed after proto-xylem vessels were stained by PI in the root hairs zone in zones along the root: zone of first root hairs formation, lateral root primordia (LRP) at stages I and II, first lateral root emergence, an intermediate zone, and a zone close to the base, on the primary root and lateral roots (Figs 1a, S2). Additional staining could be observed at the corners of the endodermal cells in myb36-1 and esb1-1 (Fig. 1c), which may relate to the deposition of ectopic cell wall, as observed in Kamiya et al. (2015). Noticeably, this ectopic cell wall material does not restore the impermeability of the stele to PI. Altogether, our analyses indicate that all the genotypes have the same high PI permeability at the root hairs zone and low PI permeability after the periderm formation. They differ in between stages I and II lateral root and intermediate zones, where the CS become fully impermeable to PI – thereafter considered as ‘functional’ – for all genotypes besides myb36, esb1, casp1/casp3, sgn3 and sgn3 esb1.

Suberin quantity and/or development in the mutant collection

Quantitative chemical analysis of suberin in 21 d-old hydroponically grown plants of myb36-1, myb36-2, esb1-1, esb1-2 and casp1/casp3-1, that are mutants with ectopic suberin, confirmed an approximate 1.9-fold increase in their root content with respect to Col-0, while sgn3-3 esb1-1 and sgn3-3 were similar to Col-0 (Fig. 1d). The group composed of gelp51-2, anac038-1 and anac038-2 was characterized by an increase in total root suberin content by 30 to 50% compared to Col-0. Within the group with unaltered CS, horst-1, horst-2, horst-1 ralph-1, and horst-1 ralph-1 gathered genotypes with disrupted CS and similar suberin content by 30 to 50% compared to Col-0. Within the group with unaltered CS, horst-1, horst-2, horst-1 ralph-1, and horst-1 ralph-1 gathered genotypes with disrupted CS and similar suberin content by 30 to 50% compared to Col-0. Within the group with unaltered CS, horst-1, horst-2, horst-1 ralph-1, and horst-1 ralph-1 gathered genotypes with disrupted CS and similar suberin content by 30 to 50% compared to Col-0. Within the group with unaltered CS, horst-1, horst-2, horst-1 ralph-1, and horst-1 ralph-1 gathered genotypes with disrupted CS and similar suberin content by 30 to 50% compared to Col-0. Within the group with unaltered CS, horst-1, horst-2, horst-1 ralph-1, and horst-1 ralph-1 gathered genotypes with disrupted CS and similar suberin content by 30 to 50% compared to Col-0.

Fig. 1 Characterization of Casparian strip permeability and suberin development. (a) Reconstituted picture of a 21 d-old primary root, and the zones that were monitored for propidium iodide (PI) permeability. Bar, 1 cm. (b) Confocal cross-sections of a 21 d-old plants, PI stained, Arabidopsis root from Col-0 at various zones: root hairs, stages I and II lateral root primordium (LRP), first lateral root (LR) emergence, intermediate, and basal. Bars, 50 µm. (c) Confocal cross-section of PI staining in roots of 21 d-old plant at stages I and II LRP development. Arrows highlight the staining related to ectopic deposition of cell wall polymers at the cell corners, stars indicate when the vessels are stained and hence, PI was able to penetrate through the stele. Bars, 50 µm. (d) Relative suberin content related to wild-type Arabidopsis plants (Col-0) of 17 Caspian strips (CS) and/or suberin mutants of 21 d-old plants. Suberin was analysed using gas chromatography after release by transesterification using boron trifluoride in methanol from solvent extracted root cell walls. Bars represent mean values in µg per mg dry weight ± SE (n = 3–5). *Suberin content taken from literature esb1-2 (Baxter et al., 2009), pCASP1::CDEF1 (Barberon et al., 2016), horst-1, horst-2 (Hofer et al., 2008), ralph-1, ralph-2 (Compagnon et al., 2009). (e) Scoring of the suberin stages along the root, as a relative position from the tip ≥ SE, after staining with the lignin/suberin dye Auramine-O (n = 3–5). Method detailed in Supporting Information Fig. S2. Asterisks indicate significant difference (P < 0.05) to Col-0 plants. Colors patterns of (c) allow to visually identify the groups that are defined in the first section of the results. They are reproduced similarly over all the figures.
suberin content: horst-1, horst-2, horst-1 ralph-1, pCASP1::CDEF1. Finally, CS(=)Sub(X) is formed by ralph-1 and ralph-2 which differ from Col-0 in their suberin composition but not necessarily in their content. From our assays, no difference in periderm development nor periderm permeability could be identified within our mutants. Altogether, we define here a collection of genotypes that covers multiple combinations of CS and suberin defects (Table S1). Although with sometimes a limited number of alleles, such as gelp51-2 in the CS(=)Sub(+) group, we would like to point out that the primary objective of this
collection is not to determine the function of each gene. Rather, we anticipate that extracting the most salient features of each group will allow us to conclude on the role of these apoplastic barriers.

