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Deciphering the role of apoplastic root barriers in the interaction between sedentary nematodes and Arabidopsis

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Abstract

The plant-parasitic nematode species *Heterodera schachtii* and *Meloidogyne incognita* infect economically important crop plants in the entire world. Their infection causes yield losses, but effective and environmentally friendly pest management strategies require high expenses or are impractical. Nematological research therefore studies plant-nematode interaction on cellular and molecular level to improve nematode control strategies. Plant-parasitic nematodes invade roots and establish feeding sites in the vascular tissue. Within root tissue, nematodes cross the endodermis, which is equipped with cell wall reinforcements and Casparian strips (CSs) in the apoplast. These cell wall modifications consist of the resilient biopolymers suberin and lignin, respectively. To examine whether suberin and the CS play a role during nematode infection of Arabidopsis, the expression of related biosynthesis genes in nematode-infected tissue was analysed. A number of suberin biosynthesis genes were significantly upregulated in infection sites, while CS related genes were downregulated. Reporter gene analysis showed differential expression of CS and suberin markers in feeding sites, indicating the presence of suberin in surrounding tissue. Histochemical staining verified the presence of a lipophilic substance, such as suberin, in an endodermis-like cell layer encircling nematode feeding sites. Finally, a typical suberin monomer composition has been verified in nematode-infected root segments. On quantitative level, this suberin monomers showed significant differences in abundance as compared to control roots. To test whether suberin or the CS affect nematode parasitism, mutants altered in suberin deposition or CS formation were used for infectivity studies. Surprisingly, not suberin alterations but defective CSs had a significant impact on nematode infection and development. The role of suberin and CSs during nematode infection and nutrient acquisition as well as the impact of the endodermal barrier surveillance system are discussed.

Zusammenfassung

Die pflanzenparasitären Nematodenarten *Heterodera schachtii* und *Meloidognye incognita* befallen ökonomisch wichtige Nutzpflanzen weltweit. Der Befall verursacht Ertragsverluste, aber effektive und umweltverträgliche Maßnahmen zur Schädlingskontrolle sind kostspielig oder inpraktikabel. Aus diesem Grund untersucht die nematologische Forschung die Pflanze-Nematode Interaktion auf zellulärer und molekularer Ebene zur Verbesserung der Pflanzenschutzstrategien. Pflanzenparasitäre Nematoden dringen in Wurzeln ein und etablieren Nährgewebe im Zentralzylinder. Dafür durchdringen Nematoden innerhalb des Wurzelgewebes die Endodermis, welche durch Zellwandverstärkungen und den Casparischen Streifen (CS) modifiziert ist. Diese Verstärkungen bestehen jeweils aus den widerstandsfähigen Biopolymeren Suberin und Lignin. Um zu untersuchen ob Suberin und der CS eine Rolle bei der Infektion von Arabidopsis mit Nematoden spielen, wurde die Expression von zugehörigen Bio-synthesegenen in infizierten Wurzelsegmenten analysiert. Eine Reihe von Suberinbiosynthesegenen war signifikant hochreguliert in Infektionsstellen, wobei CS verwandte Gene runterreguliert waren. Die Reportergenanalyse zeigte unterschiedliche Expressionsmuster von Suberin und CS verwandten Markern in Infektionsstellen, implizierte , jedoch, das Vorliegen von Suberin in umliegendem Gewebe. Histochemische Färbungen betsätigten Ablagerungen einer lipophilen Substanz, wie Suberin, in einer Endodermis-ähnlichen Zellschicht, die die Infektionsstelle umgibt. Schließlich wurde eine typische Suberin-Monomerzusammensetzung qualitativ bestätigt. Auf quantitativer Ebene zeigten sich signifikante Unterschiede im Vergleich zu Suberin aus nicht befallenen Wurzeln. Um zu untersuchen, ob Suberin oder der CS eine Auswirkung auf den Befall von Nematoden haben, wurden transgene Pflanzenlinien mit veränderter Suberinbiosynthese oder defekter Bildung des CSs für Infektionsstudien genutzt. Überraschenderweise hatten defekte CS einen signifikanten Einfluss auf den Parasitismus und nicht die Suberinveränderungen. Die Rolle von Suberin und CS insbesondere für die Nährstoffakkumulation von Nematoden sowie der Einfluss des Barriere-Überwachungssystems der Endodermis werden diskutiert.

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List of abbreviations

ASFT	Aliphatic suberin feruloyl transferase
CASP	Casparian strip membrane domain proteins
CDEF	Cuticle destruction factor (cutinase)
CN	Cyst nematode
CS	Casparian strip
DAISY	Docosanoic acid synthase
DCA	α-ω-dicarboxylic acid
ELTP	Embryo lipid transfer protein
ESB	Enhanced suberin
FA	Fatty acids
FAR	fatty acyl-CoA reductase
GPAT5	Glycerol-3-phophate acyltransferase 5
HORST	Hydroxylase of root suberized tissue
IFC	Initial feeding cell
J2	Juvenile nematode in the infective stage
KCS	β-ketoacyl-CoA synthase
LACS	acyl-CoA-esters by long-chain acyl-CoA synthase
PPN	Plant-parasitic nematode
RKN	Root-knot nematode
SGN	Schengen (protein involved in CS formation)

Chapter 1

Introduction

Nematodes

The word Nematoda is derived from the ancient Greek word *nêma,* meaning *thread*. Nematodes are roundworms, which are unsegmented animals, classified along with insects and moulting animals in the same taxonomic clade (Ecdysozoa) (Dunn *et al.*, 2008). They constitute the most abundant group of multicellular, eukaryotic organisms (Metazoa) on earth. To date approximately 27,000 species of nematodes have been described, but diversity is estimated up to one million extant organisms (Hugot *et al.*, 2001; Lambshead and Boucher, 2003; Quist *et al.*, 2015). Nematodes comprise a highly diverse group that displays a wide range of trophic ecologies. They feed on bacteria, fungi, algae, other nematodes, invertebrates, vertebrates or plants. Even though nematodes inhabit almost every possible niche on earth, they depend on moisture for their locomotion. However, in certain life cycle stages, they have the ability to survive adverse environmental conditions e.g. as dauer larvae (Decreamer and Hunt, 2013).

The majority of nematodes are microscopically small and less than 1 mm in size. However, a few animal parasites can achieve lengths of up to 8 m such as *Placentonema gigantisma*, parasite of sperm whale (Gubanov, 1951; Gibbons, 2002). The most well-known representative of free-living nematodes is the bacterivorous soil inhabitant *Caenorhabditis elegans*. *C. elegans* is the first animal whose genome was completely sequenced and is the favourite experimental model for nematode research (The *C. elegans* Sequencing Consortium, 1998; Blaxter *et al.*, 1998). Parasitic nematodes of insects can be used as biological control agents against pest breakouts (Wilson and Kakouli-Duarte, 2009). In contrast, parasitic nematodes of humans cause infections of great medical impact (for example elephantiasis, caused by *Wuchereria bancrofti*) (Dieterich and Sommer, 2009). The most important nematode affecting human health is probably the gut roundworm (*Ascaris lumbricoides*) that infects more than 1 billion people worldwide (Chan, 1997).

Many nematode species are free-living in soil, marine habitats or freshwater, feeding primarily on bacteria and fungi. In soil, these species create beneficial effects to ecology through nutrient turnover and are valuable indicators of the biological condition (Bongers and Ferris, 1999; Wilson and Kakouli-Duarte, 2009). The soil

provides optimal living conditions for a huge variety of free-living and parasitic nematode species, considering moisture and pore sizes.

The subject of this study is plant-parasitic nematodes (PPNs), which cause critical damage to agricultural yield. Each crop plant can be infected with one or more PPN species, causing root deformations and reduced plant growth (reviewed in Nicol *et al.*, 2011).

General morphology of nematodes

The body of nematodes is covered with a protective and flexible cuticle. An epidermis and a single layer of somatic muscle cells are situated beneath the cuticle. The mouth opens into a cavity, which is followed by a pharynx (or oesophagus), an intestine and a rectum with an anus in females and larvae bodies and a cloaca in males (Fig. 1). Most nematode species exhibit sexual dimorphism and have a secretory-excretory system and a complex nervous system (Gibbons, 2002; Decreamer and Hunt, 2013).

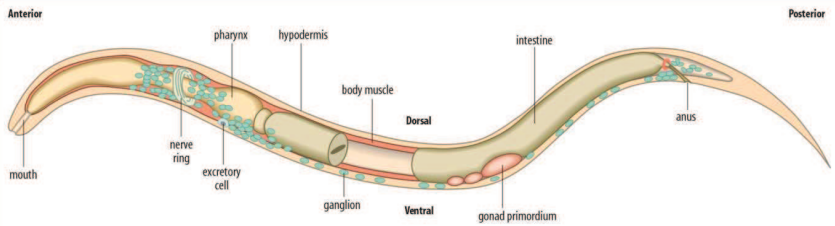

Fig. 1 The morphology of the nematode body on the basis of a *C. elegans* larva (Memorial University of Newfoundland, department of biology). (http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_06/ch06f03.jpg).

Body cuticle

The extracellular cuticle forms a flexible exoskeleton, has a multizoned structure and plays a critical role in locomotion. This outer cell layer maintains the body shape since nematodes lack a skeleton and a circular muscle system. The cuticle also functions as a barrier against the environment. Moreover the cuticle is semipermeable as it is involved in secretion and excretion processes as well as in the uptake of substances (Gibbons, 2002). The cuticle is secreted in layers and therefore consists of four distinct zones which are characterized by outgrowths such as transverse and/or longitudinal striae and ridges (Decraemer *et al.*, 2003). The

number of longitudinal incisures in distinct body regions is of taxonomic importance (Jairajpuri and Hunt, 1984). Major components of the cuticle are covalently cross-linked collagens (Johnstone, 1994). All nematodes undergo four post-embryonic moults and each time a new extracellular proteinaceous cuticle is synthesized (Gibbons, 2002; Decreamer and Hunt, 2013). Additionally, nematodes possess a glycoprotein surface coat overlaying the cuticle, which plays a role in interactions with the host plant (Spiegel and McClure, 1995; Lee, 2002).

Epidermis

The cuticle is secreted by the epidermis (hypodermis) which provides the body with a structure and protects it from osmotic stress. The epidermis is either cellular or syncytial (Gibbons, 2002; Decreamer and Hunt, 2013). A cellular epidermis is a more primitive form and mainly found in free living species and juveniles (Decreamer and Hunt, 2013).

Somatic musculature

A single layer of obliquely oriented, longitudinal somatic musculature is located underneath the epidermis. Alternate contractions of the ventral and dorsal muscle cells generate the sinusoidal locomotion of nematodes (Gibbons, 2002; Decreamer and Hunt, 2013).

Pharyngeal glands

Characteristic features for PPNs are secretions from pharyngeal glands which play an important role in plant-nematode interaction. These secretions contain cell wall-degrading enzymes which are released into plant cells (Davis *et al.*, 2011). PPNs possess three pharyngeal glands; two sub-ventral and one dorsal. Sub-ventral gland cells are large and contain secretory granules and are probably more important in the juvenile stage due to the fact that they become smaller with time. Conversely, the size of the dorsal gland cell increases with progressing life cycle (Gheysen and Jones, 2013).

Sense organs and nervous system

The anterior region is considered to be the head of the nematode and its pattern is an important diagnostic feature for species identification. Nematodes carry six lips around the mouth opening. The lip region is equipped with numerous anterior sensilla and two chemoreceptor sense organs (amphids) (Perry and Curtis, 2013). Each lip can be divided into an inner and an outer sensillum. The inner sensilla are

Fig. 2 Stomatostylet of the PPN *Globodera rostochiensis* (van Megen, Laboratory of Nematology, University Wageningen).

(https://www.wur.nl/en/Expertise-Services/Chair-groups/Plant-Sciences/Laboratory-of-Nematology/Nematode-in-the-picture/Pictures/Globodera-rostochiensis-1.htm)

chemoreceptive when openly connected to the environment and mechanoreceptive when embedded in the cuticle (Wright, 1983). Most of the cuticular sense organs are located at the nematode`s head. The largest chemoreceptors are amphids with a high concentration of receptors. Most plant-parasitic nematodes carry additional somatic sense organs, the phasmids, which are situated in the tail region. Their structure is similar to that of a sensillum. The central nervous system includes a nerve ring, which connects the sense organs and encircles the pharynx (Gibbons, 2002; Perry and Curtis, 2013).

Digestive system

The structure of the digestive system is highly diverse among nematodes. It consists of three regions: stomodeum, mesenteron and proctodeum. The stomodeum is located at the mouth opening. In PPNs, that region is equipped with a hollow spear-like feeding apparatus, the stylet (Fig. 2). The mesenteron is the intestine and is composed of a simple single-layered tube, which absorbs, stores or secretes proteins and enzymes. The proctodeum or rectum is a simple, short tube in line with the body cuticle (Gibbons, 2002; Decreamer and Hunt, 2013).

Reproductive system

The majority of nematodes are bisexual and gonochoristic (either male or female). Also uniparental reproduction takes place in the form of parthenogenesis. Sexual dimorphism is very common in PPNs, displayed by a swollen female body and a vermiform male (Gibbons, 2002; Decreamer and Hunt, 2013).

Life cycle stages

Nematode development occurs in six stages: an egg stage, four juvenile stages and one adult stage as male or female (Fig. 3). Generally, nematodes moult four times before reaching the adult stage. In PPNs the first moult happens within the egg and infective juveniles hatch in the J2 stage (Gibbons, 2002; Decreamer and Hunt, 2013).

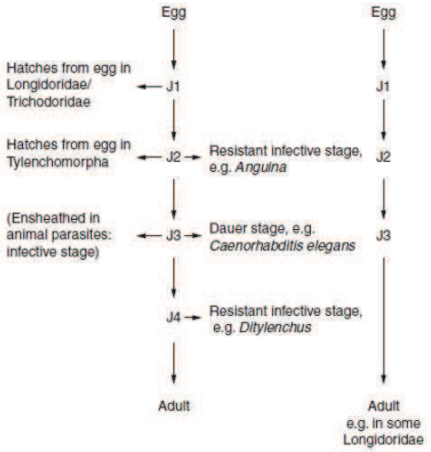

Fig. 3 Basic life cycles of parasitic nematodes (Perry and Curtis, 2013).

Molecular phylogeny: Plant parasitism in the phylum Nematoda

The evolution of the phylum Nematoda combines complex feeding habits of microphagous, saprophagous, phytophagous, omnivorous and predatory nematodes and is not yet completely decrypted. The relatively high rate of convergent evolution, the absence of fossil records and the scarcity of phylum-wide molecular data have hampered the understanding of nematode evolution for decades (van Megen *et al*., 2009; Bert *et al*., 2011). Nowadays, phylogenetic relationships among nematodes are based on the evaluation of small subunit ribosomal DNA (SSU rDNA) sequences rather than morphological characteristics. These new molecular analyses led to the development of updated phylogenetic classifications, revealing relationships between previously unconnected data. The continually increasing availability of molecular data contributes to a renewed framework of nematode evolution in the future (Blaxter *et al*., 1998; Holterman *et al*., 2006).

In the phylum Nematoda, the parasitic lifestyle has arisen multiple times. According to the phylogeny presented by Holterman *et al*. (2006), the phylum Nematoda can be divided into twelve clades (Fig. 4). Accordingly, plant parasitism arose independently at least four times in the clades one, two, ten and twelve (van Megen *et al*., 2009; reviewed in Kikuchi *et al*., 2017). In the order Tylenchida (clade 12) plant parasitism most likely evolved from fungal feeding nematodes (Holterman *et al*., 2009). This order includes the most economically important PPN species (Bert *et al*., 2011).

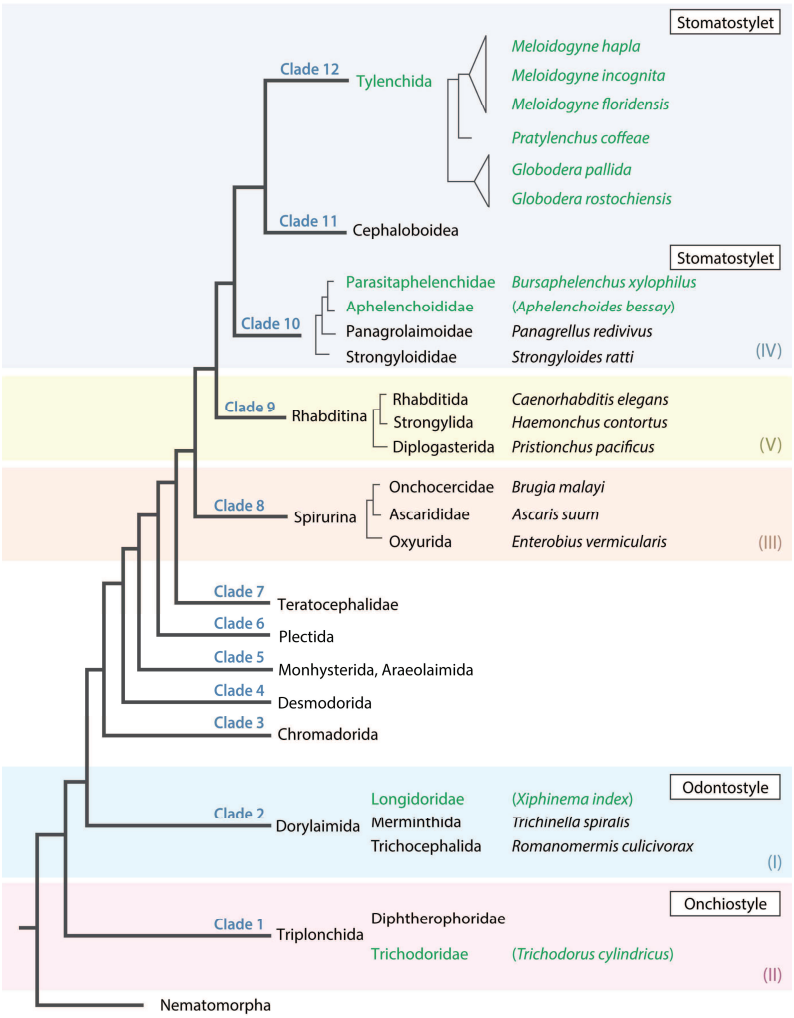

Fig. 4 Schematic overview of the phylum Nematoda (Kikuchi *et al.*, 2017). Division into 12 major clades is derived from SSU rDNA sequence data based on van Megen *et al.* (2009). For comparison, coloured boxes labelled with roman numerals indicate clades defined by Blaxter *et al.* (1998). Only representative species of major clades are given. Taxa with unavailable genome data are marked in parentheses. Plant parasitism is indicated by green colour. Types of protrusible spear-like stylets (feeding apparatus) are shown. Odontostyle: hollow tooth; Onchiostyle: curved, solid tooth; (Stomato) stylet: hollow piercing device.

Plant-parasitic nematodes

More than 4,100 species of PPNs are described to date and a relatively small range of PPNs is known to cause tremendous economic damage in agriculture (Hugot *et al.*, 2001). Phytosanitary measures especially target nematodes which entirely enter plant tissue (e.g. endoparasitic) as they can easily be transported unseen within plant parts and adhesive soil. Not surprisingly, nematodes are globally distributed. However, on species level, their occurrence can be restricted to a certain habitat and varies between high host specificity and a wide range of genera. The crop reduction caused by nematodes is highly variable and depends on nematode and host species. Carrots infested by root-knot nematodes (RKN, *Meloidogyne* spp.) cause deformations and branching, which may render them worthless on the market. Onions attacked by stem nematodes (*Ditylenchus dipsaci*) may spoil earlier than non-infested bulbs. Potatoes infected with cyst nematodes (CN, *Globodera rostochiensis*, *G. pallida*) are reduced in weight and size. Symptoms of nematode infection on the fields can be as insignificant as delayed development and reduced growth rates. Infected plants may even reach the same final weight as non-infected plants at a later time point. This mechanism of growth reduction occurs in three stages. First, nematode infection causes retarded plant growth, but at a constant rate. In the second stage, water uptake is hampered, which affects the plant's mineral balance. Finally, depending on nematode density and sensitivity of the cultivar, early death of plants occurs (Schomaker and Been, 2013). Nematodes can heavily constrain the reliability of the food supply. Crop losses due to PPNs have been estimated to cost several billion dollar per year worldwide (reviewed in Nicol *et al.*, 2011). Due to the primarily unspecific symptoms, nematode infestation can easily be confused with other diseases (e.g. wilting, growth reduction) if farmers do not receive specific training – which is the case in many developing countries (Viaene *et al.*, 2013). The calculation of economic losses is therefore only an estimation.

Management in agriculture

Until today, control strategies against nematodes are based on chemicals (nematicides) and a number of biological treatments, such as cultivation of resistant plants, crop rotation with non-hosts, fallow practices, physical soil treatment and application of nematode antagonists. Nematicides are introduced into soil to cause immobility or death of nematodes. Since chemicals are associated with a high potential for environmental contamination, their use is declining and has been

banned in increasingly more countries (Escobar *et al.*, 2015; Schomaker and Been, 2013). Natural plant resistance is a relatively safe alternative but effective resistance is not available for all crop plants (Fosu-Nyarko and Jones, 2015). Crop rotation tends to reduce the population of nematodes in the absence of the host and has proven to be very effective for species with narrow host ranges (Singh *et al.*, 2015). Additionally, fallow periods can be successful but this practice reduces income and may cause soil erosion. The effects of physical soil treatments such as heat, steam heat, soil solarization or flooding may be effective in certain agricultural system but are often uneconomic (reviewed in Viaene *et al.*, 2013). Soil amendments often suppress nematodes by releasing natural nematicides. However, large amounts are needed to cause an effective reduction and the effects are often not fully understood (reviewed in Oka, 2010). The most studied natural antagonists of nematodes are bacteria and fungi (Viaene *et al.*, 2013). Microbes or fungi often cause a suppressive effect in soil by producing toxins or inducing plant resistance. However, this effect may only reduce nematode damage in the short term. Nematode predators in soil, such as insects, mites and other nematode species, generally have a broad host range and do not exclusively feed on PPNs (Jatala, 1986; Rodriguez-Kabana *et al.*, 1987). Overall, the challenges of delivering large volumes of fungi or bacteria into soil are that the impact of produced metabolites remains unclear (reviewed in Siddiqui and Mahmood, 1996). Nevertheless, biological control agents can serve as one part of integrated nematode management, which combines biological and cultural methods and requires flexibility and understanding of PPNs (Piśkiewicz *et al.*, 2008; reviewed in Viaene *et al.*, 2013; Singh *et al.*, 2015).

Lifestyles and feeding habits

All PPNs have a hollow needle-like stylet which is used to pierce plant cell walls, excrete fluids, and take up cell contents for feeding. PPNs can be divided into four types based on the plant organ they feed on: 1) Penetration of above-ground organs like ovaries that leads to gall formation (e.g. in wheat); 2) infection of leaf buds causing malformations and necrosis in leaves (e.g. in ornamental plants); 3) feeding on stems, which may result in dry rot in above- and underground organs (occurs in many species such as, bulbs, wheat, beet); 4) attack of roots causing an overall growth reduction and malformations. In addition to direct damage caused by feeding, some nematode species also act as virus vectors (Schomaker and Been, 2013).

