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Host organelles and transporters in underground plant-pathogen interactions

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Cover: *Arabidopsis thaliana* rosettes. The left plant is healthy while the right plant has disease symptoms from *R. solani* root inoculation.

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Abstract

This thesis covers studies of three different soilborne plant pathogens, the two fungi, *Rhizoctonia solani* and *Verticillium longisporum*, and the protist *Plasmodiophora brassicae*, as well as their host responses. Based on genome sequence analysis of the pathogens and their plant hosts, different effectors and plant defence factors were predicted. Follow-up molecular studies revealed the following: In sugar beets, two genes encoding major latex protein-like (MLP) family members, *MLP1* and *MLP3,* contribute to the defence against *R. solani*. The small cysteine-rich effector RsRCP1 was highly induced in the fungus upon infection. RsRCP1 was localized to chloroplasts and mitochondria in the leaves of *Nicotiana benthamiana*. An additional *MLP* gene in oilseed rape, *MLP6*, was found to provide elevated levels of defence to *V. longisporum* together with a nitrate/peptide transporter family protein (NPF5.12). Recognition of the fungus triggered nitrate starvation and MLP-mediated defence, together reducing the lipophilic suberin barrier in the endodermal cell walls. In the genome of the clubroot pathogen *P. brassicae*, a consensus sequence led to the identification of peroxisomal targeting effectors. Arabidopsis mutants with impaired peroxisomal biogenesis demonstrated the importance of the plant peroxisomal transport proteins for *P. brassicae* establishment in the root. Host peroxisomal proteins embodied in the resting spores were also identified using a transgenic peroxisomal marker line of Arabidopsis. New technological advances and possibilities for genetic engineering of these three pathogens would greatly contribute to a deeper understanding of these different pathological systems.

Keywords: Arabidopsis, *Beta vulgaris*, *Brassica napus*, chloroplast, effectors, major latex-like protein, mitochondria, nitrate transporter, peroxisome, *Plasmodiophora brassicae, Rhizoctonia solani, Verticillium longisporum.*

Växtens organeller och transportörer vid angrepp av jordburna patogener

Sammanfattning

Många patogena arter angriper växter ovan jord men det finns också arter som har jorden som habitat. Detta avhandlingsarbete omfattar studier av tre olika jordburna patogener, de två svamparterna *Rhizoctonia solani* och *Verticillium longisporum* och protisten *Plasmodiophora brassicae* (Plasmodiophorid) och försvarsreaktioner i respektive värdväxt vid angrepp av växtrötterna. Baserat på analyser av respektive genom har olika effektorer och växtförsvar studerats på molekylär nivå. Resultat i korthet: Två så kallade ”major latex protein-like” (MLP) gener, *MLP1* och *MLP3,* bidrar till försvaret mot *R. solani* i sockerbeta. En unik effektor identifierades i genomet hos *R. solani* (*RsRCP1*), en svampgen som visades ha hög aktivitetsnivå under infektion. RsRCP1-proteinet kunde därefter lokaliseras till kloroplaster och mitokondrier i modellväxten *Nicotiana benthamiana*. En annan *MLP* gen i raps och Arabidopsis, *MLP6,* resulterade i förbättrat försvar mot *V. longisporum*. En respons som förstärktes av en kvävetransportör som begränsade mängden kväve för svampen vilket hämmade dess tillväxt. I detta fall orsakade förvaringen av nitrat och MLP-förstärkt försvar även en förtunning av suberin-skiktet i stjälkens cellväggar. I genomet hos klumprotpatogenen *P. brassicae* hittades en bevarad sekvens som resulterade i identifiering av effektorer som målsöker peroxisomerna i värdväxten. Genom analys av peroxisom-mutanter i Arabidopsis, kunde specifika transportproteiner viktiga för patogenens infektionsprocess urskiljas. Vidare kunde peroxisomproteiner från värdväxten identifieras i patogenens vilosporer. Nya tekniska framsteg och möjligheter att genetiskt förändra patogenernas genom kommer förhoppningsvis att leda till ökad kunskap av de tre växt-patogen systemen

Nyckelord: Arabidopsis, *Beta vulgaris*, *Brassica napus*, kloroplast, effektor, major latex-like protein, mitokondrie, nitrattransportör, peroxisom, *Plasmodiophora brassicae*, *Rhizoctonia solani*, *Verticillium longisporum.*

Dedication

To my children.
The three-year-old who knows everything and the nine-year-old who stays curious.

"We know more about the movement of celestial bodies
than that of the soil underfoot"
Leonardo da Vinci

In memory of my grandmother who opened my eyes to the subject of biology.
Telling me that "you will become a biologist someday" due to my interest in
chasing lizards or studying bumblebees in detail

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Holmquist L*, **Dölfors F***, Fogelqvist J, Cohn J, Kraft T, Dixelius C (2020). Major latex protein-like encoding genes contribute to *Rhizoctonia solani* defence responses in sugar beet. *Mol. Genet. Genom.* 296: 155–164.

II. **Dölfors F*,** Ilbäck J*, Bejai S, Fogelqvist J, Dixelius C. NPF5.12, a nitrate transporter protein, and MLP6, a major latex-like protein, are important defence factors against *Verticillium longisporum*. (manuscript).

III. **Dölfors F**, Stjelja Arvelius S, Dixelius C. The plant peroxisome, a hub for *Plasmodiophora brassicae* effectors. (manuscript).

IV. Tzelepis G, **Dölfors F**, Holmquist L, Dixelius C (2021). Plant mitochondria and chloroplasts are targeted by the *Rhizoctonia solani* RsCRP1 effector. *Biochem. Biophys. Res. Commun*. 544: 86-90.

Papers I and IV are reproduced with the permission of the publishers.

* These authors contributed equally

The contribution of Fredrik Dölfors to the papers included in this thesis was as follows:

I. Performed gene expression experiments, disease screening, cloning and characterizing overexpression lines and preparing mutants in Arabidopsis, data analysis. Contributed to manuscript writing and revision.

II. Experimental planning, performed all experiments in *B. napus* incl. generation of mutant lines. Performed nitrate and suberin experiments in Arabidopsis and data analysis. Contributed to manuscript writing.

III. Experimental planning, performed all cloning, confocal and disease screening experiments and contributed to finding genes of interest. Contributed to manuscript writing.

IV. Performed the virulence assays and gene expression experiments. Contributed to manuscript writing

1. Introduction

Plants host a plethora of organisms. In some cases, roots can reach depths greater than the aboveground parts reach in height (Pierret et al. 2016). Roots can also have a much larger surface area than the aboveground structures, with fine root hairs that extend it even further (Jackson et al. 1997). The root is constantly in contact with other organisms. One gram of healthy topsoil can harbour between four thousand and fifty thousand species of bacteria and up to one thousand species of fungi (Taylor et al. 2010; Raynaud & Nunan 2014). Plants influence the species composition and microbial diversity in the rhizosphere, the soil region closest to the root, by emitting root exudates (Wei et al. 2023). However, pathogenic species are also attracted by these substances. Soilborne pathogens are a major problem for the food production industry. Some, such as the fungus *Rhizoctonia solani*, which causes root rot, crown rot and damping off disease, can survive in the soil by switching to a saprophytic lifestyle. Others, such as the clubroot and stem stripe pathogens *Plasmodiophora brassicae* and *Verticillium longisporum*, produce resting structures that can survive in the soil and wait for a host for up to twenty years (Wallenhammar 1996; Zahr et al. 2021). Root diseases are difficult to detect before serious damage occurs. No efficient environmentally friendly chemical treatments are available to deter soilborne pathogens (Labrada 2008). A broad-spectrum fumigation treatment is currently being tested that impacts the microbial community (Nicola et al. 2017; Chen et al. 2023). Disease-resistant crop varieties remain in high demand, but knowledge of defence genes that can repel these pathogens is limited.

Recently, there has been an increase in attention given to studying the effects of nutrient transport and metabolism in relation to pathogenesis and disease resistance. This thesis covers plant nutrient and carrier transporter proteins in relation to the defence responses of sugar beet (*Beta vulgaris* ssp. *vulgaris*), oilseed rape (*Brassica napus*) and Arabidopsis and the infection biology of the three root-infecting pathogen species mentioned above. The pathogens described in this thesis have different lifestyles. All three of them, however, have been implicated in disturbing

host organelles involved in the metabolism of defence-related compounds, hormones and nutrients or structural defences. Part 1 serves as an introduction to all aspects that are discussed in the Results section. Part 1.1 will introduce the plant immune system and defence responses. Parts 1.2 and 1.3 introduce the fungal/protist perspective on early interactions with the host. This includes information on the different lifestyles of the three pathogens, effectors and plant organelles that are targeted. Part 1.4 introduces the types of transport systems I have studied, and Part 1.5 introduces a few hypotheses underlying my work. The illustration in part 3.5 summarizes the most important results.

1.1 Plant defence responses

1.1.1 The plant immune system

Immunity is a term that describes the state of being resistant to a disease-causing organism. Immunity includes structural attributes, autologous defence gene expression and the antimicrobial actions of endophytic microbiota (Sarenqimuge et al. 2022) and otherwise incompatible interactions. This section describes the basics of the plant immune system based on recent reviews (Li et al. 2020b; Ngou et al. 2022a; b). The terminology has recently expanded to specify the origin of the response (extra- or intracellular), the type of receptor that initiates the response and the type of molecule that triggers the response. This is because these factors influence the type of responses that are triggered. Put very simply and without signalling pathway details, plant immunity costs energy and resources and is tightly regulated by the tradeoff between growth and defence. The immune system is often described as a two-step system in which the detection of intrusion triggers a myriad of defence responses. Pattern- and effector-triggered immunity (PTI/ETI) involve local components within infected regions. Plants use membrane-bound pathogen recognition receptors (PRRs) and cytosolic nucleotide-binding leucine-rich repeat receptors (NLRs) as “self”, “altered self” and “non-self” sensors. PRRs survey cells for common, evolutionarily conserved molecular patterns displayed by pathogens (such as chitin or flagellin) that indicate an infection, while NLRs detect pathogen-secreted compounds. Recognition of microbe-associated molecular patterns or effectors triggers a response that induces PTI and/or ETI. The initiation of signalling differs depending on the receptor triggering it but may include conformational changes or the phosphorylation of signalling components. Considerable crosstalk links the two complementing defence pathways. Both lead to the activation of multiple transcription factors, signalling cascades, transcriptional reprogramming to induce defence genes, the synthesis of antimicrobial compounds and defence-related

hormones. Physiological responses include calcium influx, reactive oxygen species (ROS) accumulation and a hypersensitive response (HR) or the production of cell wall-strengthening callose. Programmed cell death, or HR, can be triggered via NLR-mediated induction of enhanced disease susceptibility (EP) domain proteins or via PRR or NLR-mediated activation of ROS bursts. NLRs may recruit helper NLRs that strengthen signals and in turn activate the production of defence-related hormones, such as ethylene (ET) and salicylic acid (SA). An increase in defence-related SA levels is often accompanied by systemic acquired resistance (SAR). SAR is a type of defence priming response. Defence signalling molecules migrate to distant parts of the plant and activate defence genes, such as pathogenesis-related (PR) genes, that protect against many types of pathogens (bacteria, fungi, viruses, and protists). The physiological response that seems to be strictly triggered by PTI is physical closure of stomata and plasmodesmata. PTI and ETI are otherwise considered to represent a continuous spectrum of responses, the strength and duration of which are dependent on the pathogen (effectors) and the plant (whose PRRs/NLRs are being activated).

Root defence hormone signalling

Hormone signalling is a minor part of this thesis. Phytohormones are still worth mentioning because the recognition of pathogenic invasion is often associated with plant hormones and transcriptional reprogramming (Kazan & Lyons 2014). Hormonal and defence responses are also context dependent and may vary depending on the plant and pathogen species, plant developmental stage (*R. solani*; Liu et al. (2019)) and pathogen infection stage (*P. brassicae*; Yang et al. (2020), *V. longisporum*; Section 3.2.6). Environmental factors such as abiotic stress (Zahra et al. 2023) or circadian rhythm (de Leone et al. 2020) also influence plant health. SA, jasmonic acid (JA) and ET are key hormones that respond to pathogens. SA is required for the induction of several PR genes (*PR1*, *PR2*, and *PR5*) and for responses such as HR and SAR (Alvarez 2000; Zheng et al. 2019a). Other PRs are induced by JA (such as members of *PR3*, *PR4*, and *PR12*). Both SA- and JA-mediated defences can be activated at different infection stages (Ali et al. 2018). Hormone signalling is not linear; extensive crosstalk links hormonal signalling pathways with synergistic or antagonistic effects (Lorenzo et al. 2004; Liu & Timko 2021). Abscisic acid (ABA) is a negative regulator of JA/ET signalling in some cases and a positive regulator in others (Anderson et al. 2004; Mauch-Mani & Mauch 2005; Bascom 2023). Context-dependent hormone crosstalk and responses can be exemplified by *V. longisporum* infection of *B. napus* and Arabidopsis. ABA biosynthesis genes are repressed at six days after inoculation of *V. longisporum* in *B. napus*. This leads to increased SA and decreased JA/ET responses, which delay the onset of disease symptoms (Behrens et al. 2019). In Arabidopsis, ABA levels

increase two days after infection (Roos et al. 2015), and JA/ET responses are not affected, leading to the full onset of symptoms (Ralhan et al. 2012; Behrens et al. 2019).

1.1.2 Structural and spatial defence regulation in roots

New undifferentiated root cells are created from the root meristematic zone above the apical root cap. These expand in the elongation zone. As the roots grow, older cells start to differentiate into different cell types in the zone of maturation. The Arabidopsis root consists of an epidermal cell layer followed by the cortex, endodermis and vascular stele (pericycle, phloem and xylem) (Fig. 1A). The undifferentiated elongation zone may be more susceptible to certain pathogens due to the lack of structural defences, such as suberin depositions in the dermal cell layers of fully mature roots. PTI defence responses are differentially regulated in a vertical developmental zone-dependent manner (Chuberre et al. 2018). For example, FLAGELLIN 22 (Flg22), a protein in the flagella of bacteria and protists, can be detected by the flagellin sensor FLS2, a protein expressed in the elongation- and maturation zones. The camalexin biosynthesis gene *CYP71A12* and callose deposition responses to Flg22 are only activated and present in the columella and elongation zone. FLS2 and a PTI activated peroxidase involved in suberization, PER5, are active in and around zones of lateral root emergence in the epidermis upon Flg22 treatment. FLS2 is otherwise most active in the elongation zone (Beck et al. 2014; Millet et al. 2010; Rich-Griffin et al. 2020; Emonet et al. 2021). Responses to chitin however are restricted to mature parts of the root (Fig. 1B) (Beck et al. 2014). Recent findings suggest that root defence responses are orchestrated differently in a horizontal, concentric cell layer-dependent manner as well. Tissue type-specific defences and even cell-specific defences enable plants to handle multiple (beneficial and pathogenic) microbial interactions simultaneously (Chuberre et al. 2018; Rich-Griffin et al. 2020; Fröschel et al. 2021). Root-infecting microbes are blocked from the xylem and nutrient-rich phloem by the endodermal barrier (Kawa & Brady 2022). In differentiated parts of the root, the apoplastic path is blocked by the Casparian strip. This is a waterproof barrier formed by lignified endodermal cell junctions. The symplastic path is blocked by hydrophobic suberin lamellae, a form of secondary cell wall formed by fatty acids, phenolics and glycerol that cover endodermal cell membranes (Doblas et al. 2017). Suberin depositions are not continuous throughout the entire root. The process starts in the zone of maturation and is initially patchy, only a few cells are suberized. This step is followed by a zone of continuous suberin depositions that extends to the root/hypocotyl junction (Fig. 1B) (Robe & Barberon 2023). To pass through the endodermal barrier, nutrients and ions can pass through unsuberized passage cells or through plasmodesmata by active

transport into neighbouring cortical cells (Robbins et al. 2014). Vascular pathogens need to infect the undifferentiated zone, enzymatically damage the barrier, disturb the suberin biosynthesis pathway, enter through a passage cell or otherwise find a weak spot in the suberin lamellae to pass through. The endodermal barrier is plastic and responds to environmental stimuli such as nutrient homeostasis (Doblas et al. 2017), biotic stress (Shukla & Barberon 2021) and wounding (Fugate et al. 2023). The vertical spread of vascular pathogens that reach the vasculature may further be blocked by protoplasts of neighbouring parenchyma cells that enter the xylem (tyloses) or polysaccharide gels (Fig. 1C) (Kashyap et al. 2020).

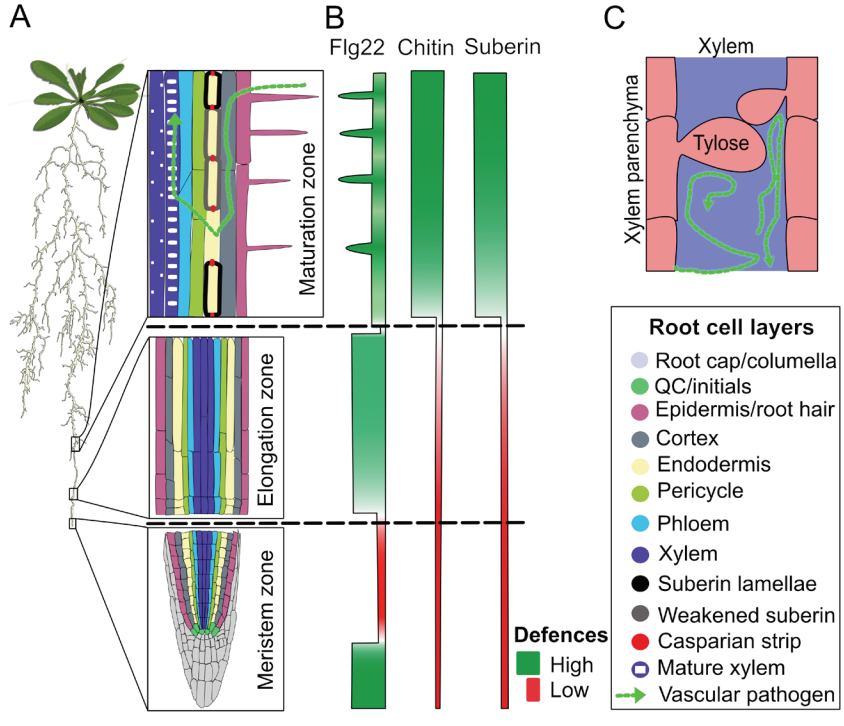


Figure 1. Cell types and defences in developing roots. A) Cell and developmental zones of Arabidopsis root. Mitotic cell division occur in the meristematic zone. Cells enlarge in the elongation zone and eventually mature into differentiated cells in the maturation zone. Vascular pathogens gain entry to the vascular system at weak points in the Casparian strip or suberin lamellae. B) PTI triggered defences vary depending on the elicitor and root development zone. Plant detection of bacterial/protist flagellin (Flg22) trigger camalexin and callose production mainly in the elongation zone and columella. FLS2 triggered PER5 is active around lateral root initiation sites and elongation zone. Chitin triggered camalexin production activates only in mature roots. Structural defences such as suberin lamella and Casparian strip are present only in mature roots. C) Vascular parenchyma cells may extend tylose (overgrowth of protoplast) into xylem in response to vascular pathogens.

1.1.3 Protein and chemical defences

Plants produce an abundance of defence-related substances against invaders, including phenolics, terpenoids, alkaloids, polyacetylenes, defensins, PRs and protease inhibitor proteins (Lacerda et al. 2014). The molecular mechanism of action of antimicrobial proteins or compounds is often not well characterized. This is true for the antifungal PR1 protein family. The *PR1* gene AT2G14610 in Arabidopsis, which is induced in response to a wide variety of pathogens, is often used as a marker for the SA-dependent SAR response (Han et al. 2023). PR proteins, such as the endoglucanases PR2; the chitinases PR3, PR8, and PR11; and RNA-degrading ribonucleases of the PR10 family (dos Santos & Franco 2023), are otherwise relatively characterized. Some known stress responses to *V. longisporum* in *B. napus* are similar to Arabidopsis responses to *P. brassicae*. Elevated levels of SA have been found after infection with *P. brassicae*, and SA was found in xylem and phloem sap after *V. longisporum* infection (Ratzinger et al. 2009; Galindo-González et al. 2020). Lignin production and the expression of camalexin, PR1 and chitinase proteins (PR3 and PR4) are induced in the early stages of infection in both *P. brassicae* and *V. longisporum*. *P. brassicae* also induces the expression of thaumatin-like proteins (PR5), while *V. longisporum* induces the expression of β1,3-glucanases (PR2) (Floerl et al. 2008; Zhao et al. 2017; Irani et al. 2018; Zheng et al. 2019a). Both pathogens trigger indole-glucosinolate production, but neither these nor their breakdown products seem to affect pathogen growth (Iven et al. 2012; Zamani-Noor et al. 2021). It is possible that these compounds can be converted to camalexin (Klein & Sattely 2017) or help maintain water homeostasis (Zamani-Noor et al. 2021). Induction of cell wall-bound phenolic compounds in the xylem of *V. longisporum*-infected *B. napus* may block the vertical spread of the pathogen (Eynck et al. 2009). Phenolic compounds are also found in cell walls early after *P. brassicae* infection (Irani et al. 2019). Both *P. brassicae* and *V. longisporum* cause JA-induced PLANT DEFENSIN 1.2 (*PDF1.2*) production (Irani et al. 2018; Li et al. 2022). Defensins are antifungal peptides encoded by the *PR12* genes that increase the permeability of pathogen cell membranes, which induces ion leakage that leads to necrotic cell death (Lacerda et al. 2014). Defence compounds against *R. solani* are mostly unknown. Common broad-spectrum defence compounds against necrotrophs include the JA precursor oxo-phytodienoic acid (OPDA). OPDA and chemically related molecules are called “death acids”. These compounds have direct fungitoxic effects but also induce callose deposition and activate JA-dependent defence genes by promoting the breakdown of JAZ repressor proteins (Christensen et al. 2015; Scalschi et al. 2015; Macioszek et al. 2023).

