Pathogens of green roof mosses and the use of a *Physcomitrella* mutant collection as a source for elucidating genes involved in the chitosan-induced signaling pathway

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ACADEMIC DISSERTATION

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ABSTRACT

Knowledge of the defense responses of mosses against pathogens has gained less attention than has our knowledge of the pathogens of vascular plants. Recently, the use of mosses has gained attention because mosses are used for greenhouse production as well as landscaping. In particular, the landscaping and greening of buildings have become popular because these initiatives offer one solution for mitigating urban problems such as heat islands and flooding. Mosses are an easy and lightweight solution for greening purposes as they can survive the harsh rooftop environment and have great stormwater retention. However, the health of plants is fundamental to achieving the benefits of greening.

Like vascular plants, mosses also are susceptible to plant diseases. Many fungi damage mosses by causing brown patches of greenish moss. Brown patches on mosses are a characteristic sign of fungal infection. However, plants have various defense mechanisms, the first of which consists of preexisting structural and chemical defenses. Second, the plant immune system uses specific receptors with which to recognize the molecular structures of microbes that are not present on the surface of the plant's own cells. Receptor-mediated sensing of these structures can trigger early defense responses of plant, which can make the plant resistant to the attacking microbe. The model moss *Physcomitrella patens*, like the vascular plants, senses the molecular structures of microbes. For example, exposure of *P. patens* to chitosan—a component of the fungal cell wall—significantly increases peroxidase activity and oxygen radical formation. Oxygen radicals in turn affect many biological events; they can directly damage the pathogen or stimulate the plant's defenses. Currently, little is known about the peroxidase-based defense as well as chitosan-induced signaling pathways of *P. patens*.

The aim of the research presented in this thesis was to study the pathogens of green roof mosses and establishes the host range of the isolated pathogens; the study also utilized a *Physcomitrella* mutant collection to identify genes involved in chitosan-induced signaling pathway. Fungal species that naturally inhabit mosses at a moss farm in Japan and on green roof environments in southern Finland were isolated and the ability of these fungi to infect and cause disease symptoms on the model moss *P. patens* was tested. In addition, the pathogenicity of fungus species towards vascular plants was also assessed. To elucidate the genes involved in chitosan-induced peroxidase activity, part of the *Physcomitrella* mutant collection was screened using the oxidation of 2,2'−azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as an indicator of peroxidase activity. Genome walking analyses were used to identify which genes were mutated within each moss line of interest.
The work described in this thesis demonstrates that mosses used for greening are colonized by many different fungal isolates. These studies reveal that several fungal genera such as *Fusarium*, *Trichoderma*, *Phoma* and *Alternaria* cause severe symptoms in *P. patens*. Moreover, our results demonstrate that mosses and vascular plants have common pathogens. The fungal isolates *Fusarium avenaceum* and *Cladosporium oxysporum* obtained from moss panels caused disease symptoms on barley and carrots, respectively, and also on two different moss species. The results also demonstrate that the *Physcomitrella* mutant collection is a valuable source for identifying genes involved in the chitosan-induced signaling pathway. Screening of part of the mutant collection and further analyses revealed that Rossmann fold protein is a significant part of the signaling chain leading to upregulated peroxidase activity induced by chitosan. In addition, this Rossmann fold protein is an important factor for normal lipoxygenase (LOX) expression and might contribute to defense against fungal pathogens.

The results from this doctoral thesis provide new insights into the pathogens of green roofs, the host range of the pathogens and the molecular mechanisms involved in disease control and defense responses in moss. The knowledge gained concerning the pathogenicity of *Trichoderma* isolates and the host range of pathogenic fungi should be considered when planting moss farms and cultivating crops in close proximity to each other or when applying biological control agents containing *Trichoderma* species to green roofs. Furthermore, these results may encourage the use of the *Physcomitrella* mutant collection to identify candidate genes for signaling pathways to elucidate the molecular mechanisms underlying the defense responses of mosses.
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ABBREVIATIONS

ABTS 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
CAD Cinnamyl alcohol dehydrogenase
CEBiP Chitin elicitor binding protein
CERK Chitin elicitor receptor-like kinase
HoMi Hochdurchsatz Minibatch
KO Knockout
LOX Lipoxygenase
MAMP Microbe-associated molecular pattern
MAPK Mitogen-activated protein kinase
MNR1 Menthone reductase1
NADP Nicotine adenine dinucleotide phosphate
PAMP Pathogen-associated molecular pattern
PR-1 Pathogenesis related
Rboh Respiratory burst oxidase homolog
RLCK Receptor-like cytoplasmic kinase
ROS Reactive oxygen species
SDR Short chain dehydrogenase/reductase
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and submitted manuscripts. The publications and manuscripts are referred to in the text by their Roman numerals.


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My contribution to the publications:

I I took part in the identification of fungi by amplification of ITS sequences by PCR and I participated in writing the manuscript together with my co-authors.

II I performed almost all the laboratory work, wrote the draft of the manuscript and revised the manuscript together with my co-authors. I mainly designed all the experiments.

III I performed the inoculation experiments with fungi and vascular plants and was involved in experimental design. I wrote the manuscript together with my co-authors.

IV I performed all the laboratory work and wrote the draft of the manuscript and revised the manuscript together with my co-authors. I mainly designed all the experiments.
1 INTRODUCTION

Bryophytes, including liverworts, hornworts and mosses, are the second largest phylum of land plants (Embryophyta) (Buck and Goffinet 2000). It has been estimated that land plants emerged in the middle Cambrian to early Ordovician interval (515.1 to 470 Ma) (Morrisa et al. 2018). The bryosphere comprises the combined community of living and dead moss tissue and associated organisms (Lindo and Gonzales 2010). Bryophytes are highly specialized in different environments and they reside in every continent (Buck and Goffinet 2000). In many places around the world, the diversity and the number of taxa are greater in mosses than in the liverworts and hornworts and only extreme wet or dry environments favor the diversity of liverworts (Tan and Pócs 2002). Mosses, which are the most species-rich group of bryophytes, include approximately 10 000 or more species (Buck and Goffinet 2000).

The life cycle of mosses can be divided into the gametophyte stage and sporophyte stage. The gametophyte is the haploid stage comprising the juvenile protonema and adult gametophores (Fig. 1). The sporophyte is the diploid stage comprising the spore capsule, in which spores are produced (Cove 2005). Germination of spores typically initiates the life cycle of mosses. After germination, spores grow to filamentous branched protonema, which differentiates into two different cell types—chloronema and caulonema (Fig. 1a). Chloronema and caulonema cells can be distinguished by the number of chloroplasts, cell length and cross wall orientation (Cove 2005). Chloronema cells are the first cell type to emerge from spores and contain well-developed chloroplasts (Bezanilla and Perroud 2009, Buck and Goffinet 2002). Caulonema cells are longer than chloronema cells and contain fewer chloroplasts (Bezanilla and Perroud 2009). Gametophores are leafy shoots that typically initiate from caulonema filaments (Cove 2005). Gametophores consist of a stem, leaves and rhizoids (Fig. 1b). Gametophores are often attached to the substratum by rhizoids, which structurally resemble protonema cells but lack chloroplasts (Cove 2005).