Another classification divides PPNs into three main types according to their feeding habit: ectoparasitic, endoparasitic and semi-endoparasitic. Ectoparasites do not enter plant tissue. They remain in the soil and feed with their stylets from the outside. Endoparasites entirely penetrate root tissue and can be categorized as either migratory or sedentary. Migratory endoparasitic nematodes are free-living in the soil and do not establish feeding sites (for example *Pratylenchus* spp.). Sedentary endoparasites induce nurse cells which provide them with nutrients throughout their lives (e.g., *Meloidogyne*, *Globodera*, *Heterodera* spp.). The nurse cell system is closely located and in some cases connected to the plant's vascular tissue and enables a continuous uptake of nutrients. Finally, semi-endoparasitic nematodes do not enter the plant tissue entirely: the posterior part remains in soil. However, these feeding types are not exclusive, as some genera also feed as migratory ecto-endoparasites for example (Decreamer and Hunt, 2013). Economically most important is a small group of sedentary endoparasitic nematodes including RKN (*Meloidogyne* spp.) and CN (*Heterodera*, *Globodera* spp.) nematodes (Jones *et al.*, 2013).

The focus of this study is laid on the CN *H. schachtii* and the RKN *M. incognita*. RKNs and CNs are root parasites with motile juveniles and sedentary, saccate adults. In both species, the juvenile stage (J2) is the infective stage. J2s establish feeding sites within the vascular root tissue which are composed of hyperactive cells providing them with nutrients throughout their life (Bohlmann, 2015; Escobar *et al.*, 2015).

Infection of sedentary endoparasitic nematodes

Sedentary endoparasitic nematodes in the mobile stage move freely through the soil and attraction towards host plants is guided by certain gradients. Soil compounds that may act as gradual attractants are amino acids, ions, pH, temperature and CO_2. However, the rhizosphere contains a complex mixture of repellent and attractant chemicals. To date, the way nematodes navigate to host roots remains unknown (Perry and Curtis, 2013). Local attractants in the rhizosphere are most likely specific allelochemicals or electrical potential gradients at the elongation zone of the root tip (Curtis, 2008). Once the nematodes reach the root surface, they adopt an exploratory behaviour accompanied by rhythmic stylet movements, increased motility and production of pharyngeal secretions (Doncaster and Seymour, 1973). Migration through plant tissue causes considerable damage depending on nematode species. Besides physical force, sedentary endoparasitic nematodes use different

tools to enhance movement within plant tissue. They release cell wall-degrading enzymes which are secreted into plant cells through the stylet (Davis *et al.*, 2011).

Inside the plant, sedentary endoparasitic nematodes migrate towards the vascular tissue where they select an initial feeding cell (Fig. 5) and induce complex feeding sites. This metabolically active tissue is their only nutrient source. CNs form syncytia and RKNs form giant cells. Syncytia are large multinucleate cells formed by local dissolution of plant cell walls and fusion of adjacent protoplasts (Jones and Northcote, 1972; Golinowski *et al.*, 1997). Giant cells are formed through repeated rounds of nuclear division and cell growth in the absence of cytokinesis (Dropkin, 1969; Jones and Goto, 2011).

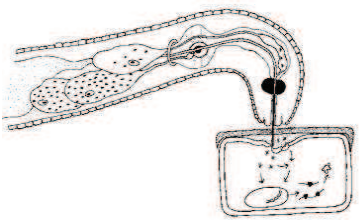

Fig. 5 Drawing of a PPN piercing a plant cell with its stylet (Davis *et al.*, 2000**).** The stylet punctures the plant cell wall but not the plasma membrane. Proteins are secreted through the stylet into the cell.

Both types of feeding sites share several features such as multiple enlarged nuclei, small vacuoles, and proliferation of the smooth endoplasmic reticulum, ribosomes, mitochondria and plastids (Gheysen and Jones, 2013). Despite these similarities, the life styles of CNs and RKNs differ in various aspects, such as the structure of stylets and pharyngeal glands as well as the migration pattern of J2s within root tissue (Wyss and Grundler, 1992; Perry and Curtis, 2013). In the following, these differences are examined in more detail.

Cyst nematodes

The largest genera of CNs are *Heterodera* with 82 species and *Globodera* with 12 species. Both genera contain many species of global agricultural importance. Most research is conducted on four CN species; *H. schachtii*, the sugar beet CN; *H. glycines*, the soybean CN; *G. rostochiensis*, the "golden" potato CN; and *G. pallida*, the "pale" potato CN (Bohlmann, 2015). Of agricultural interest are also cereal CNs, for example *H. avenae* and *H. filipjevi* (Jones *et al.*, 2013).

H. schachtii was discovered in sugar beets in 1859 by the botanist Hermann Schacht from Bonn, Germany. Only years later (1871) this new species was named and

became the first described PPN feeding on below-ground plant organs (Hallmann *et al.*, 2009). The sugar beet accounts for approximately 20 % of the global sugar production and Germany is the third largest producer in the EU (FAO, 2015; Eurostat., 2017). *H. schachtii* occurs in a broad range of climates, wherever sugar beet is grown (Baldwin and Mundo-Ocampo, 1991). Recently the transcriptome of *H. schachtii* J2s was published (Fosu-Nyarko *et al.*, 2016).

Infection of cyst nematodes

J2s of CNs penetrate the root tissue by physical disruption of cell walls caused by movements of their relatively strong and robust stylets. By thrusting their stylets, nematodes pierce epidermal cell walls at adjacent sites until the punctuations form a slit for invasion (Doncaster and Seymour, 1973; Wyss and Grundler, 1992). Along with rapid head movements and secretion of cell wall-degrading and modifying proteins, J2s move intracellularly and destructively through root tissue (Sijmons *et al.*, 1991; Gheysen and Jones, 2013). Within root tissue they move towards the vascular tissue where their behaviour becomes more exploratory. Stylet thrusts become less frequent and single cells are probed. Thereafter, the nematode carefully inserts the stylet and only retracts it in the event of an unwanted cell response, such as the deposition of callose-like material or protoplast collapse (Sobczak *et al.*, 1999; Wyss and Grundler, 1992). A cell that does not immediately respond with defence action becomes the initial feeding cell (IFC). Generally the IFC is a cambial or procambial cell (Golinowski *et al.*, 1996). The stylet remains protruded within the cell and a preparation phase follows for approximately seven hours (Wyss and Grundler, 1992). Secretions from the esophageal glands are then released into the cytoplasm of the plant cell (Wyss and Grundler, 1992). The secretions enter the cytoplasm through a small puncture in the plasmalemma in front of the opening of the hollow stylet (Sobczak *et al.*, 1999). The IFC enlarges, cytoplasmic streaming increases and the nematode begins feeding in repeated cycles. Each feeding cycle consists of three phases: uptake of nutrients by rapid pumping, retraction and reinsertion of the stylet and forward movement of secretory granules (Perry and Curtis, 2013). To withdraw food, CNs form feeding tubes that may appear between 24 and 36 hours after syncytium induction (Sobczak *et al.*, 1999; Wyss and Grundler, 1992). Feeding tubes are formed with secretions and connect the cytoplasm to the stylet orifice. Each feeding cycle requires the formation of a new feeding tube. To date the composition of the feeding tube remains elusive (Sobczak *et al.*, 1999). It is assumed that feeding tubes function as molecular sieves, since their diameter creates an exclusion size of less than 8.8 nm (Böckenhoff and

Grundler, 1994). While feeding tubes are formed by many sedentary nematode species, the formation of feeding plugs seems to be unique to CNs. Feeding plugs seal the perforated cell wall of the syncytium at the site of the inserted stylet (Sobczak *et al.*, 1999).

Syncytium development

The IFC visibly reacts to nematode induced changes within the first 24 hours (Golinowski *et al.*, 1996). The stylet tip and the cell wall of the IFC become covered with a callose-like material. The cytoplasm of the IFC increases while the size of the vacuole decreases and a number of small vacuoles forms (Golinowski *et al.*, 1996; Grundler *et al.*, 1998; Sobczak *et al.*, 1999). The expansion of existing plasmodesmata creates wall openings approximately six hours after IFC induction (Grundler *et al.*, 1998). Changes also occur in neighbouring cells before their protoplasts fuse with the IFC through the wall openings within 48 hours after induction (Golinowski *et al.*, 1996). Syncytial nuclei enlarge soon after IFC establishment and contain small nucleolar vacuoles due to their metabolic activity. Plastids increase in number and size. Also the number of mitochondria increases. The amount of endoplasmic reticulum is generally high and the cytoplasm is proliferated and electron dense (Sobczak and Golinowski, 2011). Subsequent hypertrophy of syncytial elements requires cell expansion through cell wall loosening, which is induced by upregulation of cell wall-modifying enzymes (Szakasits *et al.*, 2009; Gheysen and Jones, 2013).

A syncytium is a huge cell that may consist of more than 200 fused cells (Wyss and Grundler, 1992). The shape of a syncytium seems to be influenced by patterns of plasmodesmata distribution. They are abundant between cells of the same tissue type but appear less between two different cell types (Sobczak and Golinowski, 2011). In the early development of the syncytium, nutrients are ingested apoplastically by active transport. However, in a mature syncytium (12 to 15 days after induction), plasmodesmata connected to the phloem are re-opened and formed de novo, and the nutrients are taken up symplasmically (Hoth *et al.*, 2005, 2008; Hofmann and Grundler, 2006; Hofmann *et al.*, 2007). *H. schachtii* females infecting *Arabidopsis thaliana* (Arabidopsis) take up an amount of solutes that is four times the volume of the associated syncytia per day (Sijmons *et al.*, 1991). The syncytium induces a strong sink for plant assimilates from which the nematode draws nutrients (Jones and Northcote, 1972). Nematode males and females develop within two weeks, including three moults. Adult males stop feeding and hatch to copulate

with females while the associated syncytia degenerate. Syncytia of females remain functional until egg production is completed. Then females die and their bodies transform into brown cysts, which contain several hundreds of eggs. The cysts outlast in the soil until juveniles hatch in favourable conditions (Fig.6) (Bohlmann, 2015).

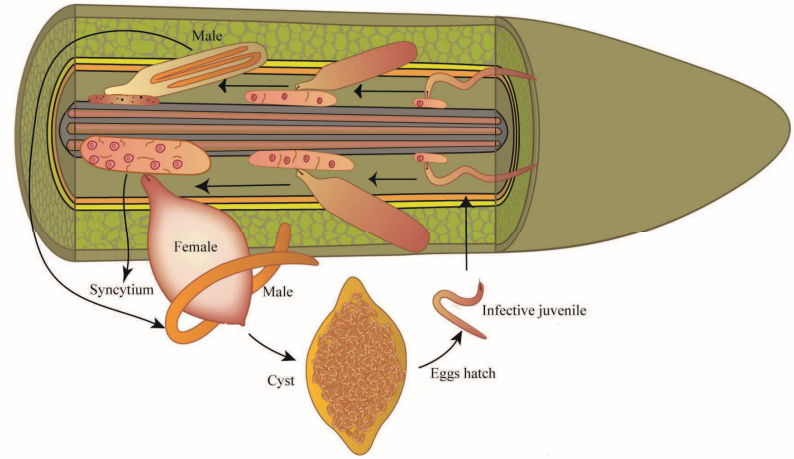

Fig. 6 Life cycle of cyst nematodes (Siddique and Grundler, 2018). Infective juveniles (J2s) hatch from a dormant cyst filled with eggs. J2s invade root tissue, initiate feeding sites and establish multinucleate syncytia. The male hatches in search for a female while its syncytium degrades. The female and its associated syncytium enlarges further. After egg production the female turns into a dead cyst.

Root-knot nematodes

RKNs infect almost all vascular plants such as crops, fruit trees and ornamental plants. A characteristic feature for RKN infection is the formation of root galls. About 90 species are identified in the genus *Meloidogyne* (= apple-shaped female) Göldi, 1887, which is a relatively small group of plant parasites. In contrast to CNs, many species of RKNs have wide host ranges infecting more than thousand plant species. They occur in temperate as well as in tropic regions and several of them are widespread. Well known tropical species are: *M. incognita*, *M. javanica* and *M. arenaria*. Other notable RKNs are *M. hapla* and *M. chitwoodi* in temperate regions (Jones *et al.*, 2013; Escobar *et al.*, 2015). RKNs are not strongly regulated through

phytosanitary measures because the economically most important species are already distributed worldwide (Hockland *et al.*, 2013). The ability of RKNs to infect so many different plant species is thought to be a result of the migration pattern in plant tissue which differs from CNs. J2s of *Meloidogyne* move intercellularly – in between cells – whereby cell walls remain intact. Hereby J2s potentially avoid an immediate defence response by the plant and a need to adapt to different cell wall compositions (Gheysen and Jones, 2013). The genomes of two RKN species, *M. incognita* and *M. hapla,* have been published, constituting the first sequenced genomes of plant parasites (Opperman *et al.*, 2008; Abad *et al.*, 2008).

Infection of root-knot nematodes

J2s of RKNs are especially attracted to growing root tips and zones of emerging lateral roots. At the meristematic and elongation region of the root, J2s enter the tissue with a combination of physical force with their comparatively delicate stylet, rubbing movements with their head and enzymatic secretions of cell wall-degrading enzymes. The J2s move mainly intercellularly through the root cortex toward the apical meristematic region of the root tip. There, they change direction to enter the emerging vascular tissue (Sijmons *et al.*, 1991; Wyss *et al.*, 1992). Even though other migration patterns have been observed such as intracellular movement (Gravato Nobre *et al.*, 1995), it is generally accepted that the characteristic migration pattern of RKN J2s is intercellular. Intercellular movement is less destructive to root tissue and therefore might reduce cell response to a minimum (Shah *et al.*, 2017). However, the J2s also secrete cell wall-degrading enzymes to enable the separation of the middle lamella. This degradation process is accompanied by mechanical pressure which ruptures plasmodesmata (Jones and Payne, 1978). Within the vascular tissue the J2s search for a potential feeding cell. Therefore a number of cells are pierced by stylet thrusting until one cell responds positively to the J2. At that point, J2s induce the formation of several giant cells (Jones and Payne, 1978; Wyss *et al.*, 1992).

Giant cell formation and gall development

At the IFC, RKN J2s perform a characteristic behavioural pattern that includes head and stylet movements, interspersed with stylet thrusting and pumping of the metacarpal bulb. The induction of the IFC is facilitated by the secretion of a protein cocktail (Sijmons *et al.*, 1991; Wyss *et al.*, 1992; Wyss and Grundler, 1992). Finally, giant cell formation is a consequence of re-programming of cellular differentiation by the J2. Part of the re-programming are stimulated cell division and the formation

of binucleate cells in adjacent cells. The nuclear division cycle, consisting of mitosis without cytokinesis, is repeated to generate more nuclei in developing giant cells (Jones and Payne, 1978). Even after establishment of the initial giant cell, the RKN retains the ability to move its head and induce other giant cells in the vicinity (Sijmons *et al.*, 1991; Wyss and Grundler, 1992). The giant cells expand rapidly within two weeks of development. Central vacuoles are thereby replaced by smaller ones. Additionally, ground cytoplasm, Golgi bodies, mitochondria and endoplasmic reticulum increase in number. Nuclei and nucleoli become enlarged. As giant cells elongate and expand, also the surrounding tissue swells and the characteristic root galls form (Jones and Goto, 2011). Cell wall ingrowths are formed at walls of giant cells adjacent to vascular tissue, approximately three days after infection. The wall ingrowths are typical for plant transfer cells and enable solute uptake from the apoplast into the symplast. As the giant cells increase in size, also the density of cell wall ingrowths increases (Jones and Dropkin, 1976; Jones and Payne, 1978; Bartlem *et al.*, 2013). Cell walls between giant cells are characterized by a large number of plasmodesmata, which are secondarily formed (Jones and Dropkin, 1976; Jones and Goto, 2011). Since giant cell formation comprises vascular continuity in the root, wound-type vascular elements often surrounds giant cells (Bartlem *et al.*, 2013).

Inside the gall, the nematode body is sedentary except for the head which feeds from the different giant cells (Sijmons *et al.*, 1991; Wyss and Grundler, 1992). To enable nutrient uptake, RKNs also form feeding tubes. The feeding tube is a cylinder that connects the stylet orifice with the cytoplasm of the plant cell. In giant cells, feeding tubes appear after stylet insertion by self-assembly of nematode secretions. The stylet protrudes through the cell wall of a giant cell without destroying the plasma membrane. The feeding tube is semi-crystalline and proteinaceous and has a diameter of less than 1 µm. Since RKNs induce several giant cells, the stylet must be withdrawn after feeding and the associate feeding tube is released. For that reason, a number of feeding tubes can be found in the cytoplasm of a giant cell. The feeding tubes serve as ultrafilter as well as pressure regulator (Hussey and Mims, 1990; Jones and Goto, 2011).

Giant cells function as sinks to provide nutrients to the nematode (McClure, 1977; Bartlem *et al.*, 2013). RKNs complete their life cycle within 20 to 40 days. *M. incognita* and most RKNs multiply by mitotic parthenogenesis (asexual reproduction). After three moults, nematodes adopt a round shape. The adult female resumes feeding and lays hundreds of eggs in a gelatinous egg mass. J2s

subsequently hatch rapidly under favourable conditions since the egg mass does not provide a protective barrier as in the case of CNs (Fig. 7) (Escobar *et al.*, 2015).

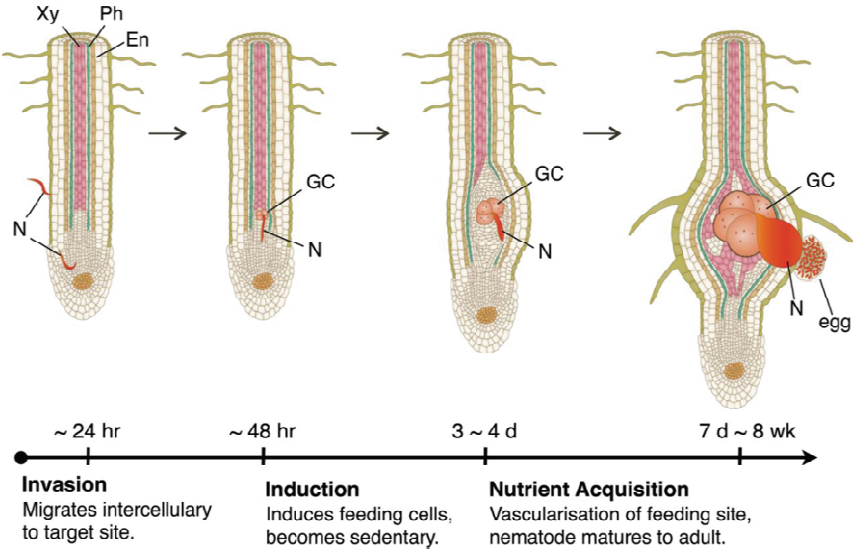

Fig. 7 The root-knot nematode life cycle in a schematic drawing (Bartlem *et al.*, 2013). A juvenile nematode (N) infects a host root, performs a U-turn close to the root tip and migrates within the vascular tissue (En, endodermis; Xy, xylem; Ph, Phloem). The nematode induces several giant cells (GC). GCs and surrounding tissue expand and a gall is formed. The nematode moults three times within the gall. The mature female lays approximately 500 eggs in a gelatinous mass into the rhizosphere.

Plant response

The plant immune system consists of two response layers. First, plants detect microbes and pathogens based on conserved molecular patterns (pathogen/ microbe-associated molecular pattern, PAMP/MAMP), which are sensed by pattern recognition receptors (PRRs) on the cell surface. Detection of PAMPs by PRRs activates a plethora of responses termed as pattern-triggered immunity (PTI). At this point, the infection of plant tissue can only occur if the parasite suppresses PTI. Therefore, successful pathogens have evolved proteinaceous and non-proteinaceous effectors, which are secreted into the infected tissue to overcome PTI. Nevertheless, plants evolved a second layer of cytoplasmic receptors (so-called R genes), which can recognize either the effectors or metabolic changes induced by effectors. The

activation of R-genes leads to an effector-triggered immunity (ETI), which may culminate in the form of programmed cell death as a hypersensitive response in infected tissue (Fig. 8) (Gheysen and Jones, 2013; Jones and Dangl, 2006). If the defence response of the plant is too weak to defeat the pathogen, the infection will be successful. In that case, the pathogen can complete its life cycle within plant tissue, which is called a compatible interaction.

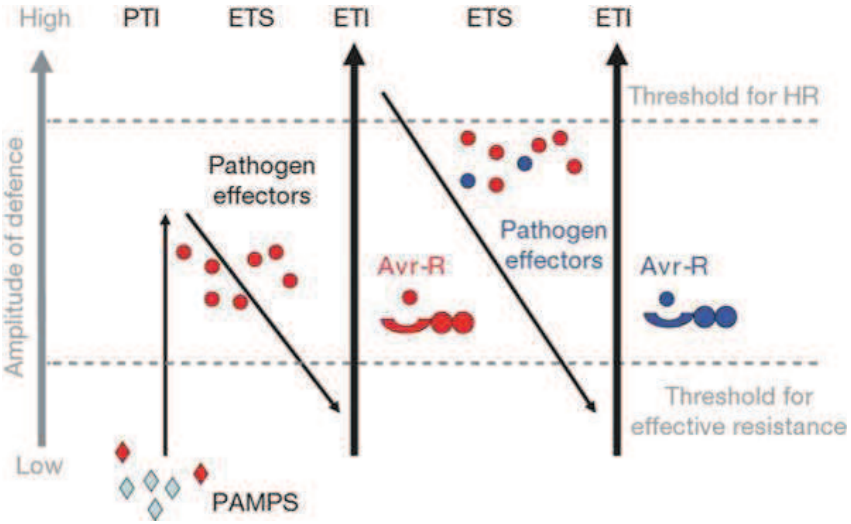

Fig. 8 Scheme of the plant immune system (Jones and Dangl, 2006). PAMP-triggered immunity (PTI) is induced when plants detect microbe/pathogen-associated molecular patterns (MAMPs/ PAMPs) by pattern recognition receptors. Pathogens overcome PTI with secreted effectors, resulting in effector-triggered susceptibility (ETS). Cytoplasmic receptors (R genes) recognize effectors and activate effector-triggered immunity (ETI), which may cause cell death. Effectors unknown to the plant's immune system suppress ETI and trigger ETS.

Arabidopsis in plant-nematode interaction

Arabidopsis became the model for molecular genetics in the 1980s. The cruciferous weed has several advantages such as a short life cycle and a small genome size which was published by Meyerowitz (2001). Another advantage of Arabidopsis is the availability of mutants, which provides an ideal resource for molecular-genetic analyses of plant processes. Already in 1991, it was reported that Arabidopsis can function as a host for several nematode species (Sijmons *et al.*, 1991). The thin and

translucent roots enable *in vivo* observation of the early stages of plant-nematode interaction (Wyss and Grundler, 1992). Just a short time later, analyses of GUS-lines showed differential gene expression in feeding sites (Goddijn *et al.*, 1993). A detailed comparison of transcriptome data revealed that the syncytium of CNs is similar to seeds and pollen (Szakasits *et al.*, 2009). The transcriptome of giant cells, however, shares similarities with that of crown-gall tissue induced by *Agrobacterium tumefaciens* (Barcala *et al.*, 2010).

