Verticillium longisporum and plant immunity responses in Arabidopsis

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Cover: Confocal image of tobacco leaves infiltrated with GFP-tagged NPF5.12 (green) and a plasma membrane mCherry marker (red), and the resulting overlap (yellow) indicating co-localization of the two proteins.
(photo: J. Roos)

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Abstract

Verticillium spp. are soil-borne ascomycete fungi belonging to a subgroup of *Sordariomycetes*, and the three major plant pathogens *Verticillium longisporum*, *V. dahliae* and *V. albo-atrum* cause disease on numerous plant species worldwide. In Sweden, *V. longisporum* poses a threat to *Brassica* oilseed crops, and is thus emphasized in this thesis. Here the early immune responses to *V. longisporum* in the model plant Arabidopsis and recent data on the *V. longisporum* genome are presented.

Three genes of importance in the Arabidopsis–*V. longisporum* interaction were studied. The genes were identified via transcriptome and single nucleotide polymorphism (SNP) analysis. RabGAP22, a RabGTPase-regulating protein, was found to contribute to *V. longisporum* resistance. Pull-down assays revealed SERINE:GLYOXYLATE AMINOTRANSFERASE (AGT1) as an interacting partner during *V. longisporum* infection and the two proteins were shown to co-localize in the peroxisomes. Unexpectedly, a role for RabGAP22 was also found in stomatal immunity. The monoterpene synthase TPS23/27 was on the other hand found to contribute to fungal invasion, by triggering germination of *V. longisporum* conidia. The third gene codes for a nitrate/peptide transporter, NPF5.12. Pull-down experiments and fluorescent imaging revealed interaction between NPF5.12 and a major latex protein family member, NPFBP1. Implications in plant immunity processes of these three genes are further discussed.

The genomes of two Swedish *V. longisporum* isolates were sequenced and found to have a size of approximately 70 Mb and harbor ~21,000 protein-coding genes. Initial analyses revealed that 86% of the *V. longisporum* genomes are shared with *V. dahliae* and *V. albo-atrum*, with a high extent of gene duplications. Large numbers of proteins were predicted to contain secretion motifs, and this group of proteins is presumed to play major roles in the interactions with *V. longisporum* host plants.

In conclusion, this thesis work has revealed new fungal and plant host genes and thereby laid the basis for new plant breeding and disease protection strategies.

Keywords: Arabidopsis thaliana, immunity, pathogen, Rab, terpene, *Verticillium longisporum*

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Dedication

To my father, who immensely proud of his son would have read this thesis in its entirety, pretending to himself and the rest of us that he actually understood what it was about. – I would have loved to play along in his performance.

If sub specie aeternitatis there is no reason to believe that anything matters, then that does not matter either, and we can approach our absurd lives with irony instead of heroism or despair.

- Thomas Nagel

True wisdom comes to each of us when we realize how little we understand about life, ourselves, and the world around us.

- Socrates

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 **Roos, J.**, Bejai, S., Oide, S. and Dixelius, C. (2014) *RabGAP22* is required for defense to the vascular pathogen *Verticillium longisporum* and contributes to stomata immunity. *PLoS ONE*, **9**, e88187.

II **Roos, J.**, Bejai, S., Dixelius, C. (2014) *Arabidopsis thaliana* monoterpene synthase *TPS23/27* triggers infection by the fungal pathogen *Verticillium longisporum* (manuscript).

III **Roos, J.**, Bejai, S. and Dixelius, C. (2014) AtNPF5.12 is a nitrate/peptide transporter involved in the defense response to the fungal pathogen *Verticillium longisporum*. (manuscript).

IV Fogelqvist, J., Bejai, S., Kamber, T., **Roos, J.**, Schwelm, A. and Dixelius, C. (2014) The first glimpse of the *Verticillium longisporum* genome. (manuscript).

Additional publications

Roos, J., Hopkins, R., Kvarnheden, A. and Dixelius, C. (2011) The impact of global warming on plant diseases and insect vectors in Sweden. *Eur. J. Plant Pathol.* **129**, 9-19.

Wallenhammar, A.-C., Almquist, C., Schwelm, A., **Roos, J.**, Marzech-Schmit, K., Jonsson, J. and Dixelius, C. (2014) Clubroot, a persistent threat to Swedish oilseed rape production. *Can. J. Plant Pathol.* (in press). doi: 10.1080/07060661.2013.870606.

The contribution of Jonas Roos to the papers included in this thesis was as follows:

I Taken part in the planning of the study, and performed a large part of the experiments. Analyzed and interpreted the data, and co-authored the paper.

II Taken part in planning of the study. Analyzed and interpreted the data, and co-authored the manuscript. Performed most of the experimental work.

III Taken part in planning of the study. Analyzed and interpreted the data, and co-authored the manuscript. Performed a large part of the experimental work.

IV Taken part in planning of the study, preparation and collection of samples for sequencing, interpretation of data. Assisted in writing of the manuscript.

1 Introduction

Soil is a complex matrix, containing anywhere between 5,000 and 50,000 unique species of microorganisms in each gram of soil (Schloss and Handelsmann, 2006; Dance, 2008). For a plant, interaction with these microorganism can be both beneficial and detrimental, for example fungal arbuscular mycorrhizas supply the plant with nutrients, and plant growth-promoting bacterial species aid in plant defense responses (Pineda *et al*., 2010; Campos-Soriano *et al*., 2012; Zamioudis and Pieterze, 2012). In contrast, interactions with soil-borne fungal, oomycete and bacterial pathogens are detrimental for the plant (Loreti *et al*., 2008; Klosterman *et al*., 2009; Akino *et al*., 2014).

In the United States it is estimated that 90% of the 2,000 major diseases on crops are caused by soil-borne pathogens (AgBioResearch, 2011). These soil-borne pathogens complete part of their lifecycle in the soil, where their resting structures persist for months or even years, and are triggered to germinate and infect plant roots whenever a susceptible plant appears. In Europe, major crop damages are cause by soil-borne plant pathogens in the genera *Sclerotinia* (Clarkson *et al*., 2012), *Phytophthora* (Jönsson *et al*., 2005), *Verticillium* (Johansson *et al*., 2006a), *Fusarium* (Peters *et al*., 2008), *Ralstonia* (Loreti *et al*., 2008), and others. With the ongoing global warming, the impact of these diseases in Europe is predicted to increase. The Nordic countries may become particularly affected, since the prolonged vegetative period will lead to an increased timespan for pathogen survival and multiplication in the soil (Roos *et al*., 2011). For example, predicted increases of Fusarium wilt (SJV, 2007) could result in higher levels of mycotoxins in grains used for both animal feed and human consumption (Fung and Clark, 2004).

This thesis is on plant immunity responses to *V. longisporum*, a soil-borne fungal pathogen of major importance for *Brassica* species grown in Sweden. The emphasis of the work is on genes invoked as part of the plant immunity

response in Arabidopsis. The intention of this summary is to highlight the latest understanding on defense responses and genomic information on plant pathogenic fungi that relates to *V. longisporum*.

1.1 *Verticillium* fungal pathogens

1.1.1 Species and plant host range

Verticillium spp. are ascomycete fungi identified to cause Verticillium wilt on a wide number of important crop species, including cotton, tomato, and olive tree (Pegg and Brady, 2002). In Sweden, *Verticillium longisporum* and *V. dahliae* are known to infect several important crop species, such as oilseed rape and sugar beet (Steventon *et al*., 2002; Johansson *et al*., 2006a). The related *V. albo-atrum* is also of some importance for strawberry (Nallanchakravarthula, 2013). In contrast to *V. dahliae*, *V. longisporum* has a preference for species within the family *Brassicaceae*, including the important crop species oilseed rape, *Brassica napus* (Johansson *et al*., 2006a; Eynck *et al*., 2007; Zhou *et al*., 2006).

Based on the phenotype of its conidia and DNA sequence analyses, *V. dahliae* var. *longisporum* was recognized as a separate species by Karapapa *et al*. (2001), and later also by Steventon *et al*. (2002). The most recent studies suggest *V. longisporum* is a diploid species that has arisen at least three times, via hybridization between different *V. dahliae* isolates and the so far unidentified ancestor species A1 and D1 (Inderbitzin *et al*., 2011a). Phylogenetic studies recognize ten different *Verticillium* species, divided into two major clades, Flavexudans and Flavnonexudans (Inderbitzin *et al*., 2011b; 2013), with *V. longisporum* placed among the Flavnonexudans.

