Cell Death and Defence Gene Responses in Plant-Fungal Interactions

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2008

Acta Universitatis Agriculturae Sueciae

2008:51

Front cover: Left, *Arabidopsis thalinana* quadruple mutant *rlm1*$_{Ler}$*pad3ein2coi1-16* inoculated with GUS tagged *Leptosphaeria maculans* 14 dpi. Middle, Barley wild-type Bowman(*Rph3*), right, barley *bst1* mutant exhibiting spontaneous lesions.

ISSN 1652-6880
ISBN 978-91-85913-84-8

Tryck: SLU Service/Repro, Uppsala 2008

Abstract

Persson, M. 2008. Cell Death and Defence Gene Responses in Plant-Fungal Interactions. Doctor's dissertation.
ISSN 1652-6880, ISBN 978-91-85913-84-8

The molecular interaction between two fungal pathogens and their hosts barley (*Hordeum vulgare*) and *Arabidopsis thaliana*, are investigated in this thesis. *Bipolaris sorokiniana* causes spot blotch and common root rot in several cereals including barley. In order to study important defence mechanisms, a set of mutants were generated and studied. The barley lesion mimic mutant *Bipolaris sorokiniana tolerant 1* (*bst1*) has an enhanced tolerance against spot blotch but not root rot. The role of H_2O_2 was found to be of importance and the gene expression of pathogenesis related (*PR*) genes were highly up regulated in *bst1* after inoculation compared to wild-type. After a new round of mutagenesis and screening, additional germplasm for both root rot and spot blotch caused by *B. sorokiniana* were identified in *bst1* background.

Leptosphaeria maculans is the causal agent of blackleg in oilseed crops. In order to study the interaction between *L. maculans* and a plant host it is more convenient to work with *Arabidopsis thaliana*. To further investigate the effects of the resistance in Arabidopsis, detailed analyses of mutants in the two TIR-NB-LRR genes in the *RLM1* locus and other *R* gene signalling mutants were made. A quantitative detection system of *L. maculans* on a genomic level with real time PCR was developed. The analyses showed that *RLM1* function is gene dose-dependent under environmental conditions. Analyses on mutants defective in hormone signaling in an $rlm1_{Ler}pad3$ background revealed significant influence of JA and ET on symptom development as well as pathogen colonization. An important resistance gene, *RLM3* was identified from a cross between the highly susceptible accession An-1 and the resistant Col-0 with the use of a microarray analysis. $RLM3_{Col}$ encodes for a TIR and NB protein and has been shown to be of importance for defence to three other necrotrophic fungi (*Botryis cinerea*, *Alternaria brassicicola* and *A. brassicae*).

Taken together, this work contributes to enhanced knowledge of interactions between hemibiotrophic fungi and their hosts with a focus on cell death and defence gene responses.

Keywords: Arabidopsis thaliana, barley, *Bipolaris sorokiniana*, *Leptosphaeria maculans, R gene* and signalling

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This thesis is dedicated to the loving memory of my mother,
Altea Persson (1953-2006). She never gave up and fought until the end.

Contents

Appendix

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

I Persson, M., Falk, A. and Dixelius, C. (2008) The barley lesion mimic mutant *bst1* is impaired in H_2O_2 production and displays enhanced *PR* and *HCP1* gene expression. (Submitted)

II Persson, M., Rasmussen, M., Falk, A and Dixelius C. (2008) Barley mutants with enhanced level of resistance to Swedish isolates of *Bipolaris sorokiniana*, casual agent of spot blotch. Plant Breeding. Epub.

III §Persson, M., §Staal, J., Oide, S. and Dixelius, C. (2008) Genetic dissection of the major resistance locus *RLM1* revealed *R* gene dependent and independent defense responses. (In manuscript)

IV Staal, J., Kaliff, M., Dewaele, E., Persson, M. and Dixelius, C. (2008) *RLM3*, a TIR domain encoding gene involved in broad-range immunity of Arabidopsis to necrotrophic fungal pathogens. Plant J. 55, 188-200.

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§Indicates shared first authorship

Introduction

Plants are part of a multifaceted community and in contact with many different organisms. Some of the organisms are beneficial, for instance mycorrhiza, whereas others are pathogenic, causing diseases, resulting in plant death or reduction in reproduction or yield (Pieterse and Dicke, 2007). Plants are not passive targets to pathogen attack. Constitutive physical mechanisms, such as waxy layers, may block the attack of pathogens. Otherwise, immediately after the presence of a pathogen has been detected defence mechanisms are initiated. The perception of pathogen by the host is the outcome of a highly coordinated and sophisticated network. Intense studies of plant defence mechanisms have taken place over the last decade and numerous reports and reviews are published. Here, recent work with relevance for the thesis work will be highlighted.

Recognition and signalling

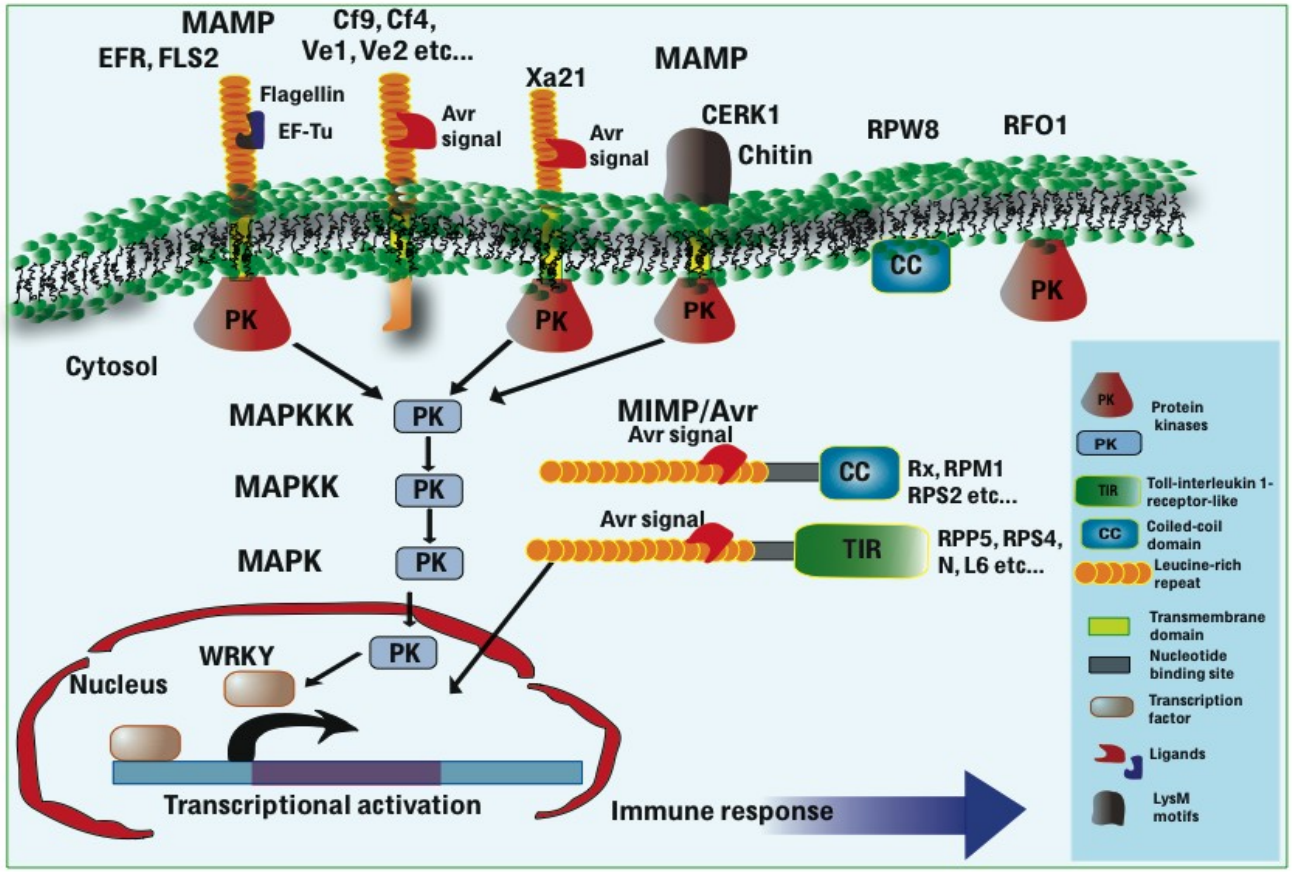
To defend themselves against pathogens, plants have developed two classes of immune receptors in the innate (non adaptive) immunity system. The first line of defence consists of a set of defined receptors referred to as pattern recognition receptors (PRRs), these recognize conserved microbe-associated molecular patterns (MAMPs), sometimes also called pathogen-associated molecular patterns (PAMP) (Nürnberger et al., 2004; Zipfel and Felix, 2005; Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). Numerous MAMPs from plant pathogens have been identified, of which flagellin, lipopolysaccharide and elongation factor Tu can be derived from Gram-negative bacteria and chitin, ergosterol and β-glucans from fungi and oomycetes (Nürnberger et al., 2004; Zipfel and Felix, 2005). Also important for the infected plant, is to establish a balance between fast and efficient response in contrast to the inappropriate expression of defence genes and uncontrolled cell death (Hofius et al., 2007). This can be compared to the overreaction of autoimmune diseases in animal innate immunity (Liew et al., 2005). Plants have developed mechanisms to sense infectious-self or modified-self molecules in addition to MAMPs/PAMPs. These molecules are either from the plant itself or direct from the invading pathogen, they are called danger-associated molecular patterns (DAMPS) (Matzinger, 2007). One example is the necrotic and ethylene- inducing peptide 1- (Nep1)- like peptide induced by several pathogens and recognized by plants (Qutob et al., 2006). The phytotoxic modification of the host is recognised and can therefore also be referred to as toxin-mediated immunity.