The Arabidopsis-nematode pathogenicity model also presents several challenges. Studies on nematode infectivity often face low infestation rates with high variations and therefore require the inclusion of a large number of plant replicates. Also, the high degree of redundancy in plant molecular mechanisms often makes it difficult to identify the specific signal transduction pathway of a certain process. Additionally, mutants with decreased infection levels often exhibit strong phenotypes. Another drawback of the Arabidopsis-nematode model is that no resistance gene has been identified. Therefore, only compatible plant-nematode interactions can be studied in Arabidopsis. Despite these limitations, Arabidopsis represents the best model plant to date to study nematode infection *in vivo* on cellular and molecular levels (Gheysen and Fenoll, 2011).

Arabidopsis roots

The root tissue of Arabidopsis follows a simple radial pattern: single layers of epidermis (trichoblasts and atrichoblasts), cortex (parenchyma cells), endodermis and pericycle, which encircle vascular cells (xylem and phloem embedded in parenchyma cells) in the centre (Fig. 9). Primary roots reveal this pattern already 1 mm from the root tip at three day old seedlings (Dolan *et al.*, 1993). The endodermis abuts the vascular tissue and has special functions regarding the nutrient household as a selective tissue (Schreiber, 2010; Geldner, 2013*a*; Nawrath *et al.*, 2013).

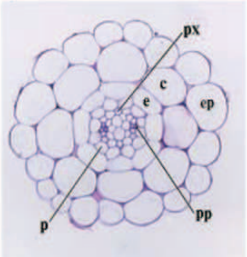

Fig. 9 Cross section of *A. thaliana* root stained with toluidine blue, seven days after germination (Scheres *et al.,* 1995). ep, epidermis; c, cortex; e, endodermis; px, protoxylem; pp, protophloem; p, pericycle.

The endodermis – A primary diffusion barrier

Terrestrial plants form diffusion barriers in the apoplast of tissues in organs above and below ground. The shoot of the epidermis is covered with the cuticle, building up the primary diffusion barrier. The cuticle is composed of cutin and wax, which is covalently linked to the cell wall of the outward facing epidermal wall. This lipid incrustation controls the exchange of water, gases and solutes and contributes to the surface structure of above-ground organs (reviewed in Schreiber, 2010). In roots, the apoplastic barrier is a compromise between nutrient uptake and protection. The diffusion barrier, therefore, is not built up in the epidermis but in the internal endodermis, which bears the function of an epithelium. The endodermis seals the vascular tissue from the environment and prevents uncontrolled diffusion of substances into or out of the stele allowing selective uptake of substances that are available in the cortex (reviewed in Enstone *et al.*, 2002; Geldner, 2013). To fulfil this vital function, the endodermis undergoes two developmental steps (Fig. 10). First, the extracellular space between adjacent endodermal cells is sealed by Casparian strips (CS) in a belt-like manner. The CS is a hydrophobic lignin-based impregnation of the primary cell wall. In addition, to impede lateral diffusion, the plasma membrane adheres tightly to the cell wall at the location of the CS. Diffusion from the cortex into the stele is thereby efficiently blocked (Alassimone *et al.*, 2010; Naseer *et al.*, 2012). In a secondary developmental step, the endodermal cells are impregnated by suberin lamellae in the primary cell wall just outside the plasma membrane. This is called the secondary stage of differentiation or “state II”. (reviewed in Nawrath *et al.*, 2013; Ranathunge *et al.*, 2011). The suberin deposition occurs in an individual manner which leads to a patchy appearance closer to the root tip. In older roots, suberin depositions become homogenous (Naseer *et al.*, 2012; Barberon *et al.*, 2016).

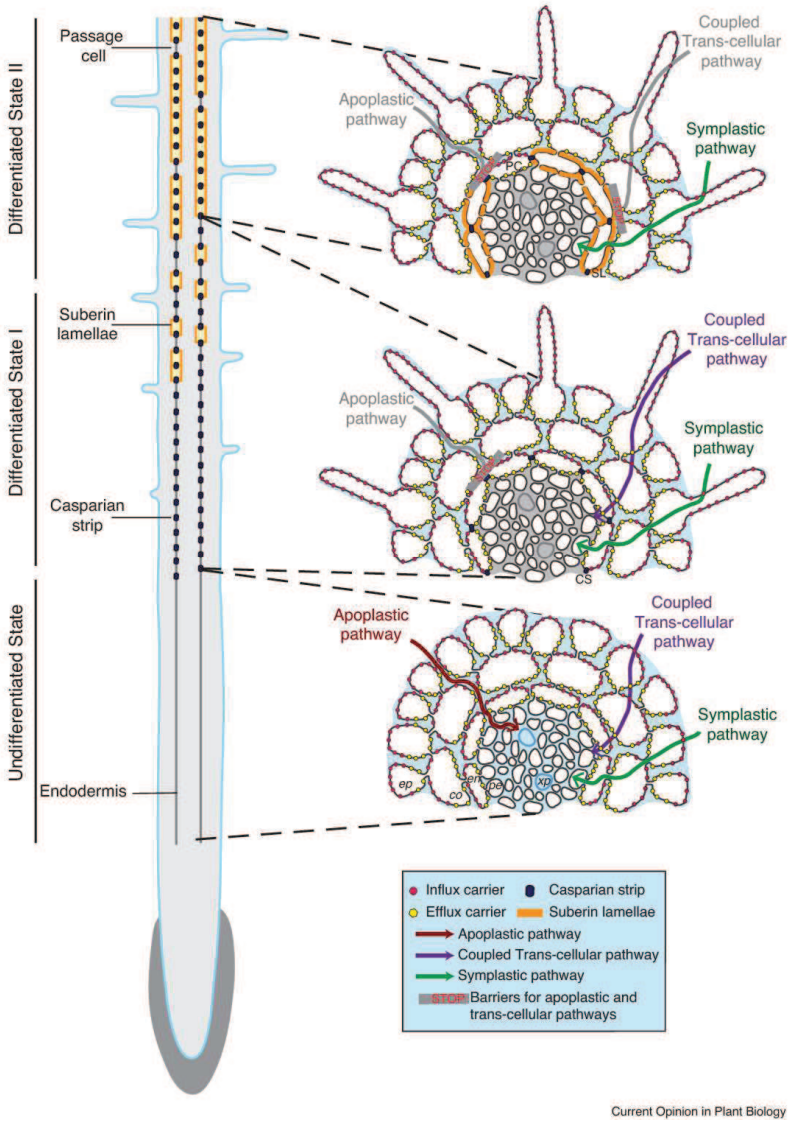

Fig. 10 Schematic drawings of the two states of endodermal differentiation and radial transport of water and solutes (Andersen *et al.*, 2015). The state I endodermis is characterized by the formation of Casparian strip and the state II by suberization of the secondary cell wall. Arrows indicates different pathways for radial transport of solutes. In the undifferentiated state, three pathways (apoplastic, symplastic and coupled trans-cellular) exist. In state I, the Casparian strip blocks the apoplastic pathway and at state II only the symplastic

pathway transport solutes across the endodermis. However, even at state II endodermis non-suberized passage cells may enable the coupled trans-cellular pathway. ep, epidermis; co, cortex; en, endodermis; pe, pericycle; xp, xylem pole; CS, Casparian strip; SL, suberin lamellae; PC, passage cell.

Suberin establishes a barrier for solute uptake from the cortex into the stele. Together CSs and suberin block the apoplastic pathway for solutes, thereby only allowing the symplastic pathway, which acts as a selective filter (Fig.10) (Barberon *et al.*, 2016).

Unlike many other plants, Arabidopsis roots do not develop an exodermis, a cell layer behind the epidermis, which is suberized and lignified in many plants (Hose *et al.*, 2001; Wilson and Peterson, 1983). The endodermis constitutes the only diffusion barrier within the roots of Arabidopsis (Dolan *et al.*, 1993; Nawrath *et al.*, 2013).

The anatomy and function of root tissue changes when they reach the secondary developmental stage (secondary growth). Secondary thickening in dicots provides essential stability to ensure longitudinal transport of water and solutes and contributes less to radial uptake. The three outer layers (epidermis, cortex and endodermis) are shed and thereby replaced by the periderm (Dolan *et al.*, 1993). This secondary tissue develops in the pericycle and consists of one or two layers of suberized cells. Suberin is deposited at the inner side of the cell wall, next to the plasma membrane with the characteristic lamellae (Nawrath *et al.*, 2013).

The Casparian strip – Endodermal lignin

For a long time, it was assumed that the CS consists mainly of suberin (Bonnett, 1968). This assumption persisted due to several factors which complicate the identification of the CS substance composition. One constraint is the location; pure CS samples are difficult to obtain and are inevitably covered by suberin. Decades later, however, several analyses of isolated CS from monocotyledoneous species indicated that lignin is a major compound (Schreiber and Riederer, 1996; Schreiber *et al.*, 1994; Schreiber *et al.*, 1999; Zeier and Schreiber, 1998). In 2012, Naseer *et* al. reported that CS in Arabidopsis roots is exclusively made of lignin. This study focused on analysing the property of the diffusion barrier in the endodermis and showed that the barrier is functional even prior to suberin depositions. Thereby it is concluded that suberin does not contribute to the establishment of an apoplastic barrier. Based on detailed observations, Naseer *et al.* (2012) showed that the CS is

deposited approximately 12 cells after onset of elongation from the root tip but suberization begins only 38 cells after onset of elongation. Lignification at the location of CS in the primary cell wall comes along with a tight attachment of the plasma membrane to the cell wall at this very location (Fig.11). This cell wall specialization leads to a solute diffusion barrier in the apoplast (Alassimone *et al.*, 2010; Alassimone *et al.*, 2012; Bonnett, 1968; Roppolo *et al.*, 2011). Isolated CSs appear like a fishnet due to their tight junctions (Fig.12).

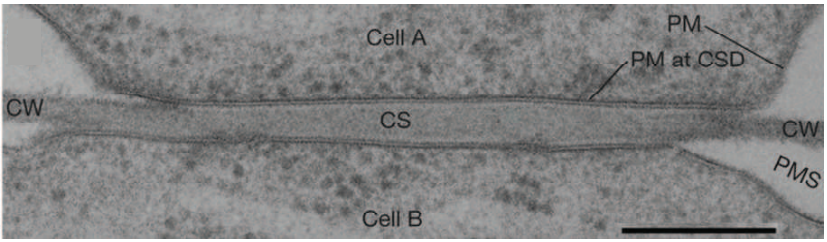

Fig. 11 Casparian strip (CS) in scanning electron micrograph (Roppolo *et al.*, 2011). CW, cell wall; CSD, Casparian strip domain; PM, plasma membrane; PMS, space generated by plasmolysis (bar= 250 nm).

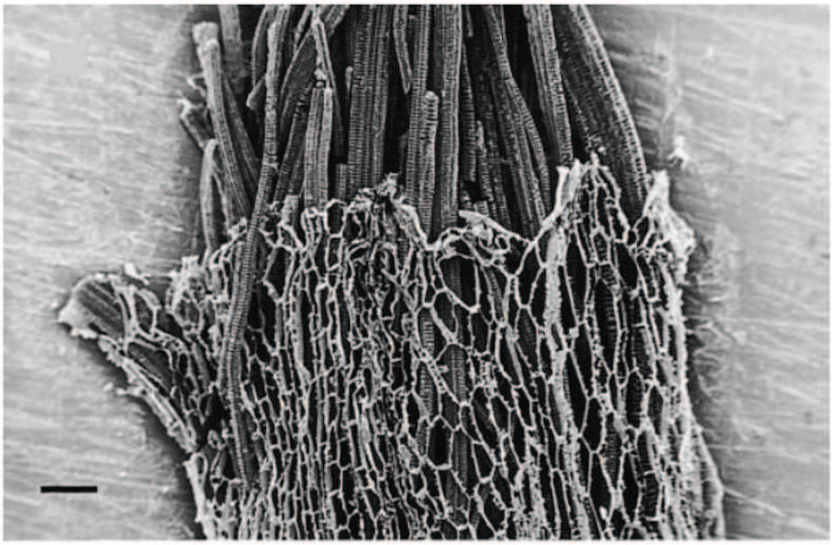

Fig. 12 Enzymatically isolated Casparian strip (CS) in scanning electron micrograph of a root from *Clivia miniata* (Schreiber *et al.*, 1999). CSs encircle vascular tissue (xylem vessels visible; bar= 100 mm).

Formation of the Casparian strip

CSs are belt-like cell wall thickenings, which impregnate primary cell walls of neighbouring endodermal cells, forming impermeable junctions (Naseer *et al.*, 2012). A protein family localized to the plasma membrane at the CS region has been characterized to play a role in the formation of CS. This *CASP* (Casparian strip membrane domain protein) family includes five proteins with transmembrane-spanning domains (*CASP1*, At2g36100; *CASP2*, At3g11550; *CASP3*, At2g27370; *CASP4*, At5g06200; and *CASP5*, At5g15290). These *CASPs* form complexes, creating membrane protein platforms, which mediate tight attachments to the cell wall. A belt-like structure spanning the entire cell is thereby formed (Roppolo *et al.*, 2011). The *CASPs* are thought to mediate CS formation by recruiting various proteins such as the *NADPH oxidase RBOHF* (*Respiratory Burst Oxidase Homolog F*, At1g64060), the peroxidase *PER64* (At5g42180) and the dirigent-domain protein *ESB1-1* (*enhanced suberin 1-1*) for lignin polymerization (Roppolo *et al.*, 2011; Hosmani *et al.*, 2013; Lee *et al.*, 2013). Other genes that play a crucial role in CS formation are a family of three receptor-like kinases, called *Schengen*. The localization of these receptor-like kinases overlaps with the Casparian strip domain and they have been shown to define a barrier surveillance system in plants (Alassimone *et al.*, 2016; Doblas *et al.*, 2017; Lee *et al.*, 2013; Pfister *et al.*, 2014). These recent advances indicate details of the complex regulatory network for CS formation and emphasize that the endodermal barrier is much more dynamic than anticipated (reviewed in von Wangenheim *et al.*, 2017).

Lignin

Next to cellulose, lignin constitutes the most abundant polymer in the biosphere. The synthesis of lignin belongs to essential evolutionary adaptations of terrestrial plants. Lignified cells provide structural integrity, affording strength as well as protection against microbial degradation. Lignin is therefore important for the reinforcement of vascular cells. This biopolymer is incorporated in the secondary cell wall at the end of cell differentiation. It is a hydrophobic and aromatic polymer consisting of 4-hydroxyphenylpropanoids, mainly deposited during secondary thickening of cells. The main building blocks are the three monolignols (hydroxycinnamyl alcohols), methoxylated to various degrees: *p*-coumaryl, coniferyl, sinapyl alcohols (Boerjan *et al.*, 2003; Vanholme *et al.*, 2010). Coniferyl and synapyl alcohols are the main compounds and give rise to the guaiacyl (G) and syringyl (S) units within the lignin polymer, respectively. The *p*-coumaryl alcohol gives rise to the *p*-hydroxyphenoyl (H) units in the biopolymer but is less abundant. These lignin

monomers contribute to the complex organization of the polymer, which is based on radical reactions that lead to covalent cross-linkages and random patterns of monolignols. This unpredictable pattern might contribute to resistance against microbial digestion (Iiyama *et al., 1994;* Sarkar *et al.*, 2009). Lignification of cell walls also occurs in response to biotic and abiotic stress (Sattler and Funnell-Harris, 2013; Malinovsky *et al.*, 2014). Even though lignin contributes to pathogen defence responses in many plant species, this study is focused mainly on the role of CS and suberin in the endodermis.

Suberin – A polyester with controversy

During evolution all terrestrial plants deposited suberin in the endodermis and many also in the exodermis (hypodermis) of roots (Enstone *et al.*, 2002). But this lipophilic polyester forms interfaces in many plant organs as a protection from the environment (Franke and Schreiber, 2007). The greatest source of suberin is the periderm of the cork oak tree (*Quercus suber*). More than 40 % of the bark is composed of suberin (Pereira, 1988). High suberin content is also found in potato (*Solanum tuberosum*) periderm as well as in other storage organs (Graça and Pereira, 2000). Also seed coats are impregnated with the polymer to prevent desiccation (Beisson *et al.*, 2007; Compagnon *et al.*, 2009).

Suberin is chemically similar to the cutin polymer of the plant cuticle, covering above ground organs. Both biopolymers, cutin and suberin, are aliphatic polyesters, but suberin is composed of higher chain lengths and greater amounts of incorporated ester-bound aromatics (Kolattukudy, 1980; Nawrath *et al.*, 2013; Schreiber, 2010). In Arabidopsis, suberin mainly occurs in the endodermis, root periderm and seed coats (Franke *et al.*, 2005; Beisson *et al.*, 2007; Compagnon *et al.*, 2009).

Suberin analysis in Arabidopsis

Since Arabidopsis plants have very fragile, faint roots, whole root sections are typically used for chemical analyses without prior isolation of suberized tissue (separating endodermis from other tissue). The method to analyse the chemical composition of suberin includes digesting the root material with polysaccharide hydrolyses to remove cell wall material. In a second step, unbound components, such as waxes are reduced by solvent extraction. It is impossible to isolate suberin as an intact polymer. Consequently, suberin monomers are extracted from the polymer and the overall organization of these must be recreated theoretically (Franke *et al.*, 2005; Nawrath *et al.*, 2013).

The suberin composition of Arabidopsis roots resembles that of other plant species. Actually, only minor differences were detected in comparison to cutin monomer composition. The quantitatively major monomer compounds of aliphatic suberin are bifunctional C18 monounsaturated ω-hydroxyacids and α-ω-dicarboxylic acids (DCA). Saturated ω-hydroxyacids, α-ω-DCAs, monofunctional C18 to C24 fatty acids and alcohols occur in lesser amount. Also detected in relatively lower quantities were aromatics (phenolic suberin), such as ferulic acid and *p*-coumeric acid as well as glycerol (Franke *et al*., 2005).

The suberin model

Based on the monomer composition gained from depolymerisation, models of the macromolecular structure of the suberin polymer have been created (Fig. 13). In general, suberin is described as a polymer containing polyaromatic and polyaliphatic domains, which are covalently linked together. Glycerol is thought to function as an anchor for long-chain diacids, which are the core of the polymer. The structure grows thereby three-dimensionally. Presumably, ferulic acids connect the aliphatic and aromatic domain, while the latter is incorporated into the primary cell wall (Bernards, 2002; Graça and Santos, 2007). The structure of suberin appears in an alternating translucent lamellar in sections of suberized cells (Kolattukudy, 1980; Serra *et al*., 2010; Nawrath *et al*., 2013).

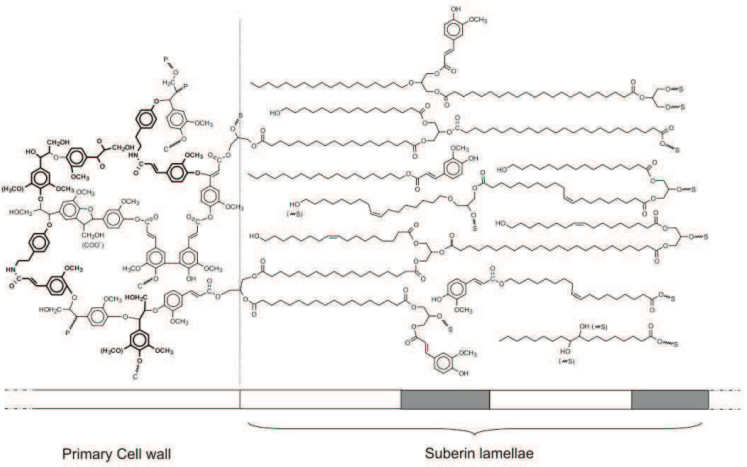

Fig. 13 Tentative model for the structure of suberin (Bernards, 2002). The phenolic suberin domain is attached to the primary cell wall by covalent bonds and the aliphatic domain generates the suberin lamellae.

However, a recent study raised doubts about this assumptions. Through the use of an inhibitor of the phenylpropanoid pathway in Arabidopsis, the formation of the phenolic suberin domain was blocked (Naseer *et al.*, 2012). This treatment, however, did not affect the formation of suberin. The result showed that the aliphatic domain of suberin forms independently from the polyaromatic domain. Nawrath *et al.* (2013) argued that if these domains are independent from each other, their ability to form one connected polymer is questionable. This assumption also supports the findings from an Arabidopsis knockout mutant (*asft/hht,* aliphatic suberin feruloyl transferase/ hydroxycinnamoyl-CoA: ω-hydroxyacid O-hydroxycinnamoyl transferase), which displays reduced ester linked ferulic acids but does not show a defective suberin lamellae (Molina *et al.*, 2009; Serra *et al.*, 2010). Taken together, these results prompted a discussion concerning the appropriate nomenclature of aliphatic and aromatic suberin polyesters (Geldner, 2013).

Suberin biosynthesis

The aromatic domain of suberin is composed of monomers from the phenylpropanoid pathway (Bernards *et al.*, 1995). Monomers of the aliphatic domain are a branch of the lipid biosynthetic pathway. Because suberin and cutin share predominant classes of compounds (ω-hydroxy acids, α-ω-DCA, alcohols, carboxylic acids and fatty acids) their biosynthesis is presumably based on similar enzymes. The following biosynthetic steps are common: oxygenation, reduction and activation of fatty acids and their transfer to glycerol-3-phosphate (Fig. 14). Differences between cutin and suberin are mainly the abundance of very long-chain fatty acid derivatives and the presence of aromatic compounds in suberin, which are predominantly ferulic acids. The step of fatty acid elongation, therefore, is elusive for suberin biosynthesis. Until now, the order in which the single reactions take place has not been completely determined. The major suberin biosynthetic steps and associated genes are described below (Beisson *et al.*, 2012; Franke *et al.*, 2012; Nawrath *et al.*, 2013; Pollard *et al.*, 2008; Vishwanath *et al.*, 2015).

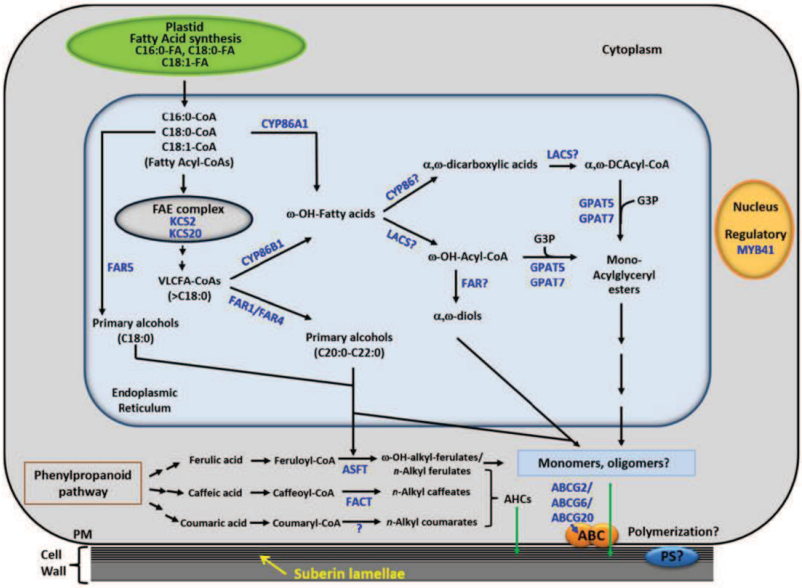

Fig. 14 Graphic overview of the suberin biosynthesis pathways (Vishwanath *et al.*, 2015). FAE, fatty acid elongation; VLCFA, very long-chain fatty acid; FAR, fatty acyl reductase; CYP, cytochrome P450 enzyme; ω-OH, ω-hydroxy fatty acid; DCA, α-ω-dicarboxylic acid; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phopsphate acyltransferase; LACS, long-chain acyl-CoA synthetases; ABC, ATP-binding-cassette; PM, plasma membrane; PS, polyester synthase; AHC, alkyl hydroxycinnamates; ASFT, Aliphatic Suberin Feruloyl Transferase; FACT, Fatty Alcohol:Caffeoyl-CoA Caffeoyl Tranferase.