Fig. 1. Gametophyte of *P. patens*. a) Juvenile gametophyte, i.e., the protonema, consists of chloronema and caulonema cells. Scale bar 100 μm. b) Adult gametophyte containing a gametophore with leaves, stem and rhizoids. c) Life cycle of moss. Scale bar 500 μm.
Over the decades, mosses have been used for many different purposes. During the world wars, *Sphagnum* spp. were used for wound dressing and before the 1930s, mosses were a very popular insulation material for buildings in Finland (Fig. 2a) (Piippo 2016, Halttunen and Kuusisto 2011). Today, the moss *Sarmentypnum trichophyllum* is used as a filtering material for wastewater treatment (Biolan 125 treatment plant) and there is growing interest in using *Sphagnum* as a substratum for the growth of greenhouse plants. Furthermore, the use of mosses for landscaping—especially for the greening of buildings (Fig. 2b)—has received increasing attention. In Japan, for example, moss mats and moss panels are manufactured for greening purposes. For the production of moss panels, gametophyte tissue of *Racomitrium japonicum* is produced in a bioreactor system and this tissue is used in a nursery to produce moss tissues that become affixed to the panels as the tissues grow.

In addition to practical applications, mosses are getting more attention as model organisms in scientific research and over the last two decades *Physcomitrella patens* has become such a model organism (Rensing et al. 2009). The *Physcomitrella* mutant collection was established in the early 2000s (Egener et al. 2002, Schween et al. 2005) and the International Moss Stock Center is managed by the Department of Plant Biotechnology, University of Freiburg. *P. patens* is the first bryophyte for which the genome has been completely sequenced (Rensing et al. 2008). The genome size is approximately 500 Mbp and it is organized into 27 chromosomes (Lang et al. 2018). *P. patens* has many features that favor its use for experimentation: it is easy to grow in the laboratory and it grows rapidly, haploid generation prevails and the high frequency of homologous recombination enables targeted gene disruption (Rensing et al. 2008, Schaefer 1991). Morphogenesis in the tip of the developing moss protonema (i.e., the juvenile gametophyte) can be followed at the single-cell level in each developmental phase (Reski 1998). *P. patens* is a widely used model organism for evolutionary-developmental studies and for studies of plant-pathogen interactions (Andersson et al. 2005, Ponce de Léon et al. 2007, Lehtonen et al. 2009, Lawton and Saidasan 2009).
Fig. 2. The use of mosses in buildings in the past and today. a) A house built in 1886, insulated with moss, in Hirvensalmi Finland. b) A modern green roof in Korkeasaari Finland.

1.1 Green roofs are important solutions for mitigating urban problems

Urbanization causes many problems such as pollution and the urban heat-island effect, which affects small and large cities alike (Li et al. 2019). Cities are also facing the greater likelihood of flooding as for porous urban land is decreasing (White 2002). In addition, urbanization continues to cause fragmentation of natural habitats and reduces biodiversity (Köhler and Ksiazek-Mikenas 2018). Furthermore, construction of new buildings increases energy consumption. In Europe (EU-28), buildings accounted for ~40 % of total final energy consumption in 2012 and are the largest end-use sector (Energy Efficiency Trends and Policies in the Household and Tertiary Sectors, 2015).

Green roofs, also called vegetated roofs, have plants on their top layer and are important solutions for mitigating problems caused by urbanization (Berardi et al. 2014). Green roofs are used to manage stormwater and to reduce the energy cost of buildings (Mentens et al. 2006, Santamouris 2014, Castleton et al. 2010). Many studies have shown that green roofs can reduce heating in winter and cooling in summer resulting in energy savings (Castleton et al. 2010). Green roofs also can sustain the biodiversity within city environments by providing habitats for different plants, animals and insect species (Köhler and Ksiazek-Mikenas 2018). However, the biodiversity of green roofs varies. Some green roofs can harbor tens of different species whereas others have only a few species (Köhler and Ksiazek-Mikenas 2018).

Green roofs can be classified into two major categories: intensive green roofs and extensive green roofs. Intensive green roofs have a thick growth layer (usually >200 mm) and relatively large plants, whereas extensive roofs have a thinner growth layer (<200 mm) and smaller plants. Extensive green
roofs are typically simple to construct and relatively easy to maintain, although intensive green roofs can support a greater number and variety of plant species (Berardi et al. 2014).

In Finland, green roofs are typically composed of vascular plants, but aging roofs become transformed into moss-dominated communities (Gabryh et al. 2016). Mosses have several beneficial properties that favor their use for green roof applications. First, mosses are poikilohydric, they can equilibrate their water content according to the water content of their surroundings by surface absorption and diffusion. Because of the poikilohydric nature of mosses, lignin is not required as a supportive structure against gravity or negative pressure generated during transpiration (Mishler and Oliver 2009). Second, some moss species can tolerate drought or desiccation and moss species such as *Antitrichia californica*, *Dicranoweisia cirrata* and *Racomitrium canescens* can survive harsh rooftop environments with limited irrigation (Charron and Quantro 2009, Anderson et al. 2010). Finally, mosses can have great capacity to retain stormwater (Anderson et al. 2010).

Plant health is an important factor for the proper functioning of green roofs. Both abiotic and biotic factors can affect plant survival. Many studies have described the influence of abiotic factors such as drought, heat and water usage on plant fitness in green roof environments (Farrell et al. 2013a, b, Rayner et al. 2016, Du et al. 2019). In addition, biological factors such as interactions with microbes might play a substantive role in roof-plant survival. Microbes associate naturally with moss communities in urban green roofs, although little is known about these interactions.

### 1.2 Bryophyte-fungi interactions

Because plants colonized land over 450 Ma, they have evolved a wide range of associations with fungi. The earliest land plants interacted with fungi, which produced mycorrhizal-like intracellular structures similar to those formed by the action of the present mycorrhiza-compatible fungi like *Glomeromycotina* and *Mucoromycotina* (Martin et al. 2017). Racoviza (1959) was among the first who described different types of interactions between Pyrenomycetes and bryophytes. He classified the interactions as ectoparasites and hemiendoparasites based on the location of interaction and based on the biological nature of interactions as harmless, moderate tolerance, or adverse interactions.

Interactions between bryophytes and symbiotic arbuscular mycorrhizal fungi belonging to the *Glomeromycotina* subphylum have been described especially in liverworts (Carella and Schornack 2018). Lignore et al. (2007) described the formation of arbuscule-like structures after the colonization of the liverwort *Fossombronia echinate* by glomeromycotean fungi. Although mosses might support the growth of arbuscular mycorrhizal fungi, the evidence for functional symbioses is unknown
(Carella and Schornack 2018). However, fungi belonging to the mucoromycotina group have beneficial interactions with bryophytes (Yu et al. 2014, Field et al. 2016).