1.2 Lifestyles of root-infecting microorganisms

All heterotrophic organisms need to acquire nutrients. This can be orchestrated in different ways depending on their lifestyle. It is common to divide pathogens into the general categories of biotrophs, hemibiotrophs or necrotrophs based on their mechanism of nutrient acquisition (Liao et al. 2022). Obligate biotrophs and biotrophs rely on stealth tactics to remain undetected in plants throughout most of their life cycle. Unlike biotrophs, obligate biotrophs cannot survive in a metabolically active state in bulk soil. "Mostly necrotrophic" fungi, including *R. solani* and *Sclerotinia sclerotiorum*, use an arsenal of necrosis-inducing effectors, destructive cell wall-degrading carbohydrate-active enzymes (CAZymes) and phytotoxins to kill host cells and feed from nutrient leakage (Aliferis & Jabaji 2010; O'Sullivan et al. 2021; Shao et al. 2021; Li et al. 2023c). Hemibiotrophs are insidious and behave like biotrophs in the colonization phase before switching to a necrotrophic lifestyle at some point in the life cycle. Infection strategies, such as the types of effectors used and host defence strategies, are usually included in categorical terms (Liao et al. 2022). A strategy of (hemi)biotrophs is to initially use immunosuppressive effectors and delay the bombardment of cell-killing agents. However, the archetypal necrotroph *S. sclerotiorum*, which targets more than 400 plant species (Laluk & Mengiste 2010), also uses this tactic. The extracellular invasive hyphae of *S. sclerotiorum* dampen initial ROS bursts via oxalic acid (Williams et al. 2011) and potentially evade PTI by secreting several chitin-binding proteins in the early stages of infection (Guyon et al. 2014). Although it is not a typical root pathogen, the necrotrophic *Cytospora chrysosperma* reduces callose deposition and inhibits programmed cell death by dampening defence gene expression (Han et al. 2021b). This indicates that immunosuppression is a commonly employed strategy among diverse pathogens with varying lifestyles. In a recent article by Rajarammohan (2021), the author argues for redefining the term "necrotroph" for these reasons. Taking this one step further, cell death induction may also apply to biotrophs. *P. brassicae* expresses cell death-inducing proteins in the resting spore release stage at the end of its life cycle (Zhan et al. 2022). Many pathogens use a spectrum of tactics through their infectious cycle and may not conform to our definitions. Since no new terms have been introduced to account for this, I will use them cautiously while describing pathogens throughout this thesis.

Unlike *R. solani* and *V. longisporum*, which grow apoplastically and intracellularly without any obvious intracellular feeding structures, *P. brassicae* is obligately intracellular and can form galls (Eynck et al. 2007; Zhang et al. 2016; Anderson et al. 2017). The severity of disease symptoms varies depending on the developmental stage of the plant during infection and the route of infection. *R. solani* causes crown

rot disease on leaves, damping off disease in young seedlings or root rot in developed beets. Responses to infection may also differ between host species. This is the case for both *P. brassicae* (Prerostova et al. 2018) and *V. longisporum* (Ralhan et al. 2012; Behrens et al. 2019) and their respective host species. This thesis covers pathogens that use a broad range of infection strategies and feeding tactics. Although the infectious cycle of *R. solani* is mostly unknown, it is classified as a necrotroph (with hemibiotrophic attributes) on sugar beet (Charova et al. 2020), *V. longisporum* as a hemibiotroph (mostly a biotroph) on oilseed rape (Depotter et al. 2016) and *P. brassicae* as an obligate biotroph (Ludwig-Müller 1999). For this reason, a brief introduction covering basic information regarding their infection cycles is given below.

1.2.1 *Rhizoctonia solani* – The root killer

The basidiomycete *R. solani* is a soilborne, multinucleate species complex that lacks asexual spores (conidia) and rarely reproduces by sexual reproduction. The species is divided into fourteen groups based on the affinity for hyphal fusion (anastomosis) and the exchange of genetic material in the form of cytoplasm/nuclei (Patil & Solanki 2016; Ajayi-Oyetunde & Bradley 2018). Although *R. solani* is capable of saprophytic growth (feeding on dead plant material in the soil), the name derives from Greek and roughly translates to "root killer" due to its aggressiveness in living host plants. As a pathogen, it has a wide host range that includes turfgrass, rice, carrot, soybean, potato, oilseed rape, radish, and others (Patil & Solanki 2016). The AG2-2IIIB subgroup, used in Paper I and Paper IV, mainly infects *B. vulgaris* (Rafiei et al. 2023a). The molecular basis for the switch between a saprophytic lifestyle and a necrotrophic lifestyle in a host is not well characterized but may briefly involve hemibiotrophic attributes, such as PTI and ETI suppressive effectors (Dölfors et al. 2019; Charova et al. 2020). Following direct penetration of the host epidermis, invasive hyphae branch out (Bashyal et al. 2018). It is likely that the secretion of CAZymes (Wibberg et al. 2016) and toxins (Zhang et al. 2021; Li et al. 2023c) aids in breaking cell walls and destabilizing cellular functions to gain access to nutrients. Symptoms of disease in seedlings appear as dark spots on hypocotyls and stems. The loss of hypocotyl integrity caused by necrotic rot results in the plant falling over, thereby leading to the name damping off disease. Infection of three-week-old sugar beet sprouts can result in death within eight days in infested soils (Dölfors et al. 2022). A partly resistant genotype may start to exhibit necrotic symptoms five days post inoculation (dpi) (Fig. 2A). A susceptible plant already has visible dark spots on the hypocotyl after two days, and at five days, the susceptible plant line dies due to a fully necrotic hypocotyl (Fig. 2B). Infection of older roots or leaves in soil or in storage results in root rot, crown rot or leaf blight, depending on

the route of infection. Either way, it causes significant losses. To date, no completely resistant cultivars of *B. vulgaris* exist. Despite the use of current plant protection strategies to reduce losses (cultivation of semi-tolerant varieties and fungicide seed treatment), an average yield loss of 20% occurs. In conductive climates, losses reach 60% (Hanson & McGrath 2011). Although this topic will not be discussed further in this thesis, biological control measures using various growth-promoting bacteria have shown promising inhibitory effects on *R. solani* AG2-2 in *B. vulgaris* (Farhaoui et al. 2023).

Figure 2. Damping off disease development in *B. vulgaris* seedlings inoculated with *R. solani*. A) Partially resistant genotype. B) Partially susceptible genotype. The plants were inoculated at twenty-one days after germination and photographed at 0 days post inoculation (dpi), 2 dpi and 5 dpi. Scale = 5 cm.

1.2.2 *Verticillium longisporum* – The latent vascular threat

V. longisporum (ascomycete) is the causal agent of stem stripe disease in *Brassica* species, was first described in Sweden 1970 (Kroeker 1970). Problems with the disease has become a major problem for the oilseed rape production (Depotter et al. 2016). Most species of *Verticillium* are haploid but *V. longisporum* is amphidiploid. It is believed to have evolved several times by relatively recent hybridization events (Inderbitzin et al. 2011). Three genetically very similar lineages of *V. longisporum* has been described. One representative dominates in Sweden (Depotter et al. 2017; Fogelqvist et al. 2018). With the development of molecular detection tools, a field can be screened for its suitability for growing brassica crops. High levels of *V. longisporum* microsclerotia have for example been found in 40% of tested soil samples from the southern parts of Sweden (Tzelepis et al. 2017). The monoterpene 1.8-cineole has a stimulative effect on microsclerotia germination (Roos et al. 2015). It is thought that root exudates in the rhizosphere trigger germination of sclerotia and attract growth of hyphae toward the root (Depotter et al. 2016). Following a swift penetration of epidermal cells, invading hyphae grow through the cortex to the endoderm. It grows along the endoderm until it penetrates the endodermal barrier to enter the xylem vessels and travel systemically. For this reason, it is described as a vascular pathogen. Although xylem is a nutrient poor environment compared to phloem, most vascular pathogens inhabit the xylem. Xylem sap contain minerals and low levels of carbohydrates, and various amino acids (Yadeta & Thomma 2013). To supplement the low levels of nutrients *V. longisporum* may enzymatically digest cell walls of neighbouring cells by employing carbohydrate active enzymes (Leonard et al. 2020). The phenotype of *V. longisporum* infection is subtle and may include reduced growth, one-sided chlorosis, early senescence, and leaf abscission. Visual symptoms clearly attributed to *V. longisporum* colonisation appear late in *B. napus* in the form of microsclerotia stem stripes (Fig. 3A,B) but can be detected growing along the root in a microscope within a week (Fig. 3C). In the field the stunting phenotype seen in experiments performed in controlled environment may be absent. This can be linked to delayed infection due to soil temperature at the time of sowing autumn/spring varieties of *B. napus* (Zheng et al. 2019b). Yield losses range between negligible to 50% with varying effect on seed oil content (Dunker et al. 2008; Depotter et al. 2019).

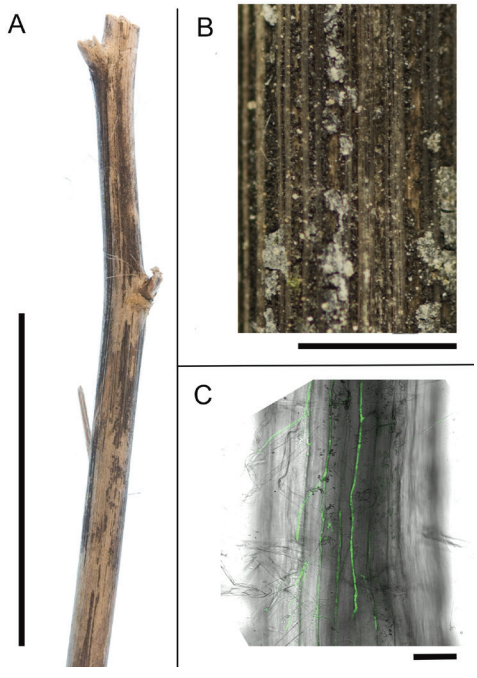


Figure 3. Stem stripe disease in *Brassica napus*. A-B) *Verticillium longisporum* microsclerotia protruding from a dried stem of a plant in the flowering stage. Scales: A = 100 mm, B = 10 mm. C) GFP tagged *V. longisporum* hyphae growing along the endodermis in the root of a plant in the vegetative stage. Scale = 0.01 mm.

1.2.3 *Plasmodiophora brassicae* – Architect and master thief

P. brassicae is a pathogenic endoparasite belonging to the supergroup Rhizaria. *P. brassicae* relies on host survival to complete its lifecycle because it is not capable of surviving in an active state outside of the host. Its genome is relatively small (25.1 Mb) (Stjelja et al. 2019) compared to that of other free-living Rhizaria (320 Mb and 100 Mb in the soil- and freshwater-living *Reticulomyxa filosa* and the marine alga *Bigelowiella natans* (Bi et al. 2019), respectively). The essential components that seem to be absent in the genome include genes encoding enzymes used in fatty acid biosynthesis and for sulfur and nitrogen uptake/assimilation (Schwelm et al. 2015; Bi et al. 2016). *P. brassicae* therefore seems to be dependent on the host to provide these services. To acquire nutrients, *P. brassicae* remodels roots to funnel nutrients to infected cells, the feeding sites. This is accomplished in part by altering host hormone homeostasis to control the rate of cell division and phloem bundle differentiation and complexity (Walerowski et al. 2018). This includes controlling the production of auxins, cytokinin, JA, SA and, at later stages, ABA (Ludwig-

Müller et al. 2009; Schwelm et al. 2015). The roots become intense carbohydrate feeding sites filled with large nutrient highways connected to the infected cells. The result is a swollen, gall-like root called a clubroot. The completion of the life cycle takes approximately three to four weeks (Liu et al. 2020). At later stages, the roots start to split, and infected cells degrade, releasing spores (Stefanowicz et al. 2021). Most brassica crops are grown for the value of their aboveground tissues, such as oil from seeds (oilseed rape), leafy vegetables (cabbage) or inflorescences (broccoli). Symptoms above ground may occur late in the disease cycle, such as chlorosis, wilting, stunting and premature senescence, leading to products being unsuitable for the market or negatively impacting seed yield (Wallenhammar et al. 2021). These symptoms may in part be attributed to the loss of water transport by *P. brassicae*-induced xylem development arrest or to the destabilization of hormone homeostasis (Malinowski et al. 2019). Yield losses are reported to be approximately 10-15% annually (Dixon 2009).

1.3 Organelle-targeting effectors

Attempts to regulate host immune responses can be accomplished either by blocking signalling pathways or regulating physiological responses. Numerous examples of effector-based immune suppression via nuclear gene regulation exist for plant pathogenic fungi, protists and bacteria (De Mandal & Jeon 2022; Tehrani & Mitra 2023). Entry to the nucleus requires the effector to either encode a nuclear localization signal or interact with a protein that does. *V. longisporum* PHOSPHOLIPASE A_2 ($VlsPLA_2$) localizes to the nucleus by binding a vesicle-associated protein. In the nucleus, the protein alters the expression of genes involved in HR signal transduction (Rafiei et al. 2023b). The obligate biotrophic *P. brassicae* encodes at least twenty-one candidate effectors that suppress cell death. However, the localization and specific function of these effectors are unknown (Zhan et al. 2022). *Xanthomonas oryzae* pv. *oryzicola*, a biotrophic bacterium, uses the TRANSCRIPTION ACTIVATOR-LIKE 7 (TAL7) effector which activates the rice gene Os09g29100, presumably by binding to the promoter region, which in turn suppresses a HR-related gene and promotes virulence (Cai et al. 2017). Hemibiotrophic *V. dahliae* has several known effectors that translocate to the nucleus. Among them, SMALL CYSTEIN RICH PROTEIN 41 (VdSCP41) binds and blocks master regulators of defence responses in cotton (Qin et al. 2018).

1.3.1 Pathogenic endomembrane and organelle targeting

Endoplasmic reticulum

The endoplasmic reticulum (ER) is part of the endomembrane system together with the Golgi apparatus, endosomes, lysosomes, and vacuoles. The ER is a common target for plant-infecting pathogens due to its involvement in monitoring the synthesis of immune receptors and delivery of antagonistic PR proteins to the extracellular space (Eichmann & Schäfer 2012). Pathogen-induced stress in plants may disturb ER function and cause the accumulation of misfolded or unfolded proteins. This triggers an ER unfolded protein response, which enforces ER quality control and protein processing, positively influencing immunity and negatively regulating root growth (Kim et al. 2022). It is also involved in triggering SAR and programmed cell death, a process that is interrupted by an effector employed by *Phytophthora sojae* to establish in plants (Moreno et al. 2012; Jing et al. 2016). *P. brassicae* employs at least nine effectors that target the ER (and nucleus). Six of these have an inhibitory effect on programmed cell death, although the exact mechanism is unknown (Hossain et al. 2021).

Peroxisome targeting

The peroxisome is a group of subpopulations of dynamic cell structures with specialized functions called microbodies (van der Klei 2014). These may form in two ways, either by budding off from the ER as immature peroxisomes or by fission from peroxules, which are elongated parts of peroxisomes (Kao et al. 2018). Intrinsic peroxisome function may vary between developmental stages and tissue types or among peroxisomes within the same cell (Bittner et al. 2022). The degree of specialization found among peroxisomes is reflected by the 163 different peroxisomal proteins found in Arabidopsis (Kaur & Hu 2011). Knowledge of the variation in function of peroxisomes according to cell type is fairly new, since all of the previous peroxisome proteome studies have used leaves as the starting material (Pan et al. 2020; Tarafdar & Chowdhary 2022). In germinating seeds, the glyoxysome is a common type of peroxisome due to its specialization in the oxidation of fatty acids from lipid bodies. In senescing tissue, a type called the gerontosome is common. However, all peroxisomes contain the same core proteome (Pan et al. 2020). Intralumenal vesicles have been found in Arabidopsis peroxisomes, which indicates that peroxisome functions may be further compartmentalized within peroxisomes (Wright & Bartel 2020).

There are many functions of peroxisomes that suggest that they are prime targets for pathogen manipulation. The peroxisome produces the defence hormones JA and benzoic acid, a precursor of SA and a major producer of ROS. The control of these

processes disturbs defence responses. It also produces the growth-regulating auxin indole acetic acid (IAA). The peroxisome is also involved in amino acid and polyamine catabolism and sulfur metabolism and is the only organelle in plant cells in which β-oxidation of fatty acids occurs (Poirier et al. 2006; van der Klei 2014; Su et al. 2019; Pan et al. 2020). These metabolic processes produce nitrogen-, sulfur- and carbohydrate-containing metabolites that can serve as nutrient sources. Regardless, only a handful of known effectors target the peroxisome. One report on peroxisome targeting in yeast (Saleem et al. 2010) and three reports on plant peroxisome targeting have, to my knowledge, been published. All known effectors have plant defence suppressive effects (Sun et al. 2017; Robin et al. 2018; Ning et al. 2022).

Chloroplast and mitochondrial targeting

Several reports have recently implicated chloroplasts and mitochondria as targets of diverse pathogens in both monocot and dicot species. These proteins mostly target functions related to ROS or SA production. Most of the early related work was from studies of bacteria (Xiang et al. 2008; Rodríguez-Herva et al. 2012). Fungal chloroplast targeting is also known (Kretschmer et al. 2020). *S. sclerotiorum* targets calcium receptors in chloroplasts to inhibit ROS accumulation and calcium influx-dependent SA signalling in Arabidopsis (Tang et al. 2020). The wheat stem stripe fungus *Puccinia striiformis* f. sp. *tritici* targets a cytochrome b complex to reduce the electron transport rate in chloroplasts, which in turn reduces ROS accumulation (Xu et al. 2019a). The first mitochondrial targeting effector was also found in bacteria (Block et al. 2010). Since then, only a few more have been located. *Magnaporthe oryzae*, the fungus that causes rice blast infection, targets both chloroplasts and mitochondria (Han et al. 2021a; Shabbir et al. 2022). Although the function of chloroplast localization is unclear, the mitochondrial effector AVR-PITA targets CYTOCHROME C OXIDOASE 11 (COX11), a key enzyme in the functional mitochondrial transport chain. It also regulates ROS production. *R. solani* AG1-1A secretes a protein that interacts with the same enzyme, which enhances virulence and may cause cell death (Zhang et al. 2023a).

Necrotrophic fungi that may benefit from cell death have evolved ways to regulate immune responses to their advantage. Most known examples have been found from stem- or leaf-infecting pathogens (Hammond-Kosack & Rudd 2008; Shao et al. 2021; Kanyuka et al. 2022). *Pyrenophora tritici-repentis,* a necrotrophic fungus of wheat, encodes a TOX-A protein that localizes to chloroplasts in wheat and induces light-dependent ROS bursts that lead to cell death (Manning et al. 2009; Andersen et al. 2021). *Parastagonospora nodorum* encodes several SnTOX proteins that trigger defence responses and may share functions with TOX-A (Richards et al.

2022). At least six TOX effectors target components of the immune system (PRRs and NLRs) to trigger programmed cell death or reduce PR defences (Friesen & Faris 2021) in favour of the pathogen. The bacterium *Pseudomonas syringae* uses coronatine, a toxin structurally related to JA, to downregulate SA-mediated defences (Brooks et al. 2005).

1.3.2 Organelle crosstalk and defence

Extensive crosstalk links peroxisomes, chloroplasts, mitochondria, the ER and the nucleus (Fig. 4). Peroxisomes supply the glyoxylate cycle and tricarboxylic acid cycle in the mitochondria with fatty acid-derived acetyl-CoA, citrate and succinate (Pan et al. 2020). Fatty acids are acquired from chloroplasts and lipid oil bodies (Su et al. 2019). The peroxisome helps detoxify chloroplast photorespiratory oxygenation with the help of mitochondria. Isoprenoid precursor production is orchestrated by crosstalk between the ER, peroxisomes, and chloroplast mevalonic acid pathway. Peroxisomes control the first and final steps in production of isoprenoid precursors (Pan et al. 2020) that are included in essential oils, gibberellic acid (GA), carotenoids and defence-related metabolites such as alkaloids or terpenes (Eigenbrode 2011). Similarly, organellar defence signalling is regulated by crosstalk between peroxisomes, mitochondria and chloroplasts and retrograde signalling to the nucleus. Hydrogen peroxide (H_2O_2) is a ROS that is generated after pathogen detection and is amplified in these organelles. Mitochondria sense ROS and SA signals and amplify the response (Norman et al. 2014). A change in the redox state that reaches the nucleus induces defence-related gene expression or induces cell death (Su et al. 2019). The specific retrograde signalling response of peroxisomes and chloroplasts is related to the coordination of defence hormone (JA and SA) production, PR gene expression and programmed cell death (Su et al. 2019; Mielecki et al. 2020; Terrón-Camero et al. 2022; Sandalio et al. 2023).

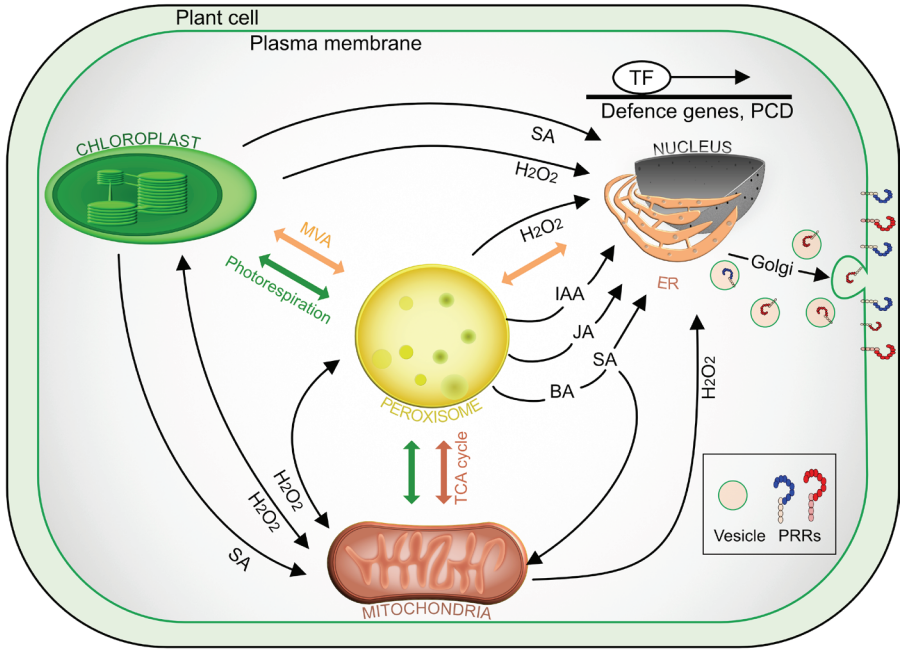


Figure 4. Organelle crosstalk and retrograde signalling. Peroxisomes may specialize to provide mitochondrial tricarboxylic acid cycle (TCA) with acetyl-CoA (brown arrow) and both organelles detoxify chloroplast photorespiration (green arrows). Chloroplasts, peroxisomes and the nucleus complete the mevalonic acid (MVA) pathway (orange arrows). Peroxisomes produce jasmonic acid (JA) and indole acetic acid (IAA) which trigger gene expression changes in the nucleus via retrograde signalling. Chloroplasts and peroxisomes generate reactive oxygen species (H_2O_2) and synthesize salicylic acid (SA) and the SA precursor benzoic acid (BA) respectively. Mitochondria senses these signalling molecules and amplifies the retrograde ROS signal to the nucleus which triggers activation of transcription factors (TF) and transcriptomic reprogramming for defence gene amplification or programmed cell death (PCD). ROS stress may force the endoplasmic reticulum (ER) into an unfolded response which strengthens the quality control of pathogen recognition receptors (PRRs).

1.4 Types of transport systems

Many types of transport mechanisms in plants are not covered in this thesis. The movement of cargo and solutes (proteins, metabolites, hormones, nutrients, ions, water) can occur locally between organelles within the cell, between adjacent cells through diffusion or plasmodesmata or over long distances through the vasculature. These processes may include uptake from the environment, vascular loading or unloading or direct interaction with carriers for physical transportation to the target. Most of these mechanisms involve transmembrane transport proteins or cytosolic

carriers to some degree. The three major types of membrane transporters in plants include passive channels and pores, active pumps and ATP-binding cassette (ABC) transporters and cotransporters (Taiz & Zeiger 2010). Specific cases of a transmembrane nitrate transporter, aquaporins, peroxisomal protein transporters and long-distance transport by cytosolic carrier proteins are discussed in this thesis and introduced below.