Specific effects of CS and endodermal suberin on root water transport

Root water transport capacity was characterized by measurement of root hydraulic conductivity (Lp) on detopped plants using the pressure chamber and exudation techniques, yielding hydrostatic Lp (Lp,), and osmotic Lp (Lp, o) conductivity, respectively (Fig. 2). Thus, Lp, varied among mutants by −73% to +48% compared to Col-0 (Fig. 2a). A significant linear correlation was observed between Lp, o and Lp, h throughout the overall set of genotypes with the exception of sgn3-3 esb1-1 (Fig. 2b). Variation in Lp, was mostly consistent with the classification of mutants according to their CS and suberin characteristics, though with very few exceptions. Mutants of the CS(−)Sub(+) group showed a significant reduction in Lp, (although not statistically significant for casp1-1 casp3-1), CS(−)Sub(−) (sgn3-3, sgn3-4 and sgn3-3 esb1-1) and CS(+)Sub(−) (gelp51-2, anac038-1, anac038-2) genotypes showed no difference in Lp, to Col-0, although Lp, h, but not Lp, o, was lower for sgn3-3 esb1-1. Finally, Col-0, and CS(−)Sub(X) mutants (horst-1, horst-1 ralph-1, pCASP1::CDEF1, ralph-1 and ralph-2) showed higher Lp, except for horst-2.

Based on these results, with the exception of three genotypes out of 16 (casp1-1 casp3-1, sgn3-3 esb1-1 and horst-2), the most important reduction in root water transport capacity occurs in plants with enhanced suberin but with defective CS. Conversely, the most important increase in root water transport capacity is found in plants with reduced or altered suberin (horst-1, horst-1 ralph-1, pCASP1::CDEF1, ralph-1, ralph-2) and non-defective CS. Defective CS were associated to both reduced and similar Lp, in our collection. Thus, suberin quantity and composition seem to influence water transport as a barrier, while CS, per se, do not.

Reduction of Lp, h, in Caspian strip defective mutants is mediated by concomitant changes in aquaporin activity

Water transport in Arabidopsis roots is considered to be mainly contributed by aquaporins (Tournaire-Roux et al., 2003). Possible interactions between endodermal barriers and aquaporin functionalities were analyzed by comparing Col-0 and a subset of mutants representing the five groups identified earlier. Excised root systems were treated with NaN3, an inhibitor of aquaporin activity (Tournaire-Roux et al., 2003; Sutka et al., 2011). By contrast to all other groups, and with the exception of casp1-1 casp3-1, CS(−)Sub(+) mutants only showed a slight inhibition of Lp, upon NaN3 addition, indicating a low contribution of aquaporins, as was already shown for esb1-1 (Wang et al., 2019) (Fig. 3). The residual Lp, h, insensitive to NaN3, was similar to that of Col-0. Thus, the low Lp, h, in this group would mainly originate from downregulation of aquaporins rather than from direct physical effects of increased suberin deposition. However, mutants from the CS(−)Sub(−) and CS(+)Sub(X) groups showed an increased absolute inhibition to NaN3, together with a significantly higher residual Lp, h, suggesting that their higher Lp, h, results from both an upregulation of aquaporins and alteration in suberin deposition or composition (Fig. 3). Finally, mutants from the CS(+)Sub(+) and CS(−)Sub(−) groups, besides sgn3-3 esb1-1, behaved similarly to Col-0.