1.1.2 Disease cycle of *V. dahliae* and *V. longisporum*

The disease cycle of *V. dahliae* and *V. longisporum* begins with the germination of specialized resting structures, microsclerotia. These consist of aggregates of melanized hyphae that are highly persistent and capable of surviving several years in the soil (Perry and Evert, 1984; Hawke and Lazarovits, 1995). Via so far unknown mechanisms, microsclerotial germination is triggered by the presence of a suitable host plant. Subsequently, fungal hyphae begin to colonize the root tissues, followed by direct penetration of root epidermal cells and finally entry into the xylem elements (Zhou *et al*., 2006; Eynck *et al*., 2007). The xylem is fairly poor of nutrients and to adapt to this, *V. longisporum* may acquire additional nutrients by digestion of cell walls and induction of ion leakage from neighboring cells (Singh *et al*., 2009; Klosterman *et al*., 2011; Yadeta and Thomma, 2013). While in the xylem,

fungal toxins and possibly occlusion of xylem tissues by the fungus lead to the characteristic wilting symptoms caused by *V. dahliae* (Hou *et al*., 2008; Laouane *et al*., 2011). In contrast, wilting symptoms are not seen on *B. napus* infected with *V. longisporum* (Dunker *et al*., 2008; Floerl *et al*., 2008; 2010; Ralhan *et al*., 2012). For both fungal species however, typical disease symptoms include stunting, chlorosis of infected leaves and premature senescence (Zhou *et al*., 2006; Eynck *et al.*, 2007). Formation of senescent and dying tissues is associated with the transition from a biotrophic to a saprophytic stage of both fungi. Here, breakdown of tissues by *V. dahliae* necrosis-inducing proteins may be of importance (Zhou *et al*., 2012; Santhanam *et al*., 2013). As the nutrient content in the senescing tissues start to decrease, both fungi begin producing microsclerotia. A process known to involve the hydrophobin gene *VDH1,* and *GARP1 in V. dahliae* (Klimes and Dobinson, 2006; Klimes *et al*., 2008; Gao *et al*., 2010). The latter, a gene coding for a glutamic acid-rich protein. The disease cycle is completed when the *V. dahliae* and *V. longisporum* microsclerotia fall to the ground together with the plant debris.

1.2 Fungal Genomes

1.2.1 Fungal genomes, transcriptomes and proteomes

The swift advances in sequencing technology and analysis (Koboldt *et al*., 2013) provide new resources to assess genes associated with fungal pathogenicity (Van de Wouw and Howlett, 2010). Since the first genome sequence of a fungal plant pathogen, *Magnaporthe oryzae* (Dean *et al*., 2005), a large number of fungal genomes have become available (Kemen *et al*., 2011; Ohm *et al*., 2012; de Wit *et al*., 2012). Genomic comparisons have shown that numerous events of gene duplications and gene losses have taken place during the evolution and host adaptation of plant pathogens (Kemen *et al*. 2011; Ohm *et al*., 2012; de Wit *et al*., 2012; Stukenbrock, 2013). Comparison of genomes from pathogenic and non-pathogenic fungi is also a direct approach to new information on effector molecules and their evolution (de Jonge *et al*., 2011; Schmidt and Panstruga, 2011; Giraldo and Valent, 2013). One interesting finding is the predicted horizontal gene transfer from fungi to oomycetes, explaining how the latter became plant pathogens (Richards *et al*., 2011).

Genome sequences of *V. dahliae* and *V. albo-atrum* isolates are now available, and these genomes vary in size between 30.3 and 35.0 Mb (Klosterman *et al*., 2011). In a whole-genome survey of the *V. dahliae* and *V. albo-atrum* genomes, ~1–4% of genomic sequences were attributed to repetitive sequences (Amyotte *et al*., 2012). Among the identified transposable

elements (TE), *Copia*, *Gypsy* and *Tc1/Mariner* were among the most frequent classes. These TEs also appeared to be more common in gene-rich areas of the *V. dahliae* VdLs.17 genome. A more recent genome comparison of eleven *V. dahliae* isolates revealed a large variation in regions enriched for long terminal repeat (LTR) retrotransposons and effector genes (de Jonge *et al*., 2013). Transposon-mediated rearrangements in these regions may help provide sequence diversity useful for host adaptation in this asexual fungus. Alongside genome sequencing, there is also increasing sets of transcriptome and proteome data for *V. dahliae* (El-Babany *et al*., 2010; Singh *et al*., 2012).

1.2.2 Effector proteins

Effectors are small proteins secreted by plant pathogens to overcome the host defenses (Hogenhout *et al*., 2009; Giraldo and Valent, 2013). Typically, these secreted proteins have low sequence homology to other known proteins and their functions are often poorly understood. Among the studied effectors is AvrPtoB from *Pseudomonas syringae* (Abramovitch *et al*., 2003), which suppress programmed cell death when delivered to the host cells via the specialized type III secretion system. The Avr2 effector from *Cladosporium fulvum,* is in contrast directed at suppressing Arabidopsis extracellular cysteine proteases, leading to increase susceptibility several fungal pathogens (van Esse *et al*., 2008).

The *V. dahliae* genome contains 780 predicted secreted proteins, the function of which are mostly unknown (Klosterman *et al*., 2011). Best studied is the *V. dahliae Ave1* effector interacting with the tomato *Ve1* disease resistance gene (de Jonge *et al*., 2012). The PevD1 effector is another protein secreted from *V. dahliae*, and its N- and C-terminal domain are responsible for triggering of systemic induced resistance (SAR) and hypersensitive response (HR) in tobacco host plants, respectively (Wang *et al*., 2012b; Liu *et al*., 2013). LysM effectors are secreted by several pathogens and bind chitin, which is thereby prevented to bind host plant receptors (de Jonge *et al*., 2010; Kombrink and Thomma, 2013; Sanchez-Vallet *et al*., 2013). The *V. dahliae* genome contains six LysM effectors, but only one of these is expressed *in planta* and required for *V. dahliae* virulence on tomato (Klosterman *et al*., 2011; de Jonge *et al*., 2013). In contrast to LysM effectors, the necrosis- and ethylene-inducing-like protein (NLP) family members in *V. dahliae* have a cytotoxic effect, and two of the seven NLPs have been shown to induce cell death in *N. benthamiana* (Santhanam *et al*., 2013). Similar to the limited information on effector function, not much is known on their transcriptional regulation. However the *V. dahliae* transcription factor VdSge1 was recently shown to be

required for expression of several putative effector genes (Santhanam and Thomma, 2013).

1.3 *Brassica* crops

1.3.1 Cultivation and economic importance

Oilseed rape (*Brassica napus*) is the third most important source of vegetable oil in the world, after soybean and palm oil (www.oilworld.de). It is also the most important oilseed crop in China, Canada, Europe and Australia. Both spring and winter types of *B. napus* and the closely related *B. rapa* (turnip rape) are grown in Europe, and produce high-quality oil used for food, feed and biodiesel. The total acreage in Europe comprises approximately 6.4 Mha.

In 2012, approximately 110,000 ha of *B. napus* and 3,000 ha of *B. rapa* were harvested in Sweden (www.svenskraps.se). Several *B. oleracea* subspecies, including white cabbage (428 ha), cauliflower (293 ha) and broccoli (255 ha) are also of importance (SJV, 2013). *Brassica* oil crops are presently grown in Sweden due to two major factors, the high market price of vegetable oil and the favorable impact they have on the crop rotation scheme. Incorporating *Brassica* species crop rotation practices lead to inhibitory effects on cereal and potato pathogens, and also lower the need of fertilizers (Kirkegaard *et al*., 1996, www.svenskraps.se).

1.3.2 Genomes and evolution of plant species in *Brassicaceae*

The *Brassicaceae* family is ancient and contains several important model and crop species, including *Amoracia rusticana* (horseradish), *Raphanus sativus* (radish), *Brassica napus* (oilseed rape) and Arabidopsis. The genome sequences of several species are available (Table 1), contributing to our understanding of evolutionary events within this plant family (Oh *et al*., 2010; Franzke *et al*., 2011; Kiefer *et al*., 2014).

The *Brassica* genus includes the cultivated diploid species *B. rapa* (turnip rape), *B. nigra* (black mustard), and *B. oleracea* (cabbage), and the allotetraploid species *B. juncea* (Indian mustard), *B. napus* (oilseed rape), and *B. carinata* (Abyssinian mustard). The relationship between these *Brassica* species was described during the 1930s (Figure 1). Following the split of *Arabidopsis* and *Brassica,* approximately 15-20 million years ago (Yang *et al*., 1999), the ancient *Brassica* genome underwent triplication followed by the divergence into *B. rapa* (A genome), *B. nigra* (B genome) and *B. oleracea* (C genome). This hexaploidization event is identified today as shared gene synteny with Arabidopsis and triplicated regions in the A, B and C genomes (Cheng *et al*., 2012; Navabi *et al*., 2013).

Table 1. Sequenced diploid plant species within the family *Brassicaceae*.

Species	Genome size (Mb)	Gene models	Reference
Arabidopsis thaliana	119	27,416	AGI, 2000
Arabidopsis lyrata	207	32,670	Hu *et al*., 2011
Schrenkiella parvula (*Thellungiella parvula)*	140	30,419	Dassanayake *et al*., 2011
Eutrema salsugineum (*Thellungiella salsuginea*)	241	26,531	Wu *et al*., 2012; Yang *et al*., 2013
Brassica rapa	285	41,174	Wang *et al*., 2011
Capsella rubella	135	26,521	Slotte *et al*., 2013

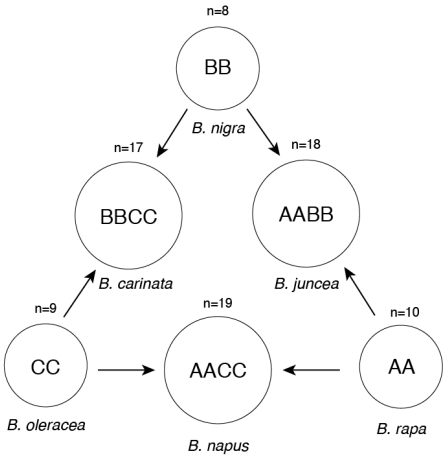

Figure 1. The three allotetraploid species *Brassica napus*, *B. carinata*, and *B. juncea* are a result of sexual crosses between the three diploid species *B. nigra*, *B. oleracea*, and *B. rapa* (U, 1935; Mizushima, 1950).