Oxygenation of fatty acids

70 to 90 % of the suberin monomers in Arabidopsis are oxygenated fatty acids (FAs) (Franke *et al.*, 2005). Oxygen is inserted into the carbon chain of FAs during an enzyme-catalysed step by cytochrome P450 oxygenases (P450s). FA hydroxylases of suberin monomers belong primarily to the CYP86 family of P450s. CYP86A1 (At5g58860), a FA ω-hydroxylase, is involved in suberin biosynthesis. The corresponding T-DNA insertion mutant *horst* (*hydroxylase of root suberized tissue*) has reduced amounts of C16 and C18 ω-hydroxy acids, which results in a 60 % decrease of total aliphatic suberin (Höfer *et al.*, 2008; Li *et al.*, 2007). Another P450 identified in suberin biosynthesis is CYP86B1 (At5g23190), whose corresponding mutant *ralph* (*root aliphatic root hydroxylase*) reveals reduced very-long-chain ω-hydroxy acids (C22-C24). CYP86B1 plays an important role in the ω-oxygenation of

polyester precursors (Compagnon *et al.*, 2009; Molina *et al.*, 2009). In both mutants the corresponding α-ω-DCAs are reduced.

Fatty acid reduction

Fatty alcohols are formed by reduction of activated fatty acids and constitute a fraction of up to 6 % of aliphatic suberin (Pollard *et al.*, 2008). The enzyme fatty acyl-CoA reductase (FAR) catalyses the reduction of FAs to primary alcohols. FARs constitute a small protein family in Arabidopsis with eight members. *far5* (At3g44550), *far4* (At3g44540) and *far1* (At5g22500) mutants show reduced levels of C18, C20 and C22 alcohols, respectively (Domergue *et al.*, 2010; Kosma *et al.*, 2012).

Fatty acid activation

Fatty acid synthesis begins with converting free fatty acids to thioesters. To date, the activation of fatty acids to the corresponding acyl-CoA-esters by long-chain acyl-CoA synthases (LACS) has not been proven. However, this modification is necessary since FARs require CoA-activated FAs. The function of LACS in this process has been shown in cutin synthesis (Nawrath *et al.*, 2013; Vishwanath *et al.*, 2015). Also, *LACS1* (At2g47249) and *LACS2* (At1g49430) are expressed in roots (Schnurr *et al.*, 2004; Weng *et al.*, 2010).

Acyl transfer to glycerol-3-phosphate

FA conjugation to form acylglycerols is catalysed by acylCoA:glycerol-3-phophate acyltransferases (GPATs) (Zheng *et al.*, 2003). In suberin biosynthesis, the enzyme GPAT5 (At3g114309) catalyses the acyl transfer to the sn-2 position of a glycerol-based acceptor (Li *et al.*, 2007). The corresponding mutant is reduced in C20-C24 fatty acids, ω-hydroxy acids and α-ω-DCAs, which accounts for a 50 % decrease in aliphatic suberin (Beisson *et al.*, 2007).

Fatty acid elongation

Fatty acid elongation is catalysed by 3-keto acyl-CoA synthases (KCS), a family that includes 21 members. KCS2/DAISY (docosanoic acid synthase, At1g04220) and KCS2.0 (At5g43760) produce fatty acids with chain length >C20 (Franke *et al.*, 2009; Lee *et al.*, 2009). Corresponding mutants have moderate suberin phenotypes, indicating functional redundancy in the KCSs family. To date, however, no further KCS enzyme involved in suberin biosynthesis has been described even though other KCSs members are showing strong expression in root tissue (Franke *et al.*, 2012).

Linking aromatic and aliphatic domains

Ferulic and coumaric acids are main compounds of the phenolic suberin domain, which are supposedly linked by ester-bounds to the aliphatic domain. These alkyl hydroxycinnamates were identified in roots and are transferred to the aliphatic compounds by different members of the BAHD acyltransferase family (Nawrath *et al*., 2013). One member responsible for the incorporation of ferulate is ASFT/HHT (At5g41040). Corresponding mutants are strongly reduced in suberin-associated ferulate but are not affected in aliphatic suberin (Gou *et al*., 2009; Molina *et al*., 2009).

Suberin and lignin in plant defence

Both suberin and lignin provide mechanical barriers that are potentially able to prevent penetration by pathogens. The role of lignin in fungi and bacteria defence as a pre-existing barrier or as pathogen-induced deposition has been reviewed by Miedes *et al.* (2014). Lignin deposition is induced upon a range of abiotic and biotic stresses in many plant species (reviewed in Moura *et al*., 2010). Suberin deposition in response to pathogen attack has especially been studied in potato tuber discs. Experiments with wound-healing suberization showed that deposition of aromatic (lignin-like) compounds provided resistance against bacterial infection. Fungal growth, however, ceased only after aliphatic suberin completed the closing layer formation on the potato discs (Lulai and Corsini, 1998). Little is known, however, about the role of endodermal suberin and lignin in pathogen defence. Nevertheless, a few studies addressed their role against nematode infection.

The resistance response of *Hordeum chilense* (wild grass) to infection with the RKN *M. naasi* includes lignin deposition in infected cortical and endodermal cells 24 hours after inoculation. Ten days after inoculation, the endodermis showed thickened walls due to suberin and lignin depositions. The resistance response of *Aegilops variabilis* (wild grass) to *M. naasi* also included the deposition of suberin or lignin in the endodermis. In both plant species, resistance begun with a hypersensitive response and deposition of callose-like material in close vicinity to the nematode, followed by lignin and/ or suberin deposition in cortical and endodermal cells. These resistance responses prevented the RKN from invading into the vascular tissue at the site of the endodermis (Balhadère and Evans, 1995). Another study on resistant banana cultivars found higher degrees of lignification in the stele and endodermis after infection with the burrowing nematode *Radopholus similis*. Interestingly, only resistant cultivars had a clearly visible suberization in the

endodermis before nematode infection. The authors concluded that the high level of lignification and suberization in the endodermis of resistant banana cultivars prevented nematode invasion into the vascular tissue (Valette *et al*., 1998). Recently, the accumulation of phenolics and lignin in cell walls of banana roots was detected in response to infection with the root lesion nematode *P. coffeae*. The authors correlated the increase of monolignol precursors and the induction of two enzymes from the phenylpropanoid pathway with higher nematode resistance in certain cultivars as compared to susceptible ones (Vaganan *et al*., 2014). The deposition of lignin and cell-wall bound ferulic acid esters after nematode infection seems to be a general defence response of banana roots. This defence response occurs in susceptible as well as in resistant cultivars (Wuyts *et al*., 2007).

Taken together the results of these studies indicate a potential role of root suberin and lignin in plant defence. Both the level of suberin and lignin deposition prior to nematode infection as well as nematode-induced deposition appear to be important. However, the impact of endodermal suberin and lignin in form of CS in plant defence against nematodes has never been closely analysed.

Goal of this study

The current study investigates the role of endodermal suberin and CSs during infection with two nematode species, *M. incognita* and *H. schachtii.* These two species perform different migration behaviour, as *M. incognita* circumvents the endodermis by entering the vascular tissue close to the root tip, whereas *H. schachtii* is able to cross the endodermis directly. This could imply that the endodermis constitutes a barrier for *M. incognita* and that suberin and CS play distinct roles for different nematode species.

First, indications for a putative role of suberin and CS are drawn from transcriptome data extracted from nematode infection sites. The specific expression of suberin and CS related genes in nematode infection sites is closer examined by qRT-PCR (quantitative reverse-transcriptase-polymerase-chain-reaction) and various reporter line assays. Taken together, the molecular-genetic data indicates if the expression of genes involved in suberin biosynthesis and CS formation is altered upon nematode infection. The presence of suberized tissue is investigated by means of histochemical staining and GC-MS (gas chromatography and mass spectrometry). Finally, infection assays with Arabidopsis lines altered in suberin biosynthesis and CS formation provide information about their function. Thereby, it is investigated if increased suberin leads to reduced infection by building a barrier against nematode

penetration. Arabidopsis lines with decreased suberin are expected to show increased infection rates. Similarly, defective CSs may aid in penetration of *M. incognita*, due to its intercellular movement. Overall, the infection assay data give hints regarding the barrier property of suberin and CS to nematode infection and their function during nutrient uptake as endodermal sealing tissue.

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Chapter 2

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REVIEW PAPER

Plant basal resistance to nematodes: an update

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Abstract

Most plant-parasitic nematodes are obligate biotrophs feeding on the roots of their hosts. Whereas ectoparasites remain on the root surface and feed on the outer cell layers, endoparasitic nematodes enter the host to parasitize cells around or within the central cylinder. Nematode invasion and feeding causes tissue damage which may, in turn, lead to the activation of host basal defence responses. Hitherto, research interests in plant–nematode interaction have emphasized effector-triggered immunity rather than basal plant defence responses. However, some recent investigations suggest that basal defence pathways are not only activated but also play an important role in determining interaction outcomes. In this review we discuss the major findings and point out future directions to dissect the molecular mechanisms underlying plant basal defence to nematodes further.

Keywords: ascarosides; callose; PAMPS; PTI; ROS; basal defence; endodermis; lignin; nematodes; suberin.

Introduction

Plant-parasitic nematodes

Plant-parasitic nematodes (PPNs) attack the majority of economically important crops, causing a global yield loss of up to 12.3% on average. In certain crops, such as bananas, losses may increase up to 30% (Sasser and Freckman, 1986). PPNs are obligate biotrophs that feed on almost all plant tissues including flowers, roots, stems, and leaves but most species of PPNs feed on roots. All PPNs, regardless of their feeding habits, possess a specialized mouth spear called a stylet which enables penetration through cell walls and facilitates feeding on plant cells.

PPNs are broadly classified on the basis of their feeding habits into either endoparasites or ectoparasites. Ectoparasitic nematodes do not enter the plants but use their stylet to feed on the plant tissue from the root surface. Depending on the length of the stylet, they may take up nutrients from different cell types or tissues, such as root hairs, epidermis, cortex, and vascular tissues. After having fed for a certain period, they move on in search of a new feeding location. Because of their lifestyle, single ectoparasitic nematodes cause relatively little damage to the plant tissues compared with endoparasitic nematodes (Hussey and Grundler, 1998). An example of ectoparasitic nematodes is *Xiphinema* sp. (dagger nematode) which feeds on a number of economically important crops such as grape vine and figs (Jones *et al.*, 2013).

Unlike ectoparasitic nematodes, migratory endoparasitic nematodes completely enter their host, moving through the different tissue layers destroying many cells during penetration. They carefully pierce the cell wall with their stylet, release saliva into the cytoplasm, and then feed on it. When feeding is completed, the cell wall is further opened with the stylet and the nematode enters the cell, thereby destroying it. Migratory endoparasitic nematodes of economic importance include the genera *Radopholus* (burrowing nematodes),

Pratylenchus (lesion nematodes), and *Hirschmanniella* (primarily rice root nematodes).

Economically, the most important group of PPNs is a small group of sedentary endoparasitic nematodes that includes root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.). The infective-stage juveniles (J2s) of both types enter the roots and migrate towards the vascular cylinder with different migration habits. For invasion, J2s of root-knot nematodes move intercellularly, whereas the J2s of cyst nematodes pierce and enter cells one by one, thereby causing more damage to the host tissue. At the vascular cylinder the J2s of both nematode groups induce a specific hypermetabolic and hypertrophic long-term feeding structure from which nutrients are withdrawn. Root-knot nematodes induce the formation of several coenocytic giant cells, whereas cyst nematodes induce the formation of a syncytium, composed of hundreds of fused root cells (see Kyndt *et al.*, 2013, for a review). After induction, the nematodes become sedentary and feed exclusively from the particular feeding structure which serves as the only source of nutrients throughout their life of several weeks.

Overview of plant basal defence

Plants have evolved a complex defence system. Preformed elements of defence, such as cell walls and their reinforcements, are the first barrier for any kind of invaders (Underwood, 2015). The plant defensive arsenal also includes a broad diversity of constitutively produced toxic phytochemicals (Broekaert *et al.*, 1997). Besides these, a sophisticated system of responses is induced upon infection that is based on the capability of plants to recognize and identify the invader.

At the molecular level, the following two-tiered detection system has evolved in plants for the purpose of pathogen recognition (Jones and Dangl, 2006; Dodds and Rathjen, 2010).

(i) So-called pattern recognition receptors (PRRs) are localized at the cell surface and recognize pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs). These PAMPs or MAMPs are evolutionarily conserved across a class of organisms and perform an important function during their life cycle. PAMPs or MAMPs activate host defence responses (PAMP-triggered immunity or PTI) through a complex signalling cascade.

(ii) Virulent pathogens are able to overcome plant defence mechanisms by secreting so-called effectors into the host. These effectors interfere with PTI responses, thereby leading to effector-triggered susceptibility (ETS). Plants may simultaneously be able to recognize effectors with intracellular nucleotide-binding leucine-rich repeat receptor proteins (NLRs). Activation of NLRs leads to a stronger and more intense defence response (effector-triggered immunity, ETI), which often culminates in a form of programmed cell death, known as the hypersensitive response (HR).

The plant's basal disease resistance is activated by virulent pathogens on a susceptible host. Therefore, at first glance, basal defence is equal to PTI minus the effects of ETS. The release of effectors to establish ETS will, however, most likely result in weak effector-triggered immunity (ETI) from a weak recognition of effectors. Accordingly, basal defence is defined as PTI plus weak ETI (Jones and Dangl, 2006).

Basal defence is considered to be the first line of defence in plants. The PRRs involved in this process are primarily receptor-like kinases (RLKs) or receptor-like proteins (RLPs), except for the extracellular glucan-binding protein which binds to the heptaglucoside released by the oomycetes *Phytophtora sojae* (Umemoto *et al.*, 1995; Boller and Felix, 2009; Monaghan and Zipfel, 2012). Structurally, RLKs consist of an extracellular receptor domain, a single membrane-spanning domain, and a cytoplasmic kinase domain (Shiu and Bleecker, 2001). PRRs survey the apoplast not only for the presence of PAMPs but also for plant-derived compounds that are released by damaged host cells. Therefore, these plant-derived compounds are referred to as damage-associated molecular patterns (DAMPs). Once PAMPs or DAMPs are detected, the PRRs initiate a broad range of downstream signalling events, including bursts of calcium and reactive oxygen species (ROS), the activation of mitogen-associated and calcium-dependent protein kinases (MAPKs and CDPKs), cell wall reinforcement, and the massive reprogramming of the host transcriptome (see Macho and Zipfel, 2014, for a review). These events can lead to restrictions in pathogen growth and development.

The downstream signalling responses in PTI as well as in ETI are regulated by a complex network of defence-related phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET). In general, SA plays a more prominent role in plant defence against biotrophs whereas JA and ET are more important in resistance against necrotrophic pathogens and herbivorous insects (Kessler and Baldwin, 2002; Glazebrook *et al.*, 2003). However, several exceptions for this general rule have been described in literature (see Denance et al., 2013, for a review). In addition, this view is mainly based on observations from leaf pathogenesis and only limited information is available on the role of defence-related phytohormones in resistance against root pathogens (Gutjahr and Paszkowski, 2009).

Plant basal defence against nematodes

The invasion of roots by nematodes causes tissue damage which is likely to generate cell wall fragments that could act as DAMPs and induce a PTI-like basal defence response. Whether such DAMP-based activation truly occurs during nematode invasion and whether it has any significance in terms of host susceptibility to nematodes remains to be clearly observed. Although extensive studies have been conducted to characterize the role of basal defence in various pathosystems, certain challenges impede the performance of such studies in plant–nematode interactions. The four major challenges are as follows: (i) The synchronization of early infected material, (ii) the isolation of infective material during the early stages of nematode infection, (iii) the differentiation of general DAMP-associated host responses from specific responses associated with nematode recognition, and

(iv) the absence of a reliable transformation system for PPNs. As a consequence, very little information is available pertaining to nematode-induced basal defence in plants.

Compared with basal defence, the second level of plant responses to nematodes, ETI, is relatively well studied. Several nematode resistance genes have been described, and their mode of action is relatively well investigated (see Goverse and Smant, 2014, for a review). Similarly, an impressive number of studies have documented the nematode's ability to suppress host defences at all levels (PTI and ETI). Since a number of older and more recent reviews have described and discussed all these studies in detail (Quentin *et al.*, 2013; Goverse and Smant, 2014; Mantelin *et al.*, 2015), we attempted to focus on the relevance of host basal defence responses during nematode infection in the present review. To provide a better overview on the topic we have divided it into three main parts. The first section discusses the function of preformed structural defences including cell walls and the endodermis as barriers to nematode entry. In the following, general damage responses associated with nematode invasion are reviewed. The final part deals with nematode-specific triggers of the basal defence response. In this context, the potential of several evolutionary conserved molecules, such as ascarosides, chitin, and the nematode cuticle as nematode-associated PAMPs (NAMPs) is discussed. At the end, we briefly explore the role of nematode effectors in manipulating host basal defence.

Preformed defences

Cell walls are barriers to all PPNs

Regardless of their feeding habits, all PPN species must penetrate the cell wall to access the cell interior for feeding. As described above, nematodes use their stylet to puncture the cell wall with physical force while simultaneously releasing a cocktail of secretions into the host to aid in penetration. Except for a few cell-wall-degrading enzymes (CWDEs) and cell wall-modifying proteins (CWMPs), the composition of this secretion cocktail is largely unknown. A list of the CWDEs and CWMPs that are released during nematode penetration was presented and discussed in a review by Bohlmann and Sobczak (2014). The list includes pectate lyases, cellulases, expansins, polygalacturonase (PG), endo-1, 4-β-xylanase, 1, 4-β-endoglucanase, and a cellulose binding protein (CBP). Among these, expansins and CBPs do not exhibit enzymatic activity, but they bind to cell wall components and thereby weaken its structure. However, these data have been collected from four different sedentary nematode species, including three cyst nematodes and one root-knot nematode. Taking into consideration that cell wall composition differs significantly in the plant kingdom, it is reasonable to assume that the composition of the secretion cocktails also differ significantly among nematode species (Zeier *et al.*, 1999). A recent review has described different aspects of cell wall modifications during plant root infection by nematodes (Wieczoreck, 2015). Therefore, we will only describe some selected aspects of cell wall changes that are associated with host basal defence activation during plant–nematode infection.

The role of PG, PG-inhibiting proteins (PGIP), and oligogalacturonan (OG) in plant–nematode interaction

An often underrated role for nematode CWDEs is their involvement in activating DAMP-triggered immunity through cell wall damage. PG is a good example for this role. PG enzymes are released by a variety of pathogens, and they hydrolyse homogalacturonan, a major pectin in the plant cell wall and thus facilitating infection (Bishop *et al.*, 1981). However, plants encode PG-inhibiting proteins (PGIPs) that block the complete hydrolysis of homogalacturonan and thus promote the formation of oligogalacturonides (OGs) (See Table 1 for all abbreviations) (Hahn *et al.*, 1981; Cervone et al., 1987, 1989). OGs are oligomers of α-1, 4-linked galacturonic acid, and they act as DAMPs to induce defence responses (Hahn *et al.*, 1981; Benedetti *et al.*, 2015). Previous work showed that nematodes produce PGs that can be secreted into a host and may play a role in parasitism (Jaubert *et al.*, 2002). Moreover, the expression of PGIPs has been shown to be induced in host plants upon nematode infection (Veronico *et al.*, 2011). At present, the contributions of PG, PGIP, and OG to plant–nematode interactions remain elusive. It will be interesting to investigate whether OGs are indeed produced upon nematode infection and whether these OGs play a role in mediating basal defence responses similar to those of other pathogenic interactions.

Specialized cell walls: the endodermis

Root vascular tissue is ensheathed by a specialized cell layer called the endodermis. The apoplast of the endodermis is sealed through cell wall reinforcements such as lignin deposition in radial and transverse cell walls, so-called casparian strips, and the impregnation of primary cell walls with suberin (Naseer *et al.*, 2012). In this way, free diffusion of water and nutrient molecules in or out of the vascular tissue and its

Table 1. *List of abbreviations*

Abbreviation	Full name
CWDE	Cell wall degrading enzyme
DAMP	Damage-associated molecular pattern
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
MAMP	Microbial-associated pattern
NAMP	Nematode-associated molecular pattern
NB-LRR	Nucleotide binding domain leucine-rich repeat protein (R-gene)
OG	Oligogalacturonides
PAMP	Pathogen-associated molecular pattern
PG	Polygalacturonase
PGIP	Polygalacturonase inhibiting protein
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
RLK	Receptor-like kinase
ROS	Reactive oxygen species

penetration by certain pathogens can be blocked (Enstone *et al.*, 2002; Ranathunge *et al.*, 2011). The incorporation of lignin and suberin into endodermal cell walls appears to be ideal for its function as a physical and physiological barrier. Lignin, an aromatic polymer, provides structural integrity and suberin, a hydrophobic polymer of an aliphatic and an aromatic domain, seals the tissue (Boerjan *et al.*, 2003; Franke and Schreiber, 2007). Together, they increase resilience against enzymatic degradation.

Is the endodermis an effective barrier against nematodes? There is currently no clear answer to this question. Nevertheless, one can draw certain conclusions from the manner in which nematodes migrate and feed inside the roots (Fig. 1). For instance, migratory endoparasitic nematodes, such as *Pratylenchus*, feed entirely from the cortex, suggesting an inability to cross the endodermis to exploit the plentiful food source that is the vascular tissue (Pitcher *et al.*, 1960). By contrast, sedentary endoparasitic root-knot nematodes, such as *Meloidogyne*, establish their feeding sites within the vascular tissue. However, these nematodes do not cross the endodermis directly. To circumvent the endodermal barriers, they enter the root at the elongation zone of the root tip, migrate within the cortex to the root meristem, which does not possess cell wall reinforcements, and enter the central cylinder from the anterior side through the differentiation zone (Wyss *et al.*, 1992; Abad *et al.*, 2009). Unlike migratory and root-knot nematodes, cyst nematodes, such as *Heterodera* and *Globodera*, are able to penetrate vascular tissues at any root zone (Wyss and Zunke, 1986; Bohlmann and Sobczak, 2014), suggesting that they have an efficient endodermis crossing ability.