1.4.1 Major latex protein-like family (MLP) (Papers I, II)

The major latex protein (MLP) family was first discovered in the latex fluid of opium poppy (Nessler et al. 1985). Since then, these or similar proteins have been identified in the moss *Physcomitrella patents* and in at least a few monocotyledons and more than 26 dicotyledon angiosperms (Radauer et al. 2008). The number of MLPs identified range from fourteen in grape vine (*Vitis vinifera*) to 135 in tetraploid peanut (*Arachis hypogaea*) (Zhang et al. 2018; Li et al. 2023b). The members of the Arabidopsis MLP family typically consist of a single, on average, 154-amino-acid-long BetV1 domain and share a weak structural relationship with PR10 proteins. MLPs are cytosolic carriers and are capable of long-distance transport through phloem sap (Thieme et al. 2015; Carella et al. 2016; Gai et al. 2018). Commonly reported attributes include a hydrophobic pocket with a ligand-binding domain for carrying hydrophobic cargo (Radauer et al. 2008; Lytle et al. 2009). Ligands that have been proposed include the SAR signal dehydroabietinal (Carella et al. 2016), alkaloids (Ozber et al. 2022), organic pollutants (Inui et al. 2013), flavonoids, hormones (cytokinin, brassinosteroid) and lipids (Radauer et al. 2008; Aglas et al. 2020). They are expressed in roots, stems, flowers, seeds and ripening fruits; are involved in the regulation of growth and development in vegetative and reproductive stages; and are involved in the response to both abiotic and biotic stress (Wang et al. 2016; Gai et al. 2018; Fujita & Inui 2021; Li et al. 2023b).

1.4.2 Nitrate peptide transporter family (NPF) (Paper II)

Nitrogen is a primary macronutrient; is an essential component of DNA, RNA amino acids and chlorophyll; and is required for basic and secondary metabolism (O'Brien et al. 2016; Zhang et al. 2020). Nitrate, one of the main sources of nitrogen, also acts as a signalling molecule for plant growth by influencing meristem dynamics and root/shoot architecture (Fredes et al. 2019). A web of functionally redundant nitrate sensors and transporters is responsible for uptake and transport locally and systemically (Hsu & Tsay 2013; Wang et al. 2018; Lu et al. 2022). Exposure to nitrogen causes a nitrogen response that affects more than one thousand genes (Contreras-López et al. 2022). Transport is orchestrated by the nitrate/peptide transporters NRT1/PTR (NPF) and NRT2 and the chloride channel (CLC) families

(Islam 2022). Most NPFs are transmembrane proton-coupled low- or high-affinity symporters that facilitate vacuolar and cellular reallocation (influx or efflux) and loading/unloading of nitrates in the vasculature (Wang et al. 2018). There are 53 NPF proteins in Arabidopsis and 193 in the sixfold larger genome of *B. napus* (Zhang et al. 2020). Several NPFs exhibit dual affinity or nonspecific ligand binding and can bind peptides, hormones (GA, ABA, jasmonates, auxins) and glucosinolates in addition to nitrate (Corratgé-Faillie & Lacombe 2017; Wang et al. 2018). Functional characterization has revealed the involvement of NPFs in pollen temperature stress, the detoxification of glycoalkaloids in ripening tomatoes, the reduction of glucosinolates in *B. napus* seeds (Kanstrup & Nour-Eldin 2022), suberin biosynthesis (Binenbaum et al. 2023; Robe & Barberon 2023), abiotic stress (Liu et al. 2023b) and biotic stress independent of nitrate transport (Wang et al. 2018).

1.4.3 Peroxins (Paper III)

The peroxisomal biogenesis PEROXIN (PEX) proteins include proteins involved in peroxisomal lumen import, membrane biogenesis, peroxisomal membrane protein import and proteins related to peroxisomal proliferation (Distel et al. 1996). The selected proteins related to Paper III are PEX3, PEX5, PEX7 PEX13 and PEX14. PEX3 is a membrane anchor for the chaperone PEX19 that helps proteins fold into the membrane. The cytosolic carrier proteins PEX5 and PEX7 aid in peroxisome maturity and bind proteins that encode conserved (but sometimes species-specific) peroxisomal targeting signals (PTSs) and transport them to the peroxisome (Pan et al. 2020; Deng et al. 2022). The transmembrane transporters PEX13 and PEX14 import the transporter-cargo complex to the lumen without the use of ATP, after which the carriers release their cargo and are ubiquitinated, exported and degraded (Barros-Barbosa et al. 2019). Mutations that result in simultaneous nonfunctional PEX5 and PEX7 proteins are plant lethal due to the importance of peroxisomes for multiple essential processes. The negative effects of a single transmembrane transporter may be only slightly deleterious, enabling mutational analysis (Woodward & Bartel 2005).

1.4.4 Aquaporins (Paper IV)

Aquaporins are channel proteins that facilitate the diffusion of water and other small uncharged molecules across lipid membranes. These genes are classified into five different subfamilies. Plasma membrane intrinsic proteins (PIPs) are an ancient protein family that regulates water homeostasis in cells and are found in most flowering plants, early mosses and green algae (Bienert et al. 2018). In addition to being found in the plasma membranes of cells, a few PIPs are also found in mitochondria and the chloroplast envelope. The PIP family of proteins is

multifunctional. Several proteins are involved in root growth, seed development, fruit development (Wang et al. 2020) and plant defence (as transporters of ROS) (Li et al. 2020a).

1.5 The war of nutrition: Transporters in plant–pathogen interactions

Nutrients can act as stress signalling molecules, and nutrient status is monitored by sensor proteins (Wang et al. 2018). Pathogen infection can cause numerous metabolic changes and the activation of nutrient transporters. One hypothesis that has been proposed regarding nutrient surveillance and defence is called impaired sugar signalling. In this model, alterations in sugar homeostasis are suggested to trigger SA-mediated defence activation in infected cells (Gebauer et al. 2017). Transcriptome profiling of *P. brassicae* infection revealed that transporters of nitrate-, sucrose-, lipid- and trehalose-related genes activate and transport nutrients towards infected cells (Irani et al. 2018; Walerowski et al. 2018). *S. sclerotiorum* in *B. napus* and *X. oryzae* in rice also enhance the transcription of sugar transporters (Jian et al. 2016; Cai et al. 2017). Upregulation of nutrient transporters results in susceptibility in these cases. Similarly, a nitrate transporter is transcriptionally upregulated only in susceptible plants after *Erysiphe necator* (powdery mildew) infection (Pike et al. 2014). This information has sparked the idea that certain susceptibility genes can be edited via genetic engineering to create a loss of susceptibility in crops (Gupta et al. 2021; Koseoglou et al. 2022). The gene editing of sugar transporter promoters has been performed to render effectors unable to bind and manipulate the rate of transcription. This results in resistance against bacterial blight caused by *X. oryzae* (Oliva et al. 2019; Xu et al. 2019b).

Pathogen starvation is a second nutrient-related hypothesis of disease resistance. Nutrient reallocation or disruption of nutrient supply is proposed to be a durable form of defence and a tactic already employed by plants (Oliva & Quibod 2017). Starvation can be accomplished by nutrient restriction (to store or reallocate nutrients away from the infected site), prevention of nutrient leakage, or impairment of pathogen-induced nutrient transport. In addition to sugars, other metabolic compounds in plants are important for pathogen growth and thus likely involved in the conflict of nutrient acquisition. One such example relates to amino acids. Amino acids, nitrates and peptides are metabolizable sources of nitrogen. Examples of pathogen/pest manipulation of amino acid transport and metabolism were reviewed by Sonawala et al. (2018). That review highlights increased pest susceptibility caused by amino acid transporter manipulation. Amino acid homeostasis seems to

be linked to pathogen-induced immune signalling, since deactivation or overexpression of such transporters is linked to constitutive expression of defence responses. Sonawala et al. (2018) proposed that amino acids be added to the list of nutrients for which nutrient surveillance systems monitor the steady state of metabolism (Fig. 5). A pathogen- or plant-induced shift in nutrient homeostasis, storage or flux across membranes can, in this model, induce defence responses. Part of this thesis touches upon this subject (mainly Paper II).

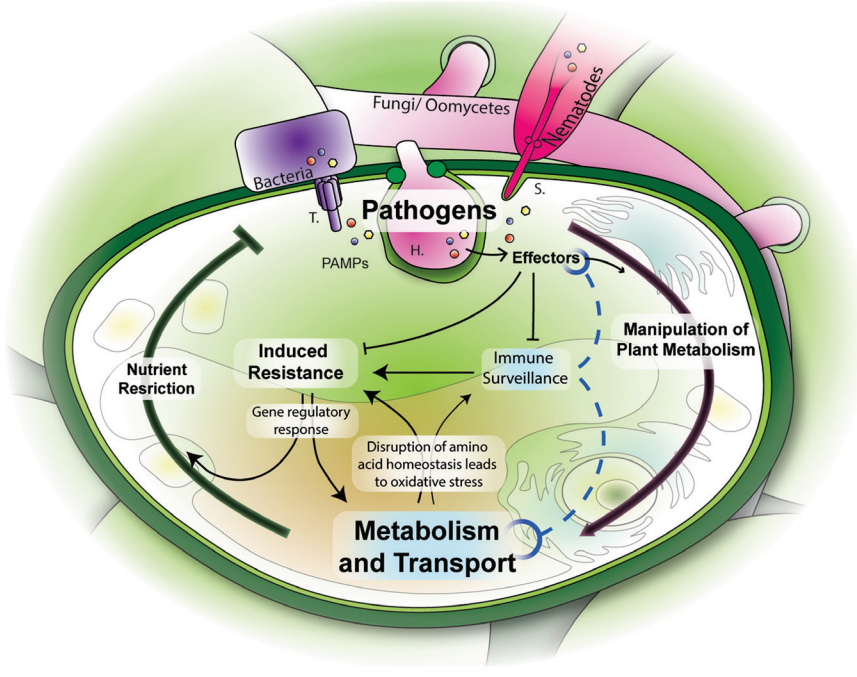


Figure 5. Model of the impaired nutrient signalling and nutrient starvation hypothesis. Pathogens excrete metabolism disturbing effectors (brown arrow). Either the pathogen, or a change in nutrient transport or homeostasis, is detected by the immune system or nutrient surveillance proteins (dashed blue line). This leads to nutrient restrictions (green arrow) and defence gene activation. In this specific case amino acids are proposed targets of pathogens and pests. Reprinted from Sonawala et al. (2010), with permission from Elsevier.

2. Aims of the study

The general aim of my work was to enhance our understanding of soilborne pathogens and their host interactions and to clarify the molecular interactions between hosts and disease-causing organisms. To accomplish this, I utilized two approaches, one from the perspective of different plant hosts and one from the perspective of the pathogens affecting them. The use of previously generated transcriptome and whole-genome sequencing data has been invaluable for the research underlying this thesis. My projects relate to effectors and defence-related genes during interactions between *B. napus* and *V. longisporum*, *B. vulgaris* and *R. solani* and Arabidopsis and *P. brassicae*. Arabidopsis was used as an alternative host for all three pathogens due to the relative ease of handling and genetic engineering and for comparison of the plant responses with those of the model plant.

The specific goals were as follows:

- Investigate the roles of differentially expressed genes in *B. vulgaris* genotypes with contrasting responses to *R. solani*
- Explore the secretome of *R. solani* to identify effectors and their subcellular targets
- Characterize defence genes in *B. napus* against *V. longisporum*
- Identify organelle-targeting *P. brassicae* effectors

3. Results and Discussion

The results obtained during my PhD candidacy are discussed in detail in the accompanying research articles and manuscripts. The purpose here is to summarize the results and to synthesize further results.

3.1 *R. solani* effector and *B. vulgaris* defence genes

Two main challenges had to be addressed while working with *B. vulgaris* and *R. solani*. (1) Plant inoculation with *R. solani* is challenging to reproduce in a controlled and uniform manner due to the lack of spore structures. This complicates the detection of plant differential responses to infection. To this end, a collaborative effort was made to develop a protocol for reproducible inoculation, which was used in research on the plant defence responses of *B. vulgaris* to *R. solani* as well as for identifying fungal effectors (Dölfors et al., 2022). Gene editing is a useful tool for studying gene function, but (2) both *R. solani* and *B. vulgaris* are recalcitrant to genetic manipulation. Although the genus *Rhizoctonia* contains uni- and binucleate species, *R. solani* AG2-2 cells are multinucleate (Li et al. 2021). Stable genetic transformation of multinuclear cells is not easy. Several genome editing challenges remain for both *B. vulgaris* and *R. solani* (Rafiei et al. 2023a). For this reason, we used *C. beticola*, a foliar pathogen of *B. vulgaris*, and Arabidopsis, an alternative *R. solani* host, to drive heterologous overexpression of our effector and defence gene candidates from *R. solani*/*B. vulgaris* system.

3.1.1 MLP-dependent resistance to *R. solani* (Paper I)

We had the opportunity to compare the transcriptomes of four *B. vulgaris* breeding lines with contrasting responses to *R. solani* AG2-2IIIB at zero, two and five dpi. Genotypes one and two displayed partial resistance ($G1^R$ and $G2^R$), and three and four displayed partial susceptibility ($G3^S$ and $G4^S$). Three defence-associated R genes on chromosome 3 with the NBS-LRR domain were transcriptionally

upregulated at two dpi. These genes were not considered candidates because previous QTL mapping has shown that R-genes in this region are not associated with *R. solani* resistance (Lein et al. 2008) and that R-genes are associated with necrotrophic susceptibility rather than resistance in several cases (Hammond-Kosack & Rudd 2008; Shao et al. 2021; Kanyuka et al. 2022). Several biotic stress scenarios, including resistance against aggressive *B. cinerea* (Gai et al. 2018), *Colletotrichum acutatum* and *C. gloeosporioides* (Adhikari et al. 2021), have implicated MLPs. Three MLP-encoding genes with differential expression upon *R. solani* challenge were found in the gene ontology group for responses to biotic stress (Paper I). Transcriptional upregulation of *BvMLP1* and *BvMLP3* in the roots of *B. vulgaris* $G1^R$ and $G2^R$ plants was verified by RT–qPCR in a follow-up experiment (Paper I, Fig. 3). These genes were subsequently transferred to Arabidopsis and subjected to disease screening and comparative DNA analysis. BvMLP protein expression in transgenic Arabidopsis plants had a slight growth-promoting effect. This difference was considered to not impact the screening results since developmental resistance to *R. solani* AG2-2 is related to plant age rather than seedling size (Liu et al. 2019). Orthologous *Atmlp1/Atmlp3* double mutants presented a marked increase in the amount of *R. solani* DNA. The *BvMLP1*- and *BvMLP3*-expressing Arabidopsis lines had significantly lower amounts of *R. solani* DNA than did the wild-type, as well as less pronounced growth reduction, chlorosis, and necrosis in the rosette (Paper I, Fig. 4, Fig. 5). These results imply that MLPs are involved in defence against *R. solani* and act within five days of attack. However, functional data on the MLP family in *B. vulgaris* are scarce, and the mechanism of action of BvMLP1 and BvMLP3 has not been characterized. Defence is likely polygenic and involves several defence pathways, such as the JA/ET and PENETRATION2 pathways, which provide Arabidopsis with resistance to *R. solani* AG8 foliar infection (Kidd et al. 2021).

3.1.2 MLPs may induce defence responses

Perhaps one of the more well-characterized MLPs, cotton GhMLP28 binds to an AP2/ERF, ETHYLENE RESPONSIVE FACTOR 6 (GhERF6). The interaction enhances the ERF binding activity and positively regulates the activation of the defence genes *PDF1.2* and a thaumatin-like, antifungal *PR5* in response to *V. dahliae* (Yang et al. 2015). AtMLP3 is the closest relative to AtMLP28 (Section 3.3.1). It is tempting to speculate that these highly similar proteins share functional homology. In particular, both an AP2/ERF transcription factor (Bv5g119300) and the chitinase *GLYCOSIDE HYDROLASE 19* (Bv8g193950) seem to be expressed with *BvMLP*s in resistant *B. vulgaris* genotypes at 2 dpi. The transcription of three glucosyltransferases related to cell wall biosynthesis was also strongly

transcriptionally upregulated together with the *MLP*s at 5 dpi in the resistant genotypes, which may or may not be related to MLP function (Paper I). *AtMLP1* and *AtMLP3* both have a distant GCC-box, an AP2/ERF binding site associated with pathogen stress (Müller & Munné-Bosch 2015), -4003 nucleotides and -1684 nucleotides upstream of the transcription start codon, respectively (unpublished). I hypothesize that JA/ET signalling upregulate AP2/ERF (Pré et al. 2008), which in turn positively influences *MLP1* and *MLP3* transcriptional activity in resistant genotypes. The MLPs may then negatively influence fungal growth by an unknown antifungal- or plant cell strengthening- activity. Perhaps MLPs induce the previously mentioned chitinase to enzymatically break down the cell wall of *R. solani* hyphae or enhance cell wall biosynthesis. Both proposed mechanisms of action would explain the decreased amount of *R. solani* DNA in the MLP-overexpressing lines. Alternatively, MLP1 and MLP3 may bind directly to the AP2/ERF protein and influence its GCC-box binding activity, thereby influencing their own transcription in a positive feedback loop and mediating expression of other defence proteins with a GCC-box, such as chitinase-encoding PR genes or PDF1.2 (Brown et al. 2003). Protein interaction assays, such as co-IP, mass spectrum analysis, yeast two-hybrid assays and BiFC analysis, could be used to investigate possible protein interactions. Transcriptional analysis of hormone response and defence gene mutants could subsequently reveal the order of signal transduction in the proposed JA/ET, AP2/ERF, MLP, and defence gene pathway. Currently, the regulatory mechanism of these MLPs is unknown and is briefly discussed in Section 3.3.2.

3.1.3 *R. solani* targets chloroplasts and mitochondria (Paper IV)

The secretome of *R. solani* AG2-2IIIB was used to identify several effector candidates (Wibberg et al. 2016). The 63-amino acid-long CYSTEIN RICH PROTEIN 1 (RsCRP1) was chosen for further analysis due to its small size and cysteine content. The relative transcript accumulation of RsCRP1 was increased 12-fold in *R. solani* mycelia at four dpi in *B. vulgaris* roots (Paper IV, Fig. 1). Wild-type and RsCRP1-augmented *C. beticola*$^{RsCRP+}$ spores were then drop inoculated on *B. vulgaris* leaves to investigate virulence changes due to the added effector candidate. Disease symptoms developed further in *C. beticola*$^{RsCRP+}$-treated plants, but there was only a marginal increase in fungal DNA in the leaves (Paper IV, Fig. 2). Another small cysteine-rich protein (RsSCR10) identified in AG1-1A is capable of inducing HR (Niu et al. 2021). We tested whether RsCRP1 has a similar function. Transgenic *N. benthamiana* plants expressing the tomato PRR Cf-4 were agroinfiltrated with the chitin-binding protein AVR4 from *Cladosporium fulvum.* The Cf-4/AVR interaction induces a strong HR. AVR4 co-agroinfiltration with

RsCRP1 had no HR suppressive effect, and infiltration of RsCRP1 alone did not cause necrosis (Paper IV, Fig. 3). The cellular localization of RsCRP1 was predicted by ProtComp 9.0 (softberry.com) to occur within peroxisomes, mitochondria, and chloroplasts. Amino acid analysis revealed a chloroplastic transit peptide at the N-terminus, further indicating chloroplastic localization. Transient expression of GFP-tagged RsCRP1 confirmed the presence of RsCRP1 in the chloroplasts and mitochondria of *N. benthamiana* leaves (Paper IV, Fig. 4).

3.1.4 The sugar beet BvPIP;1 membrane protein (unpublished)

To gain further insight into the small cysteine-rich effector gene from *R. solani*, RcCRP1 (Paper IV) was pulled down from *C. beticola*$^{RsCRP+}$-infected sugar beets, followed by MALDI–MS/MS analysis. Five interacting candidates, two ATP synthases, a peroxidase, a chlorophyll-binding protein and the PLASMA MEMBRANE INTRINSIC PROTEIN 1;1 (PIP1;1), were identified among the protein sequences. According to our RNA-seq dataset, *BvPIP1;1* was differentially regulated particularly five days after *R. solani* infection. The Arabidopsis BvPIP1;1 knockout homologues *pip1;2-1*, *pip1;3-1* and *pip1;4-1* did not show any significant differences in the amount of *R. solani* DNA compared with that in Col-0 when grown in infested soil. When the phenotypes were monitored at 4 dpi, the *pip1;3-1* knockout line tended to be more infected than was the Col-0 line. In sugar beet, *PIP1;1* and *PIP2;1* coexpression influences the pH regulatory response and enhances membrane plasticity (Bellati et al. 2010). Such changes not only impact water transfer capacity but also may facilitate the entry of effectors and/or pathogens such as *R. solani*. *AtPIP1;4*, the closest Arabidopsis homologue to *BvPIP1;1*, does not have any known substrate specificity and is not affected by drought stress (Alexandersson et al. 2005). A link to hydrogen peroxide and PTI pathways was previously suggested based on work on *P. syringae* and PIP1;4 in Arabidopsis (Tian et al. 2016). In wheat, TaPIP2;10 affects the transport of hydrogen peroxide, a function promoted by *Blumeria graminis* and *Fusarium graminearum*. Overexpression of *TaPIP2;10* significantly increased resistance to powdery mildew and Fusarium head blight through H_2O_2-induced defence (Wang et al. 2021). A link between H_2O_2 and PIPs in the *R. solani*-sugar beet system has yet to be demonstrated. It is also possible that the true target of RsCRP1 is the chlorophyll a/b binding candidate protein identified. These proteins are involved in the light harvesting complex in the chloroplast photosystem II (Xu et al. 2012). As previously mentioned, other pathogens target such proteins in photosystems I and II, which induces ROS production (Manning et al. 2009). However, these findings cannot explain the mitochondrial localization of RsCRP1.

The molecular function of RsCRP1 is unknown. We believe that targeting chloroplasts and mitochondria may affect cell function enough to give the pathogen an advantage over host defences (faster disease symptom development). It is also possible that RsCRP1 works as a complement to other effectors with similar functions. This protein may affect the mitochondrial and chloroplastic electron transport chains to either cause or inhibit oxygen species production. One can argue for both scenarios. Chloroplast and ROS metabolism are targets for at least two other *R. solani* subgroups. AG1-1A alters chloroplast integrity to increase ROS production and induce cell death in infected rice cells (Ghosh et al. 2017). Suppressing chlorophyll degradation reduces virulence (Cao et al. 2022). Two genes in AG8, *RsAG-8G_06411* and *RsAG-8G_03280*, have been linked to enhanced ROS production and correlated with increased virulence in wheat (Foley et al. 2016). The stress signal that promotes ROS propagation destabilizes the cell by altering the redox state. ROS signalling may also activate NADPH oxidases in the apoplast, thereby enhancing the stress response in neighbouring cells (Zurbriggen et al. 2010). This may explain the larger necrotic lesions of RsCRP1+ plants, despite the amount of fungal DNA not being elevated in these plants compared to that in the wild-type. Disease symptoms develop ahead of hyphal advancement.