For a long time it was thought that communication between plant cells occurs through the cell-wall spanning plasmodesmata. However, since the identification of the first plant cell surface receptor (Walker and Zhang,

1990) many plasma membrane anchored receptors have been found to play key roles in diverse processes. The PRRs are composed of different protein domains. The surface receptors mainly detect MAMPs and include receptor-like kinases (RLK), receptor-like proteins (RLP), and extracellular binding proteins (He et al., 2007) (Fig. 1). In the Arabidopsis genome, 610 RLKs and 57 RLPs are present. A recent global phenotyping of all RLP mutants revealed participation in a wide range of biological events (Wang et al., 2008). Interestingly, one RLK, the brassinosteroid-associated receptor kinase BAK1 shows multiple functions to different effectors secreted by the pathogenic bacteria *Pseudomonas syringae* (Shan et al., 2008). Recognition of pathogens leads to PAMP-triggered immunity (PTI). Other noticeable responses are cell wall alterations, deposition of callose, hormone signalling and the accumulation of pathogenesis related proteins (PR) in order to prevent further colonization by the pathogen. In the interaction between *Pseudomonas syringe* pv. *tomato* and Arabidopsis, it was found that more than 800 genes were PAMP regulated and that 96 also were up regulated during a period of 12 hours post inoculation, indicating a core PTI response (Thilmony et al., 2006; Truman et al., 2006).

A second class of immune receptors is the plant resistance (R) proteins. They are mainly intracellular and have the capability to directly or indirectly detect isolate specific pathogen effectors encoded by avirulence genes. To date numerous resistance (*R*) genes have been identified and cloned but despite the broad spectrum of pathogens they detect, they are composed of a combination of only a handful of protein domains (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). The most common R protein class harbours the leucine-rich repeat (LRR) domain. LRRs are 20 to 30 amino acids long and can also be found in animal innate immunity molecules (Nürnberger et al., 2004; Staal and Dixelius, 2007).

Figure 1. Integrated overview visualising various receptor classes detecting microbe-associated molecular patterns (MAMPs), microbe-induced molecular patterns (MIMPs) and Avr proteins (effectors). Several of the pathways converge in a MAP kinase cascade and lead to the activation of various WRKY family members. The WRKY (and possible other) transcription factors interact in a very complex network to determine the appropriate response towards the pathogen detected. Some membrane-associated proteins do not have an extracellular domain and may interact with another extracellular receptor. The RLK receptors FLS2 and EFR form heteromers with brassinosteroid associated kinase 1 (BAK1), indicating that various combinations of RLKs, analogously to mammalian TLRs, could result in a wide recognition potential of various epitopes. Intracellular receptors, here represented by different R proteins, recognise Avr signals and transduce further defence signalling. In the case of the barley powdery mildew *MLA* gene, nuclear interactions have shown to link effector-specific and MAMP-triggered immune responses. Illustration made by Fuad Bahram.


MAMP
EFR, FLS2
Flagellin
EF-Tu
Cf9, Cf4,
Ve1, Ve2 etc...
Avr
signal
Xa21
Avr
signal
MAMP
CERK1
Chitin
RPW8
RFO1
CC
PK
PK
PK
PK
PK
Cytosol
MAPKKK
MAPKK
MAPK
PK
PK
PK
MIMP/Avr
Avr signal
CC
Rx, RPM1
RPS2 etc...
Avr signal
TIR
RPP5, RPS4,
N, L6 etc...
PK
WRKY
Nucleus
Transcriptional activation
Immune response
Protein
kinases
Toll-interleukin 1-
receptor-like
Coiled-coil
domain
Leucine-rich
repeat
Transmembrane
domain
Nucleotide
binding site
Transcription
factor
Ligands
LysM
motifs

Members of the largest class of R proteins possess, in addition to the LRR, a central nucleotide binding site (NB) domain that is similar to the NB of the NODs (nucleotide binding-oligomerization domain) and the animal cell death effector proteins Apaf1 and CED4, denoted NB-ARC (Dangl and Jones, 2001). The NB-LRR class of R proteins is further sub-divided according to the N-terminal domain of these proteins. Some proteins contain a Toll-interleukin 1 receptor homology region (TIR) domain, whereas others possess a coiled-coil (CC) domain, Fig. 1. Like the LRR and NB domains, the TIR domain is found in animal innate immunity proteins, specifically Toll and the Toll-like receptors, TLRs (Soosaar et al., 2005).

Surprisingly, a grass like rice lacks TIR-NB-LRRs entirely but instead 261 X-NB-LRRs i.e. R- proteins with N-terminals with unknown function are distributed in the genome (Monosi et al., 2004). Unlike animal NB-LRRs plant NB-LRR immune receptors have evolved the ability to specifically recognize effector proteins from pathogens, the effector triggered immunity (ETI) reviewed by Chisholm et al. (2006). A co-evolutionary hypothesis was further put forward where plant defence responses and MAMPs and effectors by pathogens were illustrated as a four phased zig-zag model, where both members of the system evolve mechanisms to overcome each others defence or attack strategies over time (Jones and Dangl, 2006). Recent work has also revealed that plant NB-LRRs are very adaptive in their ways of pathogen recognition and defence initiation (Caplan et al., 2008). After the recognition phase, transcriptional activation takes place in the nucleus to induce defence-related signalling (Shen et al., 2007).

New data on indirectly recognized effectors have however emerged, that are inconsistent with the direct R-Avr protein binding and the guard model. For example, the AvrBs3 effector protein from *Xanthomonas campestris* is directly localised to the nucleus and binds to the promoter of the *Bs3* resistance gene, which leads to *Bs3* transcript accumulation followed by HR induction (Römer et al., 2007). Based on this and additional findings, the decoy model has been proposed (van der Hoorn and Kamoun, 2008). This model takes into account the evolutionary aspects of opposing selection forces on guarded effector targets. Experimental evidence in order to distinguish between variants of the guard model and the new proposed decoy model are to be expected in near future.

Hormones

Hormones are signalling molecules that have a regulatory role important for the whole lifecycle of the plant. They are produced at specific sites and in low concentrations. Defence pathways are also dependent on plant

hormones. Numerous studies have demonstrated that salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the main molecules activating defence genes (Thomma et al., 2001). However, we know that exceptions exist like defence to, *Phytophthora porri* (Roetchi et al., 2001), *Erysiphe cichoracearum* (Vogel et al., 2002) and *Leptosphaeria maculans* (Bohman et al., 2004) where these three hormones are of minor importance. The SA pathway is primarily linked to resistance to biotrophic pathogens i.e. organisms that feed and reproduce on living tissue. This is in contrast to JA and ET, which mediate resistance mostly to necrotrophic pathogens (organisms which kill their hosts and derive nutrients, live and multiply on dead tissue). This differentiation of defence signalling pathways suggests that plants detect differences between pathogen lifestyle and mode of infection. Genetic evidence for JA antagonism of SA signalling pathways is well documented, but emerging data suggest a more complex signalling network evoking both positive and negative regulatory interactions (Spoel et al., 2007; Lópes et al., 2008; Vlot et al., 2008).