In addition to its role as a physical barrier, the endodermis may also affect the development of nematodes by altering the

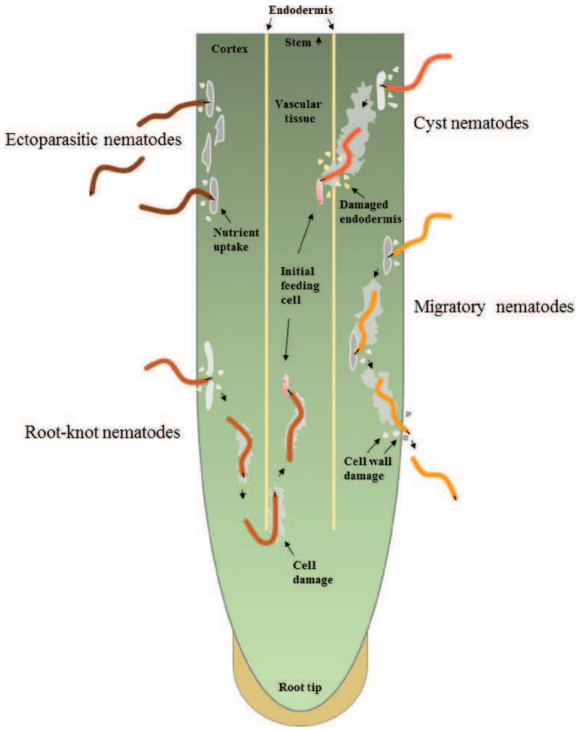

Fig. 1. Model of four different migration habits of plant-parasitic nematodes in a root. *Ectoparasitic nematodes* feed from the outside, the soil environment, on root cells. They take up the cytoplasmic content of the cell with the stylet. Because they do not enter the cell entirely they cause relatively less damage to the root tissue. Root-knot nematodes and cyst nematodes have a sedentary endoparasitic life style. Root-knot nematodes enter the root close to the tip. They move intercellularly in the root tissue towards the root tip and circumvent the endodermis in order to enter the vascular tissue where they initiate giant cells. Cyst nematodes enter the root at an undetermined point. They move intracellularly towards the vascular tissue and cross the endodermis. Within the vascular tissue they establish syncytia. Endoparasitic migratory nematodes move within the cortex while they feed on single cells, taking up the cytoplasmic content. They live freely in the soil and lay eggs within the root tissue.

flow of water and mineral nutrients in and out of the feeding sites that have been established by sedentary nematodes (Baxter *et al.*, 2009). This hypothesis again emphasizes the importance of the endodermis during nematode infection of plants.

Changes in suberin and lignin deposition upon nematode infection

In addition to their universal presence in the endodermis, the biosynthesis of suberin and lignin can also be induced through external triggers, including pathogen infection in various plant tissues (Lulai and Corsini, 1998; Bagniewska-Zadworna *et al.*, 2014). These observations raise a question as to whether suberin and lignin depositions are also induced upon nematode infection. A survey of the literature affirms that lignin may play a role in plant–nematode interactions. In fact, researchers have found that resistance to migratory nematodes correlates with increased lignin content in the cell walls of resistant banana plants. These plants also respond to nematode infection by further lignification of cell walls (Wuyts *et al.*, 2007; Dhakshinamoorthy *et al.*, 2014). Similarly, changes in the lignin composition strongly influence plants susceptibility to the root-knot nematode *Meloidogyne incognita* (Wuyts *et al.*, 2006). Furthermore, treating rice plants with a non-protein amino acid, β-aminobutyric acid (BABA), has recently been shown to delay the development of the root-knot nematode *Meloidogyne graminicola*. Histological and biochemical analyses suggested that basal defence responses, such as callose deposition, lignification, and ROS production, were activated upon BABA treatment in these plants (Ji *et al.*, 2015). Interestingly, the root-knot nematode *Meloidogyne javanica* seems to counter lignin-mediated basal defences through the repression of genes involved in lignin biosynthesis as early as 24 h after infection (Portillo *et al.*, 2013). Cytological investigations of grasses and cereals resistant to the root-knot nematode *Meloidogyne naasi* showed that cells undergoing a resistance response deposit callose as well as suberin (Balhadere and Evans, 1995). Although the contribution of suberin to nematode resistance has not been studied in this case, its co-occurrence with callose during infection hints at suberin's role as an important component of the host's basal defence. Intriguingly, most of the data about suberization and lignification have been collected on root-knot nematodes. Therefore, it will be interesting to analyse the role of these cell wall reinforcements in other types of plant–nematode interaction.

Identification of polymer-degrading enzymes released by nematodes

Nematodes are highly adaptive and sophisticated creatures. Inevitably, they may produce enzymes to degrade suberin and lignin. However, little effort has been made to identify lignin or suberin-degrading enzymes in nematodes and the existing literature pertaining to the presence of these enzymes in nematodes is scarce. This is especially true for suberin-degrading enzymes. However, fungal and bacterial cutinases that degrade suberin do appear in the literature (Ofong and Pearce, 1994; Martins *et al.*, 2014). Recently Naseer *et al.* (2012) showed that the overexpression of a cutinase gene in the endodermis of *Arabidopsis thaliana* (Arabidopsis) leads to a strong decrease in suberin in these plants, suggesting that this enzyme catalyses a broader range of substrates. Could it be possible that nematodes have adapted a similar mechanism to degrade suberin? More research is required before conclusions can be drawn concerning the capacity of PPNs to produce suberin-degrading enyzmes. Compared with suberin, more progress has been made regarding lignin-degrading enzymes. Rai *et al.* (2015) recently identified two putative lignin-degrading enzymes in the pine wood and potato cyst nematode, whereas these enzymes are absent in the majority of the other nematode species analysed. The presence of putative lignin-degrading enzymes in pine wood nematodes indicates that this species is highly adapted to overcome the relatively higher amount of degradation-resistant polymers in the cell walls of pine wood trees. These data also support the hypothesis that the composition of the secretion cocktail may vary in different nematode species and that it mirrors the defence barriers of the host plant.

Damage-associated responses to nematode infection

Callose-mediated resistance to nematodes

Callose is a plant polysaccharide made of glucose residues that are joined by β-1, 3 linkages, which are known as β-glucans. The deposition of callose between the cell wall and the plasma membrane upon wounding, pathogen infection or PAMP treatment is indicative of PTI (Luna *et al.*, 2011). In addition, callose deposition has also been shown to modify the size exclusion limit of plasmodesmata and thus contributes to the regulation of symplastic transport (Wolf *et al.*, 1991). However, the role of wound or pathogen-induced callose deposition during plant–nematode interactions is not well researched.

The ring nematode *Criconemella xenoplax* is primarily an ectoparasite that feeds on root epidermal cells. Callose-like material is deposited between the plasma membrane and the stylet of *C. xenoplax* in all cells whose cell walls were damaged by the stylet. This deposition leads to the encasement of the stylet in a thick layer of callose, where it comes into contact with the plasma membrane of the feeding cell with the exception of its aperture (Hussey *et al.*, 1992). Similar observations have been made during the incompatible interaction between the cyst nematode *Heterodera glycines* and Arabidopsis. In this instance, ultrastructure analyses indicated that many basal defence responses, including callose-like depositions, are activated upon infection (Grundler *et al.*, 1997; Waetzig *et al.*, 1999). Although callose-like depositions have been observed during compatible and incompatible interactions, the relevance of these depositions to plant susceptibility is not understood. However, a recent study has shown that the overexpression of the ethylene response transcription factor RAP2.6 in Arabidopsis decreased the susceptibility of

these plants to *Heterodera schachtii*. This decreased susceptibility was accompanied by elevated expression of JA-related genes and enhanced callose deposition at nematode infection sites (Ali *et al.*, 2013). The importance of callose in resistance to root-knot nematodes in rice, cereals, and grasses has already been mentioned in connection with suberin and lignin (Balhadere and Evans, 1995; Ji *et al.*, 2015). These findings suggest that callose depositions in and around nematode infection sites could influence the infection rate of the nematodes. Surprisingly, no difference in susceptibility to *H. schachtii* was observed in Arabidopsis callose-deficient mutants, *gsl5* (glutan synthase like 5). Interestingly, the expansion of syncytia that was induced by *H. schachtii* was significantly reduced in *At*BG_*ppap* (*Arabidopsis thaliana* β-1,3-glucanase putative plasmodesmal associated protein) mutants, which are deficient in callose degradation, supporting the importance of this process in syncytium expansion (Hofmann *et al.*, 2010).

In considering these various studies, we hypothesize that callose deposition is elicited as a basal defence response during nematode migration in host roots. Nevertheless, the amount and extent of deposition may vary depending on certain factors, such as host compatibility, nematode migration style, and feeding behaviour. Future research should aim to correlate the variations in callose deposition with the susceptibility of host plants by means of newly developed histological, microscopic, and genetic tools. Primarily, the lines that are altered in terms of callose deposition and degradation ability should be tested against different nematode species. This exploration will help to establish the relevance of callose deposition in basal resistance against nematodes.

Transcriptomic changes in the host during the nematode's migratory phase

To gain an insight into which plant basal defence responses are triggered by nematodes, it is necessary to analyse the host tissues at the time-point when they first come into contact with nematodes. However, only a handful of studies have addressed the changes in gene expression that occur during the early stages of nematode invasion. Considering the economic importance of sedentary nematodes, it is not surprising that almost all of these studies explore infection with these species.

One of the initial analyses involved tomato roots during invasion by *M. incognita* through a method that ensured synchronized infection. A differential display expression analysis revealed that eight genes were increased upon infection and most of these genes were also shown to be induced upon wounding (Lambert *et al.*, 1999). Genome-wide expression studies were performed next. The first study was performed on tomato roots upon *M. javanica* and *M. incognita* infection. These authors cut the root tips of infected plants 24 h after inoculation to ensure sampling during the migration phase. The subsequent expression analysis showed that some of the defence-related genes were up-regulated including pathogenesis-related protein 1 (PR1) (Bhattarai *et al.*, 2008). However, another study revealed contrasting results in which down-regulation of defence-related genes, including PR1, was observed in tomato root segments containing galls induced by *M. javanica* at 24 h after infection (Portillo *et al.*, 2013). This inconsistency could be explained by the fact that root samples at different infective stages were used in these studies. The samples in Portillo *et al.* (2013) were collected at a time point when gall formation had already started. By contrast, Bhattarai *et al.* (2008) collected roots tips 24 h after inoculation. In this case, the swelling of root tissue has not been addressed as an indicator for the successful induction of feeding sites. Therefore, some of the responses observed in Bhattarai *et al.* (2008) could be due to wounding. A recent transcriptomic analysis has been performed on very small Arabidopsis root segments that were infected with cyst nematodes. Importantly, only the infection sites were cut where nematodes were still in the migratory phase, as defined by lasting stylet movement. In these samples, the expression of defence-related genes, especially those related to JA, was significantly increased compared with uninfected control roots (B Mendy *et al.*, unpublished data; Kammerhofer *et al.*, 2015).

By contrast with the few studies on the migratory phase, a number of studies have analysed changes in gene expression during the later stages of nematode infection using feeding sites induced by sedentary endoparasitic nematodes. These data revealed that host defence responses are strongly suppressed (Jammes *et al.*, 2005; Szakasits *et al.*, 2009; Barcala *et al.*, 2010; Ji *et al.*, 2013). The conclusion from these studies is that nematode invasion may induce defence responses during migration which are suppressed during the later stages of nutrient acquisition and feeding in sedentary nematodes. By contrast, migratory nematodes induce a defence response that is persistent regardless of the time point after inoculation (Kyndt *et al.*, 2012; see Kyndt *et al.*, 2014, for a review). However, it is not clear whether the early defence activation in response to sedentary as well as migratory nematodes is related to a specific recognition of nematodes by the host (NAMPs) or a general response to tissue damage (DAMPs). In the future, the focus should be on developing tools and assays that can help to differentiate between the two responses.

Reactive oxygen species (ROS) in plant–pathogen interactions

The production of reactive oxygen species (ROS) in the apoplast (oxidative burst) is one of the earliest defence responses observed in various plant–pathogen interactions during PTI and ETI (Doke, 1983; Torres *et al.*, 2002, 2006). However, the quantity and amplitude of ROS production varies depending on the plant–pathogen interactions (Feng and Shan, 2014). In general, ETI is accompanied by a biphasic ROS accumulation with a low-amplitude, transient first phase, followed by a second sustained higher intensity phase. Only a low-amplitude, transient first phase occurs during PTI (Torres *et al.*, 2006).

Although ROS can be produced in organelles that have a high metabolic activity, it is generally recognized that, during an oxidative burst, the major source of ROS are the plasma membrane-bound NADPH oxidases. They are designated as Rboh (for Respiratory burst oxidase homologue)

in plants. The genetic analyses of mutants that are disrupted in Rboh function indicate that they are required for the production of a full oxidative burst in response to a variety of pathogens (Torres *et al.*, 2002). However, this lack of ROS production has variable effects on the response of plants to pathogens in terms of both cell death and resistance. On the one hand, ROS positively correlates with plant resistance by strengthening cell walls via cross linkages, lipid peroxidation, membrane damage, and the activation of defence genes (Levine *et al.*, 1994; Yoshioka *et al.*, 2003; Montillet *et al.*, 2005; Torres *et al.*, 2006) while, on the other hand, it is an important susceptibility factor for the successful infection of plants by various pathogens. The mechanistic details regarding the pathosystem-specific role of ROS is, however, as yet unknown. Nonetheless, the observation that Rboh mutants over-accumulate SA, ethylene, and other antimicrobial compounds upon pathogen infection has led to the widely accepted belief that an antagonistic interaction between ROS and SA is responsible for the reduced susceptibility of mutants to various pathogens (Torres *et al.*, 2005; Kadota *et al.*, 2014). A recent publication also suggests that Rboh may be guarded by an NLR, leading to a strong activation of immune responses in mutants that are deficient in Rboh genes (Kadota *et al.*, 2014).

ROS and the defence response in plant–nematode interactions

A few studies have highlighted the production of ROS in the context of nematode–plant interaction. The first analyses to demonstrate the involvement of ROS-related metabolites were on tomato plants infested with *M. incognita* (Zacheo *et al.*, 1982). Using enzymatic assays, the researchers detected peroxidase and superoxide dismutase (SOD) activities in resistant and susceptible tomato cultivars. They observed a slight increase upon infection in susceptible cultivars compared with control plants. Waetzig *et al.* (1999) were the first to show the plasma-membrane localization of ROS in Arabidopsis roots during an incompatible interaction with *H. glycines*. Although they did not investigate the source of ROS in this particular study, the plasma membrane localization of ROS led the authors to suggest that plasma membrane-based oxidases might be responsible for ROS production (Grundler *et al.*, 1997; Waetzig *et al.*, 1999). A recent study has shown that the generation of ROS, in the form of an oxidative burst, occurs very early during host as well as non-host interactions in resistant tomato plants carrying the nematode resistance gene *Mi* upon *M. incognita* infection. Nevertheless, the intensity and duration of the oxidative burst is enhanced in cells undergoing HR (Melillo *et al.*, 2006).

Although the occurrence of ROS has been shown to correlate positively with an enhanced resistance to nematodes, the direct involvement of ROS in basal defence remains to be seen. To fill this gap, we have recently characterized the role of Rboh-mediated ROS during a compatible interaction between Arabidopsis and *H. schachtii*. Arabidopsis encodes ten Rboh homologues (RbohA–RbohH) and our results have indicated that nematode infection triggers ROS production in the roots, which are dependent on RbohD (Siddique *et al.*, 2014). The susceptibility of mutants disrupted in RbohD or RbohD/F showed a strong decline compared with the control. Infection assays with *M. incognita* produced similar results (C. Matera *et al.*, unpublished data). Further, the plants' susceptibility to nematodes was not restored after crossing Rboh mutants with SA-deficient mutants, thereby suggesting that the role of ROS in promoting infection is independent of SA. The question that then arises is whether Rboh-mediated ROS serves to promote the formation of a feeding site in ways other than modulating immune responses. In fact, it has been found that cyst nematodes secrete the putative effector molecule 10A06 into the host that interacts with the host spermidine synthase 2 protein (SPDS2), thereby increasing the spermidine (spd) content in the infected tissues. Spd is the main substrate for polyamine oxidase (PAO) and degradation of spd by PAO results in the production of ROS (H_2O_2) which, at low concentrations, may function as a signalling molecule to stimulate the expression of antioxidant genes in the infected tissue (Hewezi *et al.*, 2010).

Future work will have to provide mechanistic details for the dual role of ROS during plant–pathogen interaction in general and plant–nematode interaction in particular. In this context, it will be important to produce transgenic lines with inducible promoters and variable capacities to produce ROS upon infection.

Nematode-specific triggers of the basal defence response

PAMPs, DAMPs, and now NAMPs

Although no NAMPs or PRRs that can detect NAMPs are currently known, some recent work suggests that plants are indeed capable of recognizing nematodes. For example, brassinosteroid-associated kinase 1 (BAK1) is a member of the leucine-rich repeats receptor-like kinase (LRR-RLK) family, and it forms receptor complexes with various PRRs to regulate their responses positively. BAK1 also regulates immune responses that are triggered by the binding of Arabidopsis PRRs to the DAMP *At*Pep1 (23-aa peptide) (Heese *et al.*, 2007). BAK1 knockout mutants show an enhanced susceptibility to a plethora of pathogens because of defects in mounting basal defence responses (Schwessinger *et al.*, 2011). A recent publication showed that silencing the Arabidopsis homologue of BAK1 in tomatoes significantly increased the susceptibility of these plants to nematodes because of defects in basal defence (Peng and Kaloshian, 2014). These data indicate that putative NAMPs or DAMPs may activate a BAK1-mediated basal defence response upon nematode recognition. However, the identity of ligands whose recognition is mediated by BAK1 and corresponding PRR remains unknown. This compound may be a protein because BAK1 has been shown to act as a co-receptor for PRRs belonging to the LRR-RLKS family which typically detects proteinaceous ligands. In the following sections, we discuss the role and potential of various conserved nematode molecules as NAMPs.

Ascarosides elicit plant defence responses and pathogen resistance

Ascarosides are an evolutionary conserved group of small signalling molecules that regulate a number of sex-specific, developmental, and social behaviours not only in *Caenorhabditis elegans* but also in many other nematode species (Ludewig and Schroeder, 2013). Structurally, ascarosides are glycosides (of dideoxy sugar ascorylase) that carry a fatty acid-derived lipophilic side chain and are secreted in the nematode's surroundings (Manosalva *et al.*, 2015; Schroeder, 2015). More than 200 different ascaroside structures have been identified from various nematode species which indicate that ascarosides are well conserved among nematodes (Schroeder, 2015). Their size and evolutionarily conserved nature and the fact that ascarosides are secreted into nematode surroundings make them good candidates for detection by nematode hosts. *Arthrobotrys oligospora*, a nematophagous fungus, recognizes and responds to ascarosides by initiating the formation of nematode-trapping devices (Hsueh *et al.*, 2013).

A recent study investigated the potential of ascarosides as NAMPs recognized by host plants. A metabolomic analysis of excretions has shown that several genera of PPNs, including *M. incognita*, *Meloidogyne hapla*, and *H. glycines,* can secrete a similar set of ascarosides. Among the ascarosides that were identified, ascr8 was the most abundant in all plant-parasitic nematodes. Ascr18 features an 11-carbon side chain and is produced in trace amounts by *C. elegans* as well as entomopathogenic nematodes (Manosalva *et al.*, 2015). Interestingly, a previous metabolomic profile of *C. elegans* acyl-CoA oxidase mutants (*acox-1*) showed an increase by 29 times in ascr 18 abundance compared with control worms (von Reuss *et al.*, 2012). To explore whether ascr18 induces a defence response in Arabidopsis, the expression of PTI genes in plants treated with ascr18 was measured via qPCR. The findings showed that local as well as systemic defence responses are activated in response to ascr18 treatments. Moreover, the plants that were treated with ascr 18 were less susceptible to a broad range of pathogens, including nematodes, when compared with untreated control plants. These authors concluded that plants may recognize ascarosides as conserved signalling molecules which leads to the activation of PTI-like basal defence responses (Manosalva *et al.*, 2015).

The discovery that ascarosides activate defence responses in plants raises a number of key questions that should be answered before conclusions can be drawn with regard to the role of ascr18 as a NAMP. One of the most fundamental questions is whether ascr18 is recognized by host surface receptors. In *C. elegans*, ascaroside perception is mediated by a diverse family of G protein-coupled receptors (GPCRs) that act upstream of conserved signalling pathways, including insulin/IGF-1 and transforming growth factor beta (TGF-β) signalling (Zwaal *et al.*, 1997; Lans and Jansen, 2007). When compared with animals, the presence of GPCRs in plants remains controversial. An example was found in Arabidopsis, in which a genome analysis has identified 56 putative GPCRs. Recent studies, however, suggest that none of them are GPCRs (Taddese *et al.*, 2014). Future research should therefore aim to characterize the receptors and pathways that mediate ascaroside signalling in host plants. Analysing nematodes with an enhanced capacity to produce ascr18 for their ability to infect plants will also be crucial. This could be achieved by manipulating the expression of receptors such as ACOX1 in plant-parasitic nematodes by performing RNAi.

Chitin as a potential NAMP

The role of chitin as a NAMP has been debated frequently. Chitin is a polymer of N-acetyl-D-glucosamine and, as such, is an important component of fungal cell walls; it is absent in plants. Nevertheless, plants secrete chitinases upon fungal infection which degrade fungal cell walls and release chitooligosaccharides. These chitooligosaccharides are perceived by plants and trigger plant defence responses and resistance against fungal pathogens (Wan *et al.*, 2008). The presence of chitin has been well documented in the eggshells of various nematode species, including the PPNs *M. javanica*, *Globodera rostochiensis*, *H. glycines*, and *H. schachtii* (Clarke *et al.*, 1967; Mcclure and Bird, 1976; Perry and Trett, 1986). More recently, chitin has been localized in the pharyngeal lumen walls of *C. elegans* (Wan *et al.*, 2008). Although chitin is detected unambiguously in the eggshells of all nematodes, its presence during the different stages of parasitism in PPNs remains to be verified, making its role as a NAMP questionable. Nonetheless, chitin is involved in the production of eggshells. Therefore, plants may be exposed to nematode-derived chitin during the reproductive stages of endoparasitic nematodes. This finding may be particularly relevant in the case of migratory endoparasitic nematodes which usually lay eggs inside plant roots. With respect to these different studies, future research should aim to characterize the host mutants that are impaired during various aspects of chitin perception.

Surface coat and cuticle

The nematode cuticle is a complex structure that performs a diverse set of functions, besides its role as an exoskeleton that maintains the nematode body's morphology. The important role of nematode cuticles during movement and growth is protection against the external environment, including microbes (Spiegel and Mcclure, 1995; Davies and Curtis, 2011). The surface coat (i.e. the outermost layer of the cuticle) is in direct contact with the nematode's environment. A number of studies have shown that nematode surfaces, as well as their secretory products, are rich in glycoproteins. Therefore, the domains of these carbohydrates are ostensibly recognized in animals by the calcium-dependent carbohydrate binding of the receptor protein family known as C-type lectins (Perrigoue *et al.*, 2008).

Compared with mammals, very few C-type lectins are characterized in plants. In fact, Arabidopsis has only one C-type lectin, the function of which remains unknown. However, Arabidopsis still contains 45 members of the lectin receptor kinase family (LecRKs), which are involved in the regulation of a number of processes (Bouwmeester and Govers, 2009).

Therefore, one can reasonably assume that some of these LecRKs may be involved in the recognition of NAMPs. Indeed, a recent publication has shown that LecRKs may play a critical role in mediating PTI responses upon insect infestation by recognizing PAMPs or DAMPs in rice plants (Liu *et al.*, 2015). Similarly, the overexpression of Arabidopsis LecRKs in potato and *Nicotiana benthamiana* resulted in significantly enhanced resistance to the pathogenic oomycete *Phytophthora infestans* (Bouwmeester *et al.*, 2014). Therefore, the identification of LecRKs that detect NAMPs in crop plants may contribute to increased resistance against nematodes in the future.