Despite the genome triplication, the *B. rapa* genome contains approximately twice the number of genes compared to *A. thaliana*, implying the *B. rapa* genome is currently undergoing a process of diploidization (Mun *et al*., 2009). Many of the triplicated genes appear to have been lost due to redundancy, except genes responding to hormones and environmental stimuli, which have been retained (Fang *et al*., 2012).

The resistance to *V. longisporum* in *B. napus* is generally low, and there have been attempts to introduce resistance by interspecific hybridization between cultivars of resistant *B. rapa* and *B. oleracea* species (Rygulla *et al*., 2007). The identification and characterization of microsatellite markers in *B. rapa, B. oleracea* and *B. napus* (Shi *et al*., 2013) and the recent construction of a consensus genetic map of *B. napus* (Raman *et al*., 2013) will likely further assist studies of genomic evolution and *B. napus* breeding efforts (Cai *et al*., 2012).

Contributing to the understanding of species evolution is the ongoing sequencing and characterization of 1001 genomes of wild Arabidopsis

accessions (Weigel and Mott, 2009, Cao *et al*., 2011). A comparative study aimed specifically at 180 Swedish accessions revealed a large variation in genome size, ranging from 160 Mb to 180 Mb, which was attributed mainly to variation in the copy number of 45S rDNAs (Long *et al*., 2013). Additional studies of the methylomes of 152 Arabidopsis accessions revealed the presence of several hundred methylated quantitative trait loci (QTL) (Schmitz *et al*., 2013). These loci were specifically targeted by RNA-directed DNA methylation and were activated and epigenetically activated in seeds and pollen.

1.4 The plant root

1.4.1 Arabidopsis root structure and vascular tissues

At the center of the Arabidopsis root are the xylem and phloem tissues, specialized for transport of water and organic nutrients respectively (Figures 2 and 3) (Lucas *et al*., 2013).

Xylem is the main water-conducting tissue, and transports certain hormones and mineral elements including nitrates (Myburg *et al*., 2013). Living parenchyma and xylem fiber cells provide structural support for the water conducting tracheary elements. These consist of two types of dead cells, tracheids and vessel elements, both with highly lignified secondary cell walls, making them impermeable to water.

The phloem carries sugars, organic nutrients, hormones, and signaling compounds. Active phloem loading and unloading of solutes by companion cells is believed to drive the transport by osmotic pressure (Turgeon and Wolf, 2009; De Schepper *et al*., 2013), and connected sieve-tube elements facilitate the transport.

1.4.2 Hormones and root development

Much of the understanding on hormones and root development comes from studies on Arabidopsis. The processes involved are highly complex and include numerous signaling pathways, plant hormones and transcription factors. In addition, these processes are influenced by light, water, gravity, nutrients and interactions with soil microorganisms (Garay-Arroyo *et al*., 2012; Jung and McCouch, 2013). Roles of small signaling peptides and small RNAs in root development are also emerging (Meng *et al*., 2010; Khan *et al*., 2011; Delay *et al*., 2013).

Among the most important plant root hormones are auxins, of which indole-3-acetic acid (IAA) is the most common naturally occurring. Establishment of a root to shoot auxin gradient as well as local auxin maxima by auxin influx

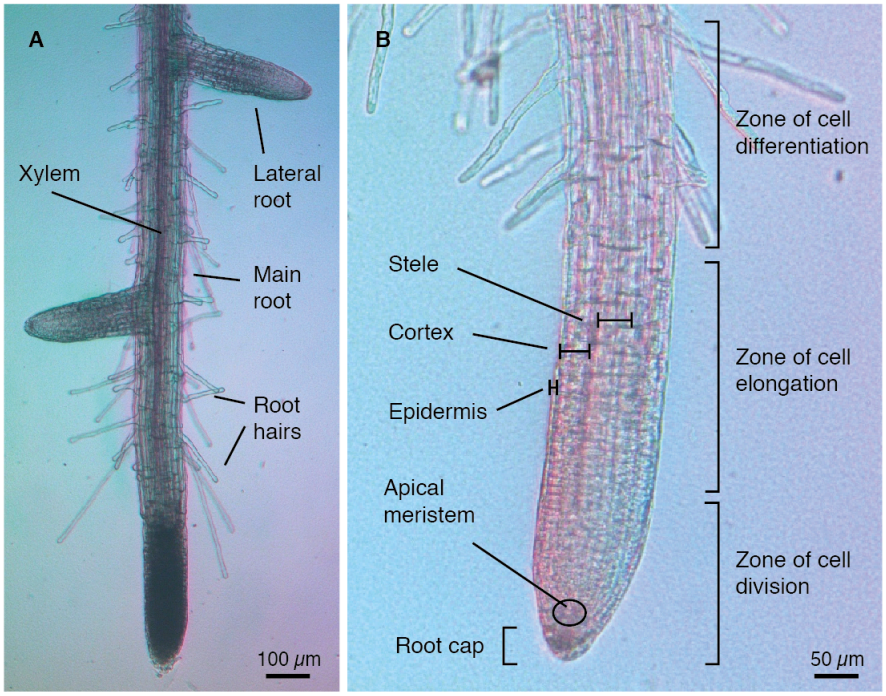

Figure 2. The Arabidopsis root. (A) Overview of root showing the main root, lateral roots, root hairs and xylem. (B) Close-up of root tip, showing the root cap and apical meristem. Further up, the epidermis, cortex, and stele are indicated. (Photo: J. Roos).

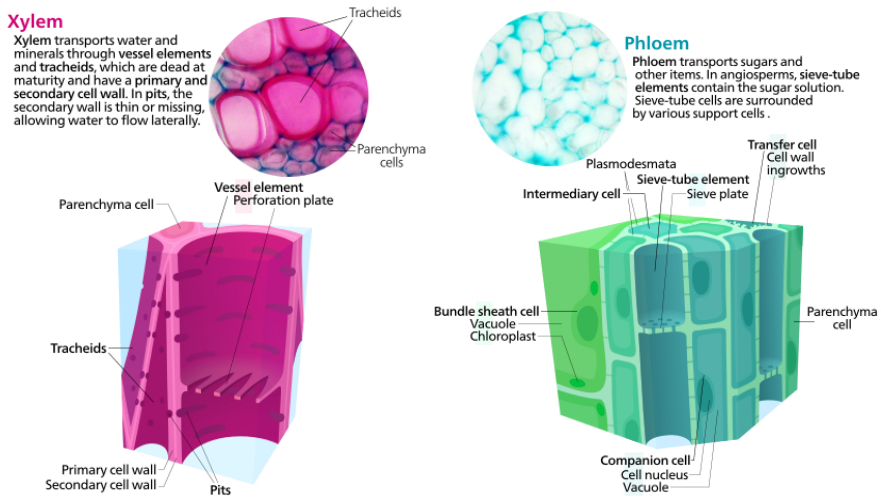

Figure 3. Schematic structure of xylem and phloem. (Image by Kelvin Song, licenced under Creative Commons Attribution-ShareAlike 3.0 Unported licence (http://creativecommons.org /licenses/by-sa/3.0/deed.en).

and efflux carriers guide the root cell division and differentiation (Grieneisen *et al*., 2007; Petersson *et al*., 2009), including the well-studied function for IAA in the formation of lateral roots from pericycle cells (Dubrovsky *et al*., 2008; Fukaki and Tasaka, 2009). The presence of a root to shoot auxin gradient, with a maximum in the root tip, is often referred to as polar auxin transport, and is mediated by the influx carriers AUXIN RESISTANCE 1 (AUX1) and LIKE AUX (LAX) proteins, whereas efflux carries are exemplified by the PIN FORMED (PIN) proteins (Petrásek *et al*., 2006; Péret *et al*., 2012).

Auxins are involved in crosstalk with both brassinosteroids (BR) and gibberrelins (GA) (Depuydt and Hardtke, 2011; Durbak *et al*., 2012). For example, auxin and repression of DELLA transcription factors are the two main components that induce GA-promoted root elongation (O'Neill *et al*., 2010; Reid *et al*., 2011). Furthermore, auxin-gibberrelin interaction has a role in lateral root formation (Farquharson, 2010), a process that is negatively regulated by cytokinin and abscisic acid (ABA) (Fukaki and Tasaka, 2009) and positively regulated by jasmonates (Sun *et al*., 2009). The negative effect of cytokinins and ABA on lateral root formation seems to be caused by disturbance of *PIN*-medated auxin transport, preventing the formation of the auxin gradient required for lateral root primordium growth (Laplaze *et al*., 2007; Shkolnik-Inbar and Bar-Zvi, 2010). Besides crosstalk with auxin in auxin-mediated root elongation (Yoshimitsu *et al*., 2011), BRs are involved in root cell division and expansion (Ibañes *et al*., 2009; Gonzáles-García *et al*., 2011), as well as crosstalk with GAs (Li and He, 2013).