SA is a strong inducer of *PR* genes, and particularly *PR-1* is used as a marker for SA-mediated defence (Gaffney et al., 1993). A different set of genes is activated by JA, such as *VSP2* and *PDF1.2* (Benedetti et al., 1995; Penninckx et al., 1998). The latter, *PDF1.2*, also responds to ET (Thomma et al., 2001).

Lately, it has become apparent that plant growth hormones and modulation of developmental processes not earlier recognised as being of importance for plant defence play important roles. A model proposed by Robert-Seilaniantz et al. (2007) shows that auxin and cytokinins promote biotrophic susceptibility by inducing necrotrophic resistance pathways via JA/ET. Plants have evolved mechanisms to suppress auxin signalling as a component of basal defence in order to hinder the invading pathogens from using the hormone as a virulence factor (Navarro et al., 2006). In parallel, gibberellic acid (GA) induces necrotrophic susceptibility, by inducing the biotrophic SA resistance pathway (Robert-Seilaniantz et al., 2007). However, most of these interactions remain to be proved.

The role of abscisic acid (ABA) in a plant stress context is complex. ABA is a well known component in abiotic stress responses, but has lately been shown to be important in defence to various pathogens (Asselbergh et al., 2008). For example, in the Arabidopsis – *L. maculans* interaction ABA is important for resistance, and both callose dependent and independent pathways are present (Kaliff et al., 2007). Data on the impact of brassionsteroids on defence responses is also emerging. The brassinosteroid signalling pathway receptor kinase protein (BAK1) has so far been shown

to be required for the control of cell death, production of ROS and the restriction of biotrophic and necrotrophic infections (Heese et al., 2007; Kemmerling et al., 2007).

Pathogenesis-related (PR) proteins

When the defence is initiated a wide range of proteins are induced in a plant. Among these a group of proteins named pathogenesis-related proteins are common (van Loon et al., 2006: Sels et al., 2008). PRs were first defined as host-specific proteins that are induced during pathological or related situations (van Loon and van Kammen, 1970) and a defined nomenclature was presented in 1994 by van Loon and co-workers (van Loon et al., 1994). Later it was discovered that the proteins could be present in uninfected tissues, depending on the species, and the "community" abandoned the definitions presented in 1994. Instead, all microbe-induced proteins were called PR proteins (van Loon and van Strien, 1999). Many PRs are also induced by various types of stress, such as senescence, cold and wounding or are present in certain tissues, such as pollen and fruits, and act as potential allergy inducers in humans. PRs accumulate after pathogen attack by virus, viroids, bacteria, fungi, nematodes, insects and herbivores and are activated by SA, JA, and ET. The specific function of many PRs is still unknown although several of them have chitinase-, peroxidase-, oxidase-, ribonuclease- and superoxidase activity (Sels et al., 2008). Not all PR types are present in all plant species and the composition is highly variable. Quantitative resistance against pathogens have in several cases been associated to the expression of PRs (Liu et al., 2004; Pflieger et al., 2001).

Induced resistance

Several kinds of plant-pathogen interactions result in the generation and emission of long-distance signals from the site of infection to healthy uninfected parts of the plant where subsequent resistance is induced. Systemic acquired resistance (SAR) is a form of inducible resistance that is triggered in systemic healthy tissues of locally infected plants. SAR is incited by avirulent pathogens (biotrophs) that attack plant aerial tissues resulting in systemic induction of a long-lasting and broad-spectrum disease resistance. SAR requires both local and systemic SA accumulation and the induction of a subset of *PR* genes, particularly *PR-1*. SA itself is not the mobile signal, but the recently discovered methyl salicylate may be the candidate (Park et al., 2007), although more data needs to be presented to finalise this conclusion (Vlot et al., 2008).

Root-colonizing rhizobacteria have also potential to induce pathogen resistance in above-ground plant tissue (van Loon, 2007). This induced

systemic resistance (ISR), in contrast to SAR, is dependent on JA and ET signalling (Pieterse et al., 1998; van Wees et al., 1999). Generally there is not a substantial change in the gene expression of the plant induced by beneficial organisms under ISR conditions (Liu et al., 2007). Instead, priming usually occurs, leading to faster response upon pathogen attack resulting in an enhanced resistance (Conrath et al., 2006).

Both in SAR and ISR, as well as in compatible pathogen interactions, it is the MAMPs that are detected and the subsequent signalling pathways activated (Bittel and Robatzek, 2007). The signalling networks that are activated by the beneficial organisms overlap the signalling pathways of the pathogens and finely tuned regulation and adaptive responses have to be balanced in order to get the proper response (van Wees et al., 2008).

Cell death

Programmed cell death (PCD) is an intrinsic mechanism that occurs in nearly all organisms. In plants endogenous stimuli induce developmental PCD processes during, for instance, embryogenesis (Bozhkov, 2005), leaf morphogenesis (Gunawardena, 2008), xylem development (Fukuda, 1996), floral development (Rogers, 2006) and organ senescence (Rogers, 2005). Exogenous stimuli elicited by abiotic and biotic factors include PCD processes exemplified by the hypersensitive response (HR) a defence reaction particularly to avirulent biotrophic pathogens. Diagnostic hallmarks of plant PCD are in analogy to animal apoptosis; chromatin condensation, DNA laddering and activation of proteases even though the occurrence of these processes is largely depending on the type of PCD. Typical for plant PCD seems to be a degradation of the cytoplasmic contents by autophagy, which involves formation of micro- and macrophagosomes that are targeted into the vacuole (Bassham, 2007). On the other hand, autophagy can also participate in processes that protect the plant from cell death (Liu et al., 2005).

To incite local cell death is a quick and efficient defence response where the plant sacrifices a few cells to prevent further tissue colonisation by an invading pathogen (Lam, 2004). However, the process must be fine tuned and tightly controlled in order to not be detrimental for the plant. HR was first described approximately 100 years ago in observations of wheat and the responses to leaf rust (Ward, 1902) and black stem rust (Stakman 1915). Thereafter, the HR phenomena have been studied in various aspects, but the entire mechanistic understanding is still elusive (Mur et al., 2008). Factors that take part in this process are *R* genes, signalling molecules like EDS1-PAD4-SAG101, SA and ET, ion fluxes e.g. Ca^{2+}, reactive oxygen species (ROS) components (superoxide, hydrogen peroxide and hydroxyl radicals) resulting in e.g. lipid peroxidation, and interaction with nitric

oxide (NO). A range of cell death mutants exists in Arabidopsis but they do also occur in several crop species as shown in this thesis (I; II). These are valuable tools to further elucidate cell death functions.

Secondary metabolites in plant defence

Secondary metabolites (SM) are organic compounds present in all higher plants. Typical for a secondary metabolite is that they are not directly involved in the growth and development of the plant. It is common that one SM is dominant or unique within a plant family or genus. The pattern of SM in individual plants is complex, it changes in tissue and organ specific ways and depends on the age of the plant and on the environment since both biotic and abiotic stresses strongly influence the metabolic profiles. Most common secondary metabolites function as defence against pathogens, insect pest and competing plants or as signalling compounds, to attract pollinators or seed distributing animals (Wink, 2003). This makes these compounds important for plant survival and reproduction and they have hence been subjected to natural selection during evolution. SM can be present in the tissue in an active state or as a precursor that becomes activated upon wounding or infection. This is important since SMs involved in defence ward off, inhibit or kill the pathogen. Two secondary metabolites that are important in Arabidopsis and in a defence perspective are callose and camalexin (Tsuji et al., 1992; Pedras and Liu, 2004).

After fungal penetration, a reinforcement of the plant cell wall is established by site directed deposition of callose and secretion of antimicrobial compounds at the site of attack. Callose is a β-1,3 glucan and deposition is a key component of defence to certain pathogens, for instance the oomycete *Pythium irregulare* (Adie et al., 2007). Synthesis and deposition of callose is triggered by both biotic and abiotic stresses.

Low molecular weight antimicrobial compounds are called phytoalexins (Paxton, 1981). Phytoalexins show great molecular structural diversity. Camalexin is important in Arabidopsis defence against both necrotrophic and biotrophic pathogens and several regulatory genes of camalexin have been identified (Glawischnig, 2007). The induction of camalexin is part of a complicated defence mechanism, which involves SA, JA and ET signalling and unknown partners.

Fungal pathogens

One group of important plant pathogenic organisms is fungi. Fungi are small, usually filamentous, eukaryotic, lack chlorophyll and are spore-bearing organisms. Of the 100,000 species known, more than 10,000 can cause disease in plants, this in contrast to 50 species that are pathogenic to humans (Agrios, 2004). All plants have at least one plant pathogenic

fungus that can cause disease, and several fungi have more than one plant host (Agrios, 2004). Fungi are sessile organisms that to be able to spread from infected tissues in one plant to another healthy plant, need assistance. The most common way to spread spores is probably assisted of the wind. Other alternatives are by water, insects, wild animals and humans.