In the second scenario, evidence of ROS, PTI and ETI inhibiting *R. solani* effectors accumulates. The lysin motif protein RsLysM, rare lipoprotein A (RsRlpa), a glycosyltransferase (RsIA_GT) and RsIA_CtaG/COX11 all suppress immune responses (Dölfors et al. 2019; Charova et al. 2020; Zhang et al. 2022, 2023a). Dampening of mitochondrial ROS signalling is a strategy proposed for use by *R. solani* AG-1A (Zhang et al. 2023a). However, whether an increase in defence-related ROS-signalling and programmed cell death or a suppression of defences is more important for the *R. solani* infection process is unclear. Perhaps both alternatives are present, but could be regulated at the transcript/translational level depending on the infection stage. Invasive hyphae may use a different set of effectors than feeding hyphae. Regardless, the regulation of ROS metabolism and the immune response functions of chloroplasts and mitochondria seem to play major roles in successful *R. solani* establishment.

3.2 B. napus defence against *V. longisporum*

As a root-infecting pathogen, *V. longisporum* is not strongly affected by fungicides applied to topsoil or on the plant surface. Although well characterized histologically (Eynck et al. 2007), the molecular events involved in the infection process are relatively unknown. Few resistance genes have been found (Su et al. 2023) and

known fungal effector proteins are rare (Rafiei et al. 2022, 2023b). Instead, identification of quantitative trait loci and disease resistance breeding are the main strategies for reducing disease risk in oilseed rape production (Depotter et al. 2016; Gabur et al. 2020). Varying levels of disease resistance can be found by screening genetically contrasting individuals followed by introgression to commercial cultivars. Resistance to *V. longisporum* has been proposed to occur differently in the early (Behrens et al. 2019) and late (Eynck et al. 2009) stages of infection. To make large-scale screening viable, differential responses to infection need to be detected during the early stages. One way to detect such responses is to measure the accumulation of defence hormone-responsive gene transcripts. However, all host species do not exhibit similar hormonal responses to infection. The same is true for *V. longisporum* and *B. napus*/Arabidopsis (see Section 1.1.1). Another way of detecting differential responses to pathogenic challenge is to quantify the relative amount of *V. longisporum* DNA in the host. At species-adjusted inoculum pressures, *V. longisporum* requires approximately fourteen days of growth in Arabidopsis to reach similar quantities as those found in *B. napus* seven days after inoculation (Fig. 6). Molecular quantification of DNA enables direct comparisons of plants while shortening screening time considerably compared to relying on disease phenotype. A fast and robust screening method developed by Jambagi and Dixelius (2023) for *Trifolium pratense* was adapted and used in disease screening of *B. napus* and *Arabidopsis* in response to *V. longisporum* in this section.

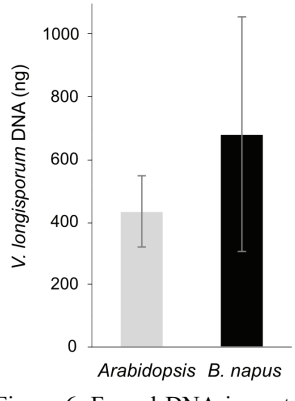


Figure 6. Fungal DNA in roots of hydroponically grown Arabidopsis (fourteen dpi) and *B. napus* (seven dpi). Inoculum pressure was $4x10^4$ and $4x10^5$ respectively. Bars represent the mean fungal DNA content in nanogram 100 mg^{-1} root material of three biological replicates ($n \geq 15$ roots each). Not statistically different (Student's t test; $P = 0.3$).

3.2.1 NPF5.12 and MLP6 contribute to *V. longisporum* defence in Arabidopsis and *B. napus*. (Paper II)

V. longisporum intrusion triggers NPF5.12 and MLP6 reprogramming

The NPF5.12 protein is one of sixteen NPF members in Arabidopsis that functions in nitrate transport across cell membranes. This transmembrane nitrate efflux transporter is located in the tonoplast (He et al. 2017) and the plasma membrane (Paper II). Differential transcriptional activity of *AtNPF5.12* was found in the roots of *V. longisporum*-susceptible plants (Can-0) compared to those of resistant plants (Sei-0) (Paper II, Fig. 1). Transcriptional downregulation of *NPF* genes upon pathogen challenge has also been documented in maize (Xia et al. 2023). As *V. longisporum* infects a susceptible plant root, transcript accumulation is attenuated for *AtNPF5.12* and its interacting partner *AtMLP6* at two dpi. *Atnpf5.12* and/or *Atmlp6* knockout mutations further increased susceptibility compared to that of the wild-type (Paper II, Fig. 3). This hints at a mechanism of defence that is activated by the change in transcription activity of these two genes and that plants lacking functional copies of the genes become more susceptible. Importantly, overexpressing lines reverted the phenotype back to the wild-type state (Paper II, Fig. 1, Fig. 3).

Orthologous B. napus mutants are susceptible to V. longisporum

To test whether the results in Arabidopsis could translate to an economically important crop grown in Sweden, I screened the responses of *B. napus*. CRISPR/Cas9 technology was applied to mutate the *BnNPF5.12* and *BnMLP6* orthologous genes. Four and fourteen proteins with very high sequence similarity to AtNPF5.12 and AtMLP6 exist in the genome of *B. napus* (Paper II). Single guide RNAs were designed to target all the *BnNPF5.12* and as many *BnMLP6* copies as possible to remove functional redundancy effects. Even though hormone signalling events differ between *Arabidopsis* and *B. napus*, the functions of NPF and MLP6 in defence may be the same in both systems. The defence of the *Bnnpf5.12* and *Bnmlp6* mutants was compromised, as revealed by relative DNA analysis. The quadruple *Bnnpf5.12*/fourteenfold *Bnmlp6* mutants contained 8.7-fold more fungal DNA than the wild-type (Paper II, Fig. 4).

3.2.2 The regulation of NPF5.12 and MLP6 is possibly JA/ET mediated in Arabidopsis (unpublished)

NPF7.2 and *NPF7.3* are suggested to be regulated by JA/ET signalling (Zhang et al. 2014). Similarly, the promoter regions of Arabidopsis *AtMLP6* and *AtNPF5.12* contain AAAG and ACGT cis-regulatory elements, which are involved in the transcriptional downregulation of JA-responsive genes (Khan et al. 2022). This

could help explain how the transcription of both *AtMLP6* and *AtNPF5.12* is downregulated in Arabidopsis during *V. longisporum* infection (Paper II). It is not known whether JA is responsible for attenuating the transcription of these genes or whether it is a host response to infection or a result of *V. longisporum* hormonal manipulation to avoid detection. In the case of *MLP6*, it is currently unknown if mobile mRNA also affects mRNA measurements.

3.2.3 Disruption of nitrate transport may trigger the MLP6 response

MLP328 (AT2G01520) has a domain structure almost identical to that of MLP6 (Section 3.3.1), and MLP328 mRNA move in a root to shoot direction during nitrate deficiency (Thieme et al. 2015). Similarly, mobile *AtMLP6* mRNA from Arabidopsis was found in the phloem sap of a phloem feeding parasite (*Cuscuta reflexa*) (Thieme et al. 2015). Analysis of root-specific transcript levels could be obscured by this RNA transportation mechanism since the rate of transcription is not measured by RT–qPCR, only transcript accumulation or abundance. Interpretation may lead to an oversimplified conclusion. The mitigated MLP6 transcriptional response in Arabidopsis roots upon inoculation with *V. longisporum* (Paper II, Fig. 3) could be an effect of root transcripts becoming mobile, resulting in less root mRNA being detected. One way to obtain more information on this topic would be to simultaneously measure *MLP6* transcript accumulation in both roots (senders) and shoots (receivers) at selected time points before and after inoculation. The AtMLP6 protein is highly enriched in the phloem during the response to *P. syringae*, as is the hydrophobic SAR signal dehydroabietinal and the antifungal PR1 protein. An *mlp6* mutant displayed a SAR defective phenotype supporting a role for MLP6 in long distance defence signalling (Carella et al. 2016). Taken together, these findings indicate that MLP6 function may be triggered by the transcriptional downregulation of the MLP6 interacting nitrate transporter NPF5.12 or the changes in cellular nitrate homeostasis during *V. longisporum* infection, and that MLP6 could migrate systemically, probably to regulate distant defence responses.

3.2.4 *NPF5.12* limits nitrate availability, which is essential for *V. longisporum* (Paper II)

Although the nitrate transport system is extensive and at least seven NPFs have nitrate efflux functions (Lu et al. 2022), we speculated that transcriptional downregulation of *NPF5.12* could be a metabolic starvation tactic to sequester nitrates from invading hyphae and limit the growth of *V. longisporum* in susceptible plants. Nitrate depletion experiments showed that *V. longisporum* is indeed dependent on host nitrate transport for proper establishment in the host (Paper II. Depleting nitrates had a large negative effect on the growth of *V. longisporum*. In

the *Atnpf5.12* mutant, which was already compromised in nitrate efflux transport, nitrate depletion did not have an equal effect on the amount of *V. longisporum* DNA (Paper II). This could mean that *Atnpf5.12* already limited the nitrate supply to the fungus in nitrogen-rich samples and that NPF5.12 has an intrinsic function to limit *V. longisporum* growth, as opposed to being monitored only by the MLP6 defence protein. Unaltered NPF5.12 nitrate efflux to the cytoplasm and apoplast would supply nitrates directly to hyphae. Theoretically, decreased mRNA accumulation could lead to a decrease in active NPF5.12 proteins, and a net flux of nitrates into storage could occur due to cytoplasmic (NPF2.9/NPF7.2) and vacuolar (CLCa/NRT2.7) influx (Raddatz et al. 2020). The activity of other nitrate transporters needs to be investigated during *V. longisporum* infection to determine anything conclusive. I hypothesize that, according to the starvation/impaired signalling hypotheses, the *Atnpf5.12* knockout mutant is more susceptible to *V. longisporum* than is the wild-type Col-0 strain under normal growth conditions because there is no change in the cellular steady state of nitrate availability or because physical interaction with MLP6 simply does not take place in the mutant. Therefore, MLP6 defence signalling is not activated by a change in NPF5.12 transcription. Thus, both NPF5.12 and MLP6 could have individual and distinct functions to limit the spread of the pathogen in susceptible plants.

3.2.5 *V. longisporum* inhibits endodermal barrier synthesis (Paper II)

V. longisporum infection has, by an unknown mechanism, an inhibitory effect on suberin deposition, which enables entry into the vasculature (Paper II, Fig. 5; Fröschel et al. (2021)). Very recently, NPFs were found to be involved in suberin deposition. NPF2.12 and NPF2.13 regulate the shoot-to-root flow of GA into phloem companion cells of the roots. NPF2.14 loads GA into the vacuole for storage. NPF3.1 then transports GA and possibly ABA to initiate suberin deposition outside the plasma membrane of the endodermis (Binenbaum et al. 2023; Robe & Barberon 2023). ABA and GA signalling are crucial for suberin deposition, and *V. longisporum* infection attenuates ABA biosynthesis in *B. napus*, which could contribute to less suberin being deposited (Behrens et al. 2019). However, in Arabidopsis, the levels of ABA increase during the early stages of infection (Roos et al. 2015), and Arabidopsis plants exhibit the same suberin-defective phenotype (Paper II). Part of the answer to how suberin-related processes are affected by *V. longisporum* infection may be attributed to the transcriptional downregulation of NPF5.12 and MLP6 in infected plants. NPF5.12 has been found to transport IBA (an IAA precursor) (Michniewicz et al. 2019) and IAA regulates several members of the GDSL-type Esterase/Lipase Protein family which are important for suberin polymerization and degradation (Shukla & Barberon 2021). Suberin was stained

with a fluorescent dye and quantified in the roots of mock-treated and *V. longisporum*-infected *npf5.12* and *mlp6* lines of *B. napus* and *Arabidopsis*. In both species, the mock-treated mutants had significantly less suberin signal in the endodermis compared to wild-type, indicating that NPF5.12 and MLP6 contribute to the suberin barrier during normal growth (Paper II, Fig. 5). As the plant switches to defence mode and *NPF5.12* and *MLP6* activity is attenuated, the suberization process is partly interrupted, leaving entry points for *V. longisporum.* The state of the endodermal suberin layer should be weakened in the whole root of the *npf5.12* and *mlp6* mutants compared to that of roots at two days after inoculation, when the genes are transcriptionally downregulated in the wild-type. This could also explain the greater fungal susceptibility of these mutants.

3.2.6 Summary of molecular events during *V. longisporum* infection

The results of this thesis and the related literature make it possible to hypothesize on an expanded model for *V. longisporum* infection. Root monoterpene exudates in the rhizosphere trigger the germination of *V. longisporum* microsclerotia (Roos et al. 2015). Hyphae enter the root through root hairs (Eynck et al. 2007) or at lateral root initiation sites by direct penetration (Paper II, Fig. 4). Transcriptome reprogramming occurs in the host, resulting in the production of secondary metabolites with antimicrobial activity, such as camalexin (Iven et al. 2012). Nitrate metabolism is altered in susceptible plants two days after the infection process is initiated. This process may constitute to a type of indirect metabolic defence to stop the growth of the pathogen. NPF7.3 (AT1G32450) is upregulated 17-fold in the susceptible *Atndr1* mutant (Roos et al. 2015), which results in root-to-shoot reallocation of nitrate (Lin et al. 2008). Transcriptional downregulation of *NPF5.12* causes nitrates to accumulate inside the central vacuole of root cells. Together, these proteins limit the amounts of nitrates that *V. longisporum* has access to in the early stages of infection, thereby further limiting its growth (Paper II, Fig. 4). Moreover, the cytoplasmic NPF5.12-interacting protein MLP6 senses changes in nitrate homeostasis and relocates towards the apex of the shoot via the phloem, presumably to regulate distant resistance responses. However, whether the local or distant function of MLP limits the growth of *V. longisporum* is unknown. In the early stages of infection, ABA biosynthesis genes are repressed in *B. napus* as *V. longisporum* penetrates the cortical layers. Increased SA (PR proteins) and decreased JA/ET responses (*ETHYLENE RESPONSE 2*, PDF1.2) are also observed (Ralhan et al. 2012; Behrens et al. 2019; Zheng et al. 2019a) whereas ABA and JA/ET signalling remain unaffected in Arabidopsis. *V. longisporum* grows vertically along the endoderm until an access point is found in the vascular system. Suberin deposition was already negatively affected by the initial downregulation of ABA in *B. napus*.

Transcriptional downregulation of *NPF5.12* and *MLP6*, and *V. longisporum* further weaken this barrier in both *B. napus* and Arabidopsis through an unknown mechanism (Paper II, Fig. 5). It is currently believed that *V. longisporum* hyphae and conidia enter the xylem and travel systemically. At this point during the infection process (14-21 dpi), early-acting SA-mediated defences are switched to late-acting defence signalling. This includes the enhanced transcription of enzymes involved in cell wall lignification (Floerl et al. 2012), phenolic compounds (Zheng et al. 2019a) and vascular occlusion substances (gums, gels and tylose depositions in xylem) (Eynck et al. 2009) to limit vertical advancement of *V. longisporum*. Vascular growth of the pathogen blocks water flow (Kashyap et al. 2020). Susceptible plants start to exhibit symptoms of stunting, chlorosis and leaf abscission (Zou et al. 2020). At the end of the growth season, hardy microsclerotia protrude from the stems of heavily infected plants, hereby making its presence known.

3.3 Major latex proteins in Arabidopsis

Several MLPs have been identified from my PhD work, including sugar beet BvMLP1 and BvMLP3 in response to *R. solani* (Paper I) and oilseed rape MLP6 in response to *V. longisporum* (Paper II). This section further synthesizes the results to better understand the functions of MLPs. For this purpose, an overview of the relationships among the MLP family members was created, and hormone responses were estimated. Possible transcriptional regulation was speculated based on the promoter sequences of the selected proteins.

3.3.1 A homology search revealed three novel MLP-like proteins in Arabidopsis (unpublished)

Previously, twenty-six *AtMLP* genes divided into three groups were identified through extensive sequence analysis of the Arabidopsis genome (Cannon & Young 2003; Cannon et al. 2004; Song et al. 2005; Radauer et al. 2008; Lytle et al. 2009; Fernandes et al. 2013; Zhang et al. 2018; Li et al. 2023b; Sun et al. 2023). Using the Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST), it is possible to identify distant relationships between proteins. Weakly similar amino acid sequences that could still have biological relevance (e.g., shared motifs) can be retrieved using this algorithm (Altschul et al. 1997). Mining the NCBI RefSeq protein database using AtMLP6 (NP_194098) as a query revealed three new, sequence-divergent MLP-like candidate genes (At1g70860, At1g24010 and At1g23910) in the Arabidopsis genome.

Multifunctionality of MLPs (unpublished)

Many *MLP*s have one gene with a similar sequence located next to it in the genome. These are likely paralogues from tandem duplication events or segmental duplications retained from a polyploidy event (Cannon et al. 2004). Multiple nonsynonymous changes have occurred among duplicated genes since the split, as visualized in Fig. 7.

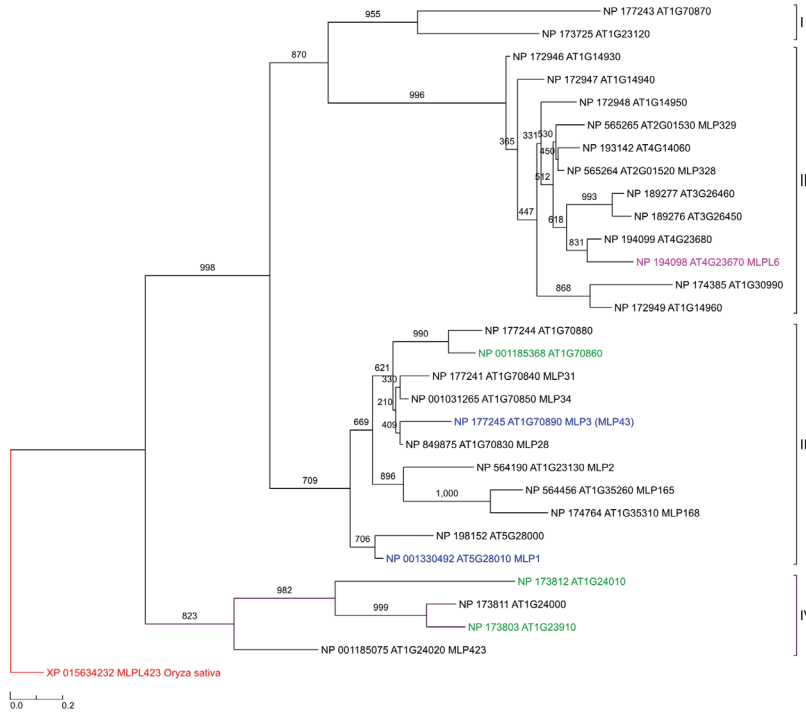


Figure 7. Phylogenetic maximum likelihood estimation of the Arabidopsis MLP family. Proteins highlighted in green, blue and magenta are novel MLP-like candidates and defence-related proteins against *R. solani* (Paper I) and *V. longisporum* (Paper II). The branch lengths are proportional to the number of amino acid substitutions/site. Nonconserved sequences were trimmed with TrimAI (Capella-Gutiérrez et al. 2009). PhyML 3.0 (Guindon et al. 2010) estimated relationships were visualized using TreeGraph (Stöver & Müller 2010).

Two of the new MLP-like proteins make up a new, divergent subgroup (IV) together with At1g24000, another MLP-like protein with unknown function. The more distantly related MLP423 in group IV is involved in multiple responses to biotic stress, including enhancing susceptibility and resistance to different pathogens (Rubio et al. 2015; He et al. 2020; Zhang et al. 2023b). No annotations exist for the

newly discovered MLP-like protein At1g70860 in Group III. However, the group does contain known multifunctional proteins, including MLP3 (MLP43), which is involved in the response to *R. solani* in Arabidopsis and sugar beet (Paper I) and is also a regulator of drought responses (Wang et al. 2016). MLP3 may also have a role in growth promotion in the vegetative phase (Paper I). The transcript accumulation of both MLP34 and MLP28 is negatively regulated upon *P. brassicae* infection in Arabidopsis (Irani et al. 2018). MLP28 is also involved in ABA-regulated leaf development (Litholdo et al. 2016) and resistance to *V. dahliae* in cotton (Yang et al. 2015). Group II contains the *V. longisporum* defence-related MLP6, which is also activated in response to gravity stimulation (Kimbrough et al. 2004; Lytle et al. 2009). The closest relative of MLP6, At4g23680, is strongly induced during drought stress (Zhou et al. 2013). The more distantly MLP6-related gene At3g26460 in group II is coexpressed with NPF5.12 (Genevestigator) and, together with *AtMLP328*, is transcriptionally downregulated by *P. brassicae* infection (Irani et al. 2018). MLP329 is a positive regulator of seed dormancy (Chong et al. 2022) and iron homeostasis (Xing et al. 2015; Martín-Barranco et al. 2020), and both AtMLP328 and AtMLP329 are involved in vegetative growth promotion (Guo et al. 2011). As more functional information is generated regarding MLPs, more examples of individual MLP multifunctionality will emerge.

Atypical BetV1 structures of MLP-like candidates (unpublished)

The common motifs of the MLP family were identified to help determine whether the novel candidates shared MLP-like features. The AtMLP BetV1 domain consists of short stretches of conserved residues interspersed with variable sites (Fig. 8). The conserved sites form eight β-sheets and three α-helixes that are held together by nine less-conserved loops. Together, these proteins form a hydrophobic cavity with a ligand binding site and a glycine-rich loop, which is similar to that of PR10 (Lytle et al. 2009; Fernandes et al. 2013).