Harmful interactions between plants and fungi result in many changes within the host plants. General symptoms such as chlorosis and necrosis followed by death of the gametophyte are common to known bryophilous pathogens, but the infection mechanisms and host responses vary (Davey and Currah 2006). Wilson (1951) was one of the first who described the symptoms and interactions between bryophytes and fungi. He reported concentric rings of dead moss in arctic moss mats of Racomitrium sp. and found that moss shoots bore fungal hyphae on the surface of the stem and inside the cells. Later, many reports described the symptoms and fungal species infecting moss tissues in nature and described moss-pathogen interactions under laboratory conditions (Redhead 1981, Kost 1988, Hassel and Kost 1998). Redhead (1981) described the interactions of Lyophyllum palustre and Galerina paludosa with the peat moss Sphagnum. Interestingly two different infection mechanisms were described: L. palustre killed Sphagnum before host-cell contact, indicating that the fungus secretes a toxin, whereas G. paludosa infection was limited to specific cell types, rhizoids and protonema. Over the last decade, several studies have described plant-fungal interactions in the model moss P. patens (Ponce de Leon et al. 2007, Lehtonen et al. 2009, Oliver et al. 2009, Lehtonen et al. 2012). Ponce de Leon et al. (2007) studied the interaction between Botrytis cinerea and P. patens and showed that P. patens was susceptible to Botrytis infection. Symptoms such as necrotic protonema tissues and browning of the stem were detected in moss tissues. Lehtonen et al. (2009) showed that the interactions with P. patens and Fusarium sp. result in discoloration of the basal part of gametophores and rhizoids followed by the death of shoots. Oliver et al. (2009) showed that the two oomycetes Pythium debaryanum and Pythium irregular infect P. patens, causing severe damage to moss.

1.3 Plant defense against pathogens

Plants continuously face attack by environmental pathogens and defend themselves with many sophisticated mechanisms, which are simply classified as preexisting or induced defense mechanisms (Agrios 2005). Plant innate immunity can be activated by specific signals that trigger numerous immune signaling pathways, leading to early and late defense responses. Activation of mitogen-activated protein kinase (MAPK) cascades and the production of reactive oxygen species (ROS) are early defense responses. Activation of MAPK cascades or production of ROS leads to the late defense responses such as the expression of several defense genes and induction of defensive structures. Plant hormones also regulate disease resistance; for example, salicylic acid mediates the defense against
biotrophic pathogens, whereas jasmonic acid and ethylene are involved in the defense against necrotrophic pathogens.

1.3.1 Preexisting structural and chemical defenses

Preexisting defense mechanisms are divided into structural and chemical defenses. The first barrier of defense is the plant surface, which is covered by a hydrophobic layer called the cuticle. The cuticle consists of cutin and waxes. Both vascular and non-vascular plants have a cuticle or similar type of surface layer (Buck and Goffinet 2000). However, Wyatt et al. (2014) showed that only gametophores of *P. patens* have a cuticle. They showed that only intracellular organelles of *P. patens* protonema cells were stained with lipid dye, whereas the entire leaf surface was uniformly stained, indicating that only leaves have a cuticle. In addition, the cuticle may function in the perception of pathogens and subsequent activation of defense responses (Serrano et al. 2014). Schweizer et al. (1996a) showed that topical spray application of cuticle monomers partially protected the leaves of a highly susceptible barley cultivar against the fungal pathogen *Erysiphe graminis f. sp. hordei*. Furthermore, addition of cutin monomers to cultured potato cells induced a transient alkalinization of the culture medium, stimulated the production of the plant stress hormone ethylene and activated defense-related genes (Schweizer et al. 1996b).

The plant cell wall is another example of a preexisting defensive structure. The wall is a complex structure consisting of a diverse mixture of proteins and polysaccharides, including cell wall–bound peroxidases and cellulose. Plant cell-wall polysaccharides of mosses and vascular plants are similar, but they differ in their side-chain composition and structure (Roberts et al. 2012). In addition, bryophytes do not contain lignin, but for example *P. patens* contains lignin-like polyphenolic material in its cell wall (Weng and Chapple 2010, Espiñeira et al. 2011). Primary and secondary cell walls surround the plant cell and can act as physical barriers to restrict the entrance of pathogens. However, recently the role of the cell wall has expanded as it can be regarded as an essential component of the plant monitoring system (Bacete et al. 2018). Plant cell-wall alterations can have a substantive impact on disease resistance. Hernández-Blanco al. (2007) reported that mutations in three types of cellulose synthetase subunits required for secondary cell-wall formation confer enhanced resistance to the soil-borne bacterium *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina*. Furthermore, Ramirez et al. (2011) demonstrated that the transcription factor MYB46, which regulates secondary cell-wall biosynthesis, has a role in disease resistance. They showed that *myb46* knockdown mutant plants had increased disease resistance to *B. cinerea*. They also showed that in *myb46* plants, the induction of cell wall–bound class III peroxidases is enhanced and *myb46* plants
react earlier and more aggressively to *B. cinerea* inoculation than wild-type *Arabidopsis* Col-0 plants. These results indicate that reinforcement of the cell wall by peroxidases may be the basis to explain the observed enhanced resistance to *B. cinerea* in *myb46* plants (Ramirez et al. 2011).

Preexisting chemical defenses include antimicrobial compounds that are present in cells before infection. For example, many phenolic compounds and fatty acid–like compounds might contribute to resistance (Agrios 2005). Phenolic compounds are secondary metabolites that contain benzene rings with different numbers of hydroxyl components and their structures may vary from a simple phenolic molecule to highly polymerized compounds (Velderrain-Rodriguez et al. 2014). Although mosses are considered non-lignified plants, they accumulate soluble phenylpropanoids such as flavonoids and lignans (Weng and Chapple 2010). In addition, moss ancestral phenolic metabolism is essential for erect plant growth and for cuticle permeability (Renault et al. 2017).

### 1.3.2 Plant immune system: recognition and activation of defense responses

The plant immune system consists of two layers. The first line of defense recognizes and responds to microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) and the second responds to pathogen virulence factors, i.e., effectors (Jones and Dangl 2006). Plant cell membranes have an important role in the immune responses. Recognition of MAMPs, the consequent release of signaling molecules and accumulation of LOX are associated with the plasma membrane (Agrios 2005). Plants have plasma membrane–localized pattern-recognition receptors and through them recognize MAMPs. Recognition of MAMPs/PAMPs by pattern-recognition receptors can trigger early defense responses that lead to PAMP triggered immunity. In response to PAMP-triggered immunity, pathogens might deliver effectors that interfere with this type of immunity. The second line of defense recognizes effectors by proteins encoded by resistance genes. Recognition of effectors results in highly specific effector-triggered immunity (Jones and Dangl 2006). Usually, effector-triggered immunity leads to localized plant cell death called the hypersensitive response, which is especially effective against biotrophic pathogens and limits the progress of infection (Koeck et al. 2011).

Chitin and chitosan are fungal cell-wall components. Chitin (C₈H₁₃O₅N)ₙ is a long-chain polymer of N-acetylglucosamine, whereas chitosan is a deacetylated form of chitin. The proportion of chitosan and chitin within the cell wall may vary during the infection process (Hadwiger and Beckman 1980, Deising and Siegrist 1995).

Plants recognize chitin through chitin elicitor receptor-like kinases (CERK) alone or together with chitin elicitor binding protein (CEBiP). CERK orthologs have been identified among vascular and
non-vascular plants (Miya et al. 2007, Shimizu et al. 2010, Bressendorff et al. 2016). Kaku et al. (2006) first showed that CEBiP has an important role in the perception of chitin and chitin-induced responses in rice. Later, Miya et al. (2007) identified chitin elicitor receptor kinase1 (CERK1) in Arabidopsis. Soon after it was shown that in rice CERK1 co-receptor is also required for chitin signaling (Shimizu et al. 2010). These studies demonstrated that disruption of normal expression of CEBiP and CERK1 resulted in suppression of defense responses. In rice, CEBiP-RNA interference cell lines have decreased levels of induced H$_2$O$_2$ after chitin treatment (Kaku et al. 2006). The Arabidopsis $cerk1$ mutant has impaired chitin-induced ROS generation and MAPK activation (Miya et al. 2007). OsCERK1 knockdown lines have reduced phytoalexin content and the expression of chitin-induced genes is suppressed (Shimizu et al. 2010). An ortholog of CERK also exists in P. patens (Bressendorff et al. 2016). Bressendorff et al. (2016) showed that P. patens encodes four orthologs of CERK, at least one of which is involved in defense responses including cell-wall modifications and phosphorylation of two MAPKs.