Fungi are divided into 7 phyla, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Zygomycota, Ascomycota, and Basidiomycota. The two fungi studied in this theses (*Bipolaris sorokiniana* and *Leptosphaeria maculans*) belong to Ascomycota. Characteristic for Ascomycota is the presence of a sexual stage called telemorph and the formation of ascospores. Ascospores are sexual spores and eight asci are formed within each ascus, which is a sac-like zygote cell. Many fungi in this phylum produce asexual spores (conidia), formed by cutting off terminal or lateral cells from special hyphae called conidiephores. This is also the case for *B. sorokiniana* and *L. maculans* (Agrios 2004; Kumar et al., 2002; Howlett et al., 2001).

Bipolaris sorokiniana

Bipolaris sorokiniana (Sacc.) Shoemaker (teleomorph: *Cochliobolus sativus*) (Ito & Kuribayashi.) Drechs. Ex Dastur. is a hemibiotrophic phytopathogenic fungus that causes disease on small grains and infects a range of wild grass species (Bakonyi et al., 1997; Pratt, 2003; Schäfer et al., 2004). Taxonomically, the fungus belongs to Ascomycotina, class Loculoascomycetes, order Pleosporales, family *Pleosporaceae*. It is characterised by thick wall elliptical conidia (60-120µm x 12-20µm) containing five to nine cells (Kumar et al., 2002), (Fig. 2, insert bottom left). Grown in the laboratory on a solid media the cultures form a dense mycelium with hyphae interwoven as a cottony mass with colours varying from white and pink to grey and black, with dark brown conidia forming at later stages. Barley and wheat are the most economically important plants that are infected. The infections usually appear as foliar spot blotch (Fig. 2 insert top left), common root rot and as black point on the seeds (Kumar et al., 2002). Foliar spot blotch decreases the photosynthetic capacity of the leaf, leading to early senescence. Common root rot decreases the water and nutrition uptake, this is important because the disease has a high potential to spread via infested seed lots. Infected seeds may rot in the soil or emerge as weak seedlings which subsequently wither and die. Yield losses varying from 20% to 80% in wheat (Duveiller and Gilchrist, 2004) and 16% to 33 % in barley (Clark, 1979) are reported. Yield losses due to this fungal pathogen are difficult to estimate in Sweden, due to co-infecting pathogens in the field. The indications are however, that in rare cases losses can be estimated to reach 20% (Waern, P. 2008, personal communication).

B. sorokiniana is present in all areas where cereals are grown, giving it a very wide distribution throughout the world. It is described that *B. sorokiniana* forms a continuous genetic pool of isolates varying in virulence and aggressiveness to various cereals and grasses (Duveiller and Garcia Altamirano, 2000; de Oliveira et al., 2002). The mechanism behind this variation is poorly understood. The sexual stage is very uncommon, thus the genetic variation may be attributed to heterokaryosis and parasexuality mechanisms (Tinline, 1962). A clear variation in virulence is usually present within a certain geographic area (I, de Oliveira et al., 2002; Meldrum et al., 2004; Arabi and Jawhar, 2004; Ghazvini and Tekauz, 2007).

The infection usually starts from seeds, infested soils or from host debris that transmits conidia via physical contact or rain splashes (Fig. 2). In a virulent *B. sorokiniana* strain, about 90% of the conidia have started to grow, forming germ tubes, germlings or hyphae already after 3h (Apoga and Jansson, 2000). The germ tubes and hyphae of *B. sorokiniana* are surrounded by extra cellular matrix (ECM) that provides a beneficial environment for the fungus. ECM contains fungal toxins, it is used for plant adhesion and as a protection from plant cuticle degrading enzymes (Åkesson et al., 1995; Apoga and Jansson, 2000). An abundant toxic compound produced by *B.* sorokiniana is prehelminthosporol (Carlson et al., 1991), which has a role in killing and weakening plant cells (Liljeroth et al., 1993).

The infection process on the leaves usually occurs through natural wounding, stomata or with the use of an appresorium-like structure through the cell wall (Yadav, 1981; Schäfer et al., 2004), (Fig. 2, insert top right) attributed to a biotrophic life style. Plant responses are usually cell wall appositions and HR-like response. If plant responses are insufficient and fail to stop the invasive growth, the fungus starts its necrotrophic way of living. This causes more dead and collapsed tissue and further uncontrolled spread leading to visible necrotic spots (Schäfer et al., 2004).

Fungicides can be used to reduce the severity of spot blotch and subsequent losses (Kiesling, 1985; Videma and Kohli, 1988). This is however costly and presents a potential threat to ecosystems and will most likely result in fungicide resistant *B. sorokiniana* isolates. The best way to avoid the fungal disease is proper agricultural practises and the use of resistant cultivars. Today no really good resistant cultivars are available against spot blotch. There are however genotypes that exhibit a lower level of susceptibility than others that are present in different breeding programs (I; Ghazvini and Tekuz, 2007).

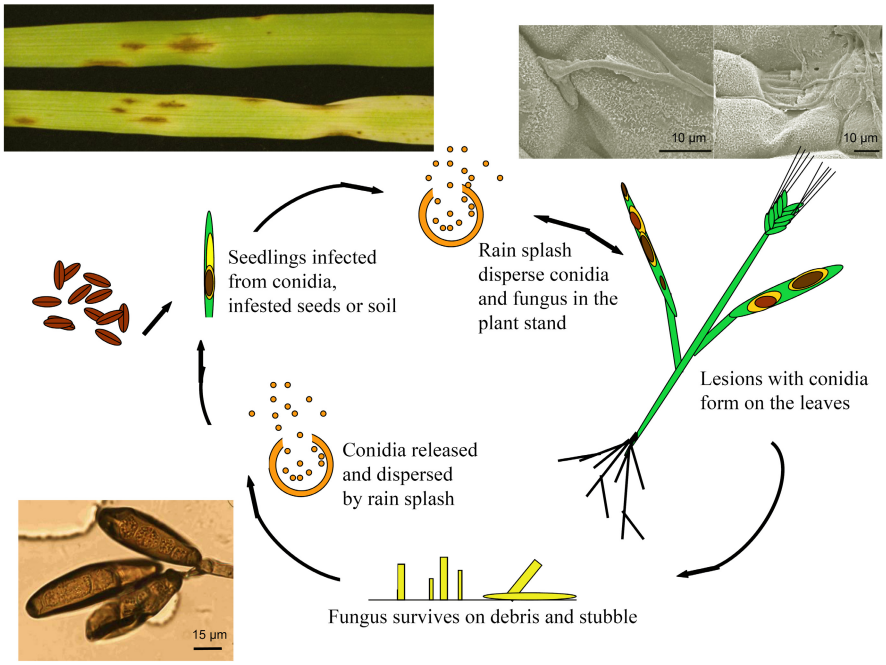


Figure 2. Disease cycle of *Bipolaris sorokiniana*.
B. sorokiniana causes leaf lesions (top left). The spread of conidia (bottom left) is usually via rain but the fungus also spreads via infected seeds. The fungal infection starts with the entering of the plant tissue via appressorium like structure or through stomata (top right). *B. sorokiniana* can stay on plant debris and in the soil, which also are sources of infection.

Leptosphaeria maculans

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph: *Phoma lingam*) (Tode ex Fr.) is a hemibiotrophic pathogenic fungus that causes blackleg on cruciferous crops, mainly Brassica species (West et al., 2001; Fitt et al., 2006a, b). Taxonomically the fungus belongs to s Ascomycotina, class Loculoascomycetes, order Pleosporales, genus *Leptosphaeria*. The most economically important plants that are infected are Brassica oilseed crops, *Brassica napus* and *B. rapa*. *L. maculans* is a haploid out-crosser with a genome size of about 34Mb and predicted to encode 10,000 genes (Plummer and Howlett, 1995; Cozijnsen et al., 2000; Howlett, 2004). The genes are localised on 15-16 chromosomes, including a non-Mendalian-transmitted mini chromosome. The sizes of the chromosomes vary between 0.7 and 3.5 Mb (Plummer and Howlett, 1995; Leclair et al., 1996). The non-Mendalian-transmitted mini chromosome may play an important role

for the fungus to cope with selection pressure and provide the pathogen with new aggressive combinations.