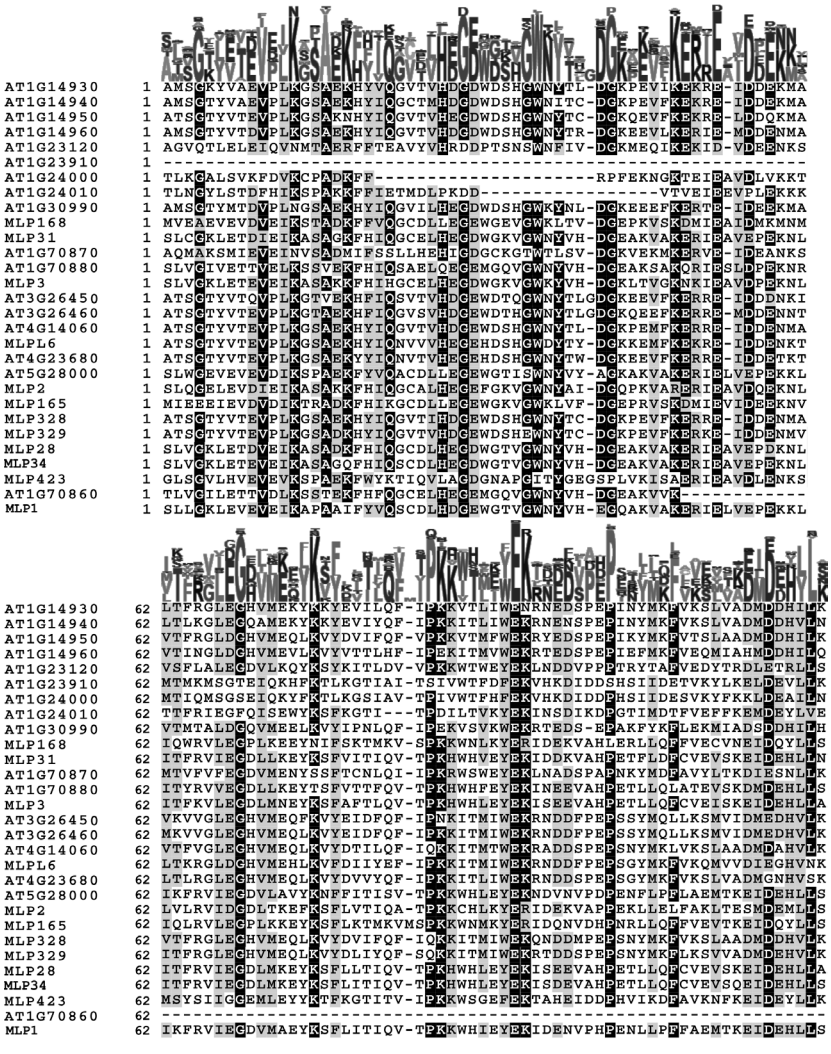


Figure 8. Sequence similarity of MLP proteins in Arabidopsis. Amino acids are shaded if > 70% of the sequences are identical. Multiple sequence alignment (MAFFT) with nonconserved regions trimmed (TrimAI).

Eleven motifs were found in the twenty-nine MLP sequences. Most MLP proteins share the motif structure of AtMLP1 and AtMLP3 (Fig. 9). The novel genes At1g70860, At1g24010 and At1g23910 are truncated versions of the BetV1 domain but show high sequence similarity to MLPs and contain conserved MLP-like motifs, thus confirming the presence of MLP-like features (Fig. 8, Fig. 9). The protein putatively encoded by At1g70860 consists of two typical MLP motifs, its secondary

structure includes two β-sheets and α-helixes, and its C-terminus contains the PR10-like loop. However, whether these proteins are functional is currently unknown.

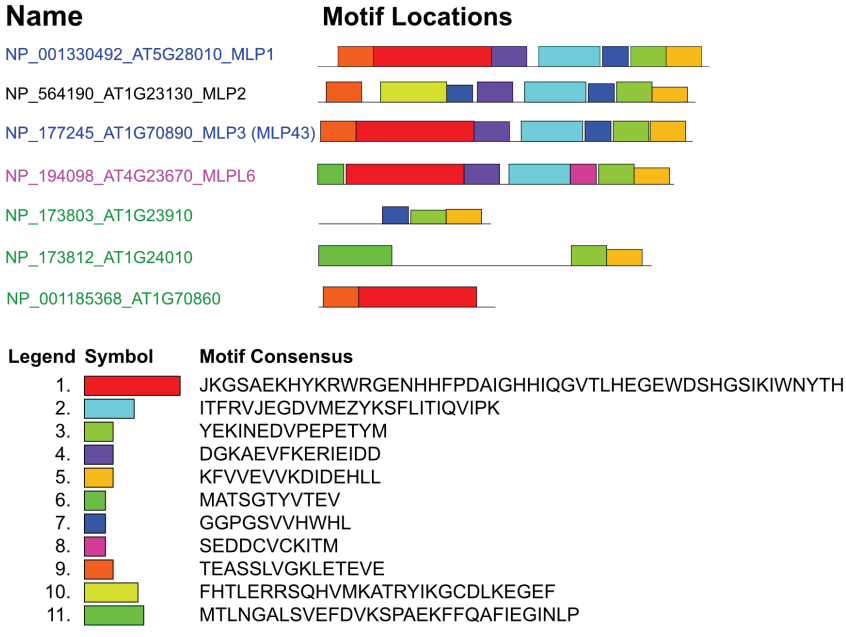


Figure 9. Conserved motifs in the Arabidopsis MLP-like protein family. Proteins with names highlighted in green, blue, and magenta are novel MLP-like candidates and defence related proteins against *R. solani* (Paper I) and the response to *V. longisporum* (Paper II).

3.3.2 Regulatory elements of *MLP* genes (unpublished)

Regulatory elements can often be found in the proximal (-1 kb) upstream region of a gene transcription start site but can regulate gene activity hundreds of thousands of bases away (Hernandez-Garcia & Finer 2014; Ricci et al. 2019). I investigated the occurrence of regulatory elements in the upstream regions of *B. vulgaris* and Arabidopsis *MLP* transcription start sites. I also investigated genetic variation in the first thousand nucleotides of the promoter regions of *BvMLP1* and *BvMLP3*. For this purpose, I used the sequenced promoter regions of the previously mentioned resistant ($G1^R$) and susceptible ($G3^S$) genotypes for *BvMLP*s and the Arabidopsis Genome Browser (TAIR) for *AtMLP*s.

ABA- and JA/ET-responsive factors may fine-tune MLP stress responses (unpublished)

The expression of several known MLPs is regulated by SA, JA and ET, and these genes contain cis-regulatory elements in their promoters (Fujita & Inui 2021). Except for the already mentioned ET-responsive element found upstream of *AtMLP1* and *AtMLP3*, Arabidopsis and *B. vulgaris*, *MLP1, MLP3,* and *AtMLP6* contain several W-box elements. WRKY75 binds to the core W-box motif (TTGAC) and is a positive regulator of JA/ET-mediated defence against fungi such as *B. cinerea* and *Alternaria brassicicola* in Arabidopsis (Du et al. 2017; Chen et al. 2021). When the *BvMLP1* promoter region was compared between the resistant $G1^R$ and $G3^S$, it became apparent that $G3^S$ had a 254 bp indel upstream of the transcription start codon. In this region, $G1^R$ contains additional core WRKY W-boxes. The greater number of potential binding sites could be one reason for the heightened activation of *BvMLP1* in resistant genotypes during *R. solani* infection.

Like those of *NPF5.12* and *MLP6*, the upstream region of *BvMLP3* contains the cis-responsive elements ACGT and AAAG (Khan et al. 2022) separated by 48 nucleotides. These elements are recognized by members of the SA- and ABA-responsive DNA-binding with one finger (DOF) transcription factor family and the SA-, ABA-, and JA-responsive basic region/leucine zipper (bZip) transcription factors. Both of these families have members that are involved in defence responses to both biotic and abiotic stress (Jakoby et al. 2002; Zou & Sun 2023). *AtMLP1, AtMLP3 and BvMLP3* also contain an ABA/JA crosstalk G-box (CACGTG), which is represented in the promoters of JA responsive genes (Fernández-Calvo et al. 2011). However, the details of the hormonal crosstalk involved are still unclear, and it is unknown whether any of these signalling pathways are related to MLP1, MLP3 or MLP6. The presence of binding sites for multiple common stress-responsive transcription factor families hints at a complex regulatory system of the *MLP* genes described in this thesis.

3.3.3 ABA sensitivity and MeJA/ACC insensitivity of *Atmlp* mutants (unpublished)

A small-scale hormone response assay was performed on *Atmlp1* and *Atmlp3* after identifying binding sites for ABA-, JA- and ET-responsive elements in the upstream regions of several MLPs (Fig. 10). Consistent with the findings of previous reports, Col-0 plants exhibited reduced lateral root initiation and primary root length in response to ABA (De Smet et al. 2003). The latter also applies to methyl jasmonate (MeJA)- and 1-aminocyclopropane-1-carboxylate (ACC)-treated Col-0 (Sun et al.

2009). MeJa and ACC are precursors that are commonly used to provoke JA and ET responses (Nandi et al. 2003; Vanderstraeten et al. 2019).

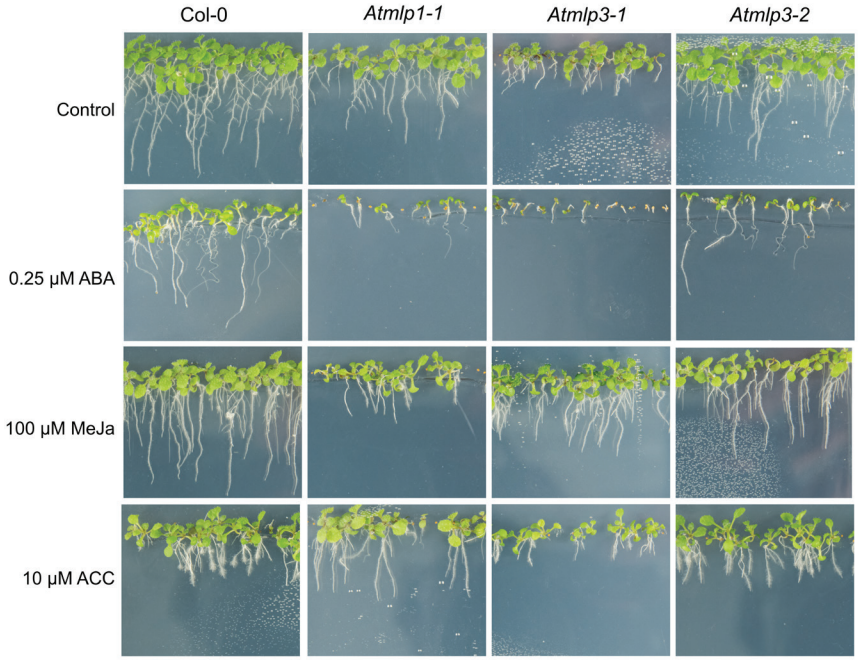


Figure 10. Hormone sensitivity screen of in vitro-grown Arabidopsis plants. The plants were germinated on MS medium (control) or MS medium supplemented with abscisic acid (0.25 µM), MeJA (µM 100) or the ethylene precursor AAC (10 µM). The photos were taken seven days after germination.

Atmlp1-1, *Atmlp3-1* and *Atmlp3-2* have mutations in their upstream promoter regions and grow more slowly than wild-type plants. Although no statistical analyses were performed, germination, root and shoot growth and lateral root initiation seem inhibited in *Atmlp1* and *Atmlp3* plants after exogenous treatment with ABA. This finding suggested that functional AtMLP1 and AtMLP3 contribute to negatively regulating ABA-induced stress responses. This contradicts previous results by Wang et al. (2016), who reported that the *AtMLP3* mutant (*Atmlp43*) was ABA insensitive. Further analysis of *Atmlp1* and *Atmlp3* is needed to elucidate the true response to ABA. However, Wang et al. (2016) used a fourfold greater concentration of ABA than I, which may have produced the different results. The *Atmlp1* mutant displayed an ACC-insensitive phenotype and *Atmlp3* a MeJa-insensitive phenotype compared to mock-treated plants. This, together with an increase in sensitivity to *R. solani*

infection, is consistent with the response of other JA- and ET-insensitive mutants to infection by *B. cinerea* (Thomma et al. 1999; Kachroo et al. 2001). The JA- and ET-responsive signalling pathways are also responsible for Arabidopsis resistance against *R. solani* AG8 (Kidd et al. 2021). A statistical analysis of a large-scale hormone treatment screen including known hormonal mutants together with RT–qPCR of hormone-responsive genes would be needed to support the observation that ET and JA regulate *AtMLP1* and *AtMLP3*. In conclusion, MLP1, MLP3 and MLP6 all respond to and act in defence against soilborne pathogen infection. The transcriptional regulation of these *MLP*s is likely complex and may involve crosstalk between several hormone pathways. Functional activation may depend on ABA- and JA/ET-responsive transcription factors and nutrient homeostasis, which together fine-tune the MLP abiotic and biotic responses.

3.4 *Plasmodiophora* organelle targeting

The molecular interactions that enable *P. brassicae* infection and prevent host defence responses are poorly understood. Few effectors and their targets have been identified. The infection biology needs to be clarified to be able to combat clubroot disease effectively. The goal of this project was to identify and characterize potential effectors used to manipulate the host. There is one place in the cell that houses defence and growth hormone synthesis, β-oxidation of fatty acids and nitrogen and sulfur metabolism, which are the types of processes that *P. brassicae* require from the host, i.e., the peroxisome (Su et al. 2019; Pan et al. 2020).

3.4.1 *P. brassicae* requires host peroxisomes to infect (Paper III)

Little is known about how *P. brassicae* use effectors, but SA is known to be important for defence responses (Pérez-López et al. 2018; Galindo-González et al. 2020). A recent study reported defence suppression by a ubiquitin ligase effector that marks RESPONSIVE TO DEHYDRATION 21A (RD21A), a positive regulator of defence responses for degradation, thereby blocking SA and ROS production (Li et al. 2023a). *P. brassicae* also targets the endomembrane system (Hossain et al. 2021). Here, the sequenced genome of *P. brassicae* e3 was used to identify novel candidate effectors (Stjelja et al. 2019). Nine out of 314 small (<400 amino acids) secreted proteins were predicted to localize to the peroxisome. Six effector candidates (PbE1 to PbE6) were transiently expressed in *N. benthamiana* leaves. PbE1 to PbE5 localized to the peroxisomal lumen (matrix) of a subset of leaf peroxisomes, while PbE6 localized to the peroxisomal membrane only (Paper III; Fig. 1). The selectiveness of the PbEs is potentially attributable to ectopic expression in the leaves and the fact that peroxisomes are a population engaged in different

tasks. The protein repertoire varies between peroxisomes and even within peroxisomal compartments (Wright & Bartel 2020; Bittner et al. 2022). It is possible that the effectors target only a subpopulation of peroxisomes that may be uncommon in the leaves of *N. benthamiana*. Additionally, the polarity and charge of sequences upstream of the PTS have recently been proposed to enhance or reduce the targeting strength of a peroxisome-targeted protein (Deng et al. 2022). It is unknown whether these regulatory features are present in *P. brassicae* effectors. It is common for peroxisomal proteins to have dual targets within the cell (Bittner et al. 2022). The *P. brassicae* peroxisomal-targeting PbEs share this attribute. This is exemplified by PbE1 and PbE6, which additionally targeted the ER and nucleus, and PbE3 and PbE4, which also targeted the ER (Paper III; Fig. S1)**.** Effectors that target parts of the endosome (the ER and Golgi) have similar target duality, as evidenced by simultaneous localization to the nucleus (Hossain et al. 2021). It is possible that ER interactions occur as new undifferentiated peroxisomes bud off from the ER.

3.4.2 *P. brassicae* steals host peroxisomal proteins (Paper III)

To obtain additional information on plant peroxisome function during *P. brassicae* infection, a plant line stably transformed with a fluorescent peroxisome marker was infected and monitored. This marker has a peroxisomal targeting signal. As the plant expresses it, peroxins transport it inside the peroxisomal lumen, where it results in peroxisome fluorescence. Lipids were also stained to clarify whether the previously reported lipid droplets were associated with peroxisomes. We identified lipids in the plasma membrane of developing zoosporangia that eventually release zoospores during primary infection stages in the epidermis (Paper III, Fig. 2). Both lipid droplets and peroxisomal proteins appeared in the secondary plasmodium as a diffuse glow. These plasmodia produce cells that are destined to mature into resting spores. Inside *P. brassicae* resting spores, these diffuse signals had been compartmentalized into spherical structures resembling peroxisomes in size and shape. Lipid droplets had also been incorporated into the spores (Paper III, Fig. 3). It is possible that the *P. brassicae* effectors identified in this study have different functions. Some may disturb host peroxisomes to aid in the release of their contents. Enzymes and metabolites, including peroxisome-associated lipid droplets, can then be stolen, taken up by plasmodia and incorporated into spore peroxisomes. We speculate that *P. brassicae* may use lipids to increase its carbohydrate reserves, perhaps for chitin production. Other peroxisomal effectors may regulate auxin or SA synthesis by disturbing IAA or benzoic acid synthesis or enhancing ROS scavenging in the peroxisomal lumen to inhibit an eventual retrograde defence response. In this case, functional PEX5 and PEX7 would be susceptibility proteins. Similarly, it is

possible that ER- and nuclear-targeted PbEs influence defence responses such as induced cell death or SAR.

3.4.3 Peroxisome transporters are essential for proper *P. brassicae* establishment (Paper III)

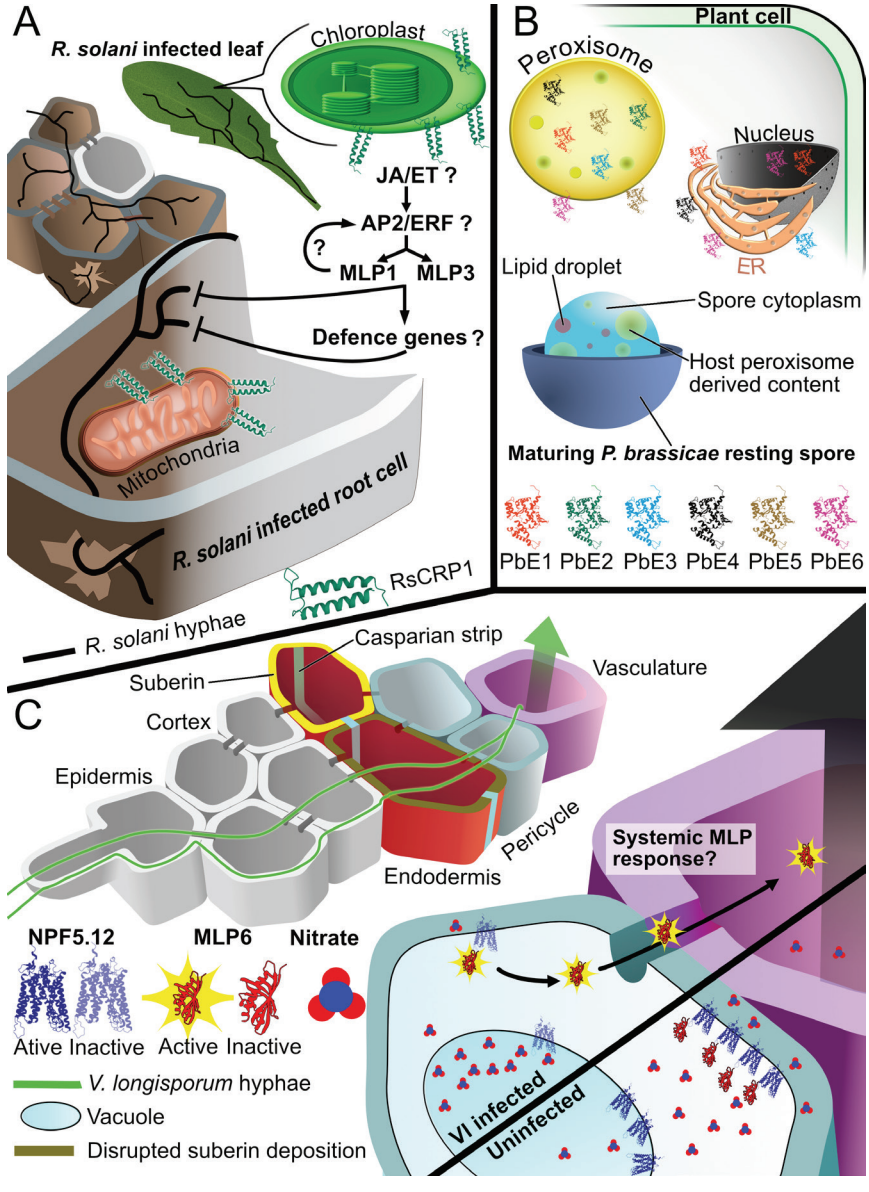
Next, Arabidopsis peroxisomal biogenesis mutants (*pex*) were screened for their responses to inoculation. All the lines carried mutations in processes involved in the transportation of cargo to the peroxisome (PEX5 or PEX7), transport across the lipid membrane (PEX13 or PEX14) or helping fold membrane proteins into the membrane (PEX3). Relative DNA analysis was used to measure the responses. All the mutants except for *pex13* had significantly less *P. brassicae* DNA than did Col-0, as well as less pronounced clubroots (Paper III, Fig. 4). These results indicate that *P. brassicae* targets peroxisomes for more than just minor benefits. The host peroxisome seems to play a vital role in *P. brassicae* establishment and in the completion of its life cycle. Multiple processes, such as reactive oxygen scavenging and the downregulation of SA-mediated immune responses, as well as carbon sources from peroxisomes, may be needed to enable and boost *P. brassicae* activities in the roots.

3.5 Summary of results obtained in this thesis

This section summarizes the most important results in this thesis based on the results from the four included papers.

Figure 11, opposite page. Summary of results from Papers I to IV. A) Paper I and Paper IV: Sugar beet root infection by *R. solani* causes damping off disease. *R. solani* initially employs effectors to dampen host immune responses followed by a switch to induce necrosis. The effector RsCRP1 localized to mitochondria and chloroplasts four days after infection and enhanced necrotic disease symptoms by an unknown mechanism. In partly resistant plants MLP1 and MLP3-mediated defences were activated at five days after infection that together reduced the spread of the fungus by an unknown mechanism. The MLPs are possibly activated by JA/ET signalling via an AP2/ERF transcription factor and may have a direct antifungal activity or induce expression of other defences. B) Paper III: *P. brassicae* infection in Brassica plants causes clubroot disease. Mutations in proteins responsible for plant peroxisomal content transport (PEX5, PEX7), a peroxisomal membrane transporter (PEX14) or a protein responsible for peroxisomal membrane protein insertion (PEX3) severely limited *P. brassicae* growth and clubroot disease symptoms. Six *P. brassicae* effectors were identified that localized to the plant peroxisomal lumen (PbE1 to PbE5) or peroxisomal membrane (PbE5 and PbE6). These effectors also target the nucleus (PbE1 and PbE6) and the ER (PbE1, PbE3, PbE4 and PbE6). At the end of the lifecycle, host derived peroxisomal proteins and lipid droplets were found embodied inside developing resting spores. C) Paper II: The vascular pathogen *V. longisporum* causes stem stripe disease. It entered the plant at lateral root initiation sites in the mature zone of the root and grew towards the endodermis via the symplast and apoplast. *V. longisporum* is dependent on nitrates from the host. Transcription activity of both the nitrate transporter *NPF5.12* and the interacting *MLP6* gene was attenuated

at two days after infection in a susceptible plant. The attenuated transcription of wild-type *NPF5.12* or changes in nitrate homeostasis may trigger MLP6-mediated defences. MLP6 may reallocate to the vasculature and travel systemically. Mutations in NPF5.12 and MLP6 in *B. napus* resulted in a significant reduction in endodermal suberin depositions and increased disease susceptibility. Adapted from Kelvinsong, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=25917225.



4. Conclusions

- Mutating multiple *BnNPF5.12* or *BnMLP6* loci increases *B. napus* susceptibility to *V. longisporum*. Simultaneous mutations in multiple *BnNPF5.12* and *BnMLP6* loci further increase susceptibility.