Fig. 2 Hydrostatic root hydraulic conductivity (Lp, ) (a), and its relation with the osmotically root hydraulic conductivity (Lp, o) (b) in Col-0, and in a collection of 16 Caspian strip (CS) and/or suberin mutants in Arabidopsis. The plants that were grown hydroponically for 19 to 21 d under environmental controlled conditions, and measured using pressure chambers (Lp, ) (means ± SE, n = 15–20, n = 3) or by the exudation method (Lp, o) (means ± SE, n = 20–25, n = 3). In (a), anac038-2 is presented at a ‘virtual’ Lp’ of 119.38 with respect to a wild-type (WT) value of 134.08 ml g−1 h−1 MPa−1, when ‘real values’ obtained during a dedicated experiment were of 205.0 and 230.2 ml g−1 h−1 MPa−1, respectively. One-way ANOVA and Tukey’s test were used to determine significant differences (α = 0.05). Data of Lp, h, for pCASP1::CDEF1 are the same as in Wang et al. (2019).
Aquaporin regulation triggered by the loss of integrity of root endodermal barriers was further investigated by testing, in roots of nine of the 17 genotypes, the relationship between the messenger RNA (mRNA) abundance of 13 PIP aquaporin genes and Lpr–h. Figure 4 shows that Lpr–h was positively correlated with the expression of AtPIP1;5 \((\rho = 0.7, P = 0.03)\) whereas it was negatively correlated with the expression of AtPIP2;1 \((\rho = -0.86, P = 2.10^{-7})\). No correlation was observed with expression of other PIP genes (Fig. S4). These results indicate that modifications in root apoplastic barriers can be accompanied with changes in expression of aquaporin genes, but which cannot simply explain their hydraulic phenotype.

Altogether, these results indicate that the two apoplastic structures at the endodermis do not simply act as physical barriers for root water or solute transport, but also functionally interact with the aquaporin-dependent pathway. Our results are in line with the results of Wang et al. (2019), where CS deficiency downregulates aquaporins activity and the deposition of ectopic suberin through a CIFS/SGN3 pathway (Doblas et al., 2017b). By contrast, the mechanism that possibly links a decrease in suberin content and/or composition with an upregulation in aquaporin activity or expression remains unknown.

Permeability to solutes at the endodermis is determined by the CS

Root permeability to solutes was determined for a subset of genotypes from each group, by supplying NaCl to detopped plants and measuring Lpr–h and root balancing pressure \((P_{Jv0})\). The value of \(P_{Jv0}\) is the hydrostatic pressure required to counteract the osmotic gradient existing between the root culture medium and the xylem sap and was taken as a proxy for root selectivity to solutes. The application of 100 mM NaCl for 1 h to the root medium typically reduces Lpr–h in WT plants (Boursiac et al., 2005). Our mutant collection followed this behavior except for the CS(–)Sub(+) group \((\text{myb36-1, myb36-2, esb1-1, esb1-2})\), where no major variation of the constitutively low Lpr–h could be detected (Fig. S5). We next determined \(P_{Jv0}\) (Fig. 5). The leakier the root to solutes, the lower the osmotic gradient across the root, and so is \(P_{Jv0}\). Hence, \(P_{Jv0}\) under NaCl treatment can be considered as an indicator of the root selectivity to Na\(^{+}\) and Cl\(^{-}\) (Boursiac et al., 2005). Only mutants with defective CS showed a marked difference in \(P_{Jv0}\) compared to Col-0, with a reduction in the CS(–)Sub(+) group even more marked in the CS(–)Sub(X) group. In the CS(=) groups, only two genotypes (anac38-1 and...
pCASp1::CDEF1) out of eight showed a significant reduction compared to Col-0. (Fig. 5). Our results highlight a clear link between defective CS and solute leakage into the root xylem. The role of suberin is less trivial since various configuration led to slight modifications in $\Pi_{\text{leaf}}$, the most robust being that the increase in suberin content in CS(−)Sub(+) provided an apparent decrease in the root selectivity. Other structural factors such as suberin macromolecular structure, crosslinking or suberin associated waxes also contribute to root selectivity. An in depth analysis of the suberin associated waxes and their physical properties would shed light on this paradox.