At Genoscope, in France, the whole genome of the isolate JN3 is undergoing sequencing. Some especially interesting genomic regions are also being sequenced in the isolates NZ-T and v29. They originate from New Zeeland and from a laboratory cross of Australian origin, respectively. This choice of isolates shows a big difference in virulence. After sequence assembly and further analysis, genes important for pathogenicity are hoped to be revealed. For more information on these matters, see www.genoscope.cns.fr/spip/Leptosphaeria-maculans-complex.html.

The infections of *L. maculans* in *B. napus* (Fig. 3) appear as leaf lesions and as blackleg (Howlett et al., 2001) but can also induce root rot (Sparague et al., 2007). The exact mechanism of infection of the roots is not fully understood. It is presumed that *L. maculans* can grow upwards in the stem as well as downwards into the hypocotyls and root. Thus, the infection of roots can occur via fungus spreading from foliar infection as well as from infested soil (Sparague et al., 2007). Lodging caused by blackleg is the most serious effect of the disease, since no harvest is possible and no yield obtained. In particularly Eastern Europe, *L. maculans* co-infects plants together with *L. biglobosa* and the yield loss in an infected field may be up to 30-50% due to lodging of the plants. Globally, the Australian isolates are considered more virulent compared to the European and so far there are only small yield losses in India and China due to different culture practices, whereas the disease is widespread in Canada. *L. maculans*, is amenable for genetic transformation, which has been useful in several studies and revealed more data of the complex infection process (Sexton and Howlett, 2001; Elliott et al., 2007; III).

In a Brassica field where the economic damage of *L. maculans* could be significant, ascospores produced in the pseudothecia on infested stubble are the main source of infection. However, infection could also start from seeds or infested soils (Fig. 3). The transmission of conidia occurs via physical contact or rain splashes. Seedlings are infected via cotyledons by ascospores that are spread through rain splash, whereas younger leaves are infected via stomata or wounds. In the initial colonisation, the fungus has a biotrophic life style. Behind the initial biotrophic hyphae front, the fungus becomes necrotrophic. It is in the necrotrophic phase in the dead plant tissue where *L. maculans* produces the asexual fruiting bodies (pycnidia) (Hammond et al., 1985; Hammond and Lewis, 1987). Pycnidiopores are thought to act as secondary inoculums, whilst the infection of neighbouring plants occurs via rain splash (Howlett et al., 2001). The spread of pycnidiospores can in rare cases reach a distance of 40 cm, even though a distance of 14 cm is more common (Travadon et al., 2007). After the initial

infection of the leaf, the fungus colonizes the intracellular spaces between mesophyll cells, growing down the petiole in the xylem vessels or between cells of xylem parenchyma and cortex. The initial stage is symptomless and biotrophic (Hammond et al., 1985), whereas later stages when the stem cortex is killed result in a blackened canker that is visible and may cause the plant to break and lodge (Fig. 3 bottom left).

During the infection, the fungus produces several toxins and other active secondary metabolites. The most prominent studied is sirodesmin PL, which is non-host specific and has antibacterial and antiviral properties and causes chlorotic lesions on plant leaves (Rouxel et al., 1988; Elliott et al., 2007). It has been shown by Elliott et al. (2007), that sirodesmin is the most important toxin produced by *L. maculans.* A fungal mutant knocked-out in sirodesmin PL coding genes was less viable in the natural host *B. napus* and performed less colonisation compared to wild type fungus.

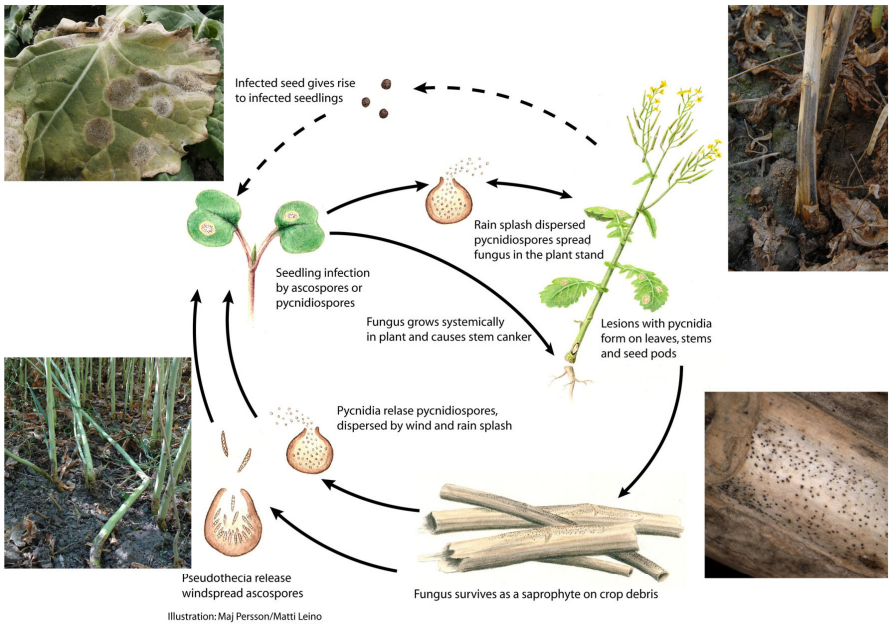


Figure 3. Life cycle of *Leptosphaeria maculans*.
L. maculans causes leaf lesions (top left), blackleg (top right), lodging (bottom left) in *B. napus*. The spread is usually via wind (ascospores) or rain splash (ascospores and pycnidiospores) and more seldom via infected seeds. The fungal grows is initially as a biotroph before switching to a necrotrophic phase later in the infection cycle to generate pycnidia (botton right). Printed with permission from Gunilla Berg, the Swedish Board of Agriculture.

The host responses of *B. napus* after infection by *L. maculans* include necrosis of guard cells and adjacent cells, production of phytoalexin, callose and lignin, accumulation of pectin as vascular plugs and induction of pathogen-related proteins (Hammond et al., 1985; Rasmussen et al.,

1992a,b; Chen and Howlett, 1996; Chen and Seguin-Swarts, 1997, 1999; Roussel et al., 1999; Brownfield and Howlett, 2001). To keep the pathogen under control the agricultural practices are important, for instance crop rotation. But there are also two types of plant resistance that can be used. The first is a qualitative that is valid through the whole lifecycles of the plants based on a gene-for-gene interaction (Ansan-Melayha et al., 1998; Delourme et al., 2004). The fungus has great possibilities to overcome this type of resistance due to its large population and high recombination frequency (Howlett et al., 2001; McDonald and Linde, 2002). The other type of resistance that could be used is quantitative based, which depends on several factors, for example callose, phytoalexin and *PR*-genes. Due to the involvement of several traits this is harder for the fungal population to overcome, since all factors should be overcome by one isolate.

In Arabidopsis it has been established that the resistance towards *L. maculans* is independent of the defence pathways involving ethylene, jasmonic acid and salicylic acid (Bohman et al., 2004). It has also been found that some *R*-genes are important, for instance *RLM1* and *RLM3*, together with both camalexin and callose are significant components in the interaction. (Staal et al., 2006; Kaliff et al., 2007; III, IV). Recently we (III) discovered that ET plays a major importance if both camalexin and RLM1 are removed from the plant defence system. If ET is produced by *L. maculans* or if it is used as a signal molecule to facilitate fungal growth is not established yet.

Barley

Barley (*Hordeum vulgare*) is an important cereal crop worldwide. It was cultivated in the Fertile Crescent over 10,000 years ago (Salamini et al., 2002) and has today spread to all temperate regions. Barley is particularly widely used and appreciated among farmers for the traits towards cold, drought and salinity (www.fao.org) and is preferentially used as animal feed and for malting i.e. beer production.

Barley is a self-pollinating diploid with seven chromosome pairs represent the large genome of 5500 Mb of which 80% is composed of repetitive DNA (Sreenivasulu et al., 2008). Due to this complexity the genome is not presently appropriate for whole genome sequencing. Instead, large scale sequencing programs for the development of expressed sequence tags (ESTs) from various cDNA libraries have been initiated. So far this has resulted in more than 400,000 ESTs deriving from various developmental stages, treatments and tissues (Sreenivasulu et al., 2008). Alignment of these ESTs led to the identification of a representative set of 50,453 unigenes with 27,094 singletons (http://compbio.dfci.harvard.edu /tgi/cgi-bin/tgi/gimain.pl?gudb=barley), representing possibly about 75% of all genes in the barley genome. There are also microarrays available for

expressional studies (Sreenivasulu et al., 2008) and transformation is feasible and has improved in recent years (Holm et al., 2000; Kumlehn et al., 2006; Hensel et al., 2008). Several mapping populations of barley have been published, for an overview see Varshney et al. (2007). Several markers can be used in comparative genomics, since markers are available in other species such as wheat, maize and the sequenced rice. Most data sets are available online at www.gramene.org and at Grain genes (http://wheat.pw.usda.gov/GG2/index.shtml). Unfortunately, different markers and different genetic backgrounds make it difficult to compare different sets. At present, the barley community has high hopes that the complex genome would be completely sequenced within five years.