1.3.3 Induced defense responses: early and late responses in chitin-induced signaling pathways

Induction of plant defenses involves both early and late defense responses. The very early defense responses occur 1–5 min after exposure to pathogen (Boller and Felix 2009). Activation of MAPK cascades and an oxidative burst are among the first defense responses after recognition of an elicitor (Asai et al. 2002, Apel and Hirt 2004). Later defense responses that occur within 5–30 min after pattern-recognition receptor activation include the activation of defense-related genes (Boller and Felix 2009).

Recognition of chitin by CEBiP and CERK1 leads to several downstream events such as the activation of the MAPK cascade and ROS production (Kawasaki et al. 2017). In rice, recognition and binding of chitin by CEBiP results in association with OsCERK followed by interaction with the receptor-like cytoplasmic kinase (RLCK) OsRLCK185 (Yamaguchi et al. 2013). OsRLCK185 is required for chitin-induced responses—for example MAPK cascade activation and ROS production (Yamaguchi et al. 2013). Recent studies with rice have also indicated that another chitin signaling pathway involving Rac/Rop GTPases is required for ROS production (Kawasaki et al. 2017). Nagano et al. (2016) showed that plasma-membrane microdomains and the localization of OsRac1 to microdomains are important for ROS production in response to chitin signaling. In rice, it is likely that OsCERK1-mediated immunity functions through pathways that branch at OsRLCK185 and OsRac1 (Kawasaki et al. 2017).
In Arabidopsis, recognition of chitin by AtCERK1 leads to the phosphorylation of receptor-like cytoplasmic kinase PBL27, which is an ortholog of OsRLCK185. Phosphorylation of PBL27 leads to the interaction of the chitin receptor complex MAPK cascades (Yamada et al. 2016). However, PBL27 is not involved in chitin-induced ROS production (Shinya et al. 2014). It might be that in Arabidopsis ROS production is achieved through the action of Botrytis Induced Kinase, which is also required for flagellin peptide flg22–induced ROS production (Kawasaki et al. 2017).

An innate immunity pathway in response to chitin also has been described in the moss *P. patens* (Bressendorff et al. 2016). In *P. patens*, recognition of chitin by the PpCERK1 complex induces phosphorylation of two MAPKs (MPK4a and MPK4b). Bressendorf et al. (2016) showed that *Ppmpk4* knockout (KO) plants are more susceptible to fungal infection, indicating that activation of the MPK4 cascade is essential for defense responses against *B. cinerea* and *Alternaria brassicola* infection.

In many host-fungus interactions, one of the earliest defense reactions is the oxidative burst, which is the rapid production of high levels of ROS (Wojtaszek 1997, Torres 2010). In plant cells, ROS are produced by plasma membrane–bound NADPH oxidases, cell wall–bound peroxidases, lipoxygenases and amine oxidases in the apoplast (Apel and Hirt 2004, Camejo et al. 2016). ROS include superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$). Plasma membrane–bound NADPH oxidases use cytosolic NADPH as the electron donor to generate O$_2^-$, whereas cell wall–bound peroxidases catalyze the single-electron oxidation of several substrates by using H$_2$O$_2$ (Czarnocka and Karpinski 2018, Almagro et al. 2009). ROS have many signaling roles in the cell; for example, they are involved in cell-to-cell communication that results in the formation of a ROS wave that carries the signal over long distances (Mittler at al. 2011).

The oxidative burst in response to PAMPs is a conserved mechanism between bryophytes and vascular plants. The first report of the oxidative burst as a response to fungal-plant interactions comes from the studies of Doke (1983), who described the rapid production of ROS in *Solanum tuberosum* plants in response to inoculation with an incompatible race of *Phytophthora infestans* but not with a compatible race. Oliver et al. (2009) first showed that the oxidative burst in *P. patens* occurs in response to plant pathogenic *Pythium*. Later, Lehtonen et al. (2009, 2012) demonstrated chitosan-induced peroxidase activity in *P. patens* and an immediate burst of ROS in the culture medium of *P. patens* and *Sphagnum capillifolium*. In *P. patens*, the chitosan-induced peroxidase activity and oxidative burst are mainly dependent on peroxidase 34 (Prx34). Lehtonen et al. (2009, 2012) showed that *P. patens Prx34* KO lines lack peroxidase activity or the oxidative response elicited by chitosan.
and therefore are more susceptible to fungal infection, indicating that this peroxidase is pivotal for defense against fungi.

Several genes that are involved in the oxidative burst, antimicrobial defense, cell-wall modifications and defense signal transduction have been found to be activated in response to pathogens (Agrios 2005). Rauyaree et al. (2001) studied the expressed genes in rice 48 h after inoculation with the fungus *Magnaporthe grisea* and showed that the largest group (21%) of expressed sequence tags contained stress- or defense-response genes, including rice peroxidase genes. Schenk et al. (2003) studied the transcriptional changes in Arabidopsis plants inoculated with the fungal pathogen *A. brassicola* and found that many of the upregulated genes encode proteins involved in β-oxidation of fatty acids, cell-wall synthesis and modifications and signal transduction. Activation of defense- and ROS-related genes also occurs in bryophytes in response to fungal infection or elicitor treatment. In *P. patens*, inoculation with *B. cinerea* induces the expression of defense-related genes such as pathogenesis related1 (PR1), phenylalanine ammonia-lyase, chalcone synthetase and LOX (Ponce de León et al. 2007). Furthermore, chitosan treatment induces the expression of an alternative oxidase, NADPH oxidase and LOX in *P. patens* (Lehtonen et al. 2012).
2 AIMS OF THE STUDY
The aims of the studies were to identify fungal species that colonize mosses at moss farms and on green roofs and then study their pathogenicity toward bryophytes and vascular plants. This thesis also aimed to find candidate genes involved in chitosan-induced signaling pathways by screening part of the *P. patens* mutant collection. The research questions were as follows:

1) What kinds of fungal genera inhabit moss panels and green roof mosses and are they pathogenic to the model moss *P. patens*? (Publications I and submitted manuscript II)
2) Do vascular and non-vascular plants have common pathogens? (Publication III)
3) Which genes are involved in chitosan-induced signaling pathways in *P. patens*? (Manuscript IV)
3 MATERIALS AND METHODS

The methods used are summarized here and described in detail in the original publications and manuscripts (I–IV).