Rosette growth is affected by the status of CS and suberin at the endodermis, and involves hydromineral nutrition

We aimed at analyzing how the defects in root endodermal barriers affect the development of the shoots under control conditions. For this purpose, a subset of mutants was selected for each group in addition to Col-0: myb36-1 and esb1-1 from CS(−)Sub(+); sgn3-3 esb1-1 and sgn3-3 from CS(−)Sub(=); anac038-1 and gelp51-2 from CS(=)Sub(+); horst-1 and horst-1 ralph-1 from CS(=)Sub(=); and ralph-1 from CS(=)Sub(X). Plants were soil-grown in the Phenopsis platform (Granier et al., 2006) for 5 wk until harvest. Plant rosette expansion as well as rosette biomass at harvest were determined (Figs 6, S6a). Shoot DW was lower than in Col-0 in the groups of mutants with altered CS, CS(−)Sub(+) and CS(−)Sub(=), while mutants with altered suberin from the CS(=)Sub(+) and CS(=)Sub(=) groups reached shoot DW similar to Col-0 (Fig. 6). Rosette area confirmed these results (Fig. S6a). It has to be noted that rosette growth after 5 wk does not predict the final rosette size, since it also depends on the cycle duration. Nevertheless, these data indicate that, under our normal soil conditions, the functionality of CS is necessary for a proper development of the aerial parts, while that of suberin layers is not.

Since both permeability to solutes and water transport capacity in roots are compromised in the CS(−) mutants with reduced growth, we aimed at identifying which one is the most influential on rosette growth and development. Previous studies showed that a change in root hydraulic conductance ($K_r$) translates into a similar change in transpiration and growth rate in the shoot of maize or peach tree (Solari & DeJong, 2006; Ehlert et al., 2009). Similarly, the rosette FW measured at harvest in our mutant collection under control conditions varied in parallel to $K_r$ (Fig. 7a). However, transpiration rate was not different from Col-0 under our low evaporative demand conditions (Fig. S6b). Such a reduction in leaf growth without change in transpiration had been reported in maize plants, where $K_r$ was downregulated using pharmacological aquaporin inhibition, provided that the evaporative demand was kept low (Ehlert et al., 2009). In the present work, we might face a similar scenario, where reduced growth of the CS(−) groups after 5 wk originates from the downregulation of $K_r$ although without provoking a major rebalancing of plant water relations.

Additionally, there was a positive correlation between rosette osmotic potential ($\Pi_{\text{leaf}}$) and growth across mutants in our experiments (Fig. 7b). As cell turgor is expected to vary inversely to $\Pi_{\text{leaf}}$ (the more negative $\Pi_{\text{leaf}}$, the more positive turgor for a given total water potential), it is unlikely that variation in $\Pi_{\text{leaf}}$ was responsible for variation in growth through changes in turgor. We therefore examined whether variation in $\Pi_{\text{leaf}}$ was rather indicative of ionome disorders which could have caused variation.
in growth. CS(−) mutants, which showed a reduced shoot growth, also exhibited ionomic differences compared to Col-0, with higher potassium (K) and lower calcium (Ca), as referenced in previous reports for esb1-1 and myb36-1 (CS(−)Sub(+) group) (Baxter et al., 2009; Kamiya et al., 2015), and a reduced K and Ca content in sgn3-3 and sgn3-3 esb1-1 (CS(−)Sub(+) group) (Pfister et al., 2014), still in agreement with previous studies (Table S2). By comparison, none of the suberin mutants (CS=Sub(+) and CS=Sub(−) groups), which did not show any growth phenotype, had any alteration in their ionome profile (Table S2), similarly to the previously described ralpb-1 (group CS=Sub(−)) (Compañón et al., 2009). Moreover, the relationship between K and \( \Pi_{\text{leaf}} \), which usually derives from the major role played by this mineral on the osmotic potential in cells, was not conserved across mutants (Fig. 7c). Thus, in our growth conditions, alteration of CS function provoked a reduction in rosette growth possibly associated to ionome variations, but not caused by the resulting change in \( \Pi_{\text{leaf}} \), the latter not being driven by K content.