A good resource that exists today is the large collections of barley mutants available at different stock centres, for instance the Nordic Genetic Resource Center. With the use of well characterised mutants further good description of barley from a molecular/genetic perspective could be initiated.

Arabidopsis thaliana

Arabidopsis is a worldwide spread dicotyledonous weed. The only economic value of Arabidopsis is as a plant model organism. It belongs to the *Cruciferae* family or *Brassicaceae* and is closely related to Brassica species such as *Brassica napus*. Several gene and sequence homologies have been identified between Arabidopsis and economic important crops (Parkin et al., 2005; Snowdon, 2007). More resources are however allocated to Arabidopsis research than to worms, fruit flies and mice. There are several important factors that make Arabidopsis the plant model organism number one (Leonelli, 2007), easy genetics, many mutants available, five chromosome pairs and a relative small genome of 125 Mb. The whole genome was sequenced in 2000 (AGI, 2000). Other very important features are the small space needed by the plant and the possibilities to grow it *in vitro* together with the short generation time (from germination to seed within 6 to 8 weeks) and the seed capacity. One specimen is enough to produce thousands of seeds within the limited lifespan. In the beginning of Arabidopsis research it was not easy to transform, but after some years, a very efficient method by using the *Agrobacterium tumifaciens*-mediated procedure was discovered (Clough and Bent, 1998). Thanks to the floral dip technique it became possible to generate a tremendous amount of different Arabidopsis mutants. Today a huge number of mutants are available at different stock centres, for instance Nottingham Arabidopsis Stock Centre (NASC). Arabidopsis is also a widespread plant, harbours general natural variation attributes shared

between many other species for a lot of different traits and functions (Leonelli, 2007). Also, very important for a research community is the network. Within the Arabidopsis community, the network works well, with large databases (www.arabidopsis.org) organizing and exhibiting data and exchange of material and ideas between different research groups.

Aims of the study

The major goal with this study was to increase knowledge on the interaction between hemibiotrophic fungi and plants and to investigate the role that cell death may play in the interactions. The specific goals were:

- Genetically characterise the barley lesion mimic mutant *bst1* and identify the mechanisms linked to the decrease in susceptibility towards infection by *Bipolaris sorokiniana*.

- Investigate important defence components in the Arabidopsis-*Leptosphaeria maculans* pathosystem.

Results and Discussion

Identification and characterization of *bst1* and germplasm for *B. sorokiniana* resistance

The *Bipolaris sorokiniana tolerant 1* (*bst1*) mutant was identified in a M_2 population. It exhibited dark brown to black conspicuous dark lesions on all above ground tissues (II). The mutation was mapped and localized to chromosome 5HL. The flanking markers of the area in 5HL, Xcln.WG644 and Xcln.BCD298 show synteny to the rice chromosome 3 (I). This chromosomal area in rice contains more than 78 genes. The putative *CNGC2* mutation in barley is a lesion mimic mutant (Rostoks et al., 2006), and the *CNGC2* gene is located in close vicinity to the mapped *bst1* mutation. The *CNGC2* gene was sequenced in wild-type and *bst1*, but unfortunately a deletion of nucleotides that usually occurs from a fast neutron treatment could not be found. To generate more detailed genetic information, additional AFLP markers were screened. The two closest markers identified were located only 0.25 and 0.23 cM on either side of the *bst1* mutation. The closest markers were sequenced (I), but the sequences did not reveal any data that could be linked to a candidate gene for the *bst1* mutation, which still remains to be identified.

To find resistance sources towards *B. sorokiniana* adapted to Swedish conditions we used *bst1* and an additional 29 barley genotypes to screen

eight different Swedish *B. sorokiniana* isolates (II). This was the first time a survey of Swedish isolates have been evaluated and classified. By the use of three barley differential lines (Bowman, ND 5883 and ND B112), two different virulence groups of *B. sorokiniana* were identified, namely isolate group 1 and 7 according to the classification system by Ghazvini and Tekauz (2007). From preliminary studies we had indications that *bst1* exhibited increased resistance compared to the wild-type. However, due to the mutagen treatment and the subsequent spontaneous forming lesions, a reduction in yield appeared. This information together with the fact that *bst1* plants look like they have symptoms caused by *B. sorokiniana*, we induced a second mutation by sodium azide to reduce or find a complete loss of the spontaneous lesions. From this new M_2 population seven additional mutants (*bst1:1-7*) were found (II). One mutant (*bst1:5)* exhibited wild-type phenotype whereas the other 6 showed various degrees of lesions.

B. sorokiniana is also the causal agent of common root rot, thus susceptibility of the roots was investigated on a subset of mutant genotypes. No difference in the root susceptibility could be distinguished in the material except for the double mutant *bst1:6* (II). The identification of sources for resistance against *B. sorokiniana* both towards root rot and spot blotch could become important. The most promising mutants to use for further studies and long-term breeding program would be *bst1*, *bst1:3*, *bst1:4* and *bst1:6*.

Plant mechanisms involved in the *B. sorokiniana* interaction

The importance of hydrogen peroxide in the interaction between barley and *B. sorokiniana* was investigated by the use of DAB staining. The *bst1* genotype displayed a high background level of H_2O_2 but had a low increase at 48 hpi (4% stained leaf area) compared to the wild-type which at the same time point exhibited a total stained leaf area of 11.7%. One interpretation of this difference may be that the biotrophic phase of *B. sorokiniana* is negatively affected due to high overall H_2O_2 levels in *bst1*.

Expression analysis at 48 hpi revealed a constitutively high expression of *PR*-genes *PR-1a, PR-2, PR-5* and *PR-10* in *bst1* plants compared to wild- type. We also discovered an oxidoreductase gene (*HCP1*) to be up-regulated in *bst1*. HCP1 have been found to have a regulatory role in the interaction between barley and brome mosaic virus (Okinaka et al., 2003). The function of the HCP1 protein is however still unknown. As a marker for the expression of the ROS system, a *RBOHB* gene was studied in our system. *RBOHB* was down regulated in *bst1*, suggesting that ROS is not a main part of a successful interaction (I).

Since primer design of well characterised defence signal marker genes in Arabidopsis is severely hampered in barley due to incomplete sequence

information further data was generated by screening a selected set of Arabidopsis mutants (Table 1).

Table 1. Response of Arabidopsis defence-signalling mutants upon challenge with *Bipolaris sorokiniana* 10 days post inoculation. Several genotypes can be included under more than one category but only presented once in the table. All genotypes are in Col-0 background if not stated otherwise.

Genotype	***B.s response****	***Genotype***	***B.s response***	***Genotype***	***B.s response***
Accessions		***ET***		*syp121-1syp122-1 eds5-3*	*S*
Col-0	*R*	*ein2-1*	*S*	*syp121-1syp122-1 NahG*	*S*
L*er*-0	*s*	*ein2NahG*	*S*	*syp121-1syp122-1 npr1-1*	*S*
An-1	*S*	*ein3-1*	*R*	*syp121-1syp122-1 sid2-1*	*S*
Ws-0	*s*	*ein4-1*	*R*	*pen2-1*	*S*
		ein6-1	*R*	*pen2eds1*	*r*
ABA responses		*eir1-1*	*R*	*pen2sag101pad4*	*r*
aba1-3[1]	*R*	*eto1-1*	*R*	*pen2sag101*	*S*
aba2-1	*S*	*etr1-1*	*R*		
abi1-1[1]	*S*	*vad1*	*r*	***Protein stability***	
abi4	*R*	*vad1ein2*	*s*	*AtHsp90.2*	*S*
		vad1ein3	*s*	*Sgt1 b*	*R*
Callose				*rar1-21*	*R*
pmr4-1	*S*	***JA***		*pad4sag101*	*R*
		eds8-1	*S*		
Camalexin		*coi1-16*	*S*	***R-gene***	
pad3-1	*R*	*esa1-1*	*r*	*ald1*	*S*
		jar1-1	*R*	*eds1-2*[2]	*s*
Cell death				*fmo1*	*s*
dnd1	*S*	***Lignin***		*mos3*	*R*
lsd1	*S*	*irx4*	*R*	*ndr1-1*	*S*
ran1-1	*R*			*pad1-1*	*S*
		Oxidative stress		*pad4-1*	*R*
Defence related		*Atnos1*	*R*	$rlm1_{Ler}rlm2_{Col}$	*R*
bik1	*S*	*AtrbohD*	*S*	$rlm1_{Ler}$*pad3*	*R*
bos1	*S*	*AtrbohF*	*S*	*rlm3-1*	*R*
bos2	*S*	*AtrbohDF*	*S*	*rlm3-2*	*R*
bos3	*S*	*nia1nia2*	*R*		
lms1[1]	*S*	*rcd1-1*	*R*	***SA***	
lms4[1]	*S*			*NahG*	*R*
lms5[1]	*R*	***Penetration***		*npr1-1*	*R*
mos5	*S*	*syp121-1*	*S*	*npr1-2*	*R*
mos5snc1npr1	*S*	*syp122-1*	*S*	*npr1-3*	*S*
pmr1-1	*R*	*syp121-1syp122-1*	*S*	*sid2*	*R*
		syp121-1syp122-1	*S*	*snc1*	*S*
		eds1-2		*mos2*	*S*