1.5 Plant immunity

1.5.1 Phytoalexins and pathogenesis-related proteins

Phytoalexins are plant antimicrobial compounds that accumulate in response to pathogen infection (Biggs, 1972; Ahuja *et al*., 2012). For example in Arabidopsis, the two tryptophan-derived phytoalexins camalexin and indole-3-carboxylic acid accumulate in roots of *V. longisporum*-infected plants (Iven *et al*., 2012). Many phytoalexins are terpene-derived and display inhibitory effects on a wide range of fungal and bacterial species (Pitarokili *et al*., 2003; Yokose *et al*., 2004; Simić *et al*., 2004; Zuzarte *et al*., 2009; Arslan and Dervis, 2010). Other phytoalexins are derived from alkaloids, flavonoids and glycosteroids (Ahuja *et al*., 2012), including the rice flavonoid sakuranetin, for which the key biosynthetic enzyme was recently identified (Shimizu *et al*., 2012).

Similar to phytoalexins, pathogenesis-related (PR) proteins are produced in plants in response to pathogen infection. PR proteins comprise proteinase inhibitors, defensins, thionins and lipid transfer proteins, and are described in a number of excellent reviews (Van Loon *et al*., 2006; Sels *et al*., 2008). Well-studied examples include the antifungal plant defensin PDF1.2 (Penninckx *et al*., 1996), the β-1,3-glucanase PR2 (Antoniw *et al*., 1980) and the chitinase PR3 (Verburg and Huynh, 1991).

1.5.2 Defense signaling

Compared to vertebrates, plants do not have a circulatory system with mobile immune cells. Instead, it is believed that each plant cell is capable of initiating a defense response (Jones and Dangl, 2006; Spoel and Dong, 2012). Plant defense responses are complex, especially due to the crosstalk between different hormone signaling pathways. Consequently, the current knowledge is based on plant interactions with a few well-studied pathogens, such as the bacterium *Pseudomonas syringae* (Xin and He, 2013).

Plants have evolved two classes of immune receptors to detect molecules from foreign organisms. The first consists of membrane-localized pattern recognition receptors (PRRs) that detect microbe-associated molecular patterns (MAMPs), leading to MAMP-triggered immunity (MTI) (Nürnberger and Brunner, 2002; Parker, 2003; Beck *et al*., 2012). PRRs recognize evolutionarily conserved and essential structures of the pathogen, such as chitin from fungal cell walls (Kaku *et al*., 2006), lipopolysaccharides (LPS) from gram-negative bacteria (Zeidler *et al*., 2004), short peptides derived from bacterial flagellin (Gómez-Gómez and Boller, 2000) or the elongation factor EF-Tu (Zipfel *et al*., 2006). Binding of MAMPs to their respective PRRs activates downstream defense signaling, ultimately leading to responses including the production of antimicrobial compounds, production of reactive oxygen species (ROS), and deposition of callose to strengthen the cell wall (Jones and Dangl, 2006; Koeck *et al*., 2011).

Cytosolic and trans-membrane plant resistance (R) proteins define the second class of immune receptors and have the capacity to detect isolate-specific pathogen effectors, encoded for by avirulence (*Avr*) genes. Typically, these *Avr* effectors are secreted by the pathogen to evade the MAMP-triggered immunity response, and therefore bind to either the PRRs or their interactors. The *Pseudomonas syringae* effector AvrPto inhibits immunity by directly binding to the MAMP receptor FLS2 (Xiang *et al*., 2008). Other examples include the tomato I-2 protein recognizing the Avr2 effector from *F. oxysporum* (Houterman *et al*., 2009). Cytosolic R proteins typically consist of a variable N-terminal domain, a nucleotide-binding (NB) domain and an N-

terminal leucine-rich repeat (LRR) domain (Meyers *et al*., 2003; Maekawa *et al*., 2011; Bonardi *et al*., 2012). The N-terminal domains are often of the coiled-coil (CC) or Toll/interleukin-1 (TIR) classes, and mediate protein-protein interactions and effector binding. The central NB domain forms an ATP-binding pocket, and is required for R protein activation. Finally, the LRR domains function mainly in recognition and binding to pathogen effectors. This recognition leads to what is referred to as effector-triggered immunity (ETI). ETI is typically more specific than MAMP-triggered immunity and often leads to a localized cell-death, described first as HR by Stakman (1915), particularly effective against biotrophic pathogens (reviewed by Zurbriggen *et al*., 2010).

1.6 Known immune responses to *V. longisporum*

1.6.1 Xylem events

Infected *B. napus* plants secrete several antifungal compounds into the xylem, and consequently xylem sap extracted from infected plants at 21 dpi inhibits the growth of *V. longisporum* (Floerl *et al*., 2008). Proteins up-regulated in the xylem sap include endochitinases and β-1,3-glucanases, indicating that the xylem defense response may include direct degradation of the fungal cell wall.

Strengthening of the xylem by increased synthesis and deposition of lignin is a well-known response to *Verticillium* infection and may contribute to increased resistance by preventing the spread of the pathogen (Eynck *et al*., 2009; Gayoso *et al*., 2010; Shi *et al*., 2012). *V. longisporum* invasion also triggers expression of the transcription factor *VASCULAR-RELATED NAC DOMAIN 7 (VND7)* in Arabidopsis, leading to *de no*vo synthesis of xylem elements (Reusche *et al.*, 2012). Newly formed xylem elements can be seen at 21 dpi, and presumably compensate for reduced water transport due to vascular obstruction by the fungus.

1.6.2 Resistance genes and defense signaling

To date, no specific resistance genes towards *V. longisporum* are identified. In contrast, the *V. dahliae*-tolerance (*VET1*) and *Ve* loci contribute to *V. dahliae* resistance in Arabidopsis and tomato respectively (Kawchuk *et al*., 2001; Veronese *et al*., 2003). The *Ve* locus in tomato contains two closely linked genes, *Ve1* and *Ve2,* coding for LRR-containing receptor-like proteins (Kawchuk *et al*., 2001). Ve1 recognizes the Ave1 effector from *V. dahliae*, as well as the Ave1 homologs from *F. oxysporum* and *Cercospora beticola* (de Jonge *et al*., 2012). When expressed in Arabidopsis, *Ve1* confers resistance to *V. dahliae*, but not to *V. longisporum* (Fradin *et al*., 2011). The *Ve1*-mediated resistance is fairly well characterized and includes increases in H_2O_2,

peroxidases and lignins (Gayoso *et al*., 2010), and requires the defense signaling components *BRI1-ASSOCIATED RECEPTOR KINASE* (*BAK1*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *NON RACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*) (Fradin *et al*., 2009; 2011). The *Ve1*-mediated response is further accompanied by HR in tobacco and tomato but not in Arabidopsis (Zhang *et al*., 2013). A *Ve1*-like gene is also identified to confer resistance to *V. dahliae* in cotton (Zhang *et al*., 2011; 2012).

The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are in general crucial components in plant defense signaling (Glazebrook, 2005; Verhage *et al*., 2010). The specific contribution of these hormones to disease is complex, not least due to the extensive crosstalk between them. *V. longisporum* is no exception to this, and results on the contribution of SA, JA and ET to immunity responses are often contradictory, which may in part be attributed to differences in infection procedures and experimental setup.

In a study of responses to *V. longisporum* in Arabidopsis, hormone pretreatments and infection studies revealed a general dependency on ET, whereas JA and SA appeared to be of lesser importance (Johansson *et al*., 2006b). More recent studies have supported a general independency of JA signaling, but some genes including *NDR1*, *JASMONATE RESISTANT 1* (*JAR1*) and *NON-EXPRESSER OF PR GENES 1* (*NPR1*) still appear important for resistance signaling (Pantelides *et al*., 2010, Fradin *et al*., 2011).

The active signaling component of JA, JA-Ile, is detected by the receptor CORONATINE INSENSITIVE (COI1) (Xie *et al*., 1998; Chini *et al*., 2009). However COI1 does not appear to have a role in JA signaling in the *V. longisporum*-specific defense, since *coi1-16* plants do not show increased susceptibility (Johansson *et al*., 2006b; Fradin *et al*., 2011). Rather, COI1 has a JA-independent root-to-shoot signaling function, and is required for completion of the *V. longisporum* disease cycle and microsclerotial development (Ralhan *et al*., 2012).

No difference in the SA content in *V. longisporum* inoculated Arabidopsis roots is found during the first few days of infection (Iven *et al*., 2012), however from 7 dpi and onwards a significant increase is found for the SA marker genes *PATHOGENESIS-RELATED 1* and *2* (*PR1* and *PR2*) (Johansson *et al*., 2006b; Pantelides *et al*., 2010). Involvement of SA in the later stages of infection is also supported by an increased SA content in the xylem of *V. longisporum*-challenged *B. napus* from 14 dpi and onwards (Ratzinger *et al*., 2009).

A contribution of ET to *V. longisporum* susceptibility is rather well established in Arabidopsis, as *etr1-1* and *ein3-1* mutants display enhanced resistance, associated with a decrease in fungal vascular colonization (Johansson *et al*., 2006b; Fradin *et al*., 2011; Pantelides *et al*., 2010).

RNA silencing is the mechanism by which the expression of RNA molecules is down-regulated or suppressed by small 21–26 nt RNAs (Baulcombe, 2004; Eamens *et al*., 2008). The process is well-studied in plant development and defense to viruses (Pumplin and Voinett, 2013; Schuck *et al*., 2013; Vargason *et al*., 2013). RNA silencing is also suggested as important for *V. longisporum* resistance in Arabidopsis, where mutation of conserved components in the RNA silencing machinery leads to increased disease development (Ellendorf *et al*., 2009). Small non-coding RNAs are also identified in cotton roots inoculated with *V. longisporum*, where resistant and susceptible cultivars activate transcription of separate RNA pools with peaks at 21 nt and 24 nt respectively (Yin *et al*., 2012).