- *V. longisporum* growth is dependent on nitrates.

- *V. longisporum* inoculation reduces suberin deposition in the endodermis.

- Mutating *BnNPF5.12* or *BnMLP6* reduces suberin in the root endodermis during normal growth in the absence of *V. longisporum*.

- MLP1 and MLP3 contribute to the defence against *R. solani.*

- The *R. solani* effector candidate RsCRP1 interacts with BvPIP;1 and localizes to chloroplasts and mitochondria.

- *P. brassicae* targets peroxisomes, the ER and the nucleus via six effector candidates.

- *P. brassicae* establishment is negatively affected in host peroxisomal biogenesis mutants.

- Host-derived peroxisomal proteins are incorporated into *P. brassicae* secondary plasmodium and resting spores.

5. Future Perspectives

5.1.1 Nitrate transporters and major latex-like proteins

Transporter activity influences disease outcome. This is the case of the nitrate transporter NPF5.12 and the carriers MLP1, MLP3 and MLP6. Other types of transporters likely also play a role in disease susceptibility/resistance. NPF5.12 and MLP6 are now known to interact, but a downstream target of MLP6 has not been identified. It would be interesting to determine whether there is another ligand bound after *V. longisporum* infection. Pull-down experiments of tagged MLP6 from phloem sap could reveal a different target compared to the cytosolic interaction with NPF5.12. Mass spectrum analysis could help identify the ligand.

Plants with differential responses to infection revealed that differences in transcriptional activity in Arabidopsis *NPF5.12* and sugar beet *MLP1* and *MLP3* have an impact on the level of disease resistance. It would therefore be of interest to look for resistance alleles in the natural genetic diversity at these loci in other cultivated beets or brassica crops. Allotetraploid crops such as oilseed rape typically contain multiple copies of genes. There is a chance to pick up resistance alleles among different genotypes of the same species or among progenitor species. For the sugar beet MLP responses to *R. solani*, it remains to be determined whether the induced mRNA accumulation of MLPs in resistant genotypes is due to differences in the promoters or upstream signalling events. This information needs to be generated by determining which transcription factors (if any) interact with the genes by protein–DNA pulldowns. Binding assays in combination with mutational analysis could perhaps help identify the specific motifs in the promoter sequences that enhance the promoter activity of these resistance genes in resistant genotypes. Gene regulation can also occur at the chromatin, protein, or RNA level.

It is possible that other nitrate transporters are involved in the response to pathogen stress. In the future, characterizing more of the 53 NPF genes and proteins in

Arabidopsis would be useful for obtaining a holistic view of nitrate transport during pathogenic stress. Measuring the simultaneous transcript accumulation of both influx and efflux proteins and xylem/phloem loading transporters could reveal additional proteins involved in the response to *V. longisporum*.

Several physiological and biochemical aspects could be investigated to help determine whether nitrate transporters play a role in pathogen-induced nutrient theft or in a putative pathogen starvation mechanism. Measuring the transcriptional responses of transporter genes during *V. longisporum* infection and chitin treatments will help determine whether the efflux transporter is influenced by the fungus or by an immune response. Infecting single and multiple mutants of other nitrate efflux transporters could then indicate a change in susceptibility due to loss of transport. Moreover, one can monitor the immune responses of marker genes (such as *NPR1* or *EIN2*) by real-time RT–qPCR. Immune responses should preferably not be different compared to those of the wild-type because this could indicate that other factors, such as the actions of interacting proteins, could influence the results. To investigate starvation tactics further, one should measure nitrate steady states, any reallocation away from roots, and storage in vacuoles by HPLC. Nitrate levels in vacuoles may be measured before and after infection by isolating vacuoles from protoplasts.

NPF transporter and MLP carrier engineering may have potential for crop improvements due to their multifunctional effects on plant growth and secondary metabolism. Overexpressing MLP1 and MLP3 have growth promoting effects which may be beneficial for production of leafy vegetables. NPF5.12 and MLP6 seem to be involved in the synthesis of the complex endodermal suberin layer. Strengthening this layer could increase tolerance to waterlogging, drought or vascular pathogen stress (Doblas et al. 2017; Kawa & Brady 2022). ABA signalling and GA are important for suberin formation. It is possible that NPF5.12 transports ABA or GA, and some MLPs are responsive to ABA signalling (Corratgé-Faillie & Lacombe 2017; Fujita & Inui 2021). To better understand the functions of these genes in suberin formation, a series of tests must be performed. For example, it needs to be determined whether NPF5.12 transports GA or ABA. Exogenous application of hormones to NPF5.12-expressing oocytes while measuring hormone accumulation could perhaps reveal such a function. The induction of NPF5.12 or MLP6 in the vascular bundle in the presence of hormones could be investigated using plants expressing the native promoter coupled to the β-glucuronidase (GUS) reporter system. The ligand binding or protein binding of MLP6 would again be important in elucidating its role in suberin formation. It could perhaps activate the expression of genes, resulting in suberin precursor production.

5.1.2 Peroxins and *P. brassicae* peroxisome targeting

Mutants of the peroxisome cargo transporters PEX5 and PEX7 and the transmembrane transporter PEX14 were nearly resistant to infection by *P. brassicae*. Understanding the reason for this phenotype would be of interest for the sake of creating clubroot-resistant crops. The presence of host peroxisomal proteins in maturing resting spores may indicate that an essential process is lacking in the *P. brassicae* metabolome that is supplied by the host, and the only way for effectors to extract these proteins may be to use intrinsic peroxin transportation to enter the lumen. However, I would like to begin on another end. It became evident that the effectors targeted only a subset of the peroxisomes. PbE1 most readily entered peroxisomes, while the others exhibited different degrees of selectiveness. I speculate that this could be due to the diverse nature of peroxisomes. Peroxisomes in leaves specialize in photorespiration (Reumann & Weber 2006), while those in a root cell, the natural environment for these effectors, would not have this function. I would like to test the idea that leaf and root peroxisomes may specialize in different functions. Perhaps the effectors can distinguish peroxisome types, or perhaps they are degraded if they enter a peroxisome without their target. The easiest way to disprove these hypotheses is to vacuum infiltrate *Agrobacterium* cells expressing fluorescently tagged effectors into roots and quantify the proportion of peroxisomes targeted between root cells and leaf cells. Differences in the proteomes of targeted peroxisomes and nontargeted peroxisomes could also be determined by first isolating a whole population of root peroxisomes from protoplasts. Then, purified fluorescently tagged effectors could be added, and a sensitive fluorescence-activated cell sorting machine could be used to separately capture only fluorescent (targeted) organelles and only nontargeted organelles. Then, the proteome could be sequenced. Such an experiment could reveal whether there is a difference among peroxisomes and whether *P. brassicae* targets specific types.

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Popular science summary

The thin layer of soil next to the root system, called the rhizosphere, is a communication hub housing thousands of microbial species. Plant–microbe interactions can be commensal or even promote plant growth or health. The colonization and feeding habits of pathogenic species, however, have deleterious effects on plant growth and physiology, leading to the development of disease symptoms. Plants have an immune system encoded by complex genetic machinery that can detect such intrusions. Receptors in the cell membrane or inside the cellular environment can interact with common molecular patterns displayed by pathogens or with signs of disturbance. A signalling cascade is sent to the nucleus and other organelles if pathogenic activities are discovered. This leads to reprogramming in the plant, which switches from growth to defence mode and starts to produce antimicrobial compounds and proteins at the site of infection. Additionally, infected roots can send stress signals to other parts of the plant to prime defences ahead of the spread of the infection. The two genes *MLP1* and *MLP3* in sugar beet are upregulated five days after infection by *Rhizoctonia solani*, a fungus that causes root rot and damping off disease. The activity of these proteins slows fungal growth. Recent findings suggest that plants may also attempt to hide nutrients from pathogens, a pathogen starvation tactic. The nitrate transporter NPF5.12 may contribute to such nutrient restriction. Normally, this protein pumps nitrate from storage pockets (vacuoles) across a series of membranes to cells with high nitrogen demand. *Verticillium longisporum*, which causes stem stripe disease, depends on these nitrates for growth. When the fungus infects a compatible plant, the activity of the *NPF5.12* gene decreases, presumably resulting in the reallocation of nitrates away from the spaces occupied by the pathogen and into storage. At the same time, MLP6, a protein that physically interacts with the nitrate transporter, becomes cell-to-cell mobile and induces defence mechanisms by a yet undetermined mechanism.

Pathogens may try to counter plant defences by secreting small virulence proteins. The specific functions of these effectors include hiding the pathogen from the plant

immune system, blocking defence signalling or facilitating nutrient release by disrupting the cell. The root killer *R. solani* secretes one of these effectors, RsCRP1, which possibly enters cells via water pores. It localizes to plant chloroplasts and mitochondria. These organelles function partly in plant defence by producing hormones or small, reactive defence signal amplifiers. Disturbances caused by RsCRP1 enhance necrotic disease symptoms, freeing nutrients for the fungus. *Plasmodiophora brassicae,* a protist that causes clubroot disease, secretes six effectors that target the nucleus, endoplasmic reticulum, and peroxisomes. The specific functions of these effectors are not known, but the host peroxisome is vital for successful establishment of the protist in the root. The clubroot pathogen steals content from peroxisomes, perhaps to increase the availability of important metabolites, and probably uses this material as a nutritional supplement to produce overwintering structures called resting spores. Knowledge about the molecular interactions between the host and pathogen is important because we need to understand the causes of disease to prevent it or enhance resistance. With knowledge about resistance genes, tolerant or resistant varieties of crops can be created via genetic engineering or conventional breeding techniques. Food security needs to be guaranteed in the future. New sources of disease resistance can help reduce the use of pesticides and help ensure crop production if integrated into breeding programmes.

Populärvetenskaplig sammanfattning

Det tunna jordlagret bredvid växters rotsystem, som kallas rhizosfären, är ett kommunikationsnav som rymmer tusentals mikrobiella arter. Interaktioner mellan växter och mikrober kan vara neutrala eller till och med främja växternas tillväxt eller hälsa. Patogena arters spridning i roten och sätt att förse sig med näring har i stället skadliga effekter för växternas tillväxt och fysiologi, vilket leder till utveckling av sjukdomssymtom. Växter har ett immunsystem som kodas av ett komplext genetiskt maskineri som kan upptäcka patogena intrång. Receptorer i cellmembranet eller inuti cellen kan binda till evolutionärt bevarade molekylära mönster som till exempel kitin som finns i svamphyfers cellvägg eller upptäcka tecken på störningar i cellens funktion. En signalkaskad skickas till cellkärnan och andra organeller i cellen om att patogena aktiviteter upptäckts. Detta leder till en omprogrammering i växten som nu växlar från tillväxt- till försvarsläge och börjar producera antimikrobiella ämnen och proteiner i infekterade celler. Dessutom kan den infekterade roten sända signaler till andra delar av växten för att aktivera ett försvar innan infektionen spridit sig dit. MLP1 och MLP3 är två proteiner som aktiveras i sockerbetor fem dagar efter en infektion av *Rhizoctonia solani*, en svampart som orsakar rotröta. Dessa proteiner bromsar svampens tillväxt. Nya rön tyder på att växter också kan försöka dölja näringsämnen från patogener. En form av utsvältningstaktik mot patogener. Nitratjoner är en av de vanligaste källorna till metaboliskt användbart kväve i växter. Nitrattransportören NPF5.12 kanske bidrar till en sådan näringsbegränsning. Normalt pumpar detta protein nitratjoner från cellens nitratlager i den vätskefyllda membranblåsan (vakuolen) genom en serie membran till celler med stort kvävebehov. *Verticillium longisporum*, ett rapskransmögel, är beroende av dessa nitrater för sin tillväxt. När den infekterar en kompatibel växt avstannar aktiviteten hos *NPF5.12*-genen, vilket leder till att strömmen av nitrater omfördelas från de utrymmen där patogenen vistas och återgår till inlagring i vakuolen. Samtidigt blir MLP6, ett protein som fysiskt interagerar med nitrattransportören, mobilt och förflyttar sig genom celler och inducerar försvarsmekanismer genom en ännu inte fastställd mekanism.

Patogener kan försöka motverka växtens försvar då den blivit upptäckt genom att utsöndra små virulensproteiner. Specifika funktioner hos dessa så kallade effektorer kan innebära att dölja patogenen från växtens immunsystem, blockera försvarssignaler eller hjälpa till att frigöra näringsämnen genom att ha sönder celler. Rotdödaren *R. solani* utsöndrar en effektor, RsCRP1, som möjligen tar sig in i cellerna via vattenporer och rör sig till växtens kloroplaster och mitokondrier. Dessa organeller deltar i växtens försvar genom att producera hormoner eller fria små reaktiva ämnen som förstärker försvarssignaler. Störningar orsakade av RsCRP1 förvärrar de nekrotiska sjukdomssymptom som svampen orsakar och frigör födoämnen till den. *Plasmodiophora brassicae*, en protist som orsakar klumprotsjuka, utsöndrar sex effektorer som söker sig till cellkärnor, det endoplasmatiska nätverket och peroxisomer. Specifika funktioner hos dessa effektorer är inte kända, men växtvärdens peroxisomer är avgörande för en framgångsrik etablering av protisten i roten. Klumprotspatogenen stjäl innehåll från peroxisomerna, kanske för att öka tillgången till viktiga metaboliter och använder det troligen som näringstillskott för att producera övervintrande strukturer som kallas vilosporer. Kunskap om molekylära interaktioner mellan värd och patogen är viktig eftersom vi måste förstå orsakerna till sjukdom för att förhindra den eller skapa förbättrad resistens. Med rätt kunskap kan detta göras antingen med hjälp av genteknik eller konventionella förädlingsmetoder. Livsmedelsförsörjningen måste garanteras för framtiden. Nya källor till sjukdomsresistens kan bidra till att minska användandet av pesticider och bidra till att säkerställa hög avkastning från våra grödor om de integreras i förädlingsprogram.

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I

Major latex protein-like encoding genes contribute to Rhizoctonia solani *defense responses in sugar beet*

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ORIGINAL ARTICLE

Major latex protein-like encoding genes contribute to *Rhizoctonia solani* defense responses in sugar beet

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Abstract
Sugar beets are attacked by several pathogens that cause root damages. Rhizoctonia (Greek for "root killer") is one of them. Rhizoctonia root rot has become an increasing problem for sugar beet production and to decrease yield losses agronomical measures are adopted. Here, two partially resistant and two susceptible sugar beet genotypes were used for transcriptome analysis to discover new defense genes to this fungal disease, information to be implemented in molecular resistance breeding. Among 217 transcripts with increased expression at 2 days post-infection (dpi), three resistance-like genes were found. These genes were not significantly elevated at 5 dpi, a time point when increased expression of three Bet v I/Major latex protein (MLP) homologous genes *BvMLP1, BvMLP2* and *BvML3* was observed in the partially resistant genotypes. Quantitative RT-PCR analysis on diseased sugar beet seedlings validated the activity of *BvMLP1* and *BvMLP3* observed in the transcriptome during challenge by *R. solani*. The three *BvMLP* genes were cloned and overexpressed in *Arabidopsis thaliana* to further dissect their individual contribution. Transgenic plants were also compared to T-DNA mutants of orthologous *MLP* genes. Plants overexpressing *BvMLP1* and *BvMLP3* showed significantly less infection whereas additive effects were seen on *Atmlp1/Atmlp3* double mutants. The data suggest that *BvMLP1* and *BvMLP3* may contribute to the reduction of the Rhizoctonia root rot disease in sugar beet. Impact on the defense reaction from other differential expressed genes observed in the study is discussed.

Keywords Arabidopsis · Defense genes · Major latex protein-like · *Rhizoctonia solani* · RNA-seq · Sugar beet

Introduction

Beta vulgaris ssp. *vulgaris*, commonly known as sugar beet, is a dicot crop grown in the temperate zone with Europe and the USA as the major production regions (Draycott 2006). The crop is cultivated for its carbohydrate-enriched taproot. In addition to sugar, sugar beet is also a source for an array of carbohydrate-based products including biofuel (Duraisam et al. 2017) and pharmaceuticals such as blood substitute (Leiva-Eriksson et al. 2014). Sugar beet is a biennial crop where carbon is translocated from the leaves to the root during the vegetative stage and vice versa during the generative phase (Fondy et al. 1989). Root crops such as sugar beet that have a relatively long growing season are particularly vulnerable to pathogens including soil microbes attracted to the carbohydrate enriched root system. The soil-borne basidiomycete *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) has become a pathogen of increasing importance on sugar beet. In the current study, our attempt was to identify defense genes against *R. solani* by comparing

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transcriptome profiles of sugar beet breeding lines known to express a differential response to this fungal pathogen.

Most *R. solani* infections are initiated by germinating sclerotia or mycelia from debris which can survive in the soil for many years (Cubeta and Vilgalys 1997). Overwintered propagules of *R. solani* germinate and start to infect sugar beet seedlings when soil temperature exceeds 12 °C (Mukhopadhyay 1987). Under optimal temperature and high humidity conditions hyphae colonize the host plant leading to seedling damping-off, crown and root rot (Sneh et al. 1996). *R. solani* AG2-2IIIB is the anastomosis group causing most problems in sugar beet production and soil inoculum is expected to increase in regions where sugar beet and maize are overlapping in the crop rotation schemes, since maize can act as a host and thus propagate the pathogen (Buddemeyer et al. 2004; Schulze et al. 2016). Further, this fungal pathogen does not produce any asexual spores and only occasionally sexual spores are formed (Cubeta and Vilgalys 1997). This lack of spore formation hampers resistance screening work because amounts of inoculum cannot be precisely controlled in field trials or when running indoor experiments. Together all these factors add to the complexity of *R. solani* disease control and work on crop improvement. The strict European regulation on use of agrochemicals prohibits treatment of the soil or the canopy to decrease *R. solani*-incited damages. The only way known to handle the disease is by implementing various cultivation practices and most importantly is the availability of resistant varieties (Buhre et al. 2009). Much work on crop improvements is presently devoted on genomic selection or marker-associated breeding where in this case the sugar beet genome is an important resource (Dohm et al. 2014; Funk et al. 2018).

Based on our transcriptome analysis, we found three major latex protein (MLP) encoding genes *BvMLP1* and *BvMLP2* and *BvMLP3* that showed elevated transcriptional activity in partly resistant genotypes of sugar beet 5 days post-inoculation with *R. solani.* Quantitative RT-PCR confirmed the *BvMLP1* and *BvMLP3* expression in infected sugar beets. Enhanced resistance against *R. solani* infection was also demonstrated when *BvMLP1* and *BvMLP3* were cloned and overexpressed in *A. thaliana*. To dissect individual contributions of the three *MLP* genes, we screened homologous T-DNA mutants in *A. thaliana*. The result showed that both *MLP1* and *MLP3* are of importance in the response to *R. solani*.

Materials and methods

Sugar beet material and *R. solani* inoculation for RNAseq

Two partially resistant (G1, line no. 11014044 09; G2, line no. 06012609 70) and two susceptible (G3, line no. 11014038 09; G4, line no. 11014072 09) sugar beet breeding-lines were used. After 13 weeks, the plants were inoculated with *R. solani* AG2-2IIIB BBA 69670 isolate by putting four infected barley kernels approximately 1 cm from the root and 1.5 cm down in the soil on four sides of the root using a tweezers. Inoculated plants were moved from 18/12 °C (day/night) regime to 24/18 °C for the infection phase. At least three roots per genotype were collected before onset of infection (day 0), and 2 and 5 days post-infection (dpi). This experimental design was chosen because it was shown in a pilot study that the fungus reaches the root 2 dpi and we estimated the infection to be in its initial phase at 5 dpi. Further, this experimental design enriches for fungal-induced genes after the inoculation procedure, and reduces the number of development-associated genes in the datasets. Roots were washed and four samples from each root were taken with a core drill. The samples were directly frozen in liquid nitrogen and stored at − 80 °C. In parallel to the infected materials, four roots from each line were harvested before inoculation as control materials.

RNA isolation

RNA samples were extracted from all four sugar beet genotypes. Three replicates for each time point, treatment and genotype were prepared. Frozen tissue was ground in a mortar to fine powder. Total RNA was isolated according to the procedure outlined by Puthoff and Smigocki (2007) and stored at − 80 °C until further use.

RNA sequencing and genome mapping

Thirty-six pair end libraries with 100 bp read length were prepared and sequenced using Illumina HiSeq 2000 technology, which generated > 20 million reads per sample. The reads were aligned using GSNAP (genome short-read nucleotide alignment) to the sugar beet genome RefBeet-1.0/Dec 2011 scaffold assembly of KWS2320. Gene IDs were translated to the RefBeet-1.1 version available at https://bvseq.molgen.mpg.de/index.shtml. Count data were generated from BAM files using standard procedures established at National Center for Genome Resources (NCGR), New Mexico, USA. Reads were apportioned (Young et al. 2011) at the gene level to avoid potential data loss associated with using only uniquely aligning reads.

Data quality control and normalization

Data were evaluated for numbers of read counts for each gene in the samples. A threshold of at least five read counts

in each set of three replicates was set. This approach generated a total of 16,768 genes for further analysis. The remaining data sets were manually checked for correct biological affiliation. The quality of samples and major sources of variance were analyzed using multivariate analysis. Data were centered and scaled to unit variance and analyzed by principal component analysis (PCA) in Simca version 13.0.0 (https://umetrics.com/products/simca). Nucleotide percentage by position, average quality (Phred) score by position and bias due to gene length was determined and count data were normalized using the R (version 3.2.3) library EDAseq (Risso et al. 2011).

Differentially expressed genes (DEG) and gene ontology (GO) enrichment analysis

Differential gene expression analysis was performed using generalized linear model methods (GLMs) implemented in the edgeR package (McCarthy et al. 2012). Absolute log2 fold change > 1 and a false discovery rate (FDR) < 0.05 settings were used to define the DEGs. A heat map was constructed using the pheatmap tool implemented in the R package (Kolde 2015). The R package topGO (Alexa and Rahnenfuhrer 2010) was used for gene ontology (GO) enrichment analysis and functional characterization of the biological processes. Fisher weight or fisherweight01 was used for statistical significance measure with a significance level of < 0.05.

Co-expression networks and visualization with Cytoscape

Expression data for the 36 samples (4 genotypes, 3 timepoints, 3 biological replicates) and 16,768 genes were used to construct weighted gene correlation networks using the WGCNA tool in R-package (Langfelder and Horvath 2008, 2012). Expression count data were converted to log2 + 1, power = 12, TOMtype = unsigned, minModuleSize = 20, reassignThreshold = 1, mergeCutHeight = 0.15, and verbose = 3. Nodes represent genes and edges are correlation coefficient values among gene pair. The network was visualized using Cytoscape version 3.3.0.

Identification and analysis of carbohydrate-related proteins

Carbohydrate active enzymes (CAZymes) in the sugar beet proteome were analyzed using the dbCAN "Data-Base for automated Carbohydrate-active enzyme Annotation" annotation pipeline (Yin et al. 2012).