**S = susceptible, s= moderately susceptibility, R = resistant, r = moderately resistant.*
[1] = Ler background, [2]= Ws background

It is not certain that the responses between barley and *B. sorokiniana* are the same as between Arabidopsis and *B. sorokiniana*, but Arabidopsis gives us a more advanced tool to gain deeper understanding of plant defence mechanisms compared to *B. sorokiniana.* Out of mutants impaired in SA, JA and ET signalling pathways *NahG*, *npr1-1*, *jar1-1*, *ein 3-1*, *ein4-1* and *ein6-1* showed no disease symptoms whereas *npr1-3, coi1-16* and *ein2-1* displayed clear susceptible phenotypes compared to the resistant wild-type Col-0. Seven additional mutants were screened to enhance the understanding of cell death related responses. *dnd1*, *lsd1*, *AtrbohD* and

AtrbohF but not *Atnos1*, *ran1-1*, *rcd1-1* showed clear disease phenotype. In addition, the early penetration events monitored on the *syp121*, *syp122* and *pen2-1* mutants are important, as was earlier found concerning responses to powdery mildew fungi (Lipka et al., 2005; Zhang et al., 2008). The susceptible *syp* triple mutants, all impaired in SA signalling, revealed an importance of SA not obvious on the single mutant level. The phytoalexin, camalexin on the other hand does not seem to play any major role since *pad3-1* was resistant. The same was true for the *R*-gene related *pad 4-1, rlm1* and *rlm3*, whereas *pad1-1, ald1* and *ndr1-1* displayed a clearly susceptible interaction.

Plant mechanisms involved in the *L. maculans* interaction

Camalexin has been established to be a very important resistance mechanism in a compatible interaction between *L. maculans* and Arabidopsis. The *resistance to L maculans 1* gene (*RLM1*) is highly important in defence in Arabidopsis. To further investigate the effects of the *RLM1* locus, in depth analyses of mutants in the two TIR-NB-LRR genes $RLM1A_{\mathrm{Col}}$ (At1g64070) and $RLM1B_{\mathrm{Col}}$ (At1g63880) and other *R* gene signalling mutants were made. Based on the assessment of disease phenotypes on offspring from an F_2 population deriving from a cross between Col_{RLM1} (resistant) and $\mathrm{L}er_{rlm1}$ (susceptible), it was concluded that the *RLM1* locus is dose dependent. In addition, a quantitative detection system of *L. maculans* at a genomic level with real time PCR was developed. The comparison between the two different methods revealed that the qPCR method was only valid at relative late time points. A decrease in fungal mass was observed during the first six days, where-after the fungal mass established on a rather constant level, followed by an increase ten days after inoculation on susceptible genotypes. Fungal DNA was also found to be present and survive for some time on non-hosts like pea and barley. Extensive washing of the leaves did not decrease the fungal DNA significantly on susceptible and resistant Arabidopsis genotypes. The latter observation might be caused due to the wound inoculation procedure which facilitates entrance to the vascular system and establishment of *L. maculans* as an endophyte. The visual disease phenotype method is widely used and enables discrimination of disease symptoms already at 7 dpi. Fungal detection with qPCR is more time consuming and more costly but more unbiased under the assumption that the sampling is done in an optimal way.

Physiological barriers were identified to be important in plant defence. A hypersensitive response (HR) at the stomata and hydathodes, and vascular plugs was found to be *R* gene dependent. In order to investigate the involvement of the classical hormones involved in plant pathogen interactions, the $rlm1_{\mathrm{L}er}$ genotype was crossed to a camalexin deficient

mutant *pad3-1* resulting in a hyper susceptible double mutant (Staal et al., 2006). This mutant was crossed to different hormone genotypes mutated in JA (*coi1-16*), ET (*ein2-1*) and SA (*NahG*). In total these combinations generated eight different mutant lines. The susceptibility and the phenotypic response were evaluated by the assessment of a GUS tagged *L. maculans* isolate, with a quantitative GUS method. The results obtained showed that JA is a repressor of fungal growth whereas both ET and SA facilitate the fungal colonisation. In the mutants studied the genotype with the greatest fungal colonisation was in the $rlm1_{Ler}pad3$ background in combination with *NahG* and *coi1-16*, which have low levels of SA and JA in a combination with a normal ET production. This genotype showed the most severe susceptibility symptoms with extensive necrosis and a purple coloured leaf. The second most susceptible genotype was *coi1-16* with normal ET production. The findings resulted in an integrated model where physical barriers such as lignin, vascular plugs, callose and HR are integrated with camalexin, and hormone signalling.

***RLM3* a gene involved in broad range immunity in Arabidopsis**

The *resistance to Leptosphaeria maculans 3* (*RLM3*) gene was identified by microarray comparisons on pools of individuals that had been classified as susceptible or resistant from a cross between Col-0 harbouring the gene of interest and An-1, a highly susceptible accession lacking the gene (IV). To keep the variance for the environmental dependent expression high, materials were sampled at different time points. Genes more highly expressed in the resistant samples had a bias towards a region on chromosome 4. By mapping, it was shown that the gene closest to marker SNP102, At4g16990 was the candidate gene for *RLM3*. The gene expression of At4g16990 was investigated 2 days after inoculation with *L. maculans* with real time PCR. The expression of Col-0 was set as a reference and the T-DNA insertion line gabi_491E04 (*rlm3-2*) had significantly lower transcript levels and expression could not be detected in An-1. In order to confirm that the gene candidate of *RLM3* was At4g16990 we complimented An-1 and *rlm3-2* using the TAC clone JAtY64O13 containing the complete genomic clone of *RLM3* and its promoter. The fungal mass in the complemented genotypes was significantly lower compared to An-1 and *rlm3-2*. The quantification was measured as ration of pg fungal versus ng plant genomic DNA with real time PCR 10 days after inoculation. By analysing the susceptibility visually in several T-DNA insertion lines in At4g16990, it was evident that the *RLM3* gene also is important in interactions between Arabidopsis and other pathogens for example *Alternaria brassicicola*, *Botrytis cinerea* and *Alternaria brassicae*. Alternative splicing of disease resistance genes has earlier been demonstrated to be of importance for *R* gene functions (Zhang and

Gassman, 2003; Zhang and Gassman, 2007). *RLM3* has been annotated as a member of the TIR-NB family of truncated *R* genes, but with putative alternative splicing leading to a TIR-X transcript. However, the exact role and importance of the different *RLM3* transcripts is currently not known. One transcript of *RLM3* (encoded by the end of exon 1 and the exons 3 and 4), however, exhibits similarity to the regulator of chromatin condensation (RCC1) family of proteins. This specific segment of 74 amino acids contains the plant- specific DZC (disease resistance, zink finger, chromosome condensation IPR013591 (DZC) domain (Staal and Dixelius, 2008). Based on *in silico* analysis one intriguing hypothesis suggests that *RLM3* could act as a TIR-DZC adaptor between specific TIR-NB-LRR receptors and downstream components harbouring the DZC domain. Future work will shed more light on this concept.

Future perspectives

Working with a system containing two participating living organism presents considerable challenges. Both the host and the pathogen need to be controlled. Since the systems of *B. sorokiniana* and *L. maculans* both are well established in our laboratory, a continuation of the work would gain further information about the interaction between plant host and hemibiotrophic fungi and what roles and effects cell death and deference gene responses have. There are several directions that I suggest to be followed.