**Table 1. Summary of the methods used in this study.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Publication or manuscript</th>
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<tr>
<td>cDNA synthesis</td>
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<td>DNA extraction</td>
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<tr>
<td>Genome walking</td>
<td>IV</td>
</tr>
<tr>
<td>Identification of fungi</td>
<td>I, II</td>
</tr>
<tr>
<td>Inoculations</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Isolation of fungi</td>
<td>I, II</td>
</tr>
<tr>
<td>Molecular cloning</td>
<td>IV</td>
</tr>
<tr>
<td>Moss transformation</td>
<td>IV</td>
</tr>
<tr>
<td>Peroxidase activity assay</td>
<td>IV</td>
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<tr>
<td>Phylogenetic analyses</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>IV</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>IV</td>
</tr>
<tr>
<td>Screening of mutant collection</td>
<td>IV</td>
</tr>
<tr>
<td>Southern blotting</td>
<td>IV</td>
</tr>
</tbody>
</table>
4 RESULTS AND DISCUSSION

4.1 Fungi isolated from moss panels and green roof mosses and their virulence toward the model moss, *P. patens*

A large number of fungal species have been found to inhabit diverse groups of bryophytes in their natural habitats (Wilson 1951, Racovitza 1959, Felix 1988, Davey and Currah 2006). However, knowledge of fungal colonization of green roof plants is limited—especially for mosses. Our study identified fungal isolates inhabiting mosses at a moss farm in Japan and on green roofs in southern Finland. In addition, our study described the ability of these fungi to infect and cause disease symptoms in the model moss, *P. patens*.

4.1.1 Fungal genera in moss panels and on green, mossy roofs

A total of 26 fungal isolates were obtained from the damaged areas of *Racomitrium japonicum* moss panels along with 64 fungal isolates and an oomycete from nine different green roofs located in southern Finland (I, II). Fungi isolated from the moss panels of *R. japonicum* belong to 11 genera of Ascomycota (*Alternaria*, *Apiospora*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Fusarium*, *Humicola*, *Myrmecridium*, *Penicillium*, *Scolecobasidium* and *Trichoderma*) (Table 2). Fungi isolated from Finnish green roofs belong to four genera of Ascomycota (*Botrytis*, *Fusarium*, *Phoma* and *Trichoderma*), three genera of Zygomycota (*Mortierella*, *Mucor* and *Rhizopus*), and one genus of Basidiomycota (*Ceratobasidium*). One genus of the fungal-like pathogens of Oomycota (*Pythium*) was also found (Table 2). *Fusarium* was the most abundant fungal genus obtained from the moss panels of *R. japonicum*, and *Trichoderma* was the most abundant genus on Finnish green roofs.

Most of the fungal genera detected in the moss panels of *R. japonicum* and on green roof mosses have been previously found to be associated with bryophytes in nature. Grandi et al. (2008) studied hyphomycetes associated with decomposing bryophytes and isolated fungi belonging to the genera *Alternaria*, *Cladosporium*, *Curvularia*, *Epicoccum* and *Humicola*. Varga et al. (2002, 2009) found that *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma* are associated with the moss *Tortella tortuosa*. *Arthrinium* (teleomorph of *Apiospora*) has been detected from *Sphagnum* species and *Scolecobasidium* on *Bryum pseudotriquetrum* (Thormann and Rice 2007, Tosi et al. 2001). Hughes et al. (2003) isolated fungi belonging to the genera *Mortierella*, *Phoma* and *Pythium* from colonies of the leafy liverwort *Cephaloziella varians*. The results from our study indicate that similar types of fungi colonize mosses in nature and on built environments, indicating that green roof mosses as well as moss farms provide a living environment for diverse fungal populations.
### Table 2. Fungal and fungal-like isolates belonging to different genera.

<table>
<thead>
<tr>
<th>Fungal division</th>
<th>Fungal genus</th>
<th>Number of isolates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td><em>Alternaria</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Apiospora</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Botrytis</em></td>
<td>1</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Cladosporium</em></td>
<td>5</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Curvularia</em></td>
<td>3</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Epicoccum</em></td>
<td>3</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Fusarium</em></td>
<td>21</td>
<td>Moss panel, Japan (6 isolates), Green roof, Finland (15 isolates)</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Humicola</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Myrmecridium</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Penicillium</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Phoma</em></td>
<td>8</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Scolecobasidium</em></td>
<td>1</td>
<td>Moss panel, Japan</td>
</tr>
<tr>
<td>Ascomycota</td>
<td><em>Trichoderma</em></td>
<td>28</td>
<td>Moss panel, Japan (3 isolates), Green roof, Finland (25 isolates)</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td><em>Ceratobasidium</em></td>
<td>1</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Oomycota</td>
<td><em>Pythium</em></td>
<td>1</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Zygomycota</td>
<td><em>Mortierella</em></td>
<td>4</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Zygomycota</td>
<td><em>Mucor</em></td>
<td>9</td>
<td>Green roof, Finland</td>
</tr>
<tr>
<td>Zygomycota</td>
<td><em>Rhizopus</em></td>
<td>1</td>
<td>Green roof, Finland</td>
</tr>
</tbody>
</table>

#### 4.1.2 Effects of isolated fungi on the model moss, *P. patens*

Many of the isolated fungi caused mild to severe symptoms on *P. patens*, and pathogenicity was scored based on the severity of the symptoms. Highly pathogenic isolates caused browning of the whole plant including the protonema filaments, stem and leaves, resulting in the death of moss plants within 2 weeks. Moderate pathogens caused browning of protonema filaments and occasionally
browning of the stem. Mild pathogens caused only browning of protonema filaments, and non-pathogenic isolates did not cause any visible symptoms.

In total, 74 isolates were selected for the pathogenicity test, 9 of which were obtained from moss panels and 65 from green roofs. A total of 66 of 73 isolated fungi and one of the fungal-like isolates caused symptoms on *P. patens*, and 33 of them were highly pathogenic and resulted in the death of the moss within 2 weeks after inoculation (Table 3). The most severe symptoms on gametophytes of *P. patens* were caused by fungi described as *Alternaria alternata*, *B. cinerea*, *Fusarium acuminatum*, *Fusarium avenaceum*, *Fusarium oxysporum*, *Fusarium triticum*, *Phoma glomerata*, *Phoma herbarum*, *Phoma tropica*, *Trichoderma atroviride*, *Trichoderma hamatum*, *Trichoderma koningiopsis*, and *Trichoderma viride* (I, II). Except for *Trichoderma*, all these fungal genera are well-known fungal pathogens of cultivated plants (Thomma 2003, Dean et al. 2012, Jimenez et al. 1993, Uhlig et al. 2007, Carrieri et al. 2014, Chohan and Chand 1980, Neuman and Boland 1999, Gullino et al. 2017).

The highly pathogenic fungal isolates caused browning and maceration of the protonema filaments, gametophore stems, and leaves (Fig. 3). Typically, the browning began from the protonema filaments and proceeded to stems and leaves, resulting in death of moss plants within 2 weeks. Cell-level alterations—including browning and discoloration—observed in infected moss plants were probably due to the accumulation of phenolic compounds. Production and release of phenolic compounds by the host is an important defense response against pathogens (Beckman 2003, Jones & Saxena 2013). The accumulation of phenolic compounds in *P. patens* as a response to fungal infection has been described by Ponce de Leon et al. (2012), who showed that *B. cinerea* infection induced the fortification of the plant-cell wall by the incorporation of the phenolic compounds, indicating this is a conserved defense mechanism among bryophytes.