1.6.3 Immunity-associated genes studied in this thesis

In this thesis, the role of three different genes in the Arabidopsis-*V. longisporum* interaction is studied: *Rab GTPase ACTIVATING PROTEIN 22* (*RabGAP22*), *1,8-CINEOLE SYNTASE* (*TPS23/27*) and *NITRATE PEPTIDE TRANSPORTER 5.12* (*NPF5.12*). Provided below is an overview of these gene families.

1.7 Small GTPases

Small GTP-binding proteins (20–40 kD) are ubiquitous in eukaryotic organisms and are divided into five families, Ras, Rho, Rab, Arf/Sar and Ran, based on their protein structures (Jiang and Ramachandran, 2006). These proteins cycle between an active GTP-bound and an inactive GDP-bound state, a process which is conserved among eukaryotic cells (Cherfils and Zeghouf, 2013). The inherent hydrolysis of GTP to GDP by the individual GTPase is slow, but is increased by several orders of magnitude by GTPase activating proteins (GAPs) (Barr and Lambright, 2010). The reverse process, dissociation of GDP and subsequent binding of GTP, is facilitated by guanine exchange factors (GEFs) (Figure 4). Addition of geranylgeranyl groups to C-terminal cysteines in Rab, Rho and Ras GTPases forms a lipid anchor that mediates their association to membranes. Guanine dissociation inhibitors (GDIs) regulate cytosol/membrane localization of small GTPases, by binding to the prenylated domain, thereby preventing membrane association (Cherfils and Zeghouf, 2013).

Ninety-three GTP-binding protein gene homologs are identified in the Arabidopsis genome, including Rab, Rho, Arf and Ran GTPases (Vernoud *et al*., 2003; Kowalczyk *et al*., 2011). The Rab GTPases constitute the largest family with 57 members, divided into eight subfamilies. Rab GTPases take part

in mechanisms underlying intracellular membrane trafficking such as vesicle budding, vesicle delivery, vesicle tethering, and vesicle fusion with the target compartment (Campanoni and Blatt, 2007). Rab proteins are also involved in other processes including hormone signaling and stress responses (Moshkov *et al*., 2003; Qi *et al*., 2005; Nielsen *et al*., 2008). GAPs are regulated in different ways, including interactions with other proteins and phospholipids, as well as phosphorylation and proteolysis of the GAPs (Bernards and Settleman, 2004).

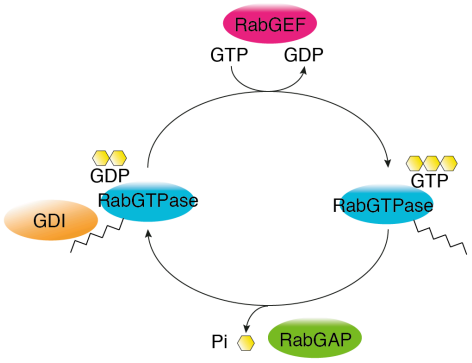

Figure 4. The RabGTPase cycle. GEF: Guanine Exchange Factor, GAP: GTPase-Activating Protein; GDI: Guanine Dissociation Inhibitor, Pi: inorganic phosphate.

1.8 Terpene secondary metabolites

1.8.1 Biosynthesis, functional diversity and gene regulation

Volatile secondary metabolites emitted from the plant are referred to as essential oils. They are typically a mixture of terpenes, alkaloids, phenols, alcohols, aldehydes and ketones, with terpenes often being a major constituent (Hyldgaard *et al*., 2012). Some of the volatile secondary metabolites attract pollinating insects (Wright *et al*., 2005; Unsicker *et al*., 2009), whereas others assist in protecting the plant from herbivores (Heil and Ton, 2009; Unsicker *et al*., 2009; Santos *et al*., 2010; Köllner *et al*., 2013) or bacterial and fungal infections (Tabanca *et al*., 2006; Gachkar *et al*., 2007; Joy *et al*., 2007; Terzi *et al*., 2007; Yi et al., 2009; Zuzarte *et al*., 2009).

Terpenes are synthesized from five-carbon isoprene units (C_5), resulting in monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and carotenoids (C_{40}). When modified by e.g. oxygenation or addition of methyl groups, they are often referred to as terpenoids. The number of plant terpene synthases is large, and many of them are multi-product enzymes (Degenhardt *et al*., 2009). In Arabidopsis, 32 functional terpene synthases are recognized (Aubourg *et al*., 2002) and about a third of these are functionally characterized (Chen *et al*., 2011). Current data

suggest that mono- and diterpenes are synthesized mainly in plastids, whereas sesquiterpenes are synthesized in the cytosol. Natural variation in this subcellular localization of terpene synthases also contributes to differences in terpene emission between Arabidopsis accessions (Huang *et al*., 2010).

1.8.2 Emission of terpenoids and effects on pathogens

Essential oils containing terpenes are shown to inhibit several pathogens, including *V. dahliae* (Arslan and Dervis, 2010). The specific inhibitory mechanism of terpenoids on bacterial and fungal pathogens vary. Disruption of cellular and organellar membranes is the most common, and other mechanisms include cell wall degradation and damage to membrane proteins (reviewed in Nazzaro *et al*., 2013). For example, the fungicidal effect of carvacrol and thymol on *Candida albicans* is suggested to be due to disruption of the plasma membrane, and inhibition of the biosynthesis of ergosterol, an essential component of the fungal cell membrane (Ahmad *et al*., 2011). Carvacrol and thymol thus function in a way similar to fluconazole (Diflucan®), a fungistatic human drug for treatment of fungal infections, to which there are signs of increasing resistance (Pfaller *et al*., 2010; Arendrup, 2014).

Emission of terpenoid secondary metabolites is an area of ongoing research and there is currently evidence for direct transport by ATP-binding cassette transporters (Yazaki *et al*., 2006; Crouzet *et al*., 2013). Similar mechanisms are used by fungal pathogens to overcome the effect of host terpenoids. For example, the fungus *Grosmannia clavigera* has an ABC-transporter GcABC-G1 capable of direct secretion of terpenes emitted by the pine host (Wang *et al*., 2012a). Consequently, deletion of this gene in the fungus makes it more susceptible to monoterpenes.

1.8.3 1,8-cineole synthase

Crystal structures are available for a few plant monoterpene synthases (Whittington *et al*., 2002; Hyatt *et al*., 2007), including the 1,8-cineole synthase from Greek sage (*Salvia fruticosa*) (Kampranis *et al*., 2007). 1,8-cineole synthase shows the typical α-helical terpene synthase fold, and conversion of a few amino acids at the catalytic site is enough to change the product specificity. The C-terminal domain contains the catalytic site whereas the N-terminal domain is suggested to have a “capping” function. The products formed by 1,8-cineole synthases are identified in several plant species, including Red Ironbark (*Eucalyptus sideroxylon*) and Arabidopsis (Chen *et al*., 2004; Keszei *et al*., 2010). In all species the main product formed is 1,8-cineole.

1.9 Transmembrane nitrate and peptide transporters

1.9.1 Transmembrane transporters

Transmembrane peptide transporters in plants are found in three different protein families. The oligopeptide transporter (OPT) family has 17 members in Arabidopsis, transporting tetra- and pentapeptides, as well as glutathione (Lubkowitz, 2011). A few members in the large ATP-binding cassette (ABC) family of transporters are capable of transporting peptides (Rea, 2007; Kang *et al*., 2011). The third family is the proton-dependent oligopeptide transporter (POT) family, recently renamed Nitrate Peptide transporter Family (NPF) (Léran *et al*., 2013). NPF members have been shown to transport nitrate, di- and tri-peptides, glucosinolate and ABA (Komarova *et al*., 2008; Lin *et al*., 2008; Kanno *et al*., 2012; Nour-Eldin *et al*., 2012). Phylogenetically, the 53 members in the NRT/PTR family are divided into four clades with no apparent substrate specificity (Tsay *et al*., 2007; Komarova *et al*., 2008; Nour-Eldin *et al*., 2012; Léran *et al*., 2013). The exception is that a specific clade seems to contain the glucosinolate transporters.

1.9.2 NPF family members

So far the function of only a few members in the Arabidopsis NPF family have been studied. For example, PTR5 (NPF8.2) is a plasma membrane-localized protein that mediates uptake of peptides during pollen germination. It also functions in nitrate transport during ovule and early seed development, as well as nitrate uptake from the rhizosphere (Komaraova *et al*., 2008). PTR3 (NPF5.2) is characterized as a di- and tri-peptide transporter, which when mutated cause increased susceptibility to *P. syringae* (Karim *et al*., 2005; 2007). Among the nitrate transporters, NRT1.8 is a low-affinity transporter responsible for uptake of nitrate from the xylem (Li *et al*., 2010), whereas NRT1.7 (NPF2.13) mediates phloem loading of nitrate in leaves (Fan *et al*., 2009).

2 Aims of the study

The emphasis of the work was to enhance the understanding of plant defense responses to *Verticillium longisporum*. The specific aims were to:

- Perform transcriptomic and SNP analyses to identify new defense-associated genes in Arabidopsis.
- Sequence the *V. longisporum* genome to assist in identification of fungal effectors.