Transgenic *A. thaliana* (*At*) materials

Total RNA was isolated from *B. vulgaris* G1 genotype (Qiagen RNeasy plant mini kit), cDNA was synthesized (qScript™ cDNA synthesis kit, Quanta Biosciences) and used as template for *MLP* gene amplifications. Sugar beet is denoted Bv. Three MLP-like protein encoding genes, *BvMLP1* (Bv7_162510_pymu), *BvMLP2*, (Bv7_162520_etow) and *BvMLP*3 (Bv_27270_xeas) were amplified (Phusion High-Fidelity PCR polymerase, New England Biolabs) and purified. Fragments were individually cloned into the pENTR/D-TOPO vector and subcloned in *E. coli*. Single colony plasmids were purified, and plasmid DNA restricted followed by Sanger sequencing (Macrogen). Confirmed inserts were introduced into pGWB405 destination vectors using the Gateway system. Primers and vectors are provided in Table S1. Final *35S:BvMLP* constructs to generate overexpressor (OE) lines were transformed into *Agrobacterium tumefaciens* strain C58, followed by transformation to *A. thaliana* Col-0 using the floral dip method (Davis et al. 2009). Twenty putative T_0 transgenic plant lines for each construct were produced followed by in vitro selection for kanamycin resistance, and PCR analysis. Two independent, homozygous T_2 lines per construct were chosen and propagated to generation T_3 to amplify enough seeds for further analysis. Following *A. thaliana* materials were used in the study: *35S:BvMLP1-1* (OE1a), *35S:BvMLP1-2* (OE1b), *35S:BvMLP2-1* (OE2a), *35S:BvMLP2-2* (OE2b), *35S:BvMLP3-1* (OE3a) and *35S:BvMLP3-2* (OE3b). Homozygous single T-DNA insertion lines: *Atmlp1-1* (SALK 018534), *Atmlp2-1* (WiscdsLox413-416K24), *Atmlp3-1* (SALK_103714C), *Atmlp3-2* (SALK_033347C) and two double mutants *Atmlp1-1/Atmlp3-1* and *Atmlp1-1/Atmlp3-2* were also included in the work.

Screening of sugar beet seedlings and Arabidopsis plantlets

R. solani AG2-2IIIB inoculum of BBA 69670 was prepared by growing fresh hyphae from a 1 cm^2 potato dextrose agar plug for 10 days on sterile maize flour medium (1:1:5 ratio of maize flour, perlite and water). Three-week-old sugar beet seedlings of the four breeding lines were grown in standard soil followed by transfer to the growth containers with a mixture of fresh soil and prepared inoculum in a ratio of 10:1. At least five roots including hypocotyls were sampled in four biological replicates at 0, 2 and 5 dpi for each of the four sugar beet breeding lines. *A. thaliana* plantlets were transferred to containers containing a 20:1 ratio of fresh soil and inoculum after cultivation in standard soil for 21 days. Six biological replicates per genotype, each comprising of at least four plants were harvested at 5 dpi. All sugar beet and *A. thaliana* plants including wildtype Col-0 were grown

under short-day conditions (8/16 h light/dark, 22/18 °C day/night).

Fungal DNA quantification and *MLP* transcript analysis

Total plant RNA was extracted and cDNA synthesis was performed as earlier described. Gene-specific primers were designed using Primer3 (Rozen and Skaletsky 2000) and expression normalized to the *TUBB4* (sugar beet) or *Ubiquitin10* (*A. thaliana*) genes. Transcript data were analyzed with the comparative C_T method (Livak and Schmittgen 2001) followed by Student's *t* test in R (version 3.16). Total DNA was extracted from inoculated samples (Möller et al. 1992). 500 µl of 3% CTAB extraction buffer per 100 mg disrupted plant material was used. The amount of fungal DNA (*RsG3PDH*) was determined with qPCR and normalized to the amount of plant DNA (*Actin2*). Primers are listed in Table S2.

Availability of data and materials

RNA-Seq data have been deposited in the National Center for Biotechnology Information (NCBI) database, and Gene Expression Omnibus under the accession number GSE92859. The sugar beet genome RefBeet 1.0, used for the mapping is converted to the RefBeet 1.1, available at https://bvseq.molgen.mpg.de/index.shtml and translations can be seen in the processed data file.

Results

Three disease resistance-type genes are expressed as an early response to *R. solani*

Sugar beet transcript data were generated for 47,713 gene models. A cut-off value was set at > five reads in at least three samples to avoid singleton bias, resulting in a final set of 16,768 genes for further analyzes. The major sources of variance in the data set were analyzed using principal component analysis. This variance was best explained by time post-inoculation followed by *R. solani* resistance level in sugar beet (Fig. S1). Data from the two partial resistant and the two susceptible genotypes were fused because no major source of variance was observed between them. This approach added statistical power to the tests of differential expression. Differential expression of the 16,768 genes was determined using a generalized linear model likelihood ratio test. During the time-course from day zero to 5 dpi, an overall increase of transcriptionally affected genes was found in partially resistant compared to susceptible genotypes (Fig. 1a). Gene ontology (GO) enrichment analysis did not find biological processes related to biotic stress over-represented in the list of genes up-regulated at 5 dpi (718) or those shared at 2 and 5 dpi (201). In contrast, 11 genes annotated as response to stress (GO term GO:0006950) were identified among the 217 transcripts up-regulated at the earlier time-point (2 dpi). Genes in this group were Bv1_007570_oxfa (abscisic stress-ripening (ASR) protein), Bv1_013700_wnij (peroxidase), Bv2_026070_scpc (unknown), Bv4_088600_cumk (NBS-LRR-type resistance protein), Bv7_178870_rzzu (peroxidase), Bv7_179080_rdtw (cationic peroxidase), Bv8u_204980_frqg (BED finger-NBS-LRR resistance protein), Bv9_206760_padn (rRNA N-glycosidase), Bv_25520_psek (peroxidase), Bv_44840_iifo (NBS-LRR-type resistance protein) and Bv2_039610_pxtp (unknown). The data suggest an early effect of three resistance-like genes to *R. solani* infection. They are Bv_44840_iifo, Bv4_088600_cumk and Bv8u_204980_frqg and located on chromosome 3, 4 and 8, respectively. These genes, encoding nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains, were not significantly elevated at 5 dpi.

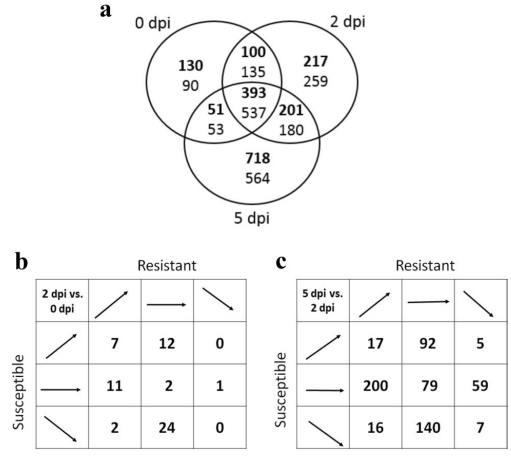

Fig. 1 Differentially expressed sugar beet genes. **a** Partially resistant and susceptible genotypes were compared at three time points; 0, 2, and 5 days post-inoculation (dpi) with *R. solani*. Bold numbers represent up-regulated genes and numbers in regular text are down-regulated genes in partially resistant genotypes. The edgeR package (Robinson et al. 2010) was used for the analysis with absolute log2 fold change > 1 and false discovery rate < 0.05. **b** Significantly differentially expressed sugar beet genes comparing genotypes and time points. 2 dpi is compared with 0 dpi, a total of 59 genes, **c** 5 dpi is compared with 2 dpi, a total of 615 genes. Arrows indicate significant up- or down-regulation or no significant differential expression between time-points. The analysis was done using the R package edgeR (McCarthy et al. 2012) with absolute log2 fold change > 1 and false discovery rate < 0.05 settings

Major latex protein-like protein encoding genes are activated in response to *R. solani* infection

To further clarify the influence of the infection-time component, a statistical test was performed to identify interaction effects between genotype and time after inoculation. In total, 660 genes were significantly different (false discovery rate (FDR) < 0.05) between partially resistant and susceptible genotypes in their response to *R. solani* inoculation (Fig. S2) Next, this set of genes was divided into functional groups using eukaryotic orthologous group (KOG) assignments. Out of the 660 genes, only 4 genes were assigned to defense mechanisms not seen in the GO enrichment analysis. Nine genes were annotated as cell wall-related genes (Table S3).

Early in the infection process (2 dpi vs. 0 dpi) 59 genes showed a significant differential response in partially resistant compared to susceptible genotypes (Fig. 1b), while the number increased at the later comparison (5 dpi vs. 2 dpi) to 615 (Fig. 1c). GO enrichment analysis showed that oxidation–reduction process (GO:0055114) genes were enriched at 2 dpi (Table S4). At 5 dpi, 19 GO groups were enriched including cell wall macromolecule catabolic process (GO:0016998), cellulose biosynthetic process (GO:0030244) and response to biotic stimulus (GO:0009607) (Table S5). In the latter GO group the three genes Bv7_162510_pymu, Bv7_162520_etow, and Bv_27270_xeas on chromosome 7 and 8, were annotated as major latex protein-like encoding genes (Table S6). Elevated levels of these three *MLP* genes, denoted as *BvMLP1*, *BvMLP2* and *BvMLP3*, were found in the partially resistant genotypes after 5 days of fungal challenge (Fig. S4). We further constructed a weighted gene co-expression network (Langfelder and Horvath 2008, 2012). A clustering of the weighted correlation network resulted in 48 modules with highly co-expressed genes (Table S7). GO enrichment analysis was performed on the genes with high correlation to each module (Data set S1). Modules 3, 4, 5, 18, 23 and 30 contained an over-representation of genes annotated as biotic stress-related genes, whereas cell wall-related genes were enriched in modules 1, 41 and 47. Out of these two main categories, only module 5 contained significantly differentially expressed biotic stress-related genes in the partially resistant genotypes in response to *R. solani*. Again, the same three *BvMLP* genes as in the GO enrichment analysis were identified.

In addition to *MLPs*, differentially expressed genes in module 5 included a MYB46 transcription factor (TF), a plant disease resistance response protein (DRR206) and a flavonoid *O*-methyltransferase protein, which are known to be involved in various stress response processes (Fig. 2). Two additional putative transcription factors, Bv2_027430_cint and Bv5_119300_wnjc, were significantly activated in the partially resistant genotype at 2 dpi, in contrast to the susceptible genotypes. These putative TFs were members of modules 6 and 14 of the co-expression network. In module 14, Bv2_027430_cint, an asymmetric leaf 2 (AS2) homolog, known as a repressive regulator, is highly correlated with six cell wall-related genes and five biotic stress-related genes significantly expressed at 5 dpi (Supplementary Table S8). In module 6, Bv5_119300_wnjc, a member of the APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily which regulates diverse plant responses, is connected with two biotic stress-related genes and two cell wall-related genes.

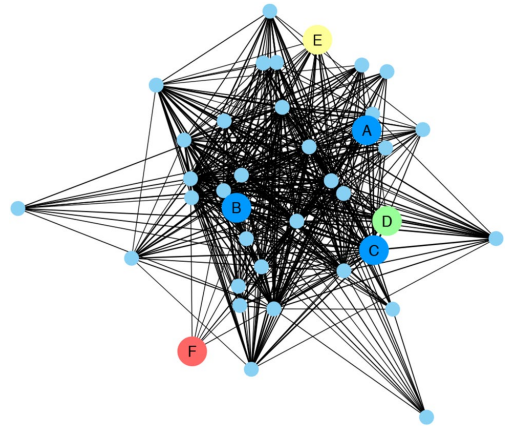

Fig. 2 Co-expression network of differentially expressed sugar beet genes in module 5. The network comprises 38 genes (nodes) where blue represents: major latex protein homologs (A, BvMLP2; B, BvMLP1; C, BvMLP3), green (D): MYB46, yellow (E): flavonoid, red (F): disease resistance response protein and light blue represents other or unknown genes. Edge weight cut-off was set at > 0.16

MLP1 and *MLP3* contributes to *R. solani* plant defense

To confirm the prediction based on the RNAseq data, qRT-PCR analysis was performed on infested sugar beet seedlings. Significant differences in transcript responses were found at 5 dpi in young sugar beet seedlings for *BvMLP1* in genotype G1 and *BvMLP3* in genotype G2, both harboring partial resistance to *R. solani* (Fig. 3). No significant response was found for *BvMLP2* (Fig. S5). To further dissect the different contributions of the *BvMLP* genes, the three coding sequences were cloned from genotype G1 and overexpressed in *A. thaliana* (Fig. S6a). In parallel, homozygous T-DNA insertion mutants in homologous *A. thaliana* genes (At5g28010, At1g23130 and At1g70890) were produced (Fig. S6b, c). These *A. thaliana* genes shared 47%, 33% and 61% amino acid sequence identity to the three sugar beet genes *BvMLP1*, *BvMLP2* and *BvMLP3*, respectively. All MLP overexpression lines developed faster and formed

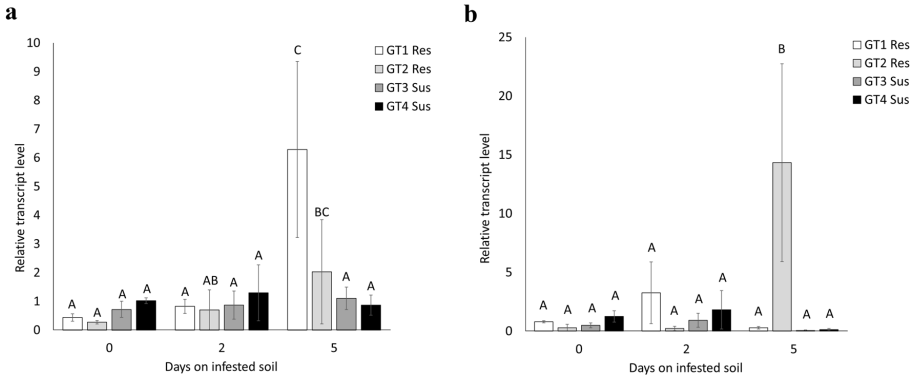

Fig. 3 Relative transcript levels of *BvMLP* genes in sugar beet. Seedlings of four genotypes were harvested for real-time qRT-PCR at 0, 2 and 5 days on infested soil. **a** *BvMLP1,* **b** *BvMLP3*. The statistics are based on a Levene's test and a Student's *t* test on three biological replicates. Different letters indicate significant difference between groups. Error bars = mean ± SD

larger rosettes than wild type (Col-0). After 5dpi, the *A. thaliana* transgenic and mutant lines were evaluated for responses to *R. solani* (Fig. 4a, b). When comparing the fungal DNA content in the different *A. thaliana* genotypes, *35S:BvMLP1-1*, *35S:BvMLP1-2* and *35S:BvMLP3-1* and *35S:BvMLP3-2* had significantly lower levels compared to Col-0, *35S:BvMLP2-1*, and *35S:BvMLP2-2* (Fig. 5a). When analyzing the T-DNA mutants, *Atmlp3-2* (*BvMLP3* homolog) showed the highest levels of *R. solani* DNA compared to Col-0 followed by *Atmlp1-1* (*BvMLP1* homolog). To clarify potential redundancy effects of the two *AtMLP* homologs, two double mutants (*Atmlp1-1/Atmlp3-1* and *Atmlp1-1/Atmlp3-2*) were made and screened against *R. solani* (Fig. 4b). Fungal DNA analysis demonstrated higher levels in *Atmlp1-1/Atmlp3-2* than in the *Atmlp1-1* and *Atmlp3-1* single mutants (Fig. 5b). Together the data suggest that the *Atmlp3-2* mutation has the largest impact but *Atmlp1-1* add some strength to the response.

Discussion

Today's sugar beet cultivars with high levels of resistance to *R. solani* are known to suffer from yield penalty or harbor less resistance to other important pathogens (Strausbaugh et al. 2013; Liu and Khan 2016). We, therefore, were interested to monitor transcript responses to this fungus on a genome-wide scale to identify defense-associated genes useful to refine the breeding work. Our transcriptome profiling identified in total 2022 differentially expressed genes at 2 dpi and slightly more (2697) at 5 dpi in the dataset. GO enrichment analysis revealed eleven defense-associated genes differentially expressed at 2 dpi. Three genes containing NBS-LRR domains characteristic for resistance *R* genes were found among the genes expressed early, all three located on chromosome 3. QTL mapping has earlier identified two major clusters of NBS-BACs on chromosome 3 (Lein et al. 2008). This quantitative *R. solani* resistance coverers 10–15% of the sugar beet genome and is associated with negative yield drag. In attempts to further optimize the breeding work, additional gene candidates were searched for.

By exploiting interaction statistics, three *MLP* like-encoding genes were identified in the partially resistant genotypes, all being increasingly activated by time. Present in all MLP proteins is a ligand-binding site for large hydrophobic molecules, hormones and secondary metabolites that allow MLPs to have multiple functions (Koistinen et al. 2005; Ma et al. 2009; Park et al. 2009). MLPs are associated with tolerance to salt and drought in *A. thaliana* (Chen and Dai 2010; Wang et al. 2016) and are activated in response to the *Alternaria brassicicola* fungus and the soil-borne plasmodiophorid *Plasmodiophora brassicae* (Schenk et al. 2000; Siemens et al. 2006). *Verticillium dahliae* is another soil-borne pathogen (ascomycete) with a broad host range that includes cotton, sugar beet and many other species (Peggy and Brady 2002). In case of cotton, the *GhMLP28* was found to enhance the activity of an ethylene response factor, *GhERF6* and thereby amplified the defense response (Yang et al. 2015). No co-activation of *ERF*-encoding genes in our sugar beet datasets was observed. The number of MLP-like proteins varies among plant species (Zhang et al. 2018). A trend seen so far is that fewer homologs are found in monocots compared to dicots. 23 *MLP* genes are present in the sugar beet genome compared to 25 in *A. thaliana*. In attempts to dissect the importance of the three *BvMLP* genes in the response to *R. solani*, we first analyzed each

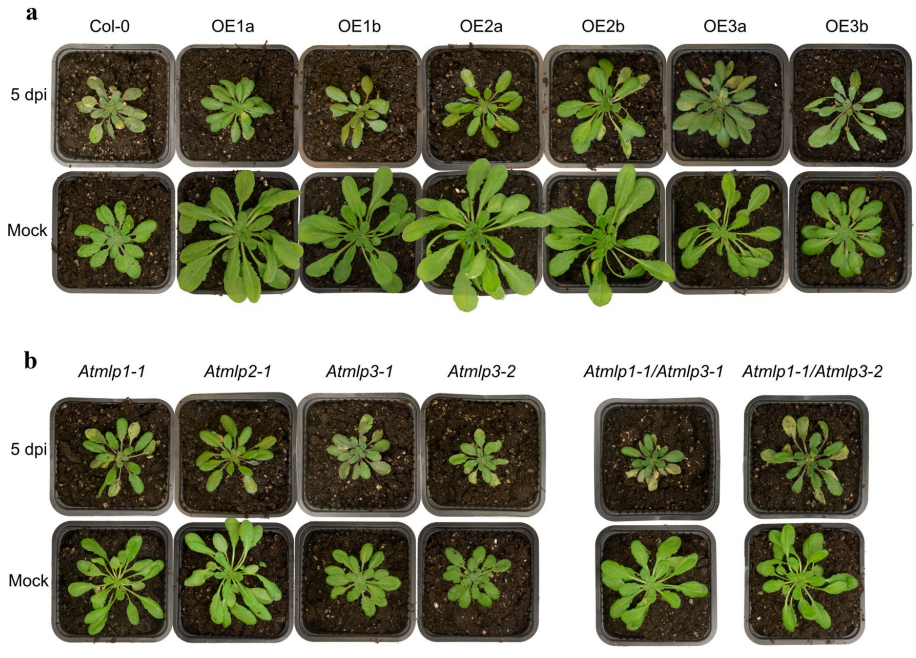

Fig. 4 Phenotypes of *A. thaliana* inoculated with *R. solani* or H_2O (mock). **a** Overexpressor lines: OE1a,b = *35S:BvML1-1*, *35S:BvML1-2*, OE2a,b = *BvML2-1*, 35S:*BvML2-2*, OE3a,b = 35S:*BvML3-1*, 35S:*BvML3-2*. **b** T-DNA insertion mutants in *BvMLP* homologues genes. Single mutants: *Atmlp1-1*, *Atmlp2-1*, *Atmlp3-1, Atmlp3-2,* and double mutants: *Atmlp1-1/Atmlp3-1* and *Atmlp1-1/Atmlp3-2*. All materials in Col-0 background. Photos taken 5 days post-inoculation

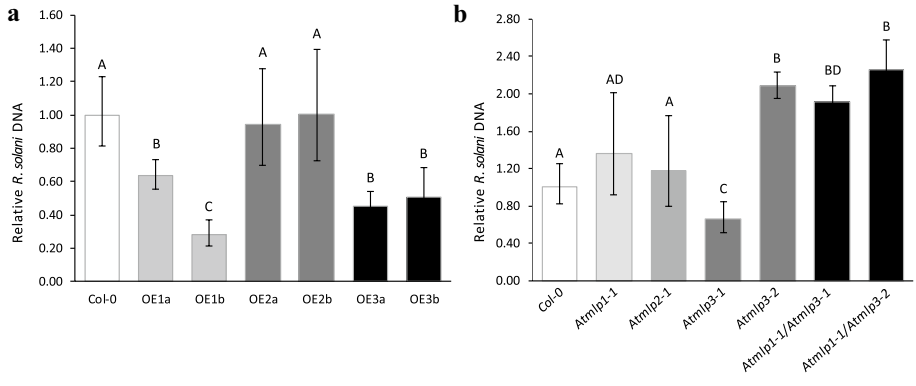

Fig. 5 Relative amount of *R. solani* DNA in *A. thaliana*. **a** Two independent *BvMLP* overexpression lines per gene, and **b** *Atmlp* single mutant and double mutant lines compared to wild-type (Col-0) at 5 days post-inoculation. OE1a and OE1b = *35S:BvMLP1-1*, *35S:BvMLP1-2*, OE2a and OE2b = *BvMLP2-1*, *35S:BvMLP2-2*, OE3a and OE3b = *35S:BvMLP3-1*, *35S:BvMLP3-2*. Statistical analysis performed with a Student's *t* test with at least four replicates. Error bars = mean ± SE

BvMLP gene independently. RNAseq gene expression levels were confirmed with qRT-PCR for two of the *MLP* genes (*BvMLP1* and *BvMLP3*). *BvMLP1 and BvMLP3* individually contributed to reduced infection levels of *R. solani* when overexpressed in *A. thaliana.* When pathogen responses of T-DNA insertion mutants in the most homologous *MLP*

genes in *A. thaliana* were monitored the *Atmlp3-2* mutant and the *Atmlp1-1/Atmlp3-2* double mutant yielded the highest level of infection. The data suggest that both *BvMLP1* and *BvMLP3* should be integrated in resistance breeding approaches to *R. solani*.