Fungi such as *Apiospora montagnei*, *Ceratobasidium* sp., *Cladosporium oxysporum*, *Epicoccum nigrum*, *Fusarium graminearum*, *Fusarium lateritium*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma koningiopsis* (isolate SS0.1-1), and *Trichoderma parariviridescens* and the oomycete *Pythium* sp. caused mild symptoms on *P. patens*. Typically, these isolates induced the browning of protonema within 2 weeks after inoculation, but the browning did not spread to the upper parts stem and leaves of *P. patens*. As phenolic compounds are one of the defense mechanisms against fungal pathogens, it might be that the accumulation of phenolic compounds in protonema filaments is sufficient to restrict the spread of weak pathogens. In addition, microscopic observation of *P. patens* inoculated with *A. montagnei* revealed the formation of the defensive structure papilla (III), which has been reported in *P. patens* in response to infection with old cultures of *Pythium debaryanum*.
(Oliver et al. 2009) and also in many mosses infected by ascomycetes and basidiomycetes (Racovitza 1959, Hassel and Kost 1998, Davey et al. 2009). Hassel and Kost (1998) reported cell-wall thickening in *Brachythecium rutabulum* in response to infection with the basidiomycete *Leptoglossum retirugum*, whereas Davey et al. (2009) described the formation of a papilla-like structure at penetration sites during the interaction between the host plant *Funaria hygrometrica* and the ascomycete *Atradidymella muscivora*. Papilla formation also has been described in hepatophytes and angiosperms (Racovitza 1959, Aist 1976), indicating that it is a highly conserved defense response against invading fungi (Davey et al. 2009).

**Fig. 3. Symptoms caused by *Fusarium avenaceum* MEL2.3 and *Trichoderma viride* TKL7.1 on *Physcomitrella patens*.** Control *P. patens* (a and b) and *P. patens* inoculated with *F. avenaceum* MEL2.3 (c and d) and *T. viride* TKL7.1 (e and f) isolates. *F. avenaceum* MEL2.3 caused browning of the plant cells within 10 days after inoculation (d). Inoculation with *T. viride* TKL7.1 caused discoloration of plant cells within 10 days after inoculation. Scale bars: 5 mm (upper row), 100 μm (lower row).

In addition, 14 fungal isolates that did not cause any apparent symptoms on *P. patens*. Those species belong to the genera *Mucor*, *Mortierella* and *Rhizopus*, all of which are members of the subphylum mucoromycotina. Carella and Schornack (2018) showed that bryophytes have beneficial endophytic interactions with members of mucoromycotina, ascomycota and basidiomycota. Field et al. (2015) demonstrated that liverwort–Mucoromycotina symbiosis is mutualistic and mycorrhiza-like. Together with our findings, these results indicate that members of mucoromycotina associate with bryophytes. As these fungi were asymptomatic on the model moss *P. patens*, they might also function as natural symbionts for mosses in green roof environments.
<table>
<thead>
<tr>
<th>Fungal description a</th>
<th>No. of isolates</th>
<th>Source of isolate b</th>
<th>Symptom severity c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternata</td>
<td>1</td>
<td>Moss panel</td>
<td>+++</td>
</tr>
<tr>
<td>Apiospora montagnei</td>
<td>1</td>
<td>Moss panel</td>
<td>+</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>1</td>
<td>Korkeasaari</td>
<td>+++</td>
</tr>
<tr>
<td>Ceratobasidium sp.</td>
<td>1</td>
<td>Korkeasaari</td>
<td>+</td>
</tr>
<tr>
<td>Cladosporum oxysporum</td>
<td>1</td>
<td>Moss panel</td>
<td>++</td>
</tr>
<tr>
<td>Epicoccum nigrum</td>
<td>2</td>
<td>Moss panel</td>
<td>++</td>
</tr>
<tr>
<td>Fusarium acuminatum</td>
<td>8</td>
<td>Korkeasaari (5), Kappelitie (3)</td>
<td>+++</td>
</tr>
<tr>
<td>Fusarium avenaceum</td>
<td>3</td>
<td>Korkeasaari, Meilahti, Moss panel</td>
<td>+++</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td>1</td>
<td>Meilahti</td>
<td>+</td>
</tr>
<tr>
<td>Fusarium lateritium</td>
<td>1</td>
<td>Korkeasaari</td>
<td>+</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>2</td>
<td>Moss panel</td>
<td>+++</td>
</tr>
<tr>
<td>Fusarium poae</td>
<td>1</td>
<td>Meilahti</td>
<td>+</td>
</tr>
<tr>
<td>Fusarium tricinctum</td>
<td>2</td>
<td>Fabianinkatu, Korkeasaari</td>
<td>+++</td>
</tr>
<tr>
<td>Mortierella elongate</td>
<td>4</td>
<td>Kaisaniemi, Onkiniemi, TKL (2)</td>
<td>Ns</td>
</tr>
<tr>
<td>Mucor circinelloides</td>
<td>1</td>
<td>Kaisaniemi</td>
<td>Ns</td>
</tr>
<tr>
<td>Mucor fragilis</td>
<td>1</td>
<td>Kappelitie</td>
<td>Ns</td>
</tr>
<tr>
<td>Mucor hiemalis</td>
<td>6</td>
<td>Fabianinkatu, Kaisaniemi, Korkeasaari, Kappelitie, Onkiniemi, TKL</td>
<td>Ns</td>
</tr>
<tr>
<td>Mucor plumbeus</td>
<td>1</td>
<td>Ikano</td>
<td>Ns</td>
</tr>
<tr>
<td>Phoma herbarum</td>
<td>6</td>
<td>Ikano (5), Meilahti (1)</td>
<td>+++</td>
</tr>
<tr>
<td>Phoma tropica</td>
<td>1</td>
<td>Meilahti</td>
<td>+++</td>
</tr>
<tr>
<td>Peyronellaea glomerata</td>
<td>1</td>
<td>Meilahti</td>
<td>+++</td>
</tr>
<tr>
<td>Pythium sp.</td>
<td>1</td>
<td>Meilahti</td>
<td>+</td>
</tr>
<tr>
<td>Rhizopus microspores</td>
<td>1</td>
<td>Ikano</td>
<td>Ns</td>
</tr>
<tr>
<td>Trichoderma atroviride</td>
<td>3</td>
<td>Kaisaniemi, Korkeasaari, Kappelitie</td>
<td>+++</td>
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<td>Metsälä</td>
<td>+++</td>
</tr>
<tr>
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<td>14</td>
<td>Fabianinkatu, Ikano, Kaisaniemi, Korkeasaari, Meilahti, Metsälä, Onkiniemi</td>
<td>++/+</td>
</tr>
<tr>
<td>Trichoderma koningii</td>
<td>1</td>
<td>Kappelitie</td>
<td>+</td>
</tr>
<tr>
<td>Trichoderma koningiiopsis</td>
<td>2</td>
<td>Onkiniemi, Moss panel</td>
<td>+++/+</td>
</tr>
<tr>
<td>Trichoderma paraviridescens</td>
<td>2</td>
<td>Onkiniemi, TKL</td>
<td>+</td>
</tr>
<tr>
<td>Trichoderma viride</td>
<td>3</td>
<td>Korkeasaari, Metsälä, TKL</td>
<td>+++</td>
</tr>
</tbody>
</table>

a The description is based on a BLAST search of the sequence (identity > 99 %) obtained with the ITS1 and ITS2 primers and with ITS4 and ITS5 primers using the NCBI database.

b Numbers in parenthesis indicate the number of isolates obtained from the roof.
Severity of symptoms: +++ severe symptoms, browning and death of moss; ++ moderate symptoms, browning of protonema and stem occasionally; + mild symptoms, browning of protonema; ns: no symptoms.