Identification of the *bst1* mutation is highly prioritized. Furthermore to rule out the possibility that *CNGC2* is not the candidate gene of *bst1,* the *CNGC2* promoter sequence needs to be examined in the *bst1* and wild-type Bowman(*Rph3*) genotypes. The *CNGC2* promoter sequence, which is not found in any public databases, can be obtained with degenerated PCR and genome walking techniques.

As our ultimate goal is to apply our knowledge on plant-pathogen interactions to actual crop breeding programmes, my findings of the present studies need to be examined under field conditions. In a preliminary field trial in Minnesota, USA, the *bst1* line showed nearly complete resistance to *B. sorokiniana*, indicating its potential breeding value. This experiment needs to be repeated and evaluated more precisely with statistical analyses including the double mutants *bst1:3*, *bst1:4*, and *bst1:6*.

High yield is another important trait for a crop plant. A cross should be made between the *bst1* and high-yielding commercial lines to test if high-yielding traits can be introduced to the *bst1* background. It is also important

to examine if the *bst1* disease resistance could be segregated from the lesion mimic phenotype.

Establishment of the Arabidopsis-*B. sorokiniana* pathosystem provides an extraordinary opportunity to investigate different defence mechanisms. This would expand our understanding on the host defence mechanisms in barley, as well as the non-host resistance in Arabidopsis. As presented in my study, the fact that the Col-0 line, which is the background for most Arabidopsis mutants, is resistant to the isolate of *B. sorokiniana* makes it possible to screen for susceptible mutant lines. With the available genome sequence of rice, identification and characterization of the rice homologs of the genes mutated in the susceptible Arabidopsis lines might shed light on differences and similarities between monocot and dicot defence mechanisms.

The collection of *coi1-16* lines generated in my study also harbours the *pen2-4* mutation (Westhpal et al., 2008). Since PEN2 play a role in Arabidopsis defence against *L. maculans* it is important to figure out how the *pen2-4* mutation affects the phenotypes of my *coi1-16* lines. The *rlm1*$_{Ler}$*pad3coi1-16* line should be identified among F_2 progeny of a cross between the quadruple mutant *rlm1*$_{Ler}$*pad3coi1-16pen2-4* and *rlm1*$_{Ler}$*pad3*. If any significant difference can be observed in disease susceptibility between the *rlm1*$_{Ler}$*pad3coi1-16* and *rlm1*$_{Ler}$*pad3coi1-16 pen2-4* lines, further crosses will be made to generate a collection of *coi1-16* lines without *pen2-4* (e.g. *rlm1*$_{Ler}$*pad3coi1-16 NahG* etc.). Since *coi1-16* is male sterile at temperatures above 20°C, this will have to be carried out in a controlled environment.

An additional interesting objective to follow up is the role of ET in Arabidopsis- *L. maculans* interaction. First, the amount of ET is difficult to determine due to its chemical properties. Therefore I suggest investigating the expression of a few genes involved in the biosynthesis and compare between inoculated plants and mock treated. Comparison of the expression should also be performed between interaction of virulent isolates and avirulent isolates in a camalexin and RML1 free background. The whole genome of *L. maculans* will soon be public and new possibilities will arise. Of high interest would be to search for ET biosynthesis genes. If present, a knockout as well as an over expression of them would make an interesting start for further detailed studies.

RLM3 has several putative alternative transcripts. Overexpression of the shortest transcript only partially restored disease resistance to the An-1 accession, while complementation by a genomic clone of *RLM3* was successful. It is interesting to examine if other transcripts can fully complement the disease susceptibility of the An-1 line. In order to evaluate the functional importance of the DZC (disease resistance, zink finger, chromosome condensation IPR013591 (DZC) domain, which has been

identified *in silico* as one of the alternative *RLM3* transcripts, a construct with end of exon 1 and the exons 3 and 4 under native and constitutive promoter can be used.

A recent study demonstrated that *L. maculans* infects roots of its natural host oilseed rape, in addition to the above ground parts. It is interesting to test if *L. maculans* is also able to infect roots of Arabidopsis. The rice pathogen, *Magnaporthe oryzae* is another example of fungal pathogens that can be both foliar and root pathogen. Studies on *M. oryzae* indicated that the R gene-dependent resistance effective in leaves does not work in the same way in roots, suggesting that different defence systems are operational to a single pathogen depending on which tissues are attacked. A problem with this, is that if the root infection is too slow, plants will start to die and become senesced, which would make visual scoring impossible. If the Arabidopsis-*L. maculans* root pathosystem is established, we could examine if *RLM1*-dependent resistance is as effective against root infection by *L. maculans* as observed for leaf infection. Furthermore, a collection of Arabidopsis mutants, including those generated in my studies would enable us to identify key components shared by the defence systems in roots and in leaves.

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Acknowledgements

This work has been carried out at the Department of Plant Biology and Forest Genetics, SLU and have been financially supported by the Interactions between Micro-Organisms and Plants (IMOP) graduate school, The Royal Swedish Academy of Agriculture and Forestry, F d Bryggareämbetets i Stockholms pensionskassa, Knut och Alice Wallenbergs stiftelse, Helge Ax:son Johnsons stiftelse and The Nilson-Ehle Foundation/ The Royal Physiographic Society in Lund.

First I would like to thank my main supervisor, Christina Dixelius, for providing new ideas and for encouraging me. Thanks for the freedom to perform work in my own directions and for steering me in the right directions when needed. I would like to thank my supervisor Ander Falk for choosing me among other candidates and for introducing me to barley and AFLP mapping and for giving me the opportunity to start on this project.

Secondly thanks to all present and past members in the Dixan group, for all scientific discussions and constructive comments during group meetings and informal gatherings, and for providing a friendly atmosphere. This work would not have been possible without your help and support. Maria Kaliff for sharing both office, personal and scientific thoughts. Jonas Roos, for friendship, readily discussing method development and for retyping protocols. Tom Martin and Vera Montiel for being fresh and positive winds in the labs and keeping the group together with after work activities. Shinichi Oide for always having an answer to my questions and discussing molecular methods. Ramesh Vetukuri for your calm personality and for keeping the lab stuff/equipment at an increasing level. Moses, Na Guan, and Tina O for covering and helping me with the last experiments. Jens Staal for all collaborations and for always having an extra idea about what would be interesting to examine. Jan Fahleson for bringing overstimulated group meetings to sense. Erica Almström, for teaching me the value of certain books and for starting the continuing and everlasting tradition of “fredags fika”. Anna Johansson, for all discussions and for general information. Johanna Holmblad, for help with the Arabidopsis mapping and for all encouragement. Mattias Myrenås for the introduction to Real Time PCR.

A special thaks to Ingrid Eriksson and Gunilla Swärd for always having a few tips and tricks to teach me. Richard Hopkins for language corrections. Jenny Carlsson, for being a rock at the department and for taking a step forward when I really needed it (det värmde mitt hjärta). Morten Rasmussen for ideas and collaboration in the barley project.

Gratitude to the heads of department Eva Sundberg, Christer Jansson and Per Bergman. For help with plant material and growth, thanks to Urban P, Kristin-Sofie M and Per L. For computer issues Björn Nicander. Thanks to Birgitta E, Lotta O and Monica B for the administration of my courses, money and different numbers. Mona M, thanks for all fast orders and price negations.

Many people at the department made my time and work better by their presence and I am grateful to all of you! Individual thanks to the ABC-corridor people Mats B for introduction in the lab and for always having time for a dark beer. Johan Meijer for all encouragements and discussions. Elke for BBQs, and barley talks, Derek and Nadiya for being good friends. Kjell S and Ulf for floor hockey fights and pub companionship. The Forest people, Johan W, Andreas H, Uddenberg, Emma L, Malin, Vestman and Luisa, for nice social gatherings and good friendship. Plant physiology people Folke, Lisa A for collaboration, Magnus E, Veronika for pub gatherings and Izabela for you optimism and your way to organize parties. To the friendly virus people Jon R, Ulrike and Anders K. Anders also for always having a good question ready and for managing to get us away from a hurricane in Mexico. Matti L for introducing me to *B. sorokiniana* and plant breeding. To Kerstin D, Per H, Dimitra, Maria M, Sara F, Cihan, Wilson, Jenni, Monica and Linn A for all friendly comments, nice chats, encouraging and comforting words given.

I would like to pay my appreciation to the people from the Interactions between Micro-Organisms and Plants (IMOP) postgraduate school and particularly to Greg, Eva and