3 Results and Discussion

This thesis spans several different areas of plant immunity. The aim is here to highlight the major findings, to describe the roles of the identified genes and proteins, and to put the results in a wider context.

3.1 *TPS23/27* contributes to *V. longisporum* susceptibility

3.1.1 Identification of the monoterpene synthase TPS23/27 (Paper II)

NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) is a plasma membrane-localized protein contributing to resistance to a number of fungal and bacterial pathogens, including *Pseudomonas syrin*gae, *Peronospora parasitica* and *V. longisporum* (Century *et al*., 1997; Johansson *et al*., 2006b). NDR1 mediates interactions with R proteins such as RESISTANCE TO *PSEUDOMONAS SYRINGAE* 2 (RPS2) and RESISTANCE TO *P. SYRINGAE PV. MACULICOLA* 1 (RPM1) (Aarts *et al*., 1998, Day *et al*., 2006). Recently, NDR1 was also shown to mediate plasma membrane cell-wall adhesion, leading to speculations on broader roles for this protein in plant immunity (Knepper *et al*., 2011). With this in mind, a microarray approach was used to identify transcripts differentially expressed in Arabidopsis Col-0 and *ndr1-1* mutant plants. Here we identified a large number of terpene synthase genes. Terpenes generally have an inhibitory effect on microorganisms, and we assumed that one or several of the identified terpene synthase genes would contribute to resistance to *V. longisporum*. From the identified genes in the microarray, the root expressed monoterpene synthase *TPS23/27* (Chen *et al*., 2004) was 2.3-fold up-regulated in *ndr1-1* compared to Col-0 in response to fungal challenge and was selected for further characterization.

3.1.2 TPS23/27 promotes *V. longisporum* invasion (Paper II)

Contrary to our assumption, we found that over-expression of *TPS23/27* lead to increased susceptibility to *V. longisporum*, whereas silencing had no significant effect on the disease progression. Most of the studied monoterpenes inhibit the growth of microorganisms, however a few are also reported to have a stimulatory effect (Kadoglidou *et al*., 2011). *TPS23/27* produces several volatile monoterpenes which are all predicted to be released into the rhizosphere (Chen *et al*., 2004). We chose to investigate the specific effect of the main product, 1,8-cineole, on the germination of *V. longisporum* conidia. We found that pure 1,8-cineole inhibited the germination, but diluted concentrations (10^{-4}x and lower) instead had a stimulatory effect. This indicated that previous investigations on the effect of terpenes and essential oils on the growth or microorganisms may be somewhat misleading, since often only high concentrations of the compounds are tested (see e.g. Rasooli *et al*., 2008; Kadoglidou *et al*., 2011). The actual concentration *in planta* is in almost all cases lower, and can thus have a very different effect on the bacteria or fungi. We therefore allowed *V. longisporum* conidia to germinate in the presence of wild-type Col-0, *35S:TPS23/27* or *TPS23/27-amiRNA* plants. In agreement with our previous observations we detected a clear increase in germination rate in the vicinity of the *TPS23/27* over-expressing plants.

3.1.3 Regulation of *TPS23/27* by MYC2-dependent JA signaling (Paper II)

In root growth experiments, roots of *35S:TPS23/27* plants were on average 18% shorter ($p<0.001$) compared to Col-0, a root phenotype not seen on *TPS23/27-amiRNA* plants (Figure 5). Treatments with MeJA are known to inhibit root growth (Staswick *et al*., 1992), and JAs are also shown to be involved in regulation of terpene synthase genes (Martin *et al*., 2003; Opitz *et al*., 2008; Köllner *et al*., 2013). We therefore decided to investigate the participation of JA, and found that treatments with MeJA resulted in a 3.4-fold up-regulation of *TPS23/27* transcript levels. The central transcription factor in JA signaling MYC2 (Kazan and Manners, 2013) binds to G-box (CACGTG) and E-box motifs (CANNTG), and inspection of the *TPS23/27* promoter sequence identified six such motifs. Subsequently we measured *TPS23/27* transcript levels in *35S:MYC2* and *myc2-1* plants. *TPS23/27* levels were significantly increased and decreased in *35S:MYC2* and *myc2-1* plants respectively, supporting a role for MYC2 in regulating *TPS23/27* expression.

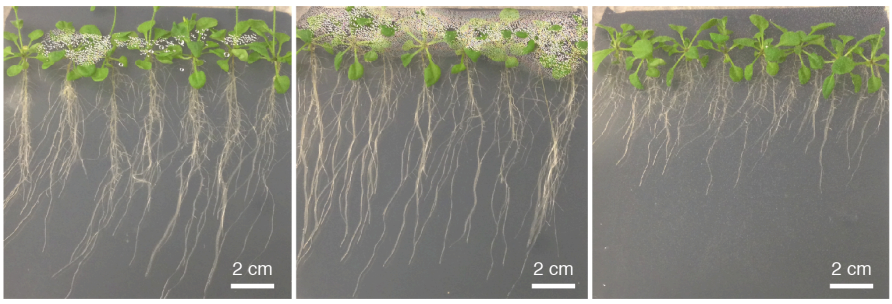

Figure 5. Root lengths of Arabidopsis Col-0, and plants silenced for (*TPS23/27-amiRNA*) and over-expressing (*35S:TPS23/27*) the monoterpene synthase *TPS23/27* genes.

3.2 Plant detection of pathogens

3.2.1 The transmembrane transporter NPF5.12 (Paper III)

In the present work we identified *NITRATE/PEPTIDE TRANSPORTER 5.12* (*NPF5.12*) as important in the immunity response to *V. longisporum*. When inoculated with *V. longisporum*, *npf5-12* mutant plants displayed increased susceptibility, correlated with a decrease in *NPF5.12* transcript levels. Treatment of nitrogen-depleted plants with the amino acids glutamine and tryptophan induced *NPF5.12* transcript levels, indicating that NPF5.12 is involved in amino acid/peptide uptake.

In pull-down and BiFC experiments we found an interaction *in planta* between NPF5.12 and NPFBP1, a member of the major latex protein (MLP) family. The NPFBP1 protein contains a relatively large hydrophobic pocket, which could potentially bind to either hydrophobic amino acids, or a *V. longisporum* effector. Detailed studies of NPF5.12 and NPFP1 in the uptake and interaction with fungal effectors or sensing of fungal-derived signals are currently ongoing.

3.2.2 Root-specific functions of RabGAP22 (Paper I)

In the present study, cDNA from a *RabGAP* gene was identified as up-regulated in response to *V. longisporum* inoculation in roots of the white cabbage accession *B. oleracea* BRA723. Via phylogenetic and qRT-PCR analyses, we showed this cDNA to be homologous to the *RabGAP22* gene in Arabidopsis. When tested for *V. longisporum* response, *rabgap22-1* knock-out mutants showed a strong increase in susceptibility, indicating this gene was important for the immunity response. GUS staining of $RabGAP22_{Pro}$*:GUS* transgenic Arabidopsis plants further revealed a clear expression of *RabGAP22* in root tissues. Regulation of the formation and growth of root and vascular

tissues is likely to be of high importance for root-invading pathogens like *V. longisporum*, and several Rab proteins are involved in such processes. The small GTPase RabA4 has a presumed involvement in secretion of cell wall components in the tips of growing root hair cells (Preuss *et al*., 2004; 2006), and similarly the RabGTPase RabG3b plays a role in tracheary element differentiation (Kwon *et al*., 2010). Finally, interesting examples come from human pathogens in the genera *Legionella, Listeria, Salmonella* and others, which are shown to interfere with Rab-mediated processes in order to establish favorable disease-promoting intracellular conditions (Brumell *et al*., 2007; Stein *et al*., 2012).

3.2.3 RabGAP22 and receptor-triggered immunity (Paper I)

Human Rab proteins regulate recycling of several membrane-bound receptor proteins (Grimsey *et al*., 2011; Goueli *et al*., 2012) and the small GTPase OsRac1 plays a role in a receptor complex recognizing fungal chitin (Akamatsu *et al*., 2013). We consequently investigated potential interactions between RabGAP22 and membrane-bound defense signaling components. BAK1 takes part in several responses initiated at the plasma membrane, including the FLS2-mediated resistance triggered by flagellin (Chinchilla *et al*., 2007) and the Ve1-mediated resistance to *V. dahliae* in Arabidopsis. Our observation that *V. longisporum*–susceptible *bak1-4* mutant plants had reduced transcript levels of *RabGAP22*, suggested that RabGAP22 could function in an early signaling complex together with BAK1 and a so far unidentified MAMP receptor.

3.3 Hormone signaling

3.3.1 Brassinosteroids (Paper I)

The identification of RabGAP22 as dependent on BAK1, together with the role of BAK1 in brassinosteroid reception (Wang *et al*., 2008), encouraged us to investigate the effect of brassinolide (BL) pretreatment on *V. longisporum* infection. This pretreatment resulted in a significant decrease in fungal colonization in both wild-type Col-0 and *rabgap22-1* mutant Arabidopsis plants. A reduction in *V. dahliae* colonization is also seen following BL treatment in tobacco (Gao *et al*., 2013), indicating the requirement for BL is shared for both *V. dahliae* and *V. longisporum*.