Lessons from animal-parasitic nematodes

Similar to PPNs, there are no universal NAMPs for animal-parasitic nematodes. However, a little bit more work has been done in animals on the recognition of worms, including nematodes. The well-described potential NAMPs in animals are glycan moieties that are present on the surface of nematodes or in extracellular secretions (ES). *Nippostrongylus brasiliensis* is a gastrointestinal parasite of rodents and a fraction of its ES fluid has been shown to be capable of inducing type II immunity in mice (Balic *et al.*, 2004). Nevertheless, the mechanistic details and the exact identity of the component in ES fluid that triggers the immune response remain unknown. Another example of a potential NAMP in an animal-parasitic nematode is ES-62, a phosphorylcholine-containing glycoprotein that is secreted by the rodent filarial nematode *Acanthocheilonema viteae.* This ES-62 glycoprotein is able to interact with a class of animal PRRs, namely the toll-like receptor TLR4, leading to an immunomodulation that is conducive to the health of both the worm and the host (Goodridge *et al.*, 2005). ES-62 is widely conserved among human filarial nematodes, and our analysis has shown that weak homologues are present in many PPNs, including *H. schachtii* (S Siddique *et al.*, unpublished data). However, the functional characterization of ES-62, including its role as a NAMP in plant–nematode interaction, remains unknown. In addition to secretions from the surface coat, nematodes release proteases into the surrounding environment that are capable of damaging cells and thus can be perceived as DAMPs. Therefore, the role of proteases in the production of DAMPs is frequently discussed in cases of animal-parasitic nematodes.

Considering that the overall structure of PAMP signalling pathways are surprisingly similar in plants and animals in that both involve membrane receptors, ROS production, Ca^{2+} fluxes, transcription factors, and inducible gene expression (Ausubel, 2005), it will be interesting to discover whether these strikingly similar systems also perceive components that are common in both groups of parasitic nematodes.

Nematode effectors suppressing host basal defence responses

Even though effectors are not the focus of this review, some current publications on their PTI-suppressing potential have to be mentioned for completeness. Given the variety of responses that may arise during basal defence in host plants, it is expected that nematodes will release a number of effectors that may suppress these responses. Moreover, the repertoire of these effectors may vary between nematodes because of their different lifestyles and host range.

Indeed, an increasing number of PTI-suppressing effectors have been characterized during the last few years (see Mantelin *et al.*, 2015, for a review). Nevertheless, the mechanistic details for most of these PTI-suppressing effectors are not fully known. One of the better characterized examples is the venom-allergen like protein 1 (VAP1) from *G. rostochiensis* (Lozano-Torres *et al.*, 2014). VAP1 is released into the apoplast of the host tissue and is thought to suppress the activation of DAMP-associated defence responses during nematode migration. The discovery that VAP1 can suppress DAMP-associated defence responses suggests that DAMP-based responses are not only activated but also play an important role in deciding the outcome of the interaction. Another example of a nematode effector that is secreted into the host apoplast and suppresses basal defence is a calreticulin that is secreted by *M. incognita* (*Mi-CRT*). The overexpression of *Mi-CRT* in Arabidopsis renders these plants more susceptible to various pathogens. Furthermore, the deposition of callose, as well as the expression of defence-related genes, was decreased significantly upon PAMP treatment in *Mi-CRT* overexpression lines. In addition to nematode effectors that suppress host basal defence, the nematodes' surface has been shown to be coated with antioxidant molecules that protect them from oxidative stress triggered by tissue damage (Mantelin *et al.*, 2015).

The identification of nematode effectors that suppress plant basal defence has proved to be challenging. However, the ongoing transcriptome and genome sequencing of several PPN species will expedite this identification which may help to explain why certain defence responses are not effective or even activated during plant infection.

Concluding remarks

PPNs are fascinating creatures that are capable of infecting and feeding on a huge number of crop plants. The activation of host basal defences during nematode infection are reviewed here (See Fig. 2 for an overview). Although nematodes seem to activate both PAMP-triggered and DAMP-triggered host responses during their migration inside the roots, the molecular details and relevance of these responses to host susceptibility remain largely unknown. Indeed, the extent to which nematode susceptibility is influenced by the activation of host basal defences may vary and depend on a number of factors including the host–nematode combination, the nematode effector repertoire, and individual infection events. Understanding the signalling events and networks that are activated during nematode migration inside the root will certainly be of great interest, potentially allowing for manipulations leading to enhanced plant resistance to nematodes. A challenge for future research would be to disconnect

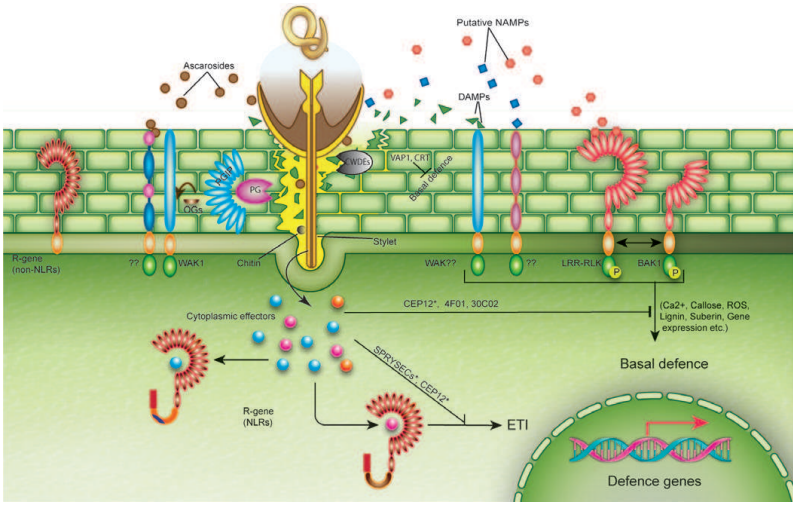

Fig. 2. A hypothetical model for molecular players involved in immune responses during plant-nematode interaction. Nematode invasion of the cell causes cell wall damage, which may produce damage-associated molecular patterns (DAMPs) to activate plant basal defence responses via DAMP-receptors such as WAK1. In addition, the nematode may secrete cell wall degrading enzymes (CWDEs) such as polygalacturonases (PG) to hydrolyse the plant cell wall. In turn, the plant produces PG-inhibiting proteins (PGIPs), which interact with PG and promote the formation of small cell wall fragments, oligogalacturonides (OGs) that may also lead to the activation of DAMP-associated immunity. Conserved nematode-associated molecular patterns (NAMPs) are released from nematodes in the apoplast and are perceived by plasma-membrane localized pattern recognition receptors (PRRs) to trigger pattern-triggered immunity (PTI) including the production of reactive oxygen species (ROS), callose, and lignin. Some PRRs may belong to the leucine-rich repeat receptor-like kinase family (LRR-RLKs), which detect typically proteinaceous ligands. LRR-RLKs recruit the BRI1-associated receptor-like kinase 1 (BAK1) as a co-receptor to initiate PTI. Nematodes, in turn, secrete apoplastic (VAP1, Lozano-Torres *et al.*, 2014; CRT, Jaouannet *et al.*, 2013) as well as cytoplasmic (CEP12, Chronis *et al.*, 2013; 4F01, Patel *et al.*, 2010; 30C02; SPRYSECs, Rehman *et al.*, 2009) effectors to perturb the DAMP- or NAMP-based immunity. Apoplastic effectors may interfere with the detection of PAMPs or DAMPs by PRRs and cytoplasmic effectors may disturb downstream signalling leading to the activation of PTI. Plants, in turn, carry R-genes (Nucleotide binding leucine rich repeat, NLRs) that recognize effectors either directly or indirectly and initiate effector-triggered immunity (ETI). In addition to NLRs, non-NLRs type R-genes also exists against nematodes. * indicates additional nuclear localization for effectors.

a specific response to nematode recognition from the general damage-associated defence response. To accomplish this goal, it will be crucial to develop assays and tools that can differentiate between the two responses. Furthermore, it will also be critical to identify additional molecular players that are involved in host defence activation in response to nematode infection. In this way, we can specifically interfere in different aspects of the host responses and study the consequence of such manipulations on the outcome of the infection process.

Supplementary data

Supplementary data can be found at *JXB* online.

Table S1. Definitions

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Chapter 3

Title: Root endodermal barriers contribute to defence against plant-parasitic cyst and root-knot nematodes

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Abstract

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 central vasculature against abiotic and biotic threats. Until now, the role of the endodermis, and especially of suberin and the CS, during plant–nematode interactions was largely unknown. Here, we analysed the role of suberin and CS during interaction between Arabidopsis plants and two sedentary nematode species, the cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. We found that suberin biosynthesis genes were differentially activated at nematode infection sites. Lipophilic staining indicated that the tissue encircling nematode infection sites is cohesively suberized. In addition, chemical suberin analysis revealed a characteristic suberization pattern in nematode-infected tissue. Notably, infection assays using Arabidopsis lines with CS defects and impaired compensatory suberization, revealed that the CS and suberization greatly impact nematode infectivity and feeding site size. To our knowledge, this study is the first example where the role of the endodermal barrier system to defence against soil-borne pathogen is described.

Introduction

Plant-parasitic nematodes (PPNs) cause tremendous yield losses in many crop plants, which are estimated to total over 80 billion USD 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 endodermis directly; RKN J2s predominantly enter the root close to the tip and then move towards the apical meristematic region, making a U-turn to enter the central vasculature without crossing the differentiated endodermis (Sijmons *et al.*, 1991; Wyss *et al.*, 1992). CN J2s are destructive, moving intracellularly and piercing cells with their stylets. By contrast, RKN J2s cause comparatively little damage, as they move intercellularly through root tissue (Sijmons *et al.*, 1991; Wyss *et al.*, 1992; Shah *et al.*, 2017).

Within the central vasculature, the J2s of both nematode groups induce characteristic nurse cell systems. The induction of these feeding sites requires cellular reprogramming, which is achieved by a cocktail of proteinaceous and non-proteinaceous compounds secreted by the J2s into the initial feeding cell. These secretions induce profound morphological and physiological changes in both the initial feeding cell and adjoining tissue (Siddique *et al.*, 2015; Smant *et al.*, 2018; Juvale *et al.*, 2018). CNs form syncytia by dissolving local cell walls and fusing the resulting protoplasts to incorporate neighbouring cells (Golinowski *et al.*, 1996; Sobczak *et al.*, 1997). RKNs induce the formation of several hypertrophied giant cells that are adjacent to each other. In the case of RKNs, the divisions of the vascular cells surrounding the nematode and the hypertrophy of giant cells lead to the formation of typical galls, which are a symptom of infection. Both types of nematode feeding sites share the following cellular features: dense cytoplasm, multiple nuclei, small vacuoles, proliferation of plastids, mitochondria, and ER, and modified cell walls. The feeding sites are highly metabolically active and are the sole nutrient source for the sedentary nematodes (Kyndt *et al.*, 2013; Siddique and Grundler, 2015). A few hours after the induction of a syncytium or giant cell, the

nematodes begin to withdraw nutrients from the modified plant tissue (Wyss *et al.*, 1992; Wyss and Grundler, 1992).

A relatively little studied aspect of plant defence against nematodes is the protective function of preformed cell wall polymers, such as suberin and lignin, in the endodermis, which encloses the central vasculature in roots (Holbein *et al.*, 2016). Both suberin and lignin confer the apoplastic barrier properties of the endodermis. Lignin-based cell wall reinforcements are deposited in radial and transversal cell walls, which encircle the cell as the Casparian strip (CS). Secondary differentiation of the endodermis proceeds with the suberization of entire cell walls. Together, suberin and the CS constitute essential cell wall reinforcements. Whereas suberin limits transmembrane transport by establishing a barrier for uptake from the apoplast into the cell interior (Barberon *et al.*, 2016), the CS seals the apoplastic pathway in and out of the endodermis, thus preventing diffusion of toxins and loss of nutrients (Naseer *et al.*, 2012). To date, only a few studies have indicated a role for endodermal 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; Franke *et al.*, 2009; Compagnon *et al.*, 2009; Molina *et al.*, 2009; Gou *et al.*, 2009). The expression pattern of promoter:GUS fusion of *CYP86A1*, *ASFT,* and *GPAT5* in transgenic Arabidopsis overlap with suberin deposition in the endodermis (Naseer *et al.*, 2012). Furthermore the transcriptional reporter *GPAT5:mCITRINE-SYP122* has been shown to act as a marker for suberization (Barberon *et al.*, 2016).

The mechanism of CS formation is relatively well-described (Roppolo *et al.*, 2011). A family of transmembrane proteins called CASPs (Casparian Strip Domain Proteins) CS formation by accumulating at the appropriate membrane locations. The expression pattern of the reporter *CASP1:NLS3xmVenus* 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 Suberin1). ESB1 is localized at the CS and mediates lignin deposition and

stabilization (Hosmani *et al.*, 2013). Another protein important for localizing CASPs is the receptor-like kinase SGN3 (Schengen3), which is responsible for forming the CASP complex in the membrane (Pfister *et al.*, 2014). CS and suberin lamella formation are co-regulated, as suberin deposition compensates for CS defects (reviewed in Barberon, 2017 and Doblas *et al.*, 2017*a*).

The identification of genes and the development of appropriate marker lines during the last few years has created new opportunities to analyse suberization and CS formation in Arabidopsis. An *in vitro* system comprising Arabidopsis, the sugar beet *(Beta vulgaris)* CN *Heterodera schachtii*, and the RKN *Meloidogyne incognita* provides optimal conditions to study the cellular and molecular aspects of plant–nematode interactions (Sijmons *et al.*, 1991). Making use of this system, we studied the role of endodermal suberin and CS in plant–nematode interactions. Our results indicate that a suberized tissue encircles nematode infection sites, thus revealing an important role for endodermal sealing in nematode development.

Results

Suberin biosynthesis genes are upregulated 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 (**Supplementary Table 1**) (Cabrera *et al.*, 2014). We found that the overall abundance of transcripts of these genes was not altered in *M. incognita* galls from 3 to 21 days after inoculation (dai). However, the transcript level of a few suberin biosynthesis genes (*ASFT*, *KCS2/ DAISY*, *FAR4*) was decreased in giant cells at 3 dai (Barcala *et al.*, 2010). By contrast, the transcript level of most suberin- and CS-related genes was 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 from micro-aspirated syncytium samples 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 transcript levels 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 *ESB1*, *CASP1*, and *SGN3* were downregulated, whereas *CYP86A1*, *KCS2/ DAISY*, *CYP86B1*, *FAR4* (encoding fatty acyl reductase4), *FAR5* (encoding fatty acyl reductase5), and *GPAT5* were upregulated in root segments containing syncytia. Furthermore, *ASFT*, *FAR1* (encoding fatty acyl reductase1), *KCS2.0* (ß-ketoacyl-CoA synthase2.0), and *LACS* (long-chain acyl-CoA synthetase) showed no significant change in expression. Taken together, many suberin biosynthesis genes showed increased expression in infection sites, while genes involved in CS formation were downregulated.

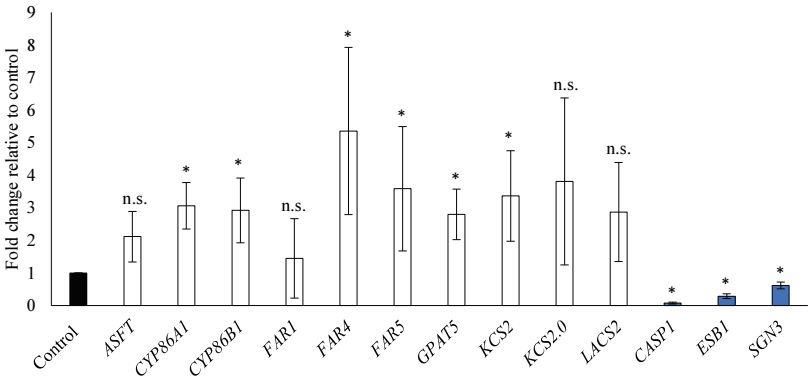


Fig. 1 Expression of suberin- and CS-related genes in dissected root tissue containing syncytia at 10 dai compared to uninfected roots as analysed via qPCR. White bars, suberin biosynthesis genes; blue bars, CS formation genes. Bars, means ± SD; asterisks, statistically significant fold change relative to the control; n. s., non-significant (t-test, $p<0.05$); n= 4.

Suberin biosynthesis genes are activated in tissue surrounding nematode infection sites

To determine the spatiotemporal patterns of suberin biosynthesis and CS formation genes during plant–nematode interactions, we analysed previously described promoter:reporter lines at various time points after inoculation (3, 5, and 10 dai) with *H. schachtii* or *M. incognita*. The activity of *pCYP86A1:GUS*, *pASFT:NLS-GFP-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) and

expression of *pCASP1:NLS3xmVenus* served as a marker of CS formation (Vermeer *et al*., 2014).

In lines expressing *pCYP86A1:GUS,* most infection sites exhibited specific GUS staining at 3 and 5 dai with *H. schachtii*. The staining was especially intense in the immediate vicinity of the nematode head at 3 dai (**Figure 2A**). At 5 and 10 dai, single cells were stained in the cell layer surrounding feeding sites, producing a highly localized, patchy pattern. To confirm that the activity of *pCYP86A1:GUS* was localized to endodermal tissue, cross-sections of feeding sites were prepared at 5 dai. No GUS staining was observed in the cortex; however, specific staining was observed in the endodermis. Occasionally, GUS staining was also observed in the central vasculature, most likely due to leakage of excessive staining from the endodermis into the vasculature (**Figure 2C**). At 3 dai with *M. incognita, pCYP86A1:GUS* expression was observed close to root swellings appearing in root tips. However, at 5 and 10 dai, GUS staining was detected throughout the gall tissue (**Figure 2B and 2C**).

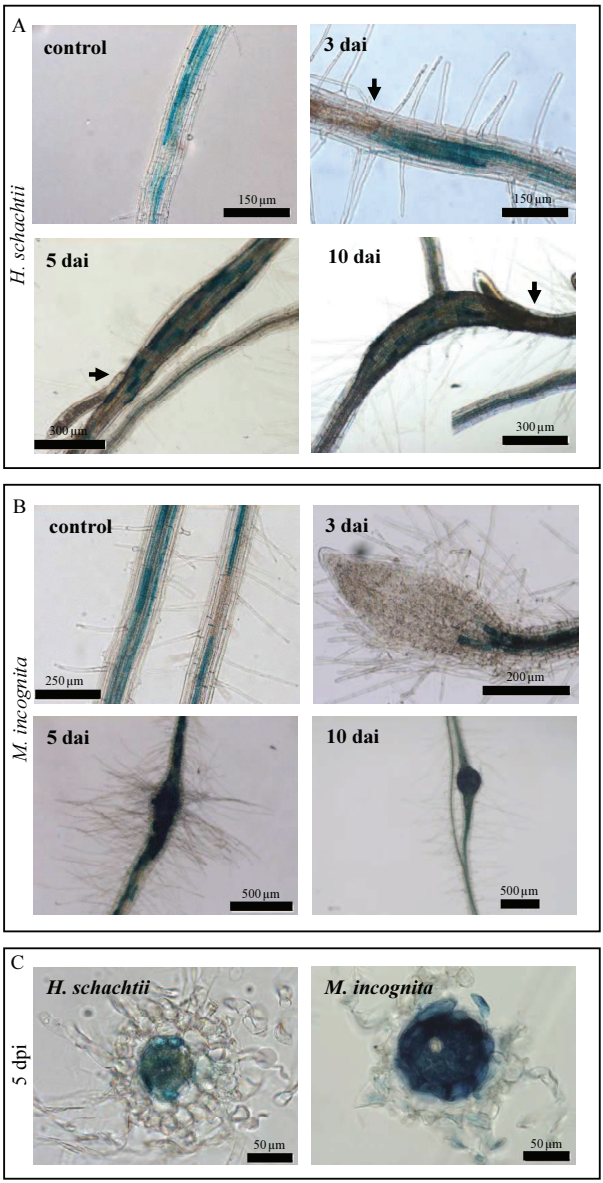


Fig. 2 Activity of *pCYP86A1:GUS* in infection sites induced by *H. schachtii* (A) and *M. incognita* (B) at 3, 5, and 10 dai and in cross-sections of infection sites at 5 dai (C). Arrows indicate location of the nematode's head.

Next we analysed the expression pattern of *pASFT:NLS-GFP-GUS* and *pGPAT5:mCITRINE-SYP122* in transgenic lines infected with *H. schachtii* and found that both constructs showed similar expression patterns as *pCYP86A1:GUS* at 3, 5, and 10 dai (**Figure 3A and Supplementary Figure S1A**). 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 sections showed weak expression at the infection sites (**Figure 3B**).

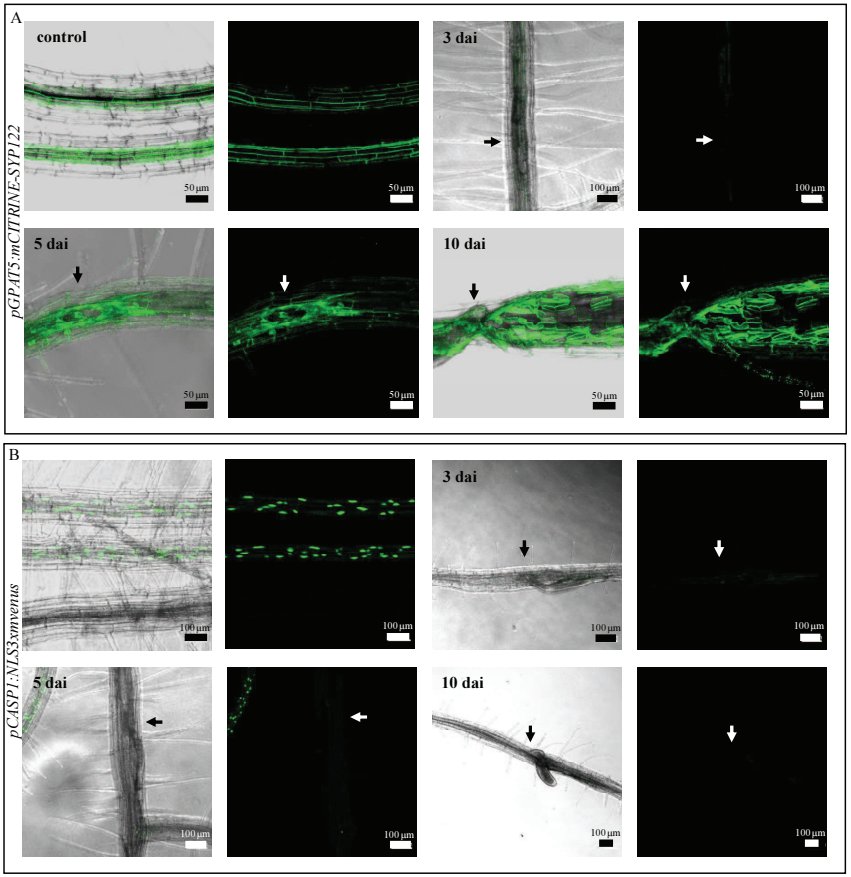


Fig. 3 Fluorescence signals of *pGPAT5:mCITRINE-SYP122* (A) and *pCASP1:NLS3xmVenus* (B) in infection sites induced by *H. schachtii* at 3, 5, and 10 dai. Arrows indicate the location of the nematode's head.