**Discussion**

The present study aimed at investigating the specific role and impact on the whole plant, of each of the two main apoplastic diffusion barriers of the root: the CS and the suberin layers. For this, we used a unique collection of Arabidopsis mutants, which we categorized according to the permeability of the CS to PI and the amount and location of suberin (Figs 1, S2, S3). The characterization of multiple mutants per group ruled out the drawbacks inherent to single-mutant analyses that could come from unforeseen genetic compensation (El-Brolosy & Stainier, 2017). The casp1-1 casp3-1 double mutant typically fits into this category,
Caspian strips do not directly block apoplastic water transport while suberin does. Yet, both act on aquaporin activity

Characterization of esb1-1 revealed that CIF/SGN3 dependent signaling, which inhibits aquaporin activity, is its primary cause of Lpₐ downregulation (Wang et al., 2019). We generalized this observation and revealed a complex interaction between apoplastic barriers and aquaporin activity and/or expression (Figs 3, 4, S4) to regulate Lpₐ (Fig. 2): CS do not directly block water transport while suberin does, but alteration of both acts on aquaporin activity. Our conclusions are based on three sets of measurements.

First, functional CS were associated with higher Lpₐ – a paradox if we only consider CS as hydrophobic barriers – while we found a correlation between suberin alteration and Lpᵢ in CS(=) Sub(=) and CS(=)Sub(X) mutants. Specifically, a substantial reduction in suberin (pCAS1::CDEF1, horst-1, horst-tralph-1), or a qualitative change in suberin composition (ralph-1), potentially affecting hydrophobicity (Schreiber et al., 2005; Kressbies et al., 2019) or crosslinking and structure (Molina et al., 2009), allowed for an increased Lpᵢ. These results extend the previous characterization of the pCAS1::CDEF1 line, for which we then observed only a trend (Wang et al., 2019). They confirm the importance of studying multiple independent mutants in a reverse genetic approach. Suberin would therefore act as a barrier to water transport. Enhanced suberin deposition, which appeared not ectopic in the CS(=)Sub(+) group, had no further effect on Lpᵢ. This suggests that regular suberin deposition already blocks efficiently the water path in WT. In vitro measurement of the water permeability of thin layers of purified suberin would help confirming such effects.

Second, from the use of the aquaporin blocker NaN₃ (Fig. 3), we derived an ‘aquaporin-mediated Lpᵢ’, and a ‘residual Lpᵢ’. The former refers to the activity of aquaporins in the root, but the latter has to be interpreted with caution since it surely reflects more than apoplastic barriers, and includes transport through lipid membranes, vessels, or communication between the radial transport pathways (Steudle, 2000; Sack et al., 2004). We found significant differences in aquaporin-mediated Lpᵢ in our collection, that confirmed a regulation of aquaporins linked to the apoplastic barrier status (see later). Qualitatively speaking, we found no difference in residual Lpᵢ in four out of six CS mutants, while mutants with a lower suberin content or different suberin compositions (CS(=)Sub(=)Sub(X)) had a higher residual Lpᵢ. We concluded that, within the root zone altered in our mutant collection, the CS is not a major barrier for water transport while suberin physically restricts this transport. In the context of disturbed CS (CS(=)Sub(+) and CS(=)Sub(=)), the comparison between esb1-1, where ectopic suberin content can be seen as a compensatory mechanism for CS deficiency, and sgn3-3 esb1-1, which lacks this response, further reinforces this conclusion. The role of suberin as a hydrophobic barrier in roots has already been highlighted by previous studies in Arabidopsis (Ranathunge & Schreiber, 2011) and other species (Frensch et al., 1996; Freundl et al., 2000; Steudle, 2000; Zimmermann et al., 2000; Ranathunge et al., 2016). The originality of our work resides in the fact that, in the root of Arabidopsis, we were able to separate the role of suberin from that of the CS, and described a complementary regulation of root aquaporins. We note that the periderm defines a zone where the organization of suberin layers and lignified cell walls would challenge our interpretations. However, the initial characterization did not reveal any difference in periderm development or permeability in our collection.