3.3.2 Jasmonic acid (Paper I)

Studies on plant hormone responses to *Verticillium* have yielded highly varied results. Nevertheless, Arabidopsis basal resistance to *V. longisporum* and *V.*

dahliae seems to require at least some of the components involved in jasmonic acid signaling (Tjamos *et al*., 2005; Johansson *et al*., 2006b; Pantelides *et al*., 2010; Fradin *et al*., 2011). In our *V. longisporum*-challenged plants, we detected an increase in JA signaling and JA and JA-Ile content at 2 dpi. Presumably this points to involvement of JA particularly in the early phase of *V. longisporum* infection, as no major impact on the JA/JA-Ile content has been found at later time-points (Ratzinger *et al*., 2009; Iven *et al*., 2012, Ralhan *et al*., 2012). RabGAP22 was found to contribute to repression of this early JA response, as both JA/JA-Ile content and JA signaling increased significantly in the *rabgap22-1* mutants plants. This effect may be attributed to RabGAP22 interfering with peroxisomal-associated JA biosynthesis. Data pointing in this direction comes from our observation that RabGAP22 interacts with the photorespiratory enzyme SERINE:GLYOXYLATE AMINOTRANSFERASE 1 (AGT1) in peroxisomes. Interaction with peroxisomal processes and JA signaling has also been seen for both Rab11 in rice and RabE1c in Arabidopsis (Cui *et al*., 2013; Hong *et al*., 2013).

3.3.3 Abscisic acid (Paper I)

Involvement of ABA in the defense to *V. longisporum* has so far not been shown to be of major importance (Veronese *et al*., 2003; Ratzinger et al., 2009; Iven *et al*., 2012). The exceptions are a specific requirement for the ABA biosynthesis gene ABA2 (Johansson *et al*., 2006b), and increased levels of ABA in Arabidopsis petioles at 15 dpi (Ralhan *et al*., 2012). Thus, our observation that the ABA content increased approximately 2-fold in Arabidopsis at 2 dpi was somewhat unexpected. A contribution of RabGAP22 in this ABA response was further identified, as the ABA content was significantly lower in the *V. longisporum*-challenged *rabgap22-1* mutant.

3.4 RabGAP22 and stomatal immunity (Paper I)

The impaired ABA response in *rabgap22-1* mutant plants, and the strong expression of *RabGAP22* in stomatal guard cells, together suggested a role for RabGAP22 in stomatal immunity. When measuring Arabidopsis stomatal apertures we found an impaired stomatal closure in *rabgap22-1* in comparison to Col-0 in response to ABA, *V. longisporum* and *P. syringae*. Heterotrimeric G proteins are involved in the stomatal responses to *P. syringae* (Zhang *et al*., 2008; Lee *et al*., 2013) and the small GTPase ROP11 negatively regulates ABA-mediated stomatal closure (Li and Liu, 2012). It could thus be speculated that several GTPases jointly regulate stomatal responses. A possible function for RabGAP22 might be regulation of the number of K^+ transporters in guard

cell membranes. Transport of K^+ into guard cell stomata leads to increased turgor pressure in the guard cells, causing stomatal closure. A similar function is seen for Rab11 in rice, which regulates transport of the OsVHA-a1 H^+-ATPase into the vacuole (Son *et al*., 2012). However we never observed RabGAP22-GFP fusion protein in the vacuoles, making this option less likely. A more tempting option is involvement of RabGAP22 in the ABA-regulated mechanism of stomatal closure. We observed significantly lower ABA levels in *V. longisporum*-inoculated *rabgap22-1* mutants compared to wild-type Col-0, suggesting that RabGAP22 contributes to the ABA increase detected in response to *V. longisporum*.

3.5 Summary

Figure 6 summarizes the major findings of this thesis, along with our current hypotheses for how the three identified genes function in the early root response to *V. longisporum*.

The Rab GTPase-regulating protein RabGAP22 displayed multiple functions. A clear repressing role of RabGAP22 on JA and JA signaling was found, and RabGAP22 interaction with the photorespiratory protein AGT1 in peroxisomes further suggested a role in peroxisomal JA biosynthesis. Increased *V. longisporum* resistance in response to BL, and analysis of *RabGAP22* transcript levels suggested a requirement of BAK1 for RabGAP22 function. We speculate that RabGAP22 functions in a receptor complex together with BAK1 and a so far unknown receptor, possibly by interfering with BAK1 phosphorylation.

The monoterpene synthase gene *TPS23/27* displayed a clear transcriptional response to MYC2-regulated JA signaling, and the TPS23/27 protein was shown to be located in chloroplasts in both roots and leaves. Plants over-expressing *TPS23/27* showed increased susceptibility to *V. longisporum*, and the plants also promoted germination of *V. longisporum* conidia. *TPS23/27* thus shows the characteristics of a novel *V. longisporum* susceptibility gene.

NPF5.12 was shown to be a plasma membrane-localized transporter in the NPF family, with a role in amino acid uptake. NPF5.12 interacted with the MLP protein NPFBP1 at the plasma membrane, and the two proteins showed potential involvement in SA signaling. The presumed role for NPF5.12 is in uptake of amino acids, small peptides, and fungal effectors.

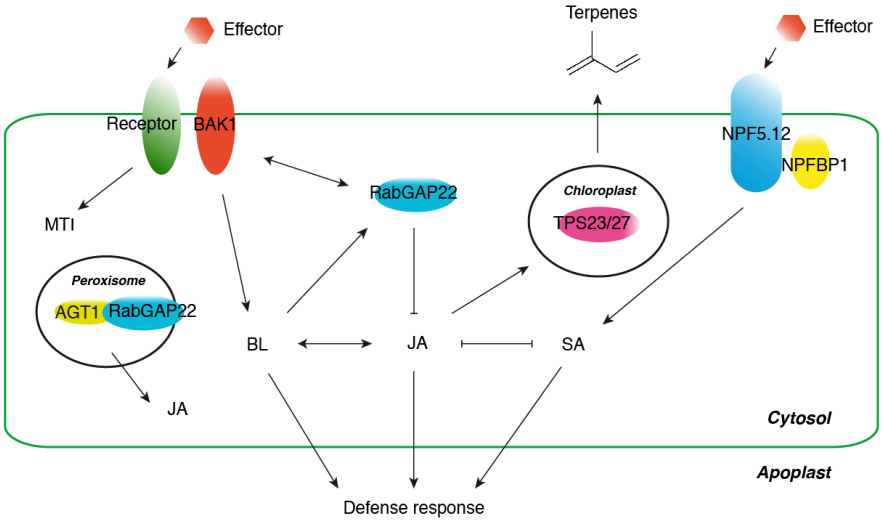

Figure 6. Summary of the major findings of this thesis. See text for details. (AGT1 = SERINE GLYOXYLATE AMINOTRANSFERASE 1; BAK1 = BRI1-ASSOCIATED RECEPTOR KINASE 1; BL = brassinolide; JA = jasmonic acid; MTI = MAMP-TRIGGERED IMMUNITY; NPF5.12 = NRT1/PTR FAMILY TRANSPORTER 5.12; NPFBP1 = NPF5.12 BINDING PROTEIN 1; SA = salicylic acid; TPS23/27 = TERPENE SYNTHASE 23/27).

3.6 *Verticillium* genomes (Paper IV)

To support future studies of *V. longisporum*-plant interactions, and to assist in discovery of effector genes, we sequenced the genomes of the two earlier described *V. longisporum* isolates VL1 (CBS110220) and 43-3 (here renamed VL2) (Steventon *et al*., 2002; Fahleson *et al*., 2003).

The sizes of the VL1 and VL2 genomes were both estimated at ~70 Mb, approximately twice the genome size of *V. dahliae* and *V. albo-atrum* (Klosterman *et al*., 2011) (Table 2). The predicted number of genes in VL1 and VL2 were roughly twice that in *V. dahliae* and *V. albo-atrum*, and a high percentage (~40%) of the genes were duplicated in the two *V. longisporum* isolates. These features could be seen as support for a hybrid origin of *V. longisporum* but could also signify a whole-genome duplication after the split from *V. dahliae*.

Transposable elements in *V. dahliae* and *V. albo-atrum* are relatively few compared to other fungal species and consist mainly of the long terminal repeat retrotransposons (LTRs) *Copia* and *Gypsy*, and DNA transposons of the *TC1/Mariner* class (Amyotte *et al*., 2012). In comparison, VL1 and VL2 contained roughly the same percentage of repetitive elements as *V. dahliae*

(Table 2), and the majority of these belonged to the LTR classes *Gypsy* and *Copia*.

On average ~250 more predicted secreted proteins were identified in VL1 and VL2 compared to *V. dahliae* and *V. albo-atrum* (Table 2). Proteins belonging to lipid transport and metabolism were twice as frequent in VL1 and VL2. This could be indicative of an enhanced capacity for pathogenicity, as lipid metabolism is involved in supplying energy for pathogen growth and in production of intra- and inter-cellular signaling molecules (LaCamera *et al*., 2005; 2009). The presence of cysteine residues is a common signature of fungal effectors (Stergiopoulos and de Wit, 2009; Koeck *et al*., 2011) and ~100 such proteins were predicted in VL1 and VL2, among which glycosyl hydrolases and proteins with cellulose-binding domains were most common.

Table 2. Comparison of the *Verticillium dahliae* VdLS.17, *V. albo-atrum* VaMs.102 and *V. longisporum* VL1 and VL2 genomes.