In the *pASFT:NLS-GFP-GUS* reporter line infected with *M. incognita*, intense *GUS* staining was present in galls at all measured time points (**Supplementary Figure S1B**). Surprisingly, *pGPAT5:mCITRINE-SYP122* showed only dim expression close to the infection sites at 3 and 5 dai (**Figure 4A**). However, at 10 dai, the cell layer surrounding galls showed intense fluorescence. By contrast, *pCASP1:NLS3xmVenus* expression was observed in *M. incognita* infection sites at 3 and 5 dai, but not at 10 dai (**Figure 4B**). Taken together, these findings show that suberin biosynthesis genes are activated in the tissue surrounding the infection sites of both CNs and RKNs.

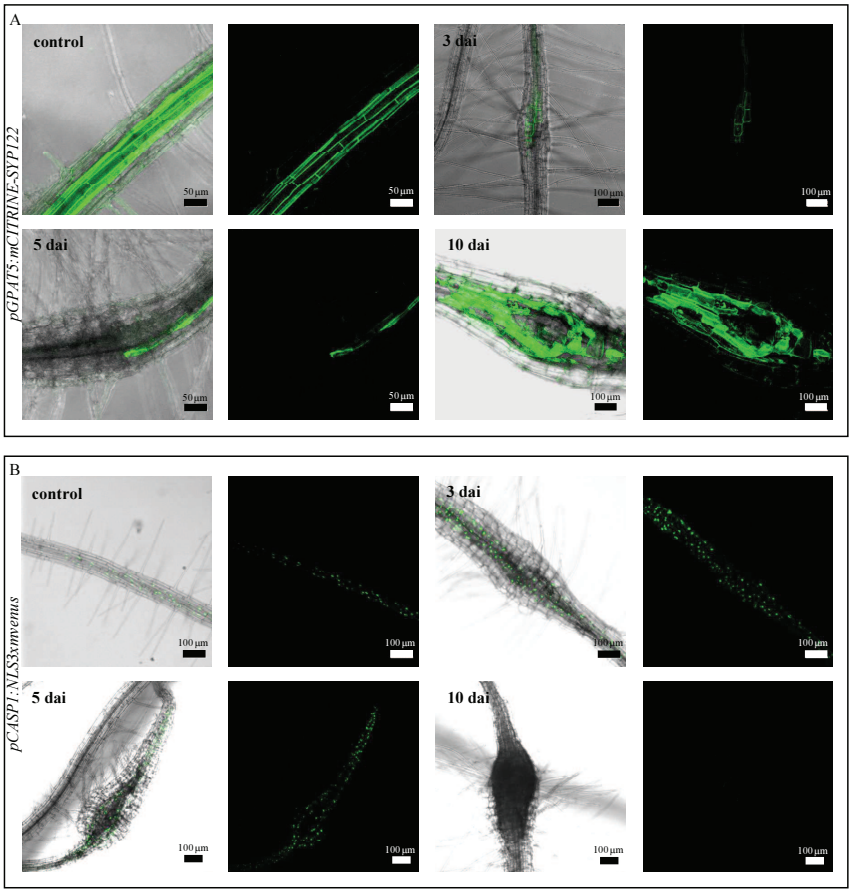


Fig. 4 Fluorescence signals of *pGPAT5:mCITRINE-SYP122* (A) and *pCASP1:NLS3xmVenus* (B) in infection sites induced by *M. incognita* at 3, 5 and 10 dai.

Nematode infection sites are encircled by a suberized tissue

To further visualize the suberin lamellae in tissue encircling nematode infection sites, we cross-sectioned galls and syncytia at 10 dai and stained them with the lipophilic stain Sudan red 7B (Brundrett *et al.*, 1991; Nawrath *et al.*, 2013). We observed red staining specifically in the cell layer surrounding syncytia and galls, indicating cohesive suberization of this tissue (**Figure 5**).

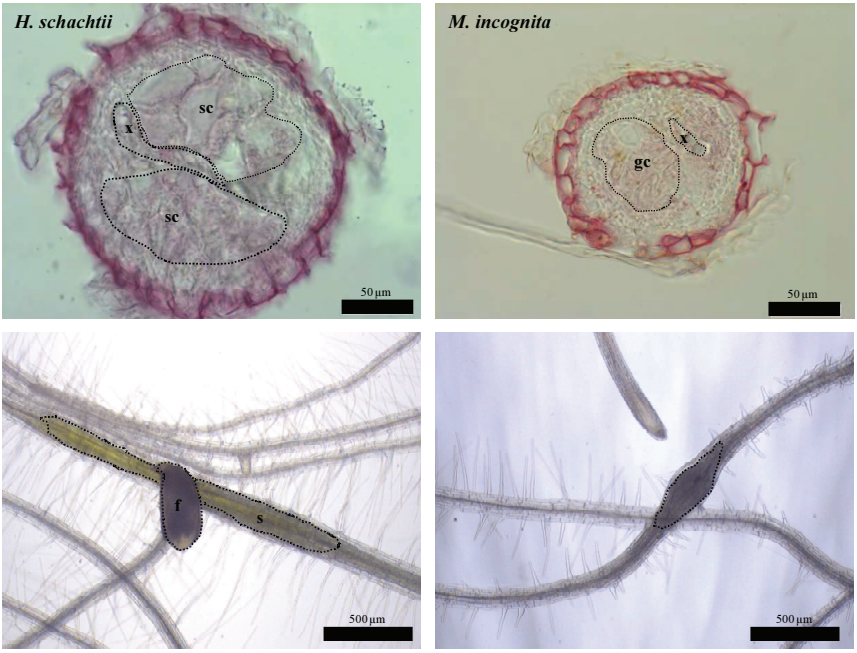


Fig. 5 Cross-sections of infection sites induced by *H. schachtii* and *M. incognita* stained with Sudan Red 7b at 10 dai. sc, syncytial cells; gc, giant cells; x, xylem; f, female; s, syncytium.

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 infection sites and control roots (**Figure 6A and 6B**). However, the abundance of single monomers was altered in nematode infection sites (**Figure 6C and 6D**). Levels of ω-hydroxyacids (ω-OH-acids) C18:1 and C16, α,ω-diacids C16 and C18, acid C20, and primary alcohol C18 were higher in aliphatic suberin extracted from *H. schachtii* infection sites than from control roots. Only the amounts of C24 primary fatty acid, alcohol, and α,ω-diacid were lower at these sites (**Figure 6C**). The monomer abundance in galls displayed a similar pattern; levels of ω-OH-acid C20, C18:1, and C16, α,ω-diacid C20, C18, and C16, primary alcohol C24, C20, and C18, and primary fatty acid C20 were higher than in control roots, whereas trans-ferulic acid levels were lower (**Figure 6D**). Our results indicate an unusual high occurrence of trans-ferulic acids in control roots for galls (**Figure 6D**). However, this peak is an artefact of several overlaying compounds, which could not be separated more clearly.

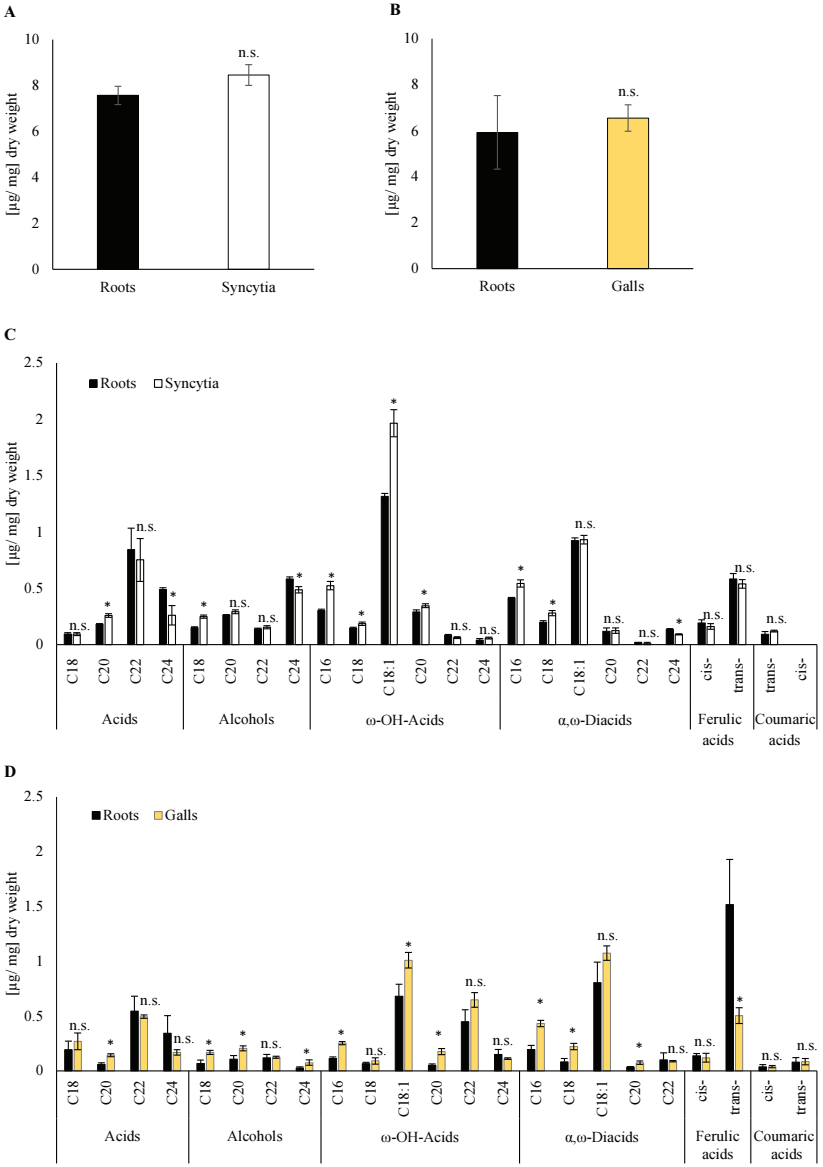


Fig. 6 Aliphatic suberin concentrations in dissected root tissue containing syncytia (A & C) and galls (B & D) at 10 dai as compared to uninfected roots. GC-MS data of total aliphatic suberin amounts and monomer composition. Bars,

means ± SD; asterisks, statistical significance relative to Col-0 wild-type; n. s., non-significant (t-test, $p<0.05$); n= 3.

Defective Casparian 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 deposition (*horst*, *cdef1* (cuticle destructing factor)), CS formation (*sgn3-3*, *sgn3-3esb1-1*), or both (*esb1-1*, *esb1-1cdef1*, *casp1-1casp3-1*) (**Figure 7**). 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 *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 s*gn3-3* 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 by enhanced suberin deposition (ectopic suberin) starting close to the root tip, accompanied by ectopic lignification in cell corners close to the CS. This abnormal suberization results in doubling of the amount of total aliphatic suberin as compared to Col-0 (Baxter *et al*., 2009; Hosmani *et al*., 2013; Lee *et al*., 2013; Li *et al*., 2017). The double mutant *sgn3-3esb1-1* exhibits 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 *esb1-1 cdef1*, suberin is degraded by CDEF1 and ectopic lignification at the site of the CS partially compensates for the lack of an apoplastic barrier. This compensatory mechanism, however, does not entirely seal the apoplast, which remains permeable, especially at lateral root emergence sites (Li *et al*., 2017). A similar phenotype to *esb1-1* is displayed by the *casp1-1casp3-1* double mutant (Roppolo *et al*., 2011; Hosmani *et al*., 2013).

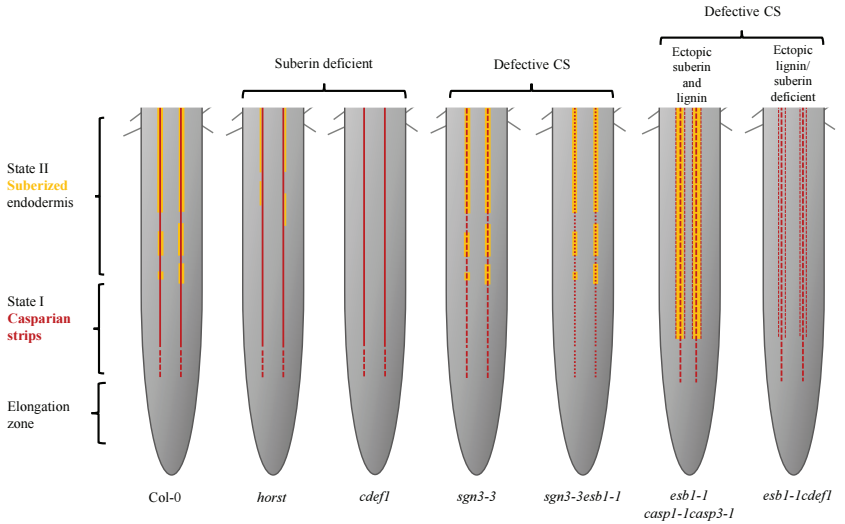
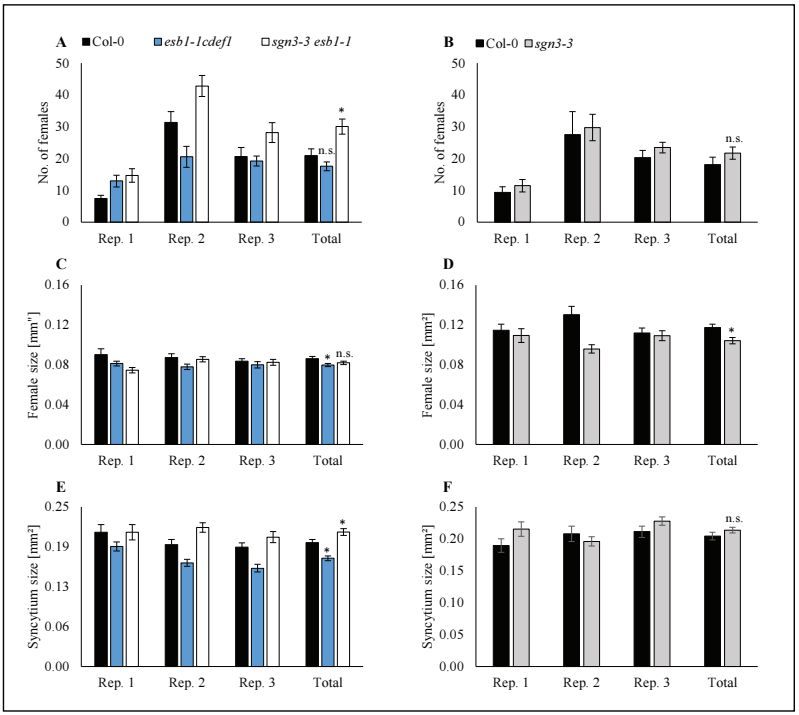


Fig. 7 Schematic of Casparian strips and suberin phenotypes in Arabidopsis roots. Col-0 and the suberin altered (*horst, cdef1*), the CS deficient (*sgn3-3, sgn3-3esb1-1*), and suberin and CS altered lines (*esb1-1, casp1-1casp3-1, esb1-1cdef1*).

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 measured their average size, and the average size of syncytia at 14 dai. For RKNs, we counted the number of galls and measured their average area at 21 dai. From the suberin altered lines, only *cdef1* showed significant reduction of average syncytium size and only in *casp1-1casp3-1* (defective CS/ ectopic suberin and lignin), but not *esb1-1* had a significantly reduced number of galls and smaller average female and syncytium sizes (**Supplementary Figure S2 and S3)**. The most remarkable results were obtained in the CS deficient lines *sgn3-3esb1-1* and *sgn3-3,* and also in *esb1-1cdef1* (CS defective/ no ectopic suberin), which all affected both nematode species (**Fig. 8**). After CN infection, we observed a significant increase in the number of females in *sgn3-3esb1-1* (defective CS), an increased average syncytium size and a decreased average female size in *sgn3-3* (defective CS), and a significant decrease in the average female and syncytium size in *esb1-1 cdef1* (defective CS/ suberin deficient) compared to Col-0 plants (**Figure 8A-F**). After RKN infection, we observed a significantly higher number of galls accompanied by a

significantly lower average gall size in *sgn3-3esb1-1* and *esb1-1 cdef1* and a significantly reduced average gall size in *sgn3-3* (**Figure 8G-J**). Taken together, our data imply that a defective CS (without ectopic suberin) renders the plant more susceptible to nematode parasitism, particularly in the case of *M. incognita*.

H. schachtii



M. incognita

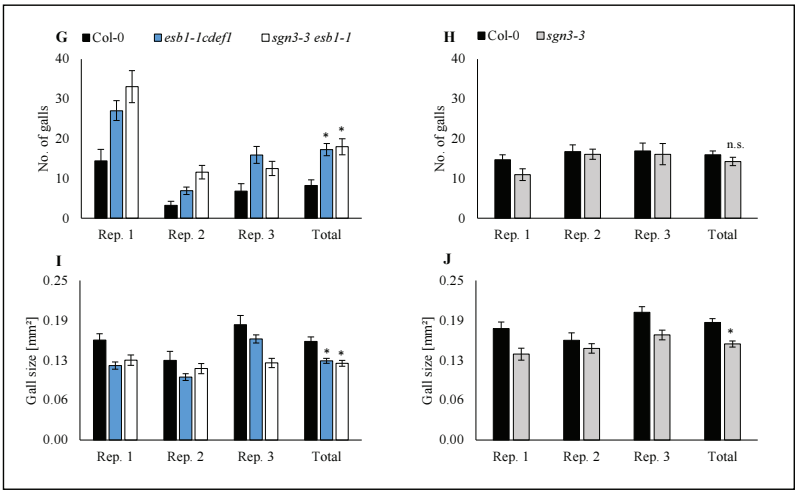


Fig. 8 Nematode infection assays. *sgn3-3*, *sgn3-3esb1-1* (defective CS), and *esb1-1cdef1* (defective CS/ suberin deficient) were inoculated with *H. schachtii* (A–F) or *M. incognita* (G–J). Rep. (repetition), independent experiments; bars, means ± SE; asterisks, statistical significance relative to Col-0; n. s., non-significant (t-test, $p<0.05$); n= 40–60.

Discussion

In the present study, we analysed the role of suberin and CS in the endodermis of Arabidopsis during CN and RKN parasitism. We examined the expression of suberin- and CS-related genes and found that the expression of suberin biosynthesis genes is strongly activated upon infection by both CN and RKN. In the initial stages of feeding site development, the expression was localized close to the head of a CN, suggesting that induction is specific to nematode infection. However, as the feeding sites developed, expression of suberin biosynthesis genes was restricted to fewer cells in the tissue surrounding the feeding site of a CN, but was homogeneous in the tissue surrounding galls (10 dai). In comparison, 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 (*pCASP1:NLS3xmVenus*) with both CN and RKN. Previously, it was shown that *pCASP1:NLS3xmVenus* is only expressed in young growing roots (Vermeer *et al*., 2014; Barberon *et al*., 2016), and we also did not detect any *pCASP1:NLS3xmVenus* signal during the later stages of CN and RKN infection (10 dai). Therefore, it is plausible that the low expression of genes involved in CS formation at 10 dai is simply due to the absence of their expression in more mature roots. Based on these observations, we propose that nematode infection causes damage to the endodermis that consequently activates suberin biosynthesis genes at the site of infection. However, once a feeding site is established and expands due to cell proliferation, a strong activity of suberin biosynthesis genes is only required at highly localized patches of still growing cells.

We found that the tissue surrounding syncytia and galls undergo a *de novo* suberization. Although the suberin monomers we detected are 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 supports our hypothesis that nematode infection induces *de novo* suberization of

endodermal tissue at the site of infection. Notably, the change in suberin monomer composition is supported by the upregulation of several genes involved in their biosynthesis. For example, we detected an increase in C18:1 and C16 ω-OH-acids in root segments containing syncytia at 10 dai. Additionally, ω-OH-acids, which are precursors of α, ω-dicarboxylic acids, and the corresponding C16 and C18 chain lengths, are 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 increases in C20 precursors, which is likely related to activation of *GPAT5* upon CN infection. The increase in the levels of the primary alcohol C18 might be due to upregulation of *FAR5*. However, there was no significant increase in C20 primary alcohol levels, despite upregulation of *FAR4* (Domergue *et al*., 2010). By contrast, galls showed a significant increase in C20 primary alcohol levels at 10 dai. Overall, especially the pattern of ω-OH-acids and primary fatty acids showed similarities to peridermal suberin as found in roots undergoing secondary development (Höfer *et al*., 2008). These changes in suberin monomer composition could represent a general pattern of re-suberization (secondary suberin) in already suberized root zones (Beisson *et al*., 2007). Our measurements of nematode-infected tissue showed similar suberin monomer abundances between syncytia and galls, which were significantly different from uninfected root tissue.

A previous study suggested that the endodermis surrounding the syncytium induced by male CNs was completely collapsed in Arabidopsis at 5 dai (Sobczak *et al*., 1997). However, our cross-sections of female-associated syncytia did not indicate endodermis collapse at 5 or 10 dai (**Fig. 2C and 5**). Furthermore, in several studies in which syncytial cross-sections were prepared for *in situ* RT-PCR, the endodermis encircling syncytial cells appeared intact in samples from 5 to 15 dai (Hofmann *et al*., 2007; Grunewald *et al*., 2008; Siddique *et al*., 2009; Szakasits *et al*., 2009). Considering the different ontogenies of male- and female-associated syncytia, we assume that this discrepancy with the previous study (Sobczak *et al*., 1997) might be related to the fact that male- and female-associated syncytia display distinct morphologies. Then again, we cannot entirely rule out the possibility that the endodermis collapses during time points not examined in this study. However, the collapse of the endodermis would have to be followed by the formation of the

periderm as our results show a cohesively suberized tissue around nematode infection sites. Another study has described the tissue surrounding infection sites as periderm-like at 14 days after invasion (Golinowski *et al.*, 1996). Our observations showed that most infection sites in ~ 25-day-old Arabidopsis plants were encircled by a translucent epidermis with root hairs (**Fig. 2A, 2B and 5**), indicative of root zones that have not yet reached the secondary developmental stage. Based on these observations we regard this tissue as an endodermis rather than a periderm.

Our expression data, microscopy observations, and biochemical measurements established that both CN and RKN infection induces a characteristic suberization pattern at the infection site, which indicates that suberin has an important role during nematode parasitism. However, alterations in endodermal suberin did not evoke 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. By contrast, nematode infection assays showed that lines with defects in CS had significant increases in the number of both CNs (*sgn3-3esb1-1*) and RKNs (*sgn3-3esb1-1* and *esb1-1 cdef1*) and significant decreases in average female (*esb1-1 cdef1*), syncytium (*esb1-1 cdef1*), and gall sizes (*sgn3-3, sgn3-3esb1-1* and *esb1-1 cdef1*) compared to the control. In addition to having defective CS formation, *sgn3-3, sgn3-3esb1-1,* and *esb1-1 cdef1* are also impaired in the compensatory suberization mechanism sealing CS defects, as these mutants are unable to deposit ectopic suberin. It was previously shown that non-functional SGN3 in *sgn3-3* and *sgn3-3esb1-1* leads to a breakdown of the compensatory barrier surveillance system (Doblas *et al.*, 2017b; Nakayama *et al.*, 2017). A similar effect is achieved by CDEF1 in *esb1-1cdef1*, which degrades newly formed suberin (Naseer *et al.*, 2012). Thus, holes in the CS barrier are not sealed by suberin in these mutants. The inability to seal newly formed holes in the endodermis has been observed in *esb1-1 cdef1* 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 *esb1-1 cdef1*), indicating a role of the endodermis during nematode nutrient acquisition. The expression pattern of *pCASP1:NLS3xmVenus* in the early biotrophic stages of nematode parasitism indicates that CS formation remains functional in infection sites and 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 increases the permeability of solutes, thereby impairing nutrient availability. The effects on the nutrient status have been previously described in detail for several suberin- and CS-affected lines (reviewed in Barberon, 2017; Doblas *et al.*, 2017*a*). 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 and phloem vessels, 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, a secondary metaphloem is formed and plasmodesmata enable symplasmic transport from sieve elements into syncytial cells (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 to syncytia, giant cells remain symplasmically isolated throughout their life cycle and a newly formed protophloem lacking companion cells surrounds the giant cells (Hoth *et al.*, 2008; Absmanner *et al.*, 2013). In galls, solutes are unloaded from sieve elements into the apoplast 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. Thus, defects in CS formation, not compensated by suberization, would lead to an outflow of solutes, which may impair nematode development.