Third, we found positive correlation between Lpᵢ and AtPIP1;5 mRNA abundance. This could fit with a putative role of this isoform in root water transport, which has yet to be proven. The function of AtPIP2;5, whose expression negatively correlated to Lpᵢ, has not been described either. In contrast, the negative correlation between Lpᵢ and the mRNA abundance of AtPIP2;1, which is among the most abundant aquaporins in roots (Boursiac et al., 2005; Monneuse et al., 2011) and acts on osmotically-driven root water flow (Javot et al., 2003; Péret et al., 2012), is contradictory to a major contribution of this aquaporin to water transport. Hence, our results highlight links between apoplastic barrier functionality and aquaporin expression and function, with a complex interplay between isoforms that remains to be elucidated. Future studies will have to take into account distinct cell-specific expression patterns of isoforms (Hacher et al., 2006a,b), their regulation at the protein level, and their possible functional interactions, at the endodermis in particular (Chau-mont & Tyerman, 2014).

Caspian strips are the primary barriers against passive solutes diffusion in roots, while suberin acts as a distinctive, compensatory barrier

Previous studies (Pfister et al., 2014; Barberon et al., 2016; Doblas et al., 2017b; Wang et al., 2019) have concluded that CS exerts a main barrier role in ion transport. With respect to these studies, the present work was carried out in a broader collection of mutants of different origin, and relied on quantitative measurements of balancing pressure (Pᵥ0, Fig. 5). Although not strictly equivalent, this parameter is indicative of the reflection coefficient (σᵥ) of the root. Measurements of σᵥ of Col-0, esb1-1 and sgn3-3 esb1-1 were reported by Wang et al. (2019) and agree with the alterations in Pᵥ0 described here. In the present study, Col-0 plants and mutants from groups with functional CS (CS(=)Sub(+) and CS(=)Sub(X)) showed very consistent Pᵥ0, in the range 0.57–0.73 of the total osmotic force due to NaCl, which fits with σᵥ values commonly in a 0.4–0.8 range (Boursiac et al., 2005; Fritz & Ehwald, 2011; Ranathunge & Schreiber, 2011; Ranathunge et al., 2017). Mutants with altered CS (CS(=)Sub(+) and CS(=)Sub(X)), exhibited a reduction in Pᵥ0, down to 0–0.47 of the total osmotic force due to NaCl (with the exception of casp1-1 casp1-3) which confirms that the CS act as the primary barrier against solute permeation towards...
inner tissues. With regard to mutants with deficient CS, the higher $P_{\text{cso}}$ of CS(−)Sub(+) members (esb1-1 and myb36-1) compared to sgn3-3 esb1-1 (CS(−)Sub(=)) suggests that deposition of ectopic suberin partially compensates for the lack of CS. This result parallels those of NaCl selectivity for esb1-1 and pCASPI::CDEF1 esb1-1 genotypes characterized in Wang et al. (2019). Altogether, these results indicate that CS are the main barriers to the free diffusion of solutes through the apoplast, whereas suberin can act secondarily as a barrier when deposited ectopically as in esb1 and myb36.