	V. dahliae[1]	*V. albo-atrum*[1]	VL1	VL2
Base coverage	7.5x	4x	64x	64x
Genome size (Mb)	35	30	~70	~70
Protein-coding genes	10,553	10,221	20,794	20,995
Repetitive sequences	4.8%	0.7%	3.0%	7.6%
Secreted proteins	780	759	1072	999

[1]**Klosterman, S.J., Subbarao, K.V., Kang, S., *et al.*** (2011) Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Path.* **7**, e1002137.

3.7 Additional information

3.7.1 Soil contaminations

During year three of this thesis work, numerous recurring soil contaminations took place in the plant growth chambers. Simultaneously, we observed a striking lack of *V. longisporum* disease development in the same facilities. Identification of the soil microorganisms was therefore pursued both in an effort to reduce the number of contaminations, but also to investigate their potential biocontrol activities against *V. longisporum*.

Microbial samples were collected directly form the soil with a sterile inoculation needle, transferred to Luria-Bertani (LB) agar plates and cultivated at 25°C in darkness. The isolates where subsequently identified by sequencing of ribosomal 16S rRNA genes. Four distinct isolates were in this way

identified; all of which were fungal species common in soil and indoor environments (Figure 7).

The potential activity of the four fungi against *V. longisporum* was evaluated both *in vitro* and by treating the soil with each fungus prior to *V. longisporum* inoculation. However, none of the four fungi suppressed growth of *V. longisporum in vitro* or in soil-grown *V. longisporum*-inoculated plants. The origin of the sudden loss of *V. longisporum* susceptibility of Arabidopsis plants was therefore unresolved.

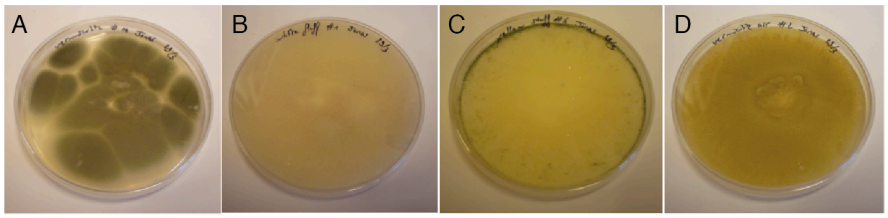

Figure 7. Microorganisms isolated from soil. A: *Penicillium corylophilum*, B: *Cunninghamella blakesleeana*, C: *Trichoderma atroviride*, D: *Paecilomyces variotii*.

3.7.2 Disease phenotypes of selected Arabidopsis mutants

Several Arabidopsis T-DNA insertion mutants screened for *V. longisporum* disease phenotype during this study are not covered in the manuscripts. A summary of additional phenotypic data is shown in Table 3.

Table 3. Phenotypes of Arabidopsis mutants grown on soil and infected with *V. longisporum*.

Locus	Gene	Mutant	Soil phenotype
At1g12110	NITRATE TRANSPORTER 1 (NPF6.3)	SALK_097431	As Col-0
At1g12110	NITRATE TRANSPORTER 1 (NPF6.3)	SALK_138710	As Col-0
At1g19250	FLAVIN-DEPENDENT MONOOXYGENASE 1	SALK_026163	As Col-0
At1g26240	Proline-rich extensin-like family protein	SAIL_535_B04	As Col-0
At1g26390	FAD-binding family protein	SALK_083228	As Col-0
At1g31950	Terpenoid cyclase	SALK_138882	As Col-0
At1g32450	NPF FAMILY PROTEIN 7.3 (NPF7.3)	SALK_043036	Susceptible
At1g64400	LONG-CHAIN ACYL-COA SYNTHETASE 3	SALK_027707	As Col-0
At1g71530	Protein kinase superfamily protein	SALK_027496	As Col-0
At1g74590	GLUTATHIONE S-TRANSFERASE TAU 10	SAIL_96_F10	As Col-0
At1g75750	GAST1 PROTEIN HOMOLOG 1	SALK_001187	As Col-0
At2g02130	CYSTEINE-RICH 68 (PDF2.3)	SALK_034705	Resistant
At2g16720	MYB DOMAIN PROTEIN 7	SALK_020256	As Col-0
At2g19990	PR-1-like	SALK_014249	Susceptible
At2g26560	PHOSPHOLIPASE A 2A	SALK_059119	Susceptible

At2g30770	CYP71A13	SAIL_505_E9	As Col-0
At2g31180	MYB DOMAIN PROTEIN 14	SALK_018565	As Col-0
At2g33050	RECEPTOR LIKE PROTEIN 26	SALK_092826	As Col-0
At2g38870	Putative protease inhibitor	SALK_111051	As Col-0
At2g44110	MILDEW RESISTANCE LOCUS O 15	SALK_078782	As Col-0
At2g47180	GALACTINOL SYNTHASE 1	SALK_128044	Susceptible
At3g09270	GLUTATHIONE S-TRANSFERASE TAU 8	SALK_150234	As Col-0
At3g09270	GLUTATHIONE S-TRANSFERASE TAU 8	SALK_049091	As Col-0
At3g12580	HEAT SHOCK PROTEIN 70	SALK_088253	As Col-0
At3g13610	2OG-Fe(II) oxygenase family protein	SALK_132418	As Col-0
At3g13610	2OG-Fe(II) oxygenase family protein	SALK_050137	As Col-0
At3g59220	ATPIRIN1	SALK_063087	Resistant
At4g12400	Carboxylate clamp-tetratricopeptide repeat protein	SALK_023494	Susceptible
At4g13020	Serine/threonine protein kinase (MHK)	SALK_037491	As Col-0
At4g16990	RESISTANCE TO *LEPTOSPHAERIA MACULANS* 3	GABI 491 E04	As Col-0
At4g21680	NPF TRANSPORTER 7.2 (NPF7.2)	SALK_024892	As Col-0
At4g23700	Putative Na+/H+ antiporter family	SALK_033417	As Col-0
At4g23810	WRKY family transcription factor 53 (WRKY53)	SALK_034157	Resistant
At5g15410	DEFENSE NO DEATH 1	*dnd1*	Susceptible
At5g24530	DOWNY MILDEW RESISTANT 6	*dmr6-1*	As Col-0
At5g24780	VEGATATIVE STORAGE PROTEIN 2	SALK_036845	As Col-0
At5g42600	MARNERAL SYNTHASE 1	SALK_152492	As Col-0
At5g48010	THALANIOL SYNTHASE 1	*thas1-1*	Resistant
At5g48010	THALANIOL SYNTHASE 1	*thas1-2*	Resistant
At5g64905	ELICITOR PEPTIDE 3 PRECURSOR	SALK_017813	As Col-0

V. longisporum symptoms on Arabidopsis Col-0 are generally mild, and include chlorosis, stunting and premature senescence. Plants scored as susceptible displayed more pronounced symptoms compared to Col-0, and a more rapid disease progression. Plants scored as resistant displayed none, or very mild disease symptoms.

4 Conclusions

- The Rab GTPase-activating protein RabGAP22 contributes to *V. longisporum* resistance.
- RabGAP22 contributes to stomatal closure in response to inoculation with *V. longisporum* and *P. syringae*, and in response to treatment with ABA and flg22.
- RabGAP22 protein localizes in the nucleus, and interacts with AGT1 in the peroxisomes.
- RabGAP22 represses early JA signaling in *V. longisporum* inoculated plants.
- Over-expression of the monoterpene synthase *TPS23/27* causes increased susceptibility to *V. longisporum*.
- Plants over-expressing *TPS23/27* promote germination of *V. longisporum* conidia.
- The TPS23/27 protein is subcellularly located in plastids.
- *TPS23/27* is under transcriptional control by MYC2-dependent JA-signaling.
- Plants mutated in the transmembrane transporter *NPF5.12* display increased colonization by *V. longisporum*.
- Amino acid treatment of nitrogen-depleted Arabidopsis plants suggests NPF5.12 may play a role in uptake of amino acids.
- NPFBP1, a member of the major latex protein (MLP) family interacts with NPF5.12 in the plasma membrane and in the peroxisomes.
- The *V. longisporum* VL1 and VL2 genomes comprise approximately 70 Mb and 21,000 genes.
- *V. longisporum* shares approximately 86% of its genes with *V. dahliae* and *V. albo-atrum*.

5 Future perspectives

Listed below are the main analyses currently being pursued in order to complete papers II, III, and IV for publication.

- Determine the amount and identity of terpenes emitted by *35S:TPS23/27* and *TPS23/27-amiRNA* transgenic plants.
- Determine the *V. longisporum* phenotype and colonization of *ndr1 TPS23/27-amiRNA* double-mutant plants.
- Study microsclerotial germination in the presence of *35S:TPS23/27* and *TPS23/27-amiRNA* transgenic plants. Also for *V. dahliae* and *F. oxysporum*.
- More detailed studies on the role of NPF5.12 and NPFP1 in the uptake and interaction with peptide substrates and potential fungal effectors.
- Determine the response of *NPF5.12* and *NPFBP1* to treatment with plant hormones.
- Identification of fungal effectors among the predicted secreted proteins in *V. longisporum*.
- Comparison of the *V. longisporum V. dahliae* and *V. albo-atrum* genomes to study their evolutionary history.
- Compare the secreted proteins and effectors from *V. longisporum V. dahliae* and *V. albo-atrum* to help identify differences in host preference.
- Select *V. longisporum* effector to be studied in more detail.

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