Based on these data, we propose that nematode feeding site development ruptures the apoplastic seal, which triggers a compensatory suberin mechanism induced by the barrier surveillance system (Nakayama *et al*., 2017; Doblas *et al*., 2017b). When this program is inoperable and the ability of the endodermis to seal itself is impaired, 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 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 nematode feeding site and *M. incognita* especially benefits from this for nutrient acquisition. Taken together, 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 triggered by the sensitive barrier surveillance system in the endodermis, which maintains its sealing properties despite infestation by nematodes (Doblas *et al*., 2017*b*; Nakayama *et al*., 2017).

Experimental procedures

Plant material and growth conditions

A. 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 (Sijmons *et al*., 1991) with 2% (w/v) sucrose and 0.1% (w/v) Gamborg´s vitamin solution 100x (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 as described previously (Hütten *et al.*, 2015). The following T-DNA insertion lines were used for infection assays: *horst-1* (*cyp86a1,* AT5G58860) (Höfer *et al.*, 2008), *sgn3-3* (AT4G20140) (Pfister *et al.*, 2014), the leaf ionomic line *esb1-1* (AT2G28670) (Baxter *et al.*, 2009), the double mutants *sgn3-3esb1-1* (Pfister *et al.*, 2014) and *casp1-1 casp3-1 (*AT2G36100/ AT2G27370) (Roppolo *et al.*, 2011), and lines *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 (*pCASP1:CDEF1*).

Nematode cultures and inoculum

H. schachtii was cultivated and second-stage juveniles (J2) were hatched as described previously (Siddique *et al.*, 2009). Freshly hatched J2 were surface sterilized with 0.05% mercury chloride. Sixty to 80 J2s in a water suspension were inoculated on 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.

M. 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% 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 *pASFT:NLS-GFP-GUS* (AT5G41040) (Naseer *et al.*, 2012) and *pCYP86A1:GUS* (Höfer *et al.*, 2008) was analysed by GUS staining. A 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (1 mM x-

Gluc) solution as described in Siddique *et al.* (2009) was poured onto the agar surface in Petri dishes and incubated at 38°C. The *pASFT:NLS-GFP-GUS* line was incubated for 5–6 h. whereas the *pCYP86A1:GUS* line was incubated overnight. Photographs were taken using a Leica DMI 4000B, Camera DFC450C (Leica Microsystems, Wetzlar, Germany).

Fluorescence microscopy

The transcriptional reporter lines *pCASP1:NLS3xmVenus* (AT2G36100) (Vermeer *et al.*, 2014; Barberon *et al.*, 2016) and *pGPAT5:mCTRINE-SYP122* (AT3G11430) (Barberon *et al.*, 2016) were examined using a Zeiss LSM 710. Plants were grown on cover slips (24 x 60 mm) covered with 3 mL agar medium. 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).

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 includes ≥300 feeding sites (per replicate). Uninfected roots excluding root tips served as controls. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer´s instructions. RNA samples were treated with DNA-free DNase Treatment & Removal (Ambion, Life Technologies) and the quality was tested with a 2100 Bioanalyzer (Agilent Technologies) on an RNA Nano Chip. The isolated RNA was transcribed into cDNA using random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The samples were analysed 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 (S. 1), 1 µL cDNA, and sterile RNAse/DNAse-free water. For internal references, ß-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 **Supplementary Table S2**.

Histochemical suberin staining

Dissected infection sites (10 dai) were incubated for 42 h at 4°C in fixation solution containing 63% (v/v) ethanol, 2% (v/v) formaldehyde, and 10x PBS buffer (without potassium) at pH 7.2. Infection sites were washed in 63% (v/v) ethanol and

in 10x PBS buffer. Washed samples were embedded in Petri dishes filled with 5% low melting agarose in water and stored at 4°C. Embedded samples were used to cut 25–30 µm cross-sections with a Leica VT1200S automated vibrating blade vibratome. Cross-sections were incubated for 30 min in Sudan Red 7B solution, washed, and mounted in 75% (v/v) glycerol on objective slides. Sudan Red 7B (Sigma) was prepared as a 0.05% (w/v) solution in PEG400:glycerol (1:1 [v/v]) (Brundrett *et al.*, 1991; Nawrath *et al.*, 2013). Samples were photographed with a Leica DMI 4000B, Camera DFC450C (Leica Microsystems, Wetzlar, Germany).

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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Supplementary material

Table S1 Expression of suberin- and CS-related genes in *M. incognita* (GC and Gall) and *H. schachtii* (Sync) infection sites. Transcriptome data extracted from NEMATIC (Cabrera et al., 2014) indicating significant fold change relative to control. "n.s." indicates no expression change. GC, giant cells; Sync, syncytia.

		GC 3 dai	Gall 3 dai	Gall 7 dai	Gall 14 dai	Gall 21 dai	Sync 5+15 dai	Root+Sync 3 dai
ASFT	AT5G41040	0.42	n.s.	n.s.	n.s.	n.s.	0.01	n.s.
CYP86A1	AT5G58860	n.s.	n.s.	n.s.	n.s.	n.s.	0.02	n.s.
CYP86B1	AT5G23190	n.s.	n.s.	n.s.	n.s.	n.s.	0.11	n.s.
FAR1	AT1G80010	n.s.	n.s.	n.s.	n.s.	n.s.	0.62	n.s.
FAR4	AT3G44540	0.39	n.s.	n.s.	n.s.	n.s.	0.27	n.s.
FAR5	AT3G44550	n.s.	n.s.	n.s.	n.s.	n.s.	0.41	n.s.
GPAT5	AT3G11430	n.s.	n.s.	n.s.	n.s.	n.s.	0.44	n.s.
KCS2	AT1G04220	0.08	n.s.	n.s.	n.s.	n.s.	0.02	n.s.
KCS2.0	AT5G43760	n.s.	n.s.	n.s.	n.s.	n.s.	0.25	n.s.
LACS2	AT1G49430	n.s.	n.s.	n.s.	n.s.	n.s.	0.35	n.s.
CASP1	AT2G36100	n.s.	n.s.	n.s.	n.s.	n.s.	0.01	n.s.
ESB1	AT2G28670	n.s.	n.s.	n.s.	n.s.	n.s.	0.02	n.s.
SGN3	AT4G20140	n.s.	n.s.	n.s.	n.s.	n.s.	0.05	n.s.

Table S2 Primers used for qPCR.

Gene	Forward primer	Reverse Primer
ASFT/ AT5G41040	GTTTCGCGGTGGGATTAGTC	ATCTGTCGTGTGGAAACCCA
β-Tubulin/ AT5G44340	TTTCCGTACCCTCAAGCTCG	GTGAAGCCTTGCGAATGGGA
CASP1/ AT2G36100	AGAGAGGTTTGGCTATATT	CTACGGCTATCACAAAGTA
CYP86A1/ AT5G58860	ACAGAACAAAAGCAAAAAGCCTAAACC	TGCAAGCACCTCACCACGAG
CYP86B1/ AT5G23190	GGTTTAGCAGCCTCAACAGC	ATCTGGACCGAGACATCCTG
ESB1/ AT2G28670	ATGTCCCTTTCCTCGTTGGA	GCCACTAGCAACAGGGAAACC
FAR1/ AT1G80010	ATTGTATCGAACCCTTGCCC	AGAAAGTTGGGGCTGGAAAG
FAR4/ AT3G44540	GCAGCAGACAATGAATCAGC	ATCAGAACCCTTCACTCCCA
FAR5/ AT3G44550	TGCATATGGAAAGGGAGTGC	ACTGGGTTCTGGTGAGATGA
GPAT5/ AT3G11430	TGTAAGAAGAGGGTCGTGGT	GCAGACTGATCAACATCCGT
KCS2.0/ AT5G43760	ACGCTCTCTACATCCTCCTC	GTGAAGTAAGCGGTGGAGAG
KCS2/ AT1G04220	CTCTGGGACCGCTAGTTCTA	TCAATCTCATCGAGCACTGC
LACS2/ AT1G49430	AGCAATTTCGGTCCAGGTTT	ACCGCAAAACACAGTAGGTT
rRNA/ AT3G41768.1	GGTGGTAACGGGTGACGGAGAAT	CGCCGACCGAAGGGACAAGCCGA
SGN3/ At4g20140	TGGCCGGTTTGACAACCTAA	CGTCTCCGGGATATCTCCAAC

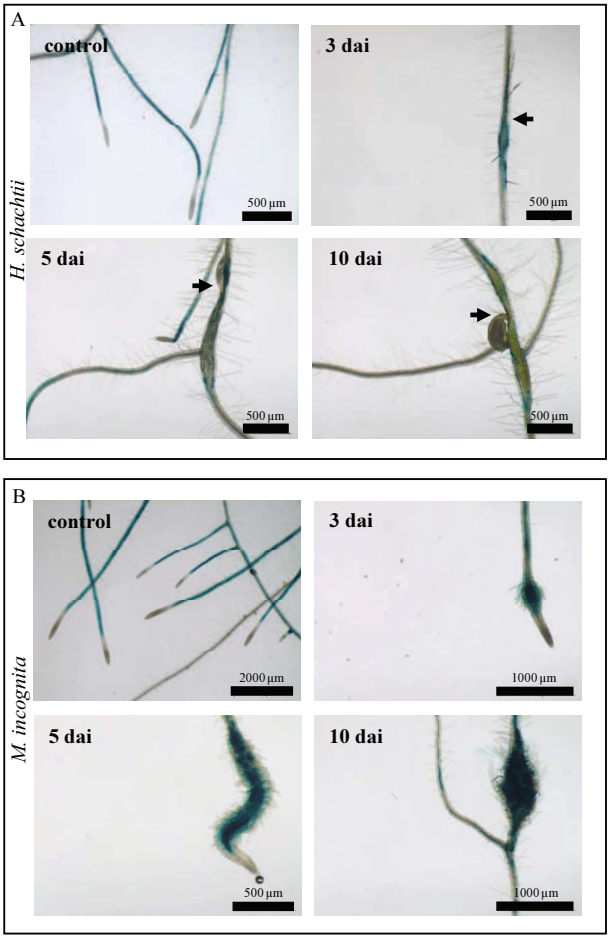

Fig. S1 Activity of *pASFT:NLS-GFP-GUS* in infection sites induced by *H. schachtii* (A) and *M. incognita* (B) at 3, 5, and 10 dai. Arrows indicate the location of the nematode's head.

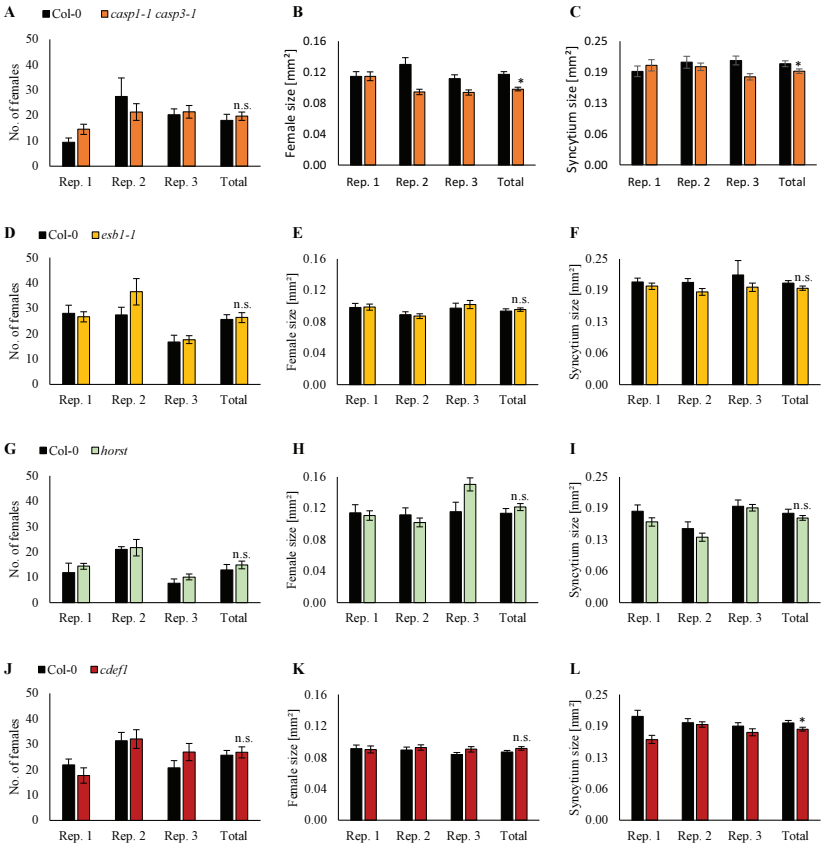

Fig. S2 Results of *H. schachtii* infection assays in CS and suberin mutants. Rep. (repetition), independent experiments; bars, means ± SE; asterisks, statistical significance relative to wild-type Col-0; n. s., non-significant (t-test, $p<0.05$); n= 20–50.

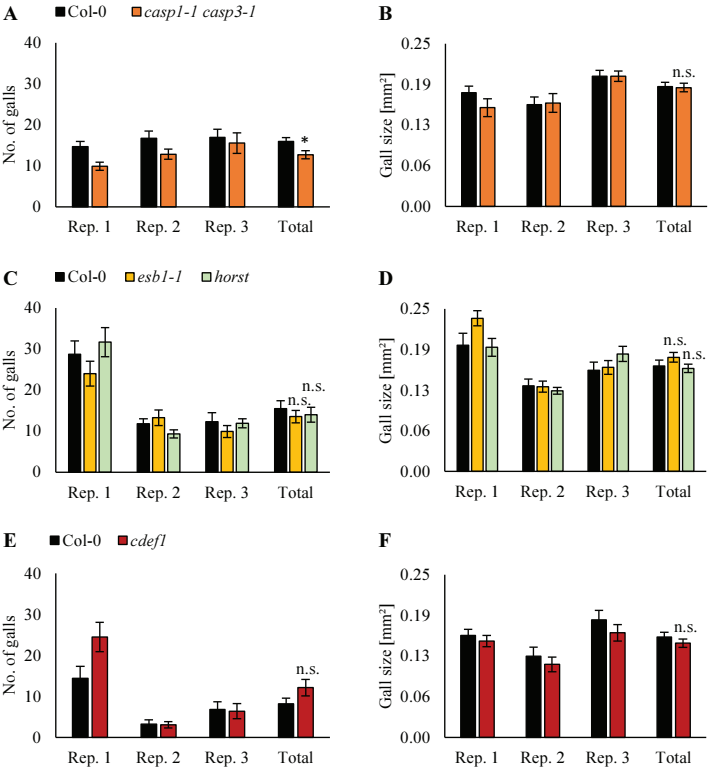

Fig. S3 Results of *M. incognita* infection assays in CS and suberin mutants. Rep. (repetition), independent experiments; bars, means ± SE; asterisks indicate statistical significance to wild-type Col-0; n. s., non-significant (t-test, $p<0.05$); n= 20–60.

Chapter 4

Discussion

The aim of this study was to investigate the role of suberin and Casparian strips (CSs) in the interaction between Arabidopsis and the sedentary endoparasitic nematodes *H. schachtii* (cyst nematode, CN) and *M. incognita* (root-knot nematode, RKN). CNs and RKNs feed from root vasculature and inevitably come in contact with the endodermis. CNs destroy endodermal cells during their migration, while RKNs are held back and circumvent this tissue. This different migration pattern of CNs and RKNs suggests that these nematode species are perfectly adapted and employ individual strategies to overcome endodermal barriers. Indeed, infection assay results presented here also support this hypothesis as mutants with enhanced barriers by ectopic suberin and lignin depositions (*esb1-1*, *casp1-1casp3-1*) predominantly showed no changes in susceptibility. Overall, the data indicate that especially CNs are well adapted to cross the endodermis unlike RKNs, which are more receptive to alterations in the endodermal barrier. This is further supported by the increased susceptibility especially to RKNs in mutants with irregularities in the apoplastic barrier (*sgn3-3esb1-1*, *esb1-1cdef1*). However, both CN and RKN benefit from a penetrable endodermis. The infection assay results show, that the success of CN and RKN infection is impacted by the endodermis and especially by the CS.

While the infection assay data indicated a role of CS in nematode parasitism, the outcome from reporter gene analyses, gene expression and chemical analyses pointed towards suberin. Also, a previous study on resistant banana cultivars concluded that preformed suberin contributes to the defence against nematodes (Valette *et al.*, 1998). Therefore, it was surprising that suberin-altered mutants had a modest effect on nematode parasitism. One explanation why the effects of an enhanced endodermal barrier were less pronounced, is the fragility of Arabidopsis roots, which are even more delicate when grown in agar medium. It is noticeable that the overall suberin levels are relatively low as compared to studies in which Arabidopsis plants were grown for longer periods and in different substrates (Franke *et al.*, 2005; Höfer *et al.*, 2008; Baxter *et al.*, 2009). This variation is not surprising as plants use endodermal suberization as a tool to respond to the availability of certain nutrients as well as to environmental conditions (Baxter *et al.*, 2009; Krishnamurthy *et al.*, 2011; Barberon *et al.*, 2016). Considering this aspects, it is possible that the age of the plants and the growth conditions may not have allowed a suberin deposition which affects nematode penetration.

Besides preformed barriers, the induced accumulation of suberin deposition can also be part of a successful defence response in some plant species (Balhadère and Evans 1995). No conclusion can be drawn from this study as suberin levels were only measured at 10 dai. However, the alterations in suberin composition and in expression pattern of suberin-biosynthesis genes at 10 dai indicate a role of suberin during feeding site development.

Regarding the function of the suberized tissue surrounding infection sites, there is an ongoing debate whether this tissue is an endodermis or a periderm. The suberized periderm is formed during secondary growth of roots and replaces endodermis, cortex and epidermis. Unlike the endodermis, the periderm does not function in selective nutrient uptake but seals and stabilizes the root. The observations in this study pointed to the presence of an intact endodermis encircling infection sites. This assumption is supported by the fact that nematodes primarily infect young root sections where periderm formation has not yet taken place. Also, microscopic observations of infected tissue showed a single layered suberized tissue encircled by cortex and epidermis. This root anatomy is indicative for a root prior to periderm formation. However, the study by Wunderling *et al*. (2018) analysed periderm formation in Arabidopsis roots and discovered that one third of the length of the primary root and the uppermost part of lateral roots are covered with periderm. The results indicate that periderm formation occurs rather extensively in major root sections. Consequently, it is likely that nematode-infected root sections may be located within these zones of periderm formation. Additionally, the study showed that plants grown in media supplemented with sugar as well as increased exposure to light triggers early periderm formation (Wunderling *et al.,* 2018). The growth conditions used in the present study include sugar-containing media and a long-day photoperiod during which roots are exposed to light. Another factor which could contribute to periderm formation is the expansion of tissue in nematode infection sites. The study of Wunderling *et al.,* (2018) also determined that the endodermis of Arabidopsis roots consist of eight cells. In cross sections of 10 dai infection sites, the suberized tissue amounts to numerous cells. Moreover, the anatomy of a root undergoing periderm formation is characterised by induced cell division of the pericycle. The cross sections of 10 dai infection sites showed a high number of small cells close to the suberized tissue, which could indicate the beginning of periderm formation. Overall, the results from Wunderling *et al*., (2018) imply that periderm formation could occur in the tissue surrounding nematode infection sites with progressing root development. It is possible that this

process is enhanced upon feeding site expansion. The replacement of the endodermis by the periderm could be the reason for the overall moderate effects of suberin and CS alterations. First of all, further experiments are needed to establish what type of tissue surrounds infection sites. This issue can be solved with the endodermis marker *pELTP::NLS3xmVenus* (embryo lipid transfer protein) in combination with the suberin specific staining Nile Red (Ursache *et al.*, 2018; Barberon *et al.*, 2016; Vermeer *et al.*, 2014). If feeding sites are surrounded by a periderm, the expression of *pELTP::NLS3xmVenus* will not be detectable at that point when the endodermis is degraded. The expression of *pELTP::NLS3xmVenus* and the overlap with Nile Red staining, however, would indicate that it is in fact a suberized endodermis.

Based on the present data, it is likely that the endodermis surrounds infection sites at the beginning of feeding site formation. However, it is plausible that the endodermis is replaced by a periderm during root growth. It is also possible that the transition from endodermis to periderm takes place after nematodes start feeding. Therefore, holes in the apoplastic barrier – even when present only during the initial phases of nematode feeding – can cause nutrient loss and both reduced suberin and defective CS can contribute to that (reviewed in Barberon *et al.*, 2017). A defective apoplastic barrier may lead to reduced nutrient accumulation in the feeding site and thereby hamper nematode growth. The nutrient status of a number of mutants has been analysed by ionomic measurements of elements in leaves, revealing distinct phenotypes (Hosmani *et al.*, 2013; Pfister *et al.*, 2014; Kamiya *et al.*, 2015; Barberon *et al.*, 2016; Li *et al.*, 2017). Considering the microscopic nature of nematode infection sites, identification of the elements which could affect nematode development is challenging. Recent development in multi-element bioimaging allows the localization of elements in the precise tissue (Persson *et al.*, 2016). In the future this technology will enable the identification of nutrients accumulated in nematode feeding sites.

Until now, the loss of nutrients through a defective CS is speculation. The properties of lignin indicate that the CS functions additionally in stabilizing the tissue. Feeding sites act as nutrients sinks, which create high internal pressure (Böckenhoff and Grundler, 1994). The CS connects the endodermal cells in a tight network and a defective CS may contribute to destabilization, which may render the whole tissue less resilient to withstand pressure.

In consideration of all studies and results discussed here, it can be concluded that suberin and lignin play a bipartite role in plant-nematode interactions. Especially when deposited in relatively high amounts in the endodermis, the biopolymers can function as a barrier to nematode infection. After feeding site establishment, however, suberin and lignin in form of CS contribute to feeding site maintenance and nutrient acquisition. In order to underpin the role of the endodermis as primary barrier to nematodes, it would be interesting to investigate the behaviour of different nematode species such as the migratory nematode *Pratylenchus* spp.. This nematode feeds from cells in the cortex and does not cross the endodermis (Sijmons *et al.*, 1991; Duncan and Moens, 2013). Infection assays with mutants displaying reduced suberin or defective CS (*esb1-1cdef1*, *sgn3-3esb1-1*, *sgn3-3* and *cdef1*) would reveal if a weakened barrier enables *Pratylenchus* spp. to access the vascular tissue. In regard to plant protection, it would be striking to see if pronounced preformed depositions of suberin and lignin in the endodermis are an indication of a defence response which induces suberization and lignification in nematode-infected tissue. Plants which already have higher levels of suberin and lignin may be more likely to involve these biopolymers in defence mechanisms. The screening of plants for increased preformed suberin and lignin may be a strategy to identify more resilient cultivars.

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