Under standard conditions, root diffusional barriers exert direct and indirect impacts on shoot development

Under control conditions, mutants of the altered CS(−) groups showed lower rosette DW and reduced surface development (Figs 6, S5). Both root hydraulic conductance and shoot solutes accumulation were correlated to rosette DW in our experiments (Fig. 7a,b). Thus, both a hydraulic defect and an alteration in solute selectivity appeared as plausible causes of the reduction in shoot growth rate.

However, the observation that plants with the lower osmotic potential are those with the lower growth rate raises an apparent paradox. Indeed, in well-watered soil conditions and with no differences in transpiration (Fig. S6), it can be assumed that the leaf water potential is similar among the genotypes tested. Hence the lower osmotic potential in the CS(−) groups should translate into an increase in the average leaf turgor pressure. According to Lockhart’s model for plant cell expansion (Lockhart, 1965), this would increase the growth rate in the CS(−) groups, for which we observed exactly the converse (Figs 6, S5). Our results therefore suggest that other parameters involved in plant cell expansion are altered when CS are not functional, namely the yield and/or the extensibility of the cell wall.

Furthermore, we looked in more details at the elemental composition of the growing rosettes of the mutant collection in order to look for the origin of the variations in osmotic potential. Our results are in accordance with previous reports (Hosmani et al., 2013; Pfister et al., 2014; Kamiya et al., 2015), and highlight that mutants of the CS(−)Sub(+) and CS(−)Sub(=) groups had opposite phenotypes with respect to K accumulation (Table S2). This implies that the significant variations in shoot osmotic potential, while related to a reduction in shoot growth in both groups, could not be attributed to K (Fig. 7c). We therefore conclude that defective CS do not limit shoot growth through K nutrition. Quantification of other osmotic potential such as NO3−, sugars, and organic acids would be required to find the origin of such osmotic potential variations.

Overall, the control of shoot growth by CS and suberin functionality is not simply mediated by variations in major nutrients or osmotic control of turgor in growing cells, but by indirect effects on other growth characteristics like cell wall mechanical properties. For example, Wang et al. (2019) identified that activation of the CIF/SGN3 signaling pathway in roots of CS deficient plants translates into an abscisic acid (ABA) dependent signaling in shoots, and such signaling could be at the origin of the growth inhibition highlighted in our study.

In conclusion, study of CS and suberin deficient mutants in Arabidopsis highlights that, in roots, suberin acts physiologically as a barrier to water transport while CS prevent the passive leakage of solutes into the stelle. However, the two components appear to control aquaporin activity. In the shoots, defect in CS provokes a reduction in growth not only via an alteration in hydromineral nutrition but also via signaling, including the CIF/SGN pathway, and perhaps also via so far undiscovered pathways.

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Author contributions

Design of the research: YB, CM, DES, RBF, TS, BM; data analysis: MC-P, YB, BM, TS; performance of the research: MC-P, ZR, MD, GR, C-HS, RBF, YB; resources: PD, MF, GR; writing-original draft: MC-P, YB; writing-review and editing: CM, BM, TS, DES, RBF, YB.

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References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** New mutants genotyping and expression information.

**Fig. S2** Propidium iodide penetration in the root of 21 d-old Casparian strips (CS) and suberin mutants.

**Fig. S3** Deconvolution of the Auramine O signal in 21 d hydroponically grown plants enables the detection and quantification of endodermal and peridermal suberin.

**Fig. S4** Expression of aquaporins genes in the mutant collection.

**Fig. S5** Effect of sodium chloride (NaCl) on the root hydraulic conductivity (Lpₒ₋ₐ) of Col-0 and of a collection of 16 Casparian strips (CS) and suberin mutants.

**Fig. S6** Kinetics of rosette development transpiration rates in Col-0 and in a selection of Casparian strips (CS) and suberin mutants grown under environmentally controlled conditions for 5 wk.

**Table S1** Table summarizing the different mutants analyzed in the present study.

**Table S2** Ionomic comparisons of the shoots of Casparian strips (CS) and suberin deficient mutants.

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