4.2 Fungi that infect cultivated moss can also cause disease in crop plants

Plant-pathogen interactions may change the diversity and structure of natural plant communities, causing ecological and economic impacts (Caseys et al. 2018). Plant pathogens can be classified as generalists or specialists based on the range of plant hosts they can infect (Barret and Heil 2012). Generalist plant pathogens such as certain *Fusarium* spp. and *B. cinerea* can infect multiple or even dozens of plant species (Caseys et al. 2018, Dean et al. 2012). Although certain generalist plant pathogens have been well described, little is known about whether mosses and vascular plants share common pathogenic fungi. Our study here revealed that certain fungi that are pathogenic to mosses are also able to infect and cause disease in vascular plants, demonstrating that the host range of these pathogens is wider than previously known.

4.2.1 Two fungal isolates pathogenic to mosses cause disease in crop plants

Our results demonstrate that *F. avenaceum* SSO1-2 isolated from moss panels could reduce the germination of tomato and carrot (III). The germination test revealed that the emergence of tomato and carrot plants was 47 % and 41 %, respectively, in soil infested with *F. avenaceum*, whereas the emergence in the respective uninoculated controls was 81 % and 77 %, and these differences were significant (p < 0.05; one-directional analysis of variance). Disease symptoms such as browning of the basal parts of the leaves and stem of barley was observed at 15 days post-inoculation, followed by maceration of the stem and basal part of leaves 44 days post-inoculation (III). In addition, *F. avenaceum* caused a few necrotic lesions on the stem of tomato seedlings 21 days post-inoculation. Moreover, *C. oxysporum* caused severe disease symptoms on carrot seedlings; discoloration, reddening, impaired growth, and premature death were observed within 5 weeks after inoculation (III). In addition, *C. oxysporum* could induce mild symptoms in *P. patens* (I).

Diseases caused by microbial pathogens, oomycetes, fungi, bacteria and viruses cause enormous crop losses, and in some cases the crop may be completely devastated (Vidyasekaran 2016). *F. avenaceum* is a fungus that commonly associates with many different host plants, and the most economically damaging impact of *F. avenaceum* is crown rot and head blight of wheat and barley (Nalim et al. 2009, Uhlig et al. 2007, Lysoe et al. 2014). The genus *Cladosporium* contains many
species that are pathogens of both plants and animals and may also destroy stored vegetables (Ho et al. 1999). Certain of the *Cladosporium* spp. are highly specialized plant pathogens. The highly specialized interaction between *Cladosporium fulvum* and tomato involves a gene-for-gene relationship i.e. for a gene for virulence in a pathogen there is a corresponding gene for resistance in the host towards that pathogen (Thomma et al. 2005, Agrios 2005). A few studies have reported that *C. oxysporum* is a pathogen of pepper and tomato, causing chlorotic spots that develop further to brown lesions on the leaves of host plants (Hammouda 1992, Lamboy and Dillard 1997). Our results from this study indicate that the host range of *F. avenaceum* and *C. oxysporum* is wider than previously known, as it covers both vascular and non-vascular plants. Navaud et al. (2018) suggested that the emergence of broad-host-range fungal pathogens results largely from host jumps. This might indicate that mosses may also play important roles in disseminating diseases among a local plant community. The results suggest that mosses might be intermediate hosts of some pathogens of crop plants and that they might contribute to plant disease epidemiology, an issue that has been largely ignored.

### 4.2.2 *Fusarium oxysporum* and *Alternaria alternata* do not cause damage to vascular plants

The results from our study showed that five isolates of *F. oxysporum* and one isolate of *A. alternata* did not cause any detectable damage in any of the vascular plants we tested, but they all were highly pathogenic to *P. patens*. *F. oxysporum* is ranked fifth in the top 10 fungal pathogens based on scientific or economic importance (Dean et al. 2012). One reason for its high importance as a fungal pathogen is its ability to infect many different plant species such as tomato, cotton and banana. The *F. oxysporum* species complex comprises different formae speciales, which together can infect more than 100 different hosts (Michielse and Rep 2009, Dean et al. 2012). Although *F. oxysporum* has an extremely broad host range at the species level, individual isolates of *F. oxysporum* cause disease in only one or a few plant species (Armstrong and Armstrong 1981, Gordon and Martyn 1997). *A. alternata* is a latent fungus, causing black spots on many fruits and vegetables and thus large post-harvest losses (Troncoso-Rojas and Tiznado-Hernandez 2014). These results indicate that, among the well-known fungal pathogens of crop plants, certain fungal isolates specialize in infecting bryophytes.
4.3 Screening of part of the Physcomitrella mutant collection reveals a role for an NAD(P)-binding Rossmann fold protein in chitosan-induced signaling

Mutations and other alterations within DNA sequences are driving forces of evolution as they bring genetic variability to populations (Loewe and Hill 2010). In agriculture, mutations have been used to develop new varieties with improved stress tolerance against abiotic and biotic factors (Ram et al. 2019). Mutational approaches also have been used widely to study functional genetics. Mutant collections provide a large source of genetic diversity for the characterization of gene function and thus are important for biological research.

A Physcomitrella mutant collection consists of more than 73,000 plants and was established in the early 2000s (Schween et al. 2000, Egener et al. 2005). This collection contains single KO plants and minibatch plants (Hochdurchsatz Minibatch; HoMi). For HoMi plants creation, several (20) transposon-tagged cDNAs are co-transformed in one experiment that is repeated five times (Schween et al. 2005). Screening of part of the P. patens mutant collection demonstrated that it is a valuable resource with which to identify genes involved in chitosan-induced peroxidase activity in mosses (IV). This work revealed the involvement of an NAD(P)-binding Rossmann fold protein in chitosan-induced peroxidase activity.

4.3.1 The Physcomitrella mutant collection as a source for candidate genes in chitosan-induced signaling pathways

Plants from the Physcomitrella mutant collection (Egener et al. 2002, Schween et al. 2005) were screened to identify transgenic moss plants that differ in their response to the fungal cell-wall component chitosan. The Moss Database (Schween et al. 2005) contains information on transgenic plants and was thus used for searching plants for the screen. The Moss Database was searched using three different requirements: 1) the appearance of transgenic plants should resemble wild-type plants (group A), 2) the number of gametophores should be like wild-type plants, and 3) transgenic plants should have a nptII selection marker integrated within their genome, indicating successful insertional mutagenesis. Plants from two different types of transformation were used; KO plants and HoMi plants.

Altogether, 2622 individual hits from KO plants and 8209 hits from HoMi plants were obtained from the Moss Database when the defined requirements were used (IV). Hits of KO plants were divided into 71 groups (different constructs) and hits of HoMi plants into 511 pools (Trafo DNA construct). Initially, one plant from each KO group and HoMi pool was thawed. Some of the thawed plants that
did not regenerate within 5 weeks were replaced with another plant from the same pool or group. Eventually, 713 plants were thawed including 3 wild-type plants, 79 KO plants, and 631 HoMi plants. Most of the KO plants (85.4 %) regenerated within 4 weeks after thawing whereas only 42.2 % of HoMi plants regenerated within those 4 weeks, and a substantial portion of HoMi plants (45.5 %) did not regenerate within 8 weeks.