The genome of *R. solani* is enriched in genes coding for carbohydrate cell wall-degrading enzymes (Wibberg et al. 2016). This knowledge formed the rational to also include genes important for cell wall biogenesis in the analysis. Several TFs are known to regulate secondary cell wall formation. Among those, *MYB46* has a key function involving biosynthesis of cellulose, hemicellulose and lignin components (Ko et al. 2014). *MYB46* was clearly activated in the present sugar beet transcripts. In the sugar beet genome, as in *A. thaliana*, only one *MYB46* gene together with its paralogue *MYB83* is present. *MYB46* homologues in poplar, maize and rice are known to possess similar function in secondary wall biosynthesis as in *A. thaliana* (Zhong et al. 2010, 2011), which leads us to believe that this function is conserved also in sugar beet. The *DRR206* gene is well studied in pea, where it is activated both in response to bacterial and fungal infections (Daniels et al. 1987). *DRR206* expression is associated with pathways involving phytoalexins and cell wall biosynthesis (Hadwiger and Chang 2015; Seneviratne et al. 2015). Interestingly transgenic *Brassica napus* plants harboring the overexpressed pea *DRR206* gene showed enhanced seedling resistance to *R. solani* (Wang and Fristensky 2001). Together these data suggest that an activated *DRR206* gene may contribute to defense in sugar beet.

Plant carbohydrate metabolism is involved in numerous processes including cell wall structure, cell shape, energy metabolism, post-translational modifications, signaling, and defense (Kubicek et al. 2014). The cell wall composition and architecture affect wall strength, which forms an important physical outer barrier to potential invading pathogens. A common theme of fungal plant pathogens is their ability to secrete cell wall-degrading enzymes (Kubicek et al. 2014). The *R. solani* AG2-2IIIB isolate BBA 69670 that preferentially attacks sugar beets is no exception and encodes a wide repertoire of carbohydrate active enzymes (Wibberg et al. 2016). Particularly, glycoside hydrolase 43 (GH-43), carbohydrate esterase 12 (CE-12) and polysaccharide lyases 1 (PL-1) families are enriched in this fungal genome. In the sugar beet genome, we found 1294 CAZyme-encoding genes and 1349 CAZyme annotated domains which are slightly higher compared to the 1200 CAZy annotated proteins in *A. thaliana* (Fig. S7). Small proportions of the CAZyme domain classes were differentially expressed during fungal challenge. In comparison to *A. thaliana*, sugar beet has fewer glycosyl transferases (GT) and about the same numbers of glycoside hydrolases (GH), carbohydrate-binding modules (CBM) and polysaccharide lyases (PL). However, an enrichment of carbohydrate esterases (CE) and particularly large numbers of auxiliary activities (AA) are annotated in the sugar beet genome compared to *A. thaliana.* Most of these AA proteins belong to the AA2 family. This family contains class II lignin-modifying peroxidases that oxidize Mn(II) to Mn(III) which in turn oxidize a variety of phenolic model compounds able to degrade and or modify lignin polymers (Levasseur et al. 2013).

In conclusion, monitoring plant responses to soil-borne pathogens is challenging due to their hidden life in the soil which is difficult to control and observe. To this end, knowledge on their modes of infection and external factors impacting the infection process is low. *Rhizoctonia solani* is no exception where disease symptoms, if seen, are represented by dead plants on heavily infested soil. Our present study has highlighted a number of gene families that could contribute to *R. solani* defense in sugar beet, maybe in an orchestrated fashion during the fungal attack and disease progression. Any biotrophic stage of *R. solani* has so far not been demonstrated but early involvement of *R*-genes may be a sign of a hemibiotrophic lifestyle. Likewise, *R. solani* produces a chitin-binding LysM effector perturbing chitin-induced immunity which adds further support to a possible presence of an initial biotrophic infection stage (Dölfors et al. 2019). *Rhizoctonia solani* has a large repertoire of carbohydrate-active enzyme (CAZy)-encoding genes in its genome suitable for cell wall degradation, important for necrotrophic growth and saprophytic survival. Involvement of *MLP* genes are observed as a plant response to other soil-borne fungi such as *V. dahliae* (Yang et al. 2015). Its function to fungal invasion is still unclear. Recently, in an RNAseq study of fungus–apple interaction, one *MLP* gene was found to impact a handful of defense-related genes including transcription factors (He et al. 2020). It seems that *MLP* genes play important roles for defense in many crops including sugar beet; details of their function remain to be elucidated.

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Author contributions LH, TK and CD designed the experiments. LH, JF and JC made the bioinformatic analysis, LH and FD performed the experimental analysis LH, FD, JF, JC and CD wrote the article.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Plant mitochondria and chloroplasts are targeted by the *Rhizoctonia solani* RsCRP1 effector

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ABSTRACT

The fungal species *Rhizoctonia solani* belongs to the Basidiomycota division and is a ubiquitous soil-borne pathogen. It is the main agent of the damping-off disease in seedlings and causes the root and crown rot disease in sugar beets. Plant pathogens deploy small secreted proteins, called effectors, to manipulate plant immunity in order to infect the host. Here, a gene (*RsCRP1*) encoded a putative effector cysteine-rich protein was cloned, expressed in *Cercospora beticola* and used for virulence assays. The *RsCRP1* gene was highly induced upon the early-infection stage of sugar beet seedlings and disease was promoted. Confocal microscopy demonstrated localization to the chloroplasts and mitochondria upon transient expression of RsCRP1 in leaves of *Nicotiana benthamiana*. Further, this effector was unable to induce necrosis or to suppress hypersensitive response induced by the Avr4/Cf4 complex in *N. benthamiana*. Overall, these data indicate that RsCRP1 is a novel effector targeting distinct plant cell organelles in order to facilitate a successful infection at the early stages of the disease development.

1. Introduction

Pathogens can enter plant hosts using various strategies; via openings and wounds, secretion of cell wall degrading enzymes or manipulation of a wide range plant defense mechanisms. Commonly, these strategies are combined to promote efficient colonization and proliferation in the host. Events involving pathogen growth and reproduction in host tissue require nutrients, which is the ultimate rational to cause disease by any organism. In order to establish a compatible interaction, pathogens must evade or suppress plant immunity [1]. To do that, among others, they secrete small proteins, called effectors. Effectors can have various functions such as inducing necrosis, protecting fungal hyphae from plant chitinases, suppressing hypersensitive response (HR), or helping fungal hyphae to stealth themselves, avoiding recognition by plant receptors [2]. Although effector biology is a growing field, still a majority are undiscovered and important aspects of their exact roles and functions are unknown. This is particularly the case for the understudied but important soil-borne pathogens.

Plants on the other hand, deploy different layers of defense including sophisticated signaling against pathogens [3]. The first layer induced by microbial elicitors, called pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) [4]. PAMPs can be essential components of fungal cell wall, such as chitin, or proteins with a crucial role in the formation of filament in a bacterial flagellum, such as flagellin. Recognition of PAMPs by the plant leads to a PAMP-triggered immunity (PTI) response. Next layer of defense involves recognition of effectors by intracellular plant resistance (*R*) genes, leading to induction of effector-triggered immunity (ETI) such as the hypersensitive response, HR [5]. However, the present understanding of the plant immune system is far more differentiated.

Rhizoctonia solani (teleomorph: *Thanatephorus cucumeris*) is a soil-borne pathogen, with a wide host range. Isolates are categorized in different anastomosis groups (AG) based on their hyphal anastomosis reactions [6]. *Rhizoctonia solani* AG2-2IIIB is the causal agent of crown root rot in sugar beets. During recent years the genomes of different R. *solani* AGs have been sequenced with the purpose to enhance our knowledge of the infection pathways [7–11]. To assist the work on sugar beet improvement we used the

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genome information of *R. solani* AG2-2IIIB [12] to search for 1) novel effector candidates, and 2) investigate their function. The latter is a challenge since *R. solani* is not amenable for genetic modifications as many other basidiomycetes.

2. Materials and methods

2.1. Fungal isolates and growth conditions

Rhizoctonia solani AG2-2IIIB isolate BBA 69670 (DSM 101808) was used in this study and cultured as earlier described [12]. The *Cercospora beticola* strain Ty1 (MariboHilleshög, Research AB) was cultured on potato dextrose agar (PDA, Difco) at 22 °C in darkness. To induce sporulation, *C. beticola* was grown on tomato extract medium at 25 °C with a photoperiod of 12 h.

2.2. RNA preparation and quantitative RT-qPCR

For gene expression analysis of the *RsCRP1* (*RSOLAG22IIIB_02432*) gene, 3-week-old sensitive sugar beet plantlets (hybrid 1604511801, MariboHilleshög Research AB) replanted in soil infested with *R. solani* mycelia. Total RNA was extracted from infected plants using the RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions, while *R. solani* mycelia grown on potato dextrose broth (PDB, Difco) were used as a control. Primers are listed in Table S1. RT-qPCR was conducted as previously described [13]. The data was normalized to the *G3PDH* expression [14] and relative transcripts were calculated according to the $2^{-\Delta\Delta Ct}$ method [15]. Statistical analysis was done using Student's t-test.

2.3. Cloning and Cercospora beticola transformation

The *RsCRP1* gene was PCR amplified from *R. solani* cDNA using high fidelity Phusion Taq polymerase (Thermo Fisher Scientific). Primers are listed in Table S1. The cDNA fragment was inserted in the pRFHUE-eGFP vector [16] using the In-Fusion HD cloning kit (Takara Bio), followed by plasmid transformation to the *Agrobacterium tumefaciens* C58C1 strain. Transformation of *C. beticola* was performed using an *A. tumefaciens*-mediated protocol [17] and three individual colonies were used for further analysis. Expression of the *RsCRP1* gene was validated using RT-PCR on hygromycin-resistant colonies (Fig. S1).

2.4. Virulence assay and fungal biomass

For the virulence assay, leaves of 3-week-old sugar beet plants (hybrid 16045118 01 MariboHilleshög Research AB) were inoculated with *C. beticola* conidia as previously described [18]. The area of disease lesions was calculated 7 days post infection (dpi). Total genomic DNA was extracted from mock (H_20) and inoculated leaves and fungal DNA was quantified using the *C. beticola* actin (*act*) gene and normalized with *B. vulgaris* elongation factor (*elf-1*), using qPCR analysis (Table S1). At least three biological replicates were used and each replicate comprised of two leaves from four inoculated plants.

2.5. Sequence analysis and confocal microscopy

Presence of conserved domains in the RsCRP1 effector was tested using the SMART 6.0 protein analysis tool [19]. Subcellular localization was investigated using the WoLF PSORT predictor [20] the ChloroP [21] and the DeepMito servers [22]

For confocal microscopy, the *RsCRP1* gene was subcloned to the pENTR/D-TOPO vector (Thermo Fisher Scientific) and inserted to the pGWB605 destination vector using the Gateway system, followed by Agro-transformation in C58C1 cells and transiently expressed in *N. benthamiana* leaves. Imaging was performed using an LSM 800 confocal microscope (Zeiss). The green fluorescence was excited/emitted at 488/516 nm and detected at 411–553 nm. The red fluorescence was excited/emitted at 633/684 and detected at 645–700 nm. For the HR suppression assay, the *RsRCP1* gene was entered to the pGWB602 binary vector and transiently expressed in *N. benthamiana* plants harboring the Cf-4 receptor protein from tomato plants. The HR was triggered 24 h after RsRCP1 Agro-infiltration with the *Cladosporium fulvum* Avr4 effector.

3. Results and discussion

3.1. The RsCRP1 gene is highly induced upon early infection stages

The current wealth of pathogen genomes led to prediction of effector proteins which in general builds on the presence of a secretion signal, size (>400 aa) and content of cysteines [2]. To narrow down the effector candidates in the *R. solani* genome we compared the data from five different strains resulting in eleven genes unique for the AG2-2IIIB strain [12]. The small cysteine-rich protein-encoding gene *RsCRP1* was chosen for further studies based on its transcription patterns. It was highly induced already 4dpi in sugar beet seedlings, followed by reducing levels at 5 dpi as compared to fungal mycelia grown in PDB (Fig. 1).

3.2. Heterologous expression of RsCRP1 in Cercospora beticola promotes disease development

To take the next step involving further gene analysis, we evaluated the options among fungal sugar beet pathogens. We finally chose the ascomycete *C. beticola* causing Cercospora leaf spot disease which per se is a serious problem particularly in countries with strict fungicide restrictions. *C. beticola* produces rich amount of conidia another feature that further simplify its use compared to *R. solani*. The *RsRCP1* gene was ligated to the pRFHUE-eGFP vector driven by the constitutively expressed PgdpA promoter from *Aspergillus nidulans,* transformed to *C. beticola* and used for sugar beet infection. Increased necrotic lesions was observed for the *RsCRP1* + strains as compared to the wild type (WT) and the strain where only the empty vector was inserted (Fig. 2a). In parallel, DNA was extracted from infected leaf regions 7dpi and fungal biomass was calculated. No significant difference in the amount of fungal DNA was observed among WT and strains where the *RsCRP1* gene was overexpressed (Fig. 2b). Taking together, these data indicate

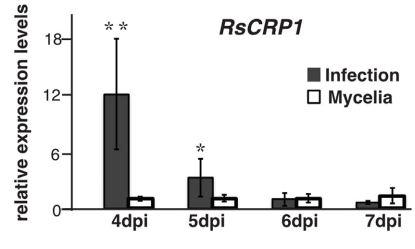

Fig. 1. The *RsCRP1* gene is highly induced upon early infection stages. Relative transcript levels were analyzed in sugar beets 4, 5, 6 and 7 dpi. *R. solani* mycelia was grown in PDB medium and used as a control. Data were normalized to the expression levels of the *G3PDH* gene according to the $2^{-\Delta\Delta Ct}$ method. Asterisks (*p < 0.05, **p < 0.01) indicate statistically significant differences between columns at the same time point according to Student's t-test. Error bars represent SD and is based on at least three biological replicates.

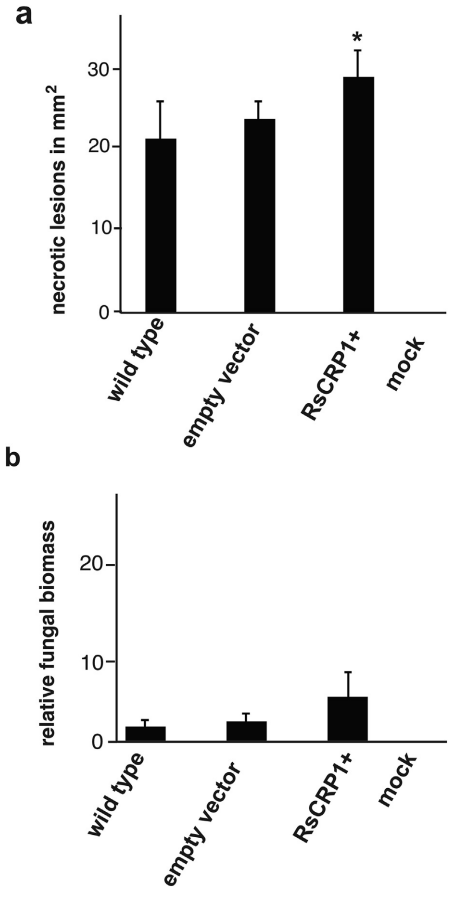

Fig. 2. Overexpression of the *RsCRP1* gene promotes *C. beticola* disease development. a) Symptoms in sugar beet leaves. b) Area (mm^2) of necrotic lesions in sugar beet leaves. c) *C. beticola* DNA biomass in infected leaves. Data show the average of three independent overexpression strains each includes three biological replicates 7dpi. Asterisk (*p < 0.05) indicates statistically significant differences according to Student's t-test.

that RsCRP1 is involved in disease development at the early stages of the infection process.

3.3. Transient expression of RsCRP1 does not suppress PTI-related HR

A broad variety of effectors have been found in secretomes of different *R. solani* strains. In the rice sheath blight disease pathogen, *R. solani* AG1 IA, three effectors associated with necrosis are found among other categories such as carbohydrate-active enzymes [8]. A cell death-inducing effector was later identified in this genome together with RsIA_NP8 [23,24]. In *R. solani* AG8, AG1-IA and AG3 secretomes, a xylanase and a protease are involved in the cell death process as well [25]. Further, our previous data showed that *R. solani* deploys LysM effectors to suppress chitin-induced immunity similar to hemibiotrophic ascomycete pathogens [18].

To generate additional functional data on RsCRP1, it was transiently expressed in *N. benthamiana* plants using a construct where *RsCRP1* was driven by the *35S* promoter. No necrosis was observed in the Agro-infiltrated area, indicating that the RsCRP1 effector is not involved in this process (Fig. 3). It is also known that certain effectors suppress immune responses such as HR [26]. In case of *R. solani* the newly found effector RsRlpA (a rare lipoprotein A) has this feature, suggesting that *R. solani* deploys effectors to suppress basal immune responses [27]. To investigate whether RsCRP1 functions as a suppressor of programmed cell death, the Avr4/Cf4 complex was used. The Avr4 is a chitin-binding effector from the tomato pathogen *Cladosporium fulvum* and recognized by the tomato PRR Cf4 leading to a strong HR [28,29]. Our data showed that RsRlpA was not able to suppress PTI-related HR induced by this complex (Fig. 3).

3.4. RsCRP1 targets plant mitochondria and chloroplasts

Knowledge on fungal effectors has expanded over the last years and it is known that they can be localized in different parts of host cells such as apoplast, nucleus and vacuoles [2]. To get insights to the subcellular localization of RsCRP1 in host cells, it was fused with the GFP fluorescence protein at the C-terminus, keeping its signal peptide intact followed by transiently expression in *N. benthamiana* leaves. Examination under confocal microscope 48 h post infiltration showed that RsCRP1 targeted distinct cell compartments, a novel feature for this pathogen (Fig. 4). To clarify localization, RsCRP1-GFP was co-expressed with the ScCOX4-mCherry, a marker of mitochondria [30]. Co-localization was observed, indicating accumulation of RsCRP1-GFP in this organelle (Fig. 4). In addition, co-localization between RsCRP1-GFP and chlorophyll was also seen, suggesting chloroplasts targeting as well (Fig. 4). Analysis of the RsCRP1 amino acid sequence revealed presence of a chloroplast transit peptide (cTP) at the N-terminus, and prediction of localization to the mitochondrial matrix, further support organellar accumulation of this effector.

Effector localization to chloroplasts and mitochondria of host plants has been mostly reported in host-bacteria interactions [31–34]. Similar observations have also emerged from the poplar rust fungal basidiomycete *Melampsora larici-populina* [35]. This fungal pathogen is thought to use its chloroplast-targeted protein 1 (CTP1) effector to subvert host cell machinery for protein sorting [36]. CTP1 also accumulates in the mitochondria [35]. The N-terminus of CTP1 facilitate the organelle targeting. Whether targeting domains that mimics the plant transit system may have evolved via

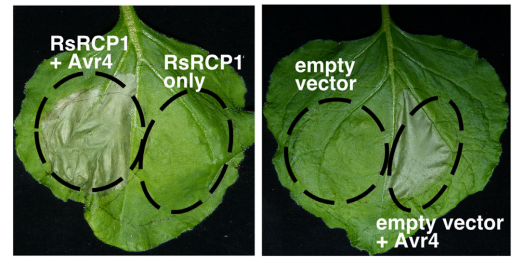

Fig. 3. RsCRP1 does not suppress PTI-induced HR. Leaves were Agro-infiltrated first with the RsCRP1 effector ligated to the pGWB602 binary vector driven by the 35S promoter, followed by HR challenge 24hpi with the Avr4 effector derived from *Cladosporium fulvum* in Cf-4 transgenic *N. benthamiana* plants. Agro-infiltration with empty vector was used as a control. Images taken 3dpi.

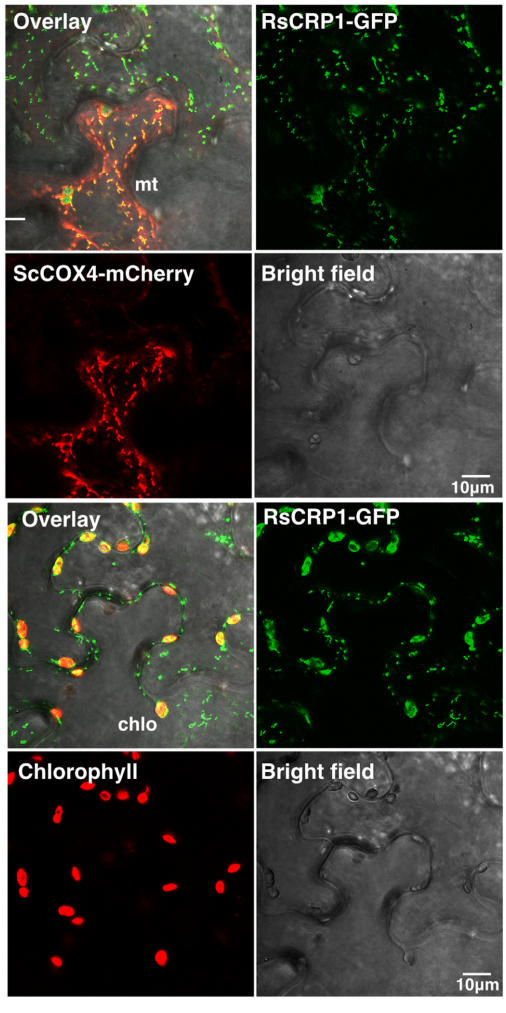

Fig. 4. RsCRP1 is localized to plant mitochondria and chloroplasts. Live-cell imaging of C-terminal GFP-tagged RsCRP1 in *N. benthamiana* leaf epidermal cells. Proteins were expressed in *N. benthamiana* leaves by Agro-infiltration. Monitoring was performed using a laser-scanning confocal microscope with a sequential scanning mode 48 h post infiltration. The GFP and the chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (red) fluorescent signals were collected at 505–525 nm and 680–700 nm, respectively. Mitochondrial localization was assayed using the ScCOX4-mCherry marker (red) and excited at 561 nm and collected at 580–620 nm. (cp): chloroplasts, (mt): mitochondria.

sequence exchange with fungal mitochondrial or horizontal gene transfer process is presently unclear.

Exploitation of an endogenous plant system is an efficient strategy to abate plant defense. To this end, impact on plant cellular compartments added to the list of resistance genes, different phytohormones and gene regulatory pathways that could be targeted by pathogen effectors. Impaired photosynthesis or functions channeled via mitochondria such as production of reactive oxygen species [37] could be an important complement of the *R. solani* effector repertoire affecting other functions than those related to biotrophic or necrotrophic infection stages.

Authors contribution

CD, GT, FD and LH conceived the experiments, GT performed the transformation, confocal microscopy and transient expression in plants and FD performed the virulence assays. All authors contribute to writing of the manuscript and text revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.01.019.

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This thesis is covering analysis of three soilborne pathogens and defence responses in their host plants. New effectors, their plant targets, and additional plant defence components were found. These findings will be a valuable source for future studies on plant disease resistance and pathogen infection biology.

Fredrik Dölfors received his graduate training at the Department of Plant Biology, Swedish University of Agricultural Sciences, Uppsala and obtained a MSc in Genetic and Molecular Plant Science at Uppsala University.

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