Root endodermal barrier system contributes to defence against plant-parasitic cyst and root-knot nematodes

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SUMMARY

Plant-parasitic nematodes (PPNs) cause tremendous yield losses worldwide in almost all economically important crops. The agriculturally most important PPNs belong to a small group of root-infecting sedentary endoparasites that includes cyst and root-knot nematodes. Both cyst and root-knot nematodes induce specialized long-term feeding structures in root vasculature from which they obtain their nutrients. A specialized cell layer in roots called the endodermis, which has cell walls reinforced with suberin deposits and a lignin-based Casparian strip (CS), protects the vascular cylinder against abiotic and biotic threats. To date, the role of the endodermis, and especially of suberin and the CS, during plant–nematode interactions was largely unknown. Here, we analyzed the role of suberin and CS during interaction between Arabidopsis plants and two sedentary root-parasitic nematode species, the cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. We found that nematode infection damages the endodermis leading to the activation of suberin biosynthesis genes at nematode infection sites. Although feeding sites induced by both cyst and root-knot nematodes are surrounded by endodermis during early stages of infection, the endodermis is degraded during later stages of feeding site development, indicating periderm formation or ectopic suberization of adjacent tissue. Chemical suberin analysis showed a characteristic suberin composition resembling peridermal suberin in nematode-infected tissue. Notably, infection assays using Arabidopsis lines with CS defects and impaired compensatory suberization, revealed that the CS and suberization impact nematode infectivity and feeding site size. Taken together, our work establishes the role of the endodermal barrier system in defence against a soil-borne pathogen.

Keywords: nematodes, endodermis, Casparian strip, apoplastic barriers, suberin, plant.

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INTRODUCTION

Plant-parasitic nematodes (PPNs) cause tremendous yield losses in many crops, which are estimated to a total over US$80 billion per year (Nicol et al., 2011). Different species of PPNs infect different plant tissues, including flowers, stems, and leaves. However, the most complex and economically important group of PPNs comprises root-infecting sedentary endoparasites that includes cyst nematodes (CNs; *Heterodera* spp. and *Globodera* spp.) and root-knot nematodes (RKNs; *Meloidogyne* spp.). While both CNs and RKNs have a sedentary lifestyle, they differ in their migration and feeding characteristics. Infective second-stage juveniles (J2s) of CNs enter the root at any location and have developed mechanisms to cross the cortex tissue (including endodermis) directly to reach the vascular cylinder (Marhavy et al., 2019) where their feeding site is induced. RKNs J2s predominantly enter the root close to the tip and then move towards the apical
meristematic region, making a U-turn to enter the vascular cylinder without crossing the differentiated endodermis (Sijmons et al., 1991; Wyss et al., 1992). CNs J2s are destructive, moving intracellularly and piercing cells with their stylets. In contrast, RKNs J2s cause comparatively little damage, as they move intercellularly and piercing cells with their stylets. Unlike the endodermis, the periderm is not equipped with apoplastic pathway in and out of the endodermis, there-
fore preventing diffusion of toxins and loss of nutrients.

The periderm is formed during later stages of root development and replaces the endodermis as a sealing tissue. Instead the periderm is fully suberized. Studies carried out so far showed a rather fast transition from endodermal to peridermal protection in Arabidopsis roots and hypocotyls (Doilan and Roberts, 1995; Wunderling et al., 2018). To date, only a few studies have indicated a role for endodermal/peridermal suberin and lignin in the resistance response to nematode infection (Balhadère and Evans, 1995; Valette et al., 1998).

Many enzymes involved in suberin biosynthesis have been identified, including a fatty acid ω-hydroxylase (CYP86A1/HORST, hydroxylase of root suberized tissue), a β-ketoacyl-CoA synthase (KCS2/DAISY, docosanoic acid synthase), an acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT5), and a hydroxy-cinnamoyl-CoA transferase (ASFT, aliphatic suberin feruloyl transferase) (Beisson et al., 2007; Höfer et al., 2008; Compagnon et al., 2009; Franke et al., 2009; Gou et al., 2009; Molina et al., 2009). The expression pattern of promoter:GUS fusion of CYP86A1, ASFT, and GPAT5 in transgenic Arabidopsis overlaps with suberin deposition in the endodermis (Naseer et al., 2012). Furthermore, the transcriptional reporter GPAT5:mCitrine

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The mechanism of CS formation is relatively well described (Roppolo et al., 2011; Lee et al., 2013). A family of transmembrane proteins called CASPs (Casparian Strip Domain Proteins) drives CS formation by accumulating at the appropriate membrane locations (Roppolo et al., 2011). The expression pattern of the reporter pCAS1:NLS3xmVe-
nus was shown to coincide with CS formation during root development (Vermeer et al., 2014; Barberon et al., 2016). CS establishment also depends on the dirigent domain-containing protein ESB1 (ENHANCED SUBERIN 1). ESB1 is localized at the CS and mediates lignin deposition and stabilization (Rosman et al., 2013). Another protein important for localizing CASPs is the receptor-like kinase SGN3 (SCHENGEN 3), which is responsible for forming CASP complexes in the membrane (Pfister et al., 2014). Previous studies have shown that ectopic depositions of suberin compensates for CS defects (reviewed in Barberon, 2017 and Doblas et al., 2017a).

The identification of genes and the development of appropriate marker lines during the last few years has created new opportunities to analyze suberization and CS formation in Arabidopsis. An in vitro system comprising Arabidopsis, the beet CN Heterodera schachtii, and the RKN Meloidogyne incognita provides optimal conditions to study the cellular and molecular aspects of plant–ne-
matode interactions. Making use of this system, we studied the role of suberin and CS in plant–nematode interactions (Sijmons et al., 1991). Our results indicated an important role for endodermal sealing in nematode development.
RESULTS

Transcripts of suberin biosynthesis-related genes are increased at sites of nematode infection

We surveyed transcriptome data of nematode infection sites available in NEMATIC (NEMatode–Arabidopsis Transcriptomic Interaction Compendium) to gain insight into the expression pattern of a number of suberin- and CS-related genes (Table S1) (Cabrera et al., 2014). We found that the overall expression of these genes was not altered in M. incognita galls from 3 to 21 days after inoculation (dai). However, the transcript levels of a few suberin biosynthesis genes (ASFT, KCS2/DAISY and FAR4) were reduced in giant cells at 3 dai (Barcala et al., 2010). In contrast, the transcript levels of most suberin- and CS-related genes were found to be significantly reduced in syncytia induced by H. schachtii at 5 and 15 dai (Szakasits et al., 2009). The transcriptome analysis by Szakasits et al. (2009) was conducted on RNA isolated from micro-aspirated syncytia protoplasts excluding the root tissue surrounding syncytia. As suberin- and CS-related genes are almost exclusively expressed in the endodermis, we hypothesized that the strong reduction in transcripts of suberin- and CS-related genes reported in Szakasits et al. (2009) might be due to the exclusion of surrounding root tissue. Therefore, to investigate whether the selected genes are differentially expressed in infected tissue, we dissected root segments containing H. schachtii infection sites and quantified gene expression in these segments (Figure 1). We found that transcript abundance of ESB1, CASP1 and SGN3 was reduced, whereas transcript abundance of CYP86A1, KCS2/DAISY, CYP86B1, FAR4 (encoding fatty acyl reductase4), FAR5 (encoding fatty acyl reductase5), and GPAT5 was increased in root segments containing H. schachtii infection sites and quantified gene expression in these segments (Figure 1). We found that transcript abundance of ESB1, CASP1 and SGN3 was reduced, whereas transcript abundance of CYP86A1, KCS2/DAISY, CYP86B1, FAR4 (encoding fatty acyl reductase4), FAR5 (encoding fatty acyl reductase5), and GPAT5 was increased in root segments containing syncytia. Furthermore, although not statistically significant, ASFT, FAR1 (encoding fatty acyl reductase 1), KCS2 (β-ketoacyl-CoA synthase 2), and LACS (long-chain acyl-CoA synthetase) showed a slight increase in transcript abundance. Taken together, many suberin biosynthesis genes showed increased expression in infection sites, while expression of genes involved in CS formation was decreased.

Figure 1. Expression of suberin- and Casparian strip (CS)-related genes in root segments containing syncytia. 10–12-day old Arabidopsis plants were inoculated with H. schachtii J2s and gene expression in dissected root tissue containing syncytia 10 days after inoculation (dai) was compared with uninfected roots as analyzed via qPCR. White bars, suberin biosynthesis-related genes; blue bars, CS formation-related genes. Bars, means ± SD; asterisks, statistically significant fold change relative to the control (t-test, P < 0.05); n = 4.

Suberin biosynthesis genes are expressed in tissue surrounding nematode infection sites

To determine the spatiotemporal patterns of expression of suberin biosynthesis- and CS formation-related genes during plant–nematode interactions, we analyzed previously described promoter:reporter lines at three different time points after inoculation (3, 5, and 10 dai) with H. schachtii or M. incognita. The activity of pCYP86A1:GUS and pGPAT5:mCitrine-SYP122 was used as an indicator of suberin biosynthesis (Höfer et al., 2008; Naseer et al., 2012; Barberon et al., 2016). Expression of pELTP:mCitrine-SYP122 was used as a marker for endodermis and pCASP1:NLS3xmVenus served as a marker of CS formation (Vermeer et al., 2014).

In lines expressing pCYP86A1:GUS, we found that most infection sites exhibited GUS staining upon H. schachtii infection. The staining appeared slightly more intense in the infected area as compared with the surrounding tissue at 3 dai (Figure S1a). At 5 and 10 dai, single cells were stained in the cell layer surrounding syncytium, producing a highly localized, patchy pattern. At 3 dai with M. incognita, pCYP86A1:GUS expression was observed close to root swellings appearing in root tips (Figure S1b). However, at 5 and 10 dai, intense GUS staining was detected throughout the gall tissue (Figure S1b). To confirm that the activity of pCYP86A1:GUS was localized to endodermal tissue, cross-sections of feeding sites were prepared at 5 dai (Figure S1c). No GUS staining was observed in the cortical parenchyma, however specific staining was present in the tissue surrounding feeding sites induced by CN or RKN. Occasionally, GUS staining was also observed in the central vasculature, most likely due to leakage of excessive staining from the endodermis into the vasculature.

Previous studies have shown that CS defects lead to the escape of CASPARIAN STRIP INTEGRITY FACTORS (CIF) peptides from the stele, leading to the continuous activation of the SGN3–SGN1 signalling module, causing lignification/suberization of the defected tissue and therefore sealing the endodermal barrier (Allassimone et al., 2016; Doblas et al., 2017b). SGN3 loss-of-function leads to strong...
CS defects without deposition of compensatory suberin (Hosmani et al., 2013). Based on activation of suberin biosynthesis-related genes, we hypothesized that nematode damage may as well cause leakage of CIF from the stele, leading to the enhanced suberization as a part of a CIF/SGN3-dependent signalling cascade for CS integrity maintenance. To test this hypothesis, we analyzed the suberization of infected tissues using pGPAT5:mCitrine-SYP122 expression in Col-0 and sgn3. We observed a clear expression of pGPAT5:mCitrine-SYP122 in cell layer surrounding the nematode feeding sites induced by CN or RKN (Figures 2 and 3). We found that expression of pGPAT5:mCitrine-SYP122 was only slightly reduced in the sgn3 background upon CN infection. However, expression of pGPAT5:mCitrine-SYP122 appeared to be reduced strongly at all time points in RKN infected tissues as compared with CN (Figures 2c,d and 3c,d).

We next monitored the fate of endodermis upon infection with H. schachtii or M. incognita using the expression of pELTP:mCitrine-SYP122. In uninfected roots, we found a clear expression of pELTP:mCitrine-SYP122 at all time points (Figure 4a,c). Upon infection, we observed expression of pELTP:mCitrine-SYP122 in the cell layer around syncytia or galls at 3 dai (Figure 4b,d). However, beginning at 5 dai, we observed a decrease in expression of pELTP:mCitrine-SYP122 at the infection site as compared with adjacent non-infected tissues, indicating the degradation of endodermis (Figure 4b,d). Interestingly, the extent of endodermal degradation (as indicated by lack of pELTP:mCitrine-SYP122 expression) appeared to be particularly strong in regions infected by CN as compared with RKN. At 10 dai, expression of pELTP:mCitrine-SYP122 was absent in infected, as well as in non-infected, tissues indicating complete degradation of endodermis (Figure 4b,d). For pCASP1:NLS3xmVenus, the punctate expression pattern was not detectable at 5 and 10 dai, although a few sites at 3 dai in young root samples showed weak expression at the infection sites for both syncytia and galls (Figure S2).

Taken together, these findings showed that both syncytia and galls are surrounded by endodermis during early stages of infection and feeding site development. However, the endodermis becomes degraded between 3 and 5 dai, this change indicates periderm formation or ectopic suberization. Regardless of the identity of the tissue, we found

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**Figure 2.** 3D Tile-scan (xyz) maximum projection images monitoring pGPAT5:mCitrine-SYP122 in Col-0 (a, b) and sgn3 (c, d) background upon cyst nematode (CN) infection. Here, 10-12-day old Arabidopsis plants were inoculated with second-stage juveniles (J2a) of Heterodera schachtii and images were taken at 3, 5 and 10 days after inoculation (dai). Asterisks indicate cyst nematode-induced feeding site (syncytium). Representative images are shown. Scale bar, 200 μm.
that suberin biosynthesis genes are activated in cells surrounding the infection sites of both CNs and RKNs.

Periderm is formed during the later stages of infection

Some previous studies have mentioned degradation of the endodermis and the presence of periderm-like tissue around syncytia (Golinowski et al., 1996; Sobczak et al., 1997; Radakovic et al., 2018; Różańska et al., 2018). To analyze this anatomical problem in more detail, we conducted serial sectioning of samples with nematode infection sites at 3, 5 and 10 dai (Figures 3 and 5). Our examinations confirmed results provided by Wunderling et al. (2018) who showed that in non-infected roots, endodermis with relatively clearly recognizable CSs (Figure S3a) starts to degenerate when primary xylem bundles are fully differentiated and pericyclic cells start to divide (Figure S3b). Based on the numbers of primary xylem vessels and the presence of secondary xylem vessels, we were able to identify basipetal and acropetal ends of syncytia induced by *H. schachtii* (Figure 5). The endodermis was well preserved at the basipetal region of infection sites containing syncytia at 3 dai (Figure 5a,b). But it was partially destroyed or sometimes even hardly recognizable along syncytia and close to the acropetal region behind the syncytium (Figure 5c–f). In the same samples, cellular divisions were absent in the pericycle located at the basipetal region of infection sites (Figure 5a), but occurred along the syncytium, except in the region next to the nematode head where most cells were destroyed, and at the acropetal part of the infection site (Figure 5b–f). In samples from infection sites collected at 5 dai, the endodermis was found at both regions but only when the syncytium and dividing pericyclic cells were absent on sections (Figure 5g–l). When the syncytium appeared on sections it was surrounded by dividing pericycle, and degraded and detaching endodermal, cortical parenchyma and epidermal cells (Figure 5h–k). Transmission electron microscopy observations confirmed the degradation of endodermis around syncytia (Figure S3c) and formation of the periderm from dividing pericycle cells (Figure S3d). The cells in the outer layer of the periderm started to differentiate into dead phellem (cork) cells with suberized cell walls (Wunderling et al., 2018). No functional endodermal cells were found in samples collected at 10 dai (Figure 5m–r). Sections taken at basipetal and acropetal regions of samples contained roots that were at early stages of secondary thickening and had well developed periderm. It is worth to note that the number of cells forming the periderm and the diameter of the root at distal regions of...
collected infection sites (Figure 5m,r) were less and smaller than around the syncytium (Figure 5n–q). These findings clearly indicate that CNs infection increases root development and formation of periderm (secondary cover tissue).

Although the endodermis was usually partly destroyed in roots infected with RKNs, it was still well recognizable around invaded J2s at 5 dai (Figure S3e). There was usually also no cell division in the pericycle in this region and at this time point. However, divisions of pericycle cells were fewer compared with 10 dai infection sites of CNs, and no recognizable endodermis was found in 10 dai samples of RKN infection sites (Figure S3f). Taken together, these observations supported our microscope data indicating degradation of the endodermis in nematode-infected regions around 5 dai. These data also explain differences observed in the expression of pELTP:mCitrine-SYP122, which is a marker for endodermis and showing that, in infected regions, degradation of the endodermis occurs earlier in CN infection sites than RKN sites.

Nematode infection sites are encircled by suberized tissue

To visualize suberin deposits, we employed a previously described protocol to stain infected and uninfected roots at 10 dai with the fluorescent dye Nile Red. Dissected galls and syncytia were cleared according to the ClearSee protocol (Kurihara et al., 2015), stained and viewed under a confocal laser scanning microscope (Ursache et al., 2018). We observed a specific red staining in non-infected control roots, as described previously (Figure 6). Upon infection, we found that the cell layer surrounding syncytia was stained specifically, indicating cohesive suberization of this tissue. In comparison with syncytia, staining of the cell layer surrounding the infection site induced by RKN appeared to be patchy (Figure 6).

Figure 5. Comparison of anatomy of infection sites containing syncytia induced by Heterodera schachtii. (a–f) Selected sections from single representative infection site with syncytium at 3 days after inoculation (dai); (g–l) selected sections from single representative infection site with syncytium at 5 dai; (m–r) selected sections from single representative infection site with syncytium at 10 dai. Parts (a), (g) and (m) (upper line) are directed towards the root base (ba- sipetally), whereas parts (f), (l) and (r) (bottom line) are directed towards the root tip (acropetally). C, cortex; DC, detached/degraded cortex and epidermis; E, endodermis; Ep, epidermis; N, nematode; P, pericycle; Pd, periderm; S, syncytium; X, primary xylem. Asterisks indicate secondary xylem vessels. Scale bar: 20 μm. All figures were taken at the same magnification.
Suberin monomer composition is altered upon infection with nematodes

We next compared the chemical composition of aliphatic suberin in hand-sectioned root tissue containing syncytia and galls at 10 dai with that of uninfected control roots. There was no statistical difference in total aliphatic suberin content between nematode-infected root segments and control roots (Figure 7a). However, the abundance of single monomers was altered in nematode infection sites (Figure 7b,c). Levels of ω-hydroxyacids (ω-OH-acids) C18:1 and C16, ω,ω-di acids C16 and C18, acid C20, and primary alcohol C18 were higher in aliphatic suberin extracted from H. schachtii-infected root segments than from control roots. Only the amounts of C24 primary fatty acid, alcohol, and ω,ω-diacid were lower at these sites (Figure 7b). The monomer abundance in galls displayed a similar pattern; levels of ω-OH-acid C20, C18:1, and C16, ω,ω-diacid C20, C18, and C16, primary alcohol C24, C20, and C18, and primary fatty acid C20 were higher than in control roots (Figure 7c).

Defective Caspian strips influence nematode infection and development

To characterize the role of the endodermis during plant–nematode interactions, we performed nematode infection assays with Arabidopsis lines altered in suberin deposits (horst, pCASP1:CDEF1 (cuticle destroying factor 1)), CS formation (sgn3-3, sgn3-3esb1-1), or both (esb1-1, esb1-1CDEF1, casp1-1casp3-1) (Figure 8). The suberin mutant horst has 60% less aliphatic suberin than the wild-type and shows delayed suberin deposition (Höfer et al., 2008; Naseer et al., 2012), whereas pCASP1:CDEF1 (expressing the suberin-degrading enzyme CDEF1 under the endodermis-specific CASP1 promoter (pCASP1:CDEF1)) lacks suberin throughout the root (Naseer et al., 2012; Barberon et al., 2016; Li et al., 2017). The CS mutant sgn3-3 is defective in CS formation, displaying a non-functional apoplastic barrier throughout the root system (Pfister et al., 2014; Barberon et al., 2016; Li et al., 2017). In comparison, the mutant esb1-1 only shows a delay in CS formation, which is compensated for by enhanced suberin deposition (ectopic suberin) starting close to the root tip, and accompanied by ectopic lignification in cell corners close to the CS. This abnormal suberization results in doubling of the amount of total aliphatic suberin compared with Col-0 (Baxter et al., 2009; Hosmani et al., 2013; Lee et al., 2013; Li et al., 2017). The double mutant sgn3-3esb1-1 exhibited a more severe CS phenotype without ectopic suberization or lignification, due to the regulatory role of SGN3 in compensatory mechanisms (Pfister et al., 2014). In esb1-1CDEF1, suberin was degraded by CDEF1 and ectopic lignification at the site of the CS, partially compensating for the lack of an apoplastic barrier. This compensatory mechanism, however, did not entirely seal the apoplast, which remained permeable, especially at lateral root emergence sites (Li et al., 2017). A similar phenotype to esb1-1 was displayed by the casp1-1casp3-1 double mutant (Roppolo et al., 2011; Hosmani et al., 2013).

To perform nematode infection assays, plants were grown in vitro for 12 days and then infected with CN or RKN J2s. For CNs, we counted the number of females, and calculated their average size, and the average size of syncytia at 14 dai (Figure S4). For RKNs, we counted the number of galls and calculated their average area at 21 dai (Figure S5). From the suberin altered lines, pCASP1:CDEF1 showed significant reduction of average syncytium size (Figure S4). Similarly casp1-1casp3-1 (defective CS/ectopic suberin and lignin) but not esb1-1 showed a significantly reduced number of galls and smaller average female and syncytium sizes (Figures S4b,c and S5a). The most marked results were obtained in the CS-deficient lines sgn3-3esb1-1, and also in esb1-1CDEF1 (CS defective/no ectopic suberin), which both affected infection by both nematode...
species (Figure 9). After CN infection, we observed a significant increase in the number of females and a significant increase in average syncytium size in sgn3-3esb1-1 (defective CS/no ectopic suberin; Figure 9a,e). Similarly, we observed a significant decrease in the average female and syncytium size in esb1-1CDEF1 (defective CS/suberin deficient but ectopic lignin) compared with Col-0 plants (Figure 9c,e). After RKN infection, we observed a significantly higher number of galls accompanied by a significantly lower average gall size in sgn3-3esb1-1 and esb1-1CDEF1 (Figure 9g) and a significantly reduced average gall size in sgn3-3esb1-1, esb1-1CDEF1, and sgn3-3 (Figure 9i,j). Taken together, our data implied that a defective CS (without ectopic suberin) renders the plant more susceptible to nematode parasitism, particularly for *M. incognita*.

**DISCUSSION**

In the present study, we analyzed the role of suberin and CS, and the fate of endodermis in Arabidopsis roots during CN and RKN parasitism. We found that the expression of suberin biosynthesis genes was activated upon infection by both CN and RKN. In comparison with suberin biosynthesis genes, both CN and RKN infection showed only modest expression of the CS marker gene (pCASP1: NLS3xmVenus) during the early stages of infection (3 and 5 dai). Intriguingly, the expression of CS formation genes (ESB1, SGN3, CASP1) was downregulated or undetectable at 10 dai in both CN and RKN. This finding was supported by our microscope data and anatomical observations showed that feeding sites induced by both CN and RKN were surrounded by endodermis during early stages of infection. However, the endodermis was degraded during later stages of feeding site development, indicating periderm formation. Based on our observations, we proposed that nematode infection causes damage to the endodermis and induced periderm development that consequently activated suberin biosynthesis genes at the site of infection. However, once a feeding site was established and expanded, increased expression of suberin biosynthesis genes was only required at highly localized patches where phellogen cells differentiate into phellem cells that had suberized cell walls.

We found that the tissue surrounding syncytia and galls underwent *de novo* suberization. Although the detected suberin monomers were characteristic of Arabidopsis roots (Franke et al., 2005), we found a consistent change in suberin composition in nematode infection sites. This change in monomer composition and anatomical observations supported our hypothesis that nematode infection induces periderm formation that encompass *de novo* suberization of phellem cells at the site of infection. Notably, the change in suberin monomer composition was supported by upregulation of several genes involved in their biosynthesis. For example, we detected an increase in C18:1 and C16 x-OH-acids, which are precursors of ω-xylo-carboxylic acids, and the corresponding C16 and C18 chain lengths, were significantly increased. Changes in these...
monomers have previously been shown to be associated with the expression of CYP86A1 (Höfer et al., 2008) and we also observed a significant increase in CYP86A1 expression upon CN infection. Similarly, we found significant increase in C20 precursors that is likely to be related to activation of GPAT5 upon CN infection. The increase in levels in the primary alcohol C18 might be due to upregulation of FAR4. However, there was no significant increase in C20 primary alcohol levels, despite an increase in FAR4 transcript levels (Domergue et al., 2010). Recently it has been shown that most esterified fatty alcohols are a major compound of suberin-associated waxes (Delude et al., 2016). Although, the suberin extraction method used here did not allow detection of associated waxes, upregulation of FAR5 indicated that the level of C20 primary alcohol could be increased in the associated waxes. In contrast, galls showed a significant increase in C20 primary alcohol levels at 10 dai. Overall, especially the patterns of ω-OH-acids and primary fatty acids showed similarities to peridermal suberin as found in roots undergoing secondary development (Höfer et al., 2008) and supported our anatomical observations that development of nematode feeding site induced and enhanced the formation of the secondary state of growth (including formation of periderm) in infected root regions. Our measurements of nematode-infected tissue showed similar suberin monomer abundances between syncytia and galls, which were significantly different from uninfected root tissue.

Our expression data, microscopy observations, and biochemical measurements established that both CN and RKN infection induced a characteristic suberization pattern at the infection site, and indicated that suberin has an important role during nematode parasitism. However, alterations in endodermal suberin did not result in significant changes in infection assays. It is possible that reduced suberin levels cause subtle changes in aspects of parasitism (such as feeding site initiation, nutrient availability, parasite maturation, and reproductive success), which might not lead to significant effects in the parameters used in this study. In contrast, nematode infection assays showed that lines with defects in ‘CS formation without ectopic suberin’ had significantly increase in the numbers of both CNs (sgn3-3esb1-1) and RKNs (sgn3-3esb1-1 and esb1-1CDEF1) and significantly decreased average sizes of females (esb1-1CDEF1), syncytia (esb1-1CDEF1), and galls (sgn3-3, sgn3-3esb1-1 and esb1-1CDEF1) compared with the control. In addition to having defective CS formation, sgn3-3, sgn3-3esb1-1, and esb1-1CDEF1 were also impaired in the compensatory suberization mechanism sealing CS defects, as these mutants are unable to deposit ectopic suberin. It has been previously shown that non-functional SGN3 in sgn3-3 and sgn3-3esb1-1 led to a breakdown of the compensatory barrier surveillance system (Doblas et al., 2017b; Nakayama et al., 2017). A similar effect was achieved by CDEF1 in esb1-1CDEF1, which degrades newly formed suberin (Naseer et al., 2012). Therefore, holes in the CS barrier were not sealed by suberin in these mutants. Intriguingly, esb1-1CDEF1 showed ectopic lignification close to the corner of the cells, which can be seen as a reinforced apoplastic barrier line. Nevertheless, the inability of ectopic lignin to seal newly formed holes in the endodermis has been observed in esb1-1CDEF1 at lateral root emergence sites, which were the only entry points for the apoplastic tracer propidium iodide (Li et al., 2017). These holes in the apoplastic barrier might make it easier for nematodes to reach the vascular cylinder and establish their feeding site. Intriguingly, we found that CS defects had a much more pronounced effect on RKNs than on CNs, unravelling what may be a key difference in migration habit between the two nematode species. CNs move intracellularly and are able to cross the endodermis directly, whereas RKNs move intercellularly and circumvent the CS by migrating to the meristematic region and then making a U-turn to enter the vasculature (Sijmons et al., 1991; Wyss et al., 1992). Therefore, unsealed holes in the endodermis might provide additional entry points into the vasculature, contributing to the higher infectivity of RKNs.

However, our infection assays also revealed reduced average feeding site sizes (sgn3-3, sgn3-3esb1-1, and esb1-1CDEF1), indicating a role for the endoderm during nematode nutrient acquisition. The expression pattern of pCAS1p:NLS3xmVenus in the early biotrophic stages of nematode parasitism indicated that CS formation remains functional in infection sites and that CS may play a role in nutrient homeostasis for nematodes. The CS seals the apoplast and plays a vital role in plant nutrient homeostasis by preventing diffusion out of the central vasculature. A discontinuous CS increased the permeability of solutes, therefore impairing nutrient availability. These effects on nutrient status have been previously described in detail for several suberin- and CS-affected lines (reviewed in Berberon, 2017; Doblas et al., 2017a). Giant cells and syncytia act as metabolic sinks and share features of nutrient transfer cells. Both types of feeding sites are surrounded by a dense network of xylem vessels and phloem sieve tubes, which are formed de novo. Although similarities have been drawn between the function of syncytia and galls, there are fundamental differences in how nutrients are transported towards and into these two different feeding sites (Jones and Northcote, 1972; Hoth et al., 2008; Siddique and Grundler, 2015). Young syncytia are symplasmically isolated and nutrients are supplied by active transport (Hofmann and Grundler, 2006; Hofmann et al., 2007). During syncytium development, secondary phloem elements are formed and plasmodesmata enable symplastic transport from sieve elements into syncytium (Hoth et al., 2005; Hofmann et al., 2007; Absmanner et al., 2013). Therefore, nutrient uptake into syncytia changes from active to
symplasmic transport during nematode development (Hofmann et al., 2007; Hoth et al., 2008). In comparison with syncytia, giant cells remain symplasmically isolated throughout their life cycle and newly formed secondary phloem sieve tubes that lack companion cells surround the giant cells (Hoth et al., 2008; Absmanner et al., 2013). In galls, solutes are unloaded from sieve elements into the apoplastic space from which the nutrients are further transported into the symplasmically isolated giant cells (Bartlem et al., 2013). Hence, in galls, nutrients temporarily reside in the apoplastic space between sieve elements and giant cells. Normally, the loss of these nutrients into surrounding tissue or the soil is prevented by the CS barrier. Therefore, defects in CS formation, not compensated for by suberization, would lead to an outflow of solutes, which may impair nematode development.

Based on these data, we proposed that nematode feeding site development ruptures the apoplastic seal of the endodermis, and triggers a compensatory suberin mechanism induced by the barrier surveillance system (Doblas et al., 2017b; Nakayama et al., 2017). When this programme is inoperable and the ability of the endodermis to seal itself is impaired, and nutrients leak out of the vasculature, leading to deficiencies for both the plant and the parasite. A consequence of this reduced nutrient state is reduced growth rates, reflected in the reduced size of the feeding sites.

CONCLUSION

Our results imply that a functional endodermis constitutes a hurdle to nematode penetration and can therefore be considered as part of preformed defences. The endodermal tissue is maintained around the nematode feeding site during early stages of infection and M. incognita especially benefits from this for infection and nutrient acquisition. Taken together, the endodermis plays a dual role in nematode parasitism, a barrier to nematode entrance and a regulator of nutrient acquisition for nematodes from feeding sites. The overall modest impact of the CS and suberin alterations on nematode infection possibly underlies compensatory mechanisms such as the upregulation of influx carriers (Pfister et al., 2014; Barberon et al., 2016). Changes in suberin monomer composition are most likely to be triggered by the sensitive barrier surveillance system in the endodermis that maintains its sealing properties despite infestation by nematodes (Doblas et al., 2017b; Nakayama et al., 2017).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used for all experiments. Plants were grown in Petri dishes containing agar medium. *H. schachtii* infection assays were performed on 0.8% (w/v) Daichin (Duchefa Biochemie) agar medium enriched with modified Knop nutrient solutions as described previously (Stijmons et al., 1991) with 2% (w/v) sucrose and 0.1% (w/v) 100× Gamborg’s vitamin solution (Sigma-Aldrich). Infection assays with *M. incognita* were conducted in Murashige and Skoog medium including vitamin and MES buffer (Duchefa Biochemie) supplemented with 0.5% (w/v) Gelrite (Roth) and 2% (w/v) sucrose. Seeds were surface sterilized and plants were grown under conditions described previously (Hütten et al., 2015). The following T-DNA insertion lines were used for infection assays: *horst-1* (cyp86a1, AT5G58860) (Hofer et al., 2008), *sgn3-3* (AT2G20140) (Pfister et al., 2014), the leaf ionomic line *esb1-1* (AT2G28970) (Baxter et al., 2009), the double mutants *sgn3-3esb1-1* (Pfister et al., 2014) and *casp1-casp3-1* (AT2G36100/AT2G23730) (Roppolo et al., 2011), and lines *pCASP1:CDEF1* (Naseer et al., 2012) and *esb1-1CDEF1* (Li et al., 2017), containing the suberin-degrading enzyme CDEF1 expressed under the endodermis-specific CASP1 promoter. *pGAP7:mCitrine-SYP122* was crossed into the *sgn3-3* background.

Nematode cultures and inoculum

*Heterodera schachtii* was propagated and second-stage juveniles (J2) were hatched as described previously (Siddique et al., 2009). Freshly hatched J2 were surface sterilized with 0.05% (w/v) mercury chloride. Here, 60–80 J2s in a water suspension were inoculated onto the agar surface above the root system of 10– to 12-day-old plants. Petri dishes were stored under standard growth conditions, as described above.

*Meloidogyne incognita* was propagated and eggs were extracted as described previously (Mendy et al., 2017). To separate freshly extracted eggs from soil particles, the eggs were suspended in a 35% (w/v) sucrose solution and centrifuged. Eggs and freshly hatched J2s were surface sterilized as described previously (Mendy et al., 2017). The roots of 10– to 12-day-old plants were inoculated with 90–100 sterile J2s and the plants were stored under standard growth conditions in darkness (see above).

Infection assays and measurements

For each experiment, 10–20 plants were used per genotype, and experiments were repeated three times independently. For *H. schachtii* infection assays, the number of female nematodes was counted at 14 dai and females and syncytia were photographed. For *M. incognita* infection, the number of galls was counted at 21 dai and galls were photographed. Photographs were used for the area measurements of galls, syncytia, and *H. schachtii* females using Leica Application Suite software. In total, 90–150 individual infection sites were photographed for each genotype with a Leica M165C, Camera Leica DFC450C (Leica Microsystems, Wetzlar, Germany).

GUS staining procedure

The activity of previously described promoter:GUS fusions *pCYP86A1:GUS* (Hofer et al., 2008) was analyzed by GUS staining. A 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (1 mM X-Gluc) solution was poured onto the agar surface in Petri dishes and incubated at 38°C as described in Siddique et al. (2009). The *pCYP88A1:GUS* line was incubated overnight. Photographs were taken using a Leica DMI 4000B camera DFC450C (Leica Microsystems).

Fluorescence microscopy

The transcriptional reporter lines *pCASP1:NLISxVenus* (AT2G36100) (Vermeer et al., 2014; Barberon et al., 2016) and
pGPA15:TomCitrine-SYP122 (AT3G11430) (Barberon et al., 2016) were examined using a Zeiss LSM 710 (Zeiss, Oberkochen, Germany) confocal laser scanning microscope. Plants were grown on coverslips (24 × 60 mm) covered with 3 mL agar medium. Pictures were taken with a ×40 water immersion objective. Maximum intensity projections of confocal Z-stacks were obtained using ImageJ software (NIH; http://rsb.info.nih.gov/ij) and Zeiss Zen2.1 (black edition) software.

A Zeiss LSM 880 inverted confocal scanning microscope was used for Tile scanning. Pictures were taken with a ×40 water immersion objective. For more detailed analyses, imaging was performed with Z-scans plus Tile-scans (overlap 20%). Fluorescence signals for mCitrine (excitation 514 nm, emission 515–550 nm), was detected. Images were analyzed and processed/edited using ImageJ software (NIH; http://rsb.info.nih.gov/ij) and Zeiss Zen2.1 (black edition) software.

Reverse-transcription quantitative PCR (qPCR)

Infection sites induced by H. schachtii and M. incognita on Col-0 plants were hand-dissected at 10 dai and immediately frozen in liquid nitrogen. Three biological replicates were collected, each containing ~100 mg fresh root weight, which included ≥300 feeding sites (per replicate). Uninfected roots, excluding root tips, served as controls. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA samples were treated with DNA-free DNase Treatment & Removal (Ambion, Life Technologies, Darmstadt, Germany) and the quality was tested using a 2100 Bioanalyzer (Agilent Technologies, Heidelberg, Germany) on an RNA NanoChip. The isolated RNA was transcribed into cDNA using the High Capacity cDNA Reverse-Transcription Kit (Qiagen, Hilden, Germany) and the quality was tested using a 2100 Bioanalyzer (Agilent Technologies, Heidelberg, Germany) on an RNA NanoChip. The isolated RNA was transcribed into cDNA using random primers and the High Capacity cDNA Reverse-Transcription Kit (Applied Biosystems, Darmstadt, Germany). The samples were analyzed with the StepOne Plus Real-Time PCR System (Applied Biosystems) in 20 µl reactions containing 10 µl Fast SYBR Green qPCR Master Mix, 0.75 µl forward and reverse primer (10 µM) each, 1 µl cDNA, and sterile RNase/DNase free water. For internal references, β-tubulin 4 and 18S rRNA were used with a 1:100 dilution of cDNA. qPCR was carried out as described previously (Hütten et al., 2015). Relative expression was calculated according to Pfaffl (2001). Primers are listed in Table S2.

Anatomy of infected roots

A. thaliana Col-0 plants were grown and inoculated with J2s of H. schachtii or M. incognita as described above. Root segments (~5 mm long) containing feeding sites at 3, 5 and 10 dai were dissected and processed for microscopic examinations as described by Golinski et al. (1996) and Sobczak et al. (1997). Semi-thin sections were stained with hot 0.1% (w/v) toluidine blue (pH 6.8) and examined under an Olympus AX70 “Provis” (Olympus, Tokyo, Japan) light microscope equipped with an Olympus UC90 digital camera (Olympus). Ultra-thin sections were examined under an FEI 268D ‘Morgagni’ (ThermoFisher Scientific, Waltham, MA, USA) transmission electron microscope operating at 80 kV. Images were taken with an Olympus-SIS ‘Morada’ digital camera. Images were processed for similar contrast and brightness using Adobe Photoshop (Adobe Corp., San Jose, CA, USA) software.

Histochemical suberin staining

Dissected infection sites (10 dai) were fixed in 4% (w/v) paraformaldehyde in 1× PBS buffer for 1 h at RT and washed twice for 1 min in PBS buffer. Fixed root samples were incubated for 10 days at RT in ClearSee solution in dark (Kurihara et al., 2015). Cleared root sections were incubated overnight in fresh ClearSee solution containing 0.05% Nile Red (Sigma, CAS-No: 7385-67-3) and examined using a Zeiss LSM 710 (Ursache et al., 2018). Maximum intensity projections of confocal Z-stacks were obtained using ImageJ software (version 1.48v, National Institutes of Health, USA, http://imagej.nih.gov/ij).

Suberin analyses

For aliphatic suberin monomer extraction, three technical replicates of dissected infection sites (10 dai) from ≥30 plants (~450–600 mg fresh weight) and control roots of uninfected plants were frozen in liquid nitrogen. To remove unbound lipids, samples were extracted for 24 h in methanol and chloroform, dried, and weighed. Samples were depolymerized and injected on-column on an Agilent 6890N gas chromatograph (GC) combined with an Agilent 5973N quadrupole mass-selective detector for monomer identification and for quantitative analysis based on an internal standard using an identical GC system coupled with a flame ionization detector, as described previously (Franke et al., 2005).

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CONFLICT OF INTERESTS

The authors declare there is no conflict of interest.

AUTHOR CONTRIBUTIONS

SS conceptualized the study and designed the experiments with input from JH, FMWG, LS, RBF, MS and NG. JH performed the majority of the experiments. PM performed the confocal microscopy experiments with help from SF. MS and MG performed anatomical observations. JH curated the data and performed the statistics. SS and JH wrote the manuscript with input from all authors.

DATA STATEMENT

All data referred to are included in the manuscript or supplemental materials of this manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Expression of suberin- and Casparian strip (CS)-related genes in Meloidogyne incognita (GC and Gall) and Heterodera schachtii (Sync) infection sites.

Table S2. Primer list for qPCR.
REFERENCES