Altogether, 385 transgenic plants were screened for chitosan-induced peroxidase activity, and activity could not be induced for 23 of these plants. To verify the results from the peroxidase activity screen, additional peroxidase activity assays were carried out with wild-type and 23 transgenic moss lines. The experiment was conducted twice with three replicates in each experiment. According to Mann Whitney U-test, four mutant lines had significantly lower peroxidase activity than wild-type plants when a p-value of 0.02 was used. In all, 1.04 % of the screened plants had significantly lower chitosan-induced peroxidase activity in comparison with wild type.

4.3.2 One mutation is located within an NAD(P)-binding Rossmann fold protein

Our studies with *P. patens* mutant lines demonstrated that genome walking analysis was a suitable method for detecting mutated genes within the genome of *P. patens*. To determine which genes had been mutated within the transgenic moss lines, *P. patens* line 58174 together with a wild-type line were chosen for the genome walking analysis. Line 58174 was chosen because its chitosan-induced peroxidase activity was significantly lower than that of wild type, and line 58174 was stable in all experiments (IV). The genome walking profile of line 58174 identified many nptII integrations, and some of them were located within genes encoding, for example, 28S ribosomal RNA, sucrose phosphatase, and a protein of the NAD(P)-binding Rossmann fold superfamily.

The Rossmann fold is very common fold and the functional roles of Rossmann fold superfamily proteins are versatile (Lesk 1995). The Rossmann fold is found in many metabolic enzymes that bind cofactors such as NAD and NADP. NADP is an essential molecule for energy metabolism and is involved in signaling pathways that regulate many cellular processes including transcription and apoptosis (Berger et al. 2004, Ying 2006). Many enzymes including dehydrogenases, ATPases and GTPases have a Rossman fold (Rao and Rossmann 1973, Burton 2018). As the Rossman fold has many candidate biological functions, we aimed to further elucidate the role of this Rossmann fold protein in the chitosan-induced signaling pathway.
4.3.3 An NAD(P)-binding Rossmann fold protein is involved in chitosan-induced peroxidase activity and lipoxygenase expression

According to JGI Phytozome v12.1 ontology annotation, the NAD(P)-binding Rossmann fold protein is predicted to be a dehydrogenase. To elucidate the role of this Rossmann fold protein in the chitosan-induced signaling pathway, single KO plants lacking a gene (Pp3c26_9450V3.1) for this Rossmann fold protein were generated (IV). The peroxidase activity assay showed that the KO lines lacking the gene for this Rossmann fold protein had significantly lower chitosan-induced peroxidase activity than wild-type plants, thus demonstrating a role for the Rossmann fold protein in the chitosan-induced signaling pathway (IV).

Dehydrogenases positively regulate the resistance to pathogens in vascular plants (Choi et al. 2008, Hwang et al. 2008, Rong et al. 2016). Choi et al. (2008) showed that the Rossman fold–containing enzyme menthone reductase1 (MNR1), a member of the short-chain dehydrogenase/reductase (SDR) superfamily, positively regulates the defense against pathogens in pepper (*Capsicum annuum*). *CaMNR1*-silenced pepper plants were significantly more susceptible to infections by *Xanthomonas campestris pv vesicatoria* and *Colletotrichum coccodes*. In addition, the expression of each of salicylic acid–responsive (basic PR1 and PR10) and jasmonic acid–responsive (defensin1) proteins was reduced in silenced *CaMNR1*-plants. Hwang et al. (2008) showed that SDR3 is directly involved in plant defense. Five days after spray inoculation with *Pseudomonas syringae* DC300, the bacterial population in *Arabidopsis* plants that overexpressed SDR3 was 4- to 6-fold lower than that of wild type (Hwang et al. 2008). In addition, the bacterial population of the *Atsdr3* mutant lines was 3- to 5-fold higher than that of wild-type plants. Rong et al. (2016) showed that cinnamyl alcohol dehydrogenase (CAD) contributes to host resistance against the fungus *Rhizoctonia cerealis* in wheat (*Triticum aestivum* L.). Knockdown of *TaCAD12* significantly repressed resistance against *R. cerealis* and downregulated defense genes (PR10, PR17c and chitinase1). Our results showed that *P. patens* NAD(P)-binding Rossmann fold protein is, at least in part, be required for peroxidase activation in non-vascular plants and thus it might contribute also to pathogen defense.

Infection by a pathogen can induce the expression of LOX genes (and hence upregulation of lipid peroxidation; Casey and Hughes 2004) in both vascular and non-vascular plants (Porta and Rocha-Sosa 2002, Ponce de León et al. 2007, Lehtonen et al. 2012, Ponce de León et al. 2015). Because studies have shown that LOXs are among the defense-responsive genes of *P. patens*, we aimed to test the expression of LOX7 in KO lines lacking the gene for the Rossmann fold protein we identified and in wild-type plants before and after 40 min of chitosan treatment. Interestingly, compared with wild-type plants, the level of *P. patens* LOX7 mRNA was significantly lower in all Rossmann fold protein
KO lines before and after chitosan treatment. Kumar et al. (2008) showed that the *Saccharomyces cerevisiae* transcriptional regulator Gal80p, which is involved in the repression of galactose metabolism genes, contains an N-terminal Rossmann fold and binds to NADP, which in turn regulates galactose-metabolizing genes in *S. cerevisiae*. Our results reveal that, in the absence of the Rossmann fold protein we identified, LOX7 expression in *P. patens* decreases compared with wild type, indicating that this Rossmann fold protein may be required for normal LOX7 expression in *P. patens*. 
5 CONCLUSION AND FUTURE PROSPECTS

The main findings of this thesis are the description of fungal pathogens of green roof mosses, their host ranges, and the role of a Rossmann fold protein in chitosan-induced peroxidase activity and for normal LOX expression. Build environments such as moss green roofs and moss farms are inhabited by many different fungal species, and many of these fungi can cause disease in bryophytes. *Fusarium* and *Trichoderma* species were the most predominant genera isolated from a moss farm in Japan and from moss green roofs in Finland, respectively. Many members belonging to the genus *Fusarium* are well-known plant pathogens, but *Trichoderma* spp. are typically considered as avirulent plant symbionts or parasites of other fungi and thus are widely used as biocontrol agents against fungal diseases of plants. Surprisingly, all of the isolated *Trichoderma* spp. in our study were potent, moderate or mild pathogens of *P. patens*, indicating their ability to infect mosses. These results indicate that *Trichoderma* spp. might also be primary pathogens of bryophytes. As *Trichoderma* spp. are typically considered non-plant pathogenic species and are widely used for biological control agents, their pathogenicity should be considered if used close to areas of moss cultivation or for moss green roofs. Our results also demonstrate that mosses and vascular plants share common pathogens. This finding is important as it suggests that mosses may contribute to the disease epidemiology of crop plants and may have played a role in the evolution of a broad host range of fungal pathogens.

Screening part of the *Physcomitrella* mutant collection enabled us to describe a role for a previously unknown component, namely a Rossmann fold protein, in the chitosan-induced signaling pathway of *P. patens*. Our results also demonstrate that this Rossmann fold protein is important for the normal expression of *P. patens* LOX7 and thus is also might contribute to defense against fungal infection. The results from our study may encourage the use of the *Physcomitrella* mutant collection in the field of functional genomics, toward the goal of revealing the roles of specific genes in signaling pathways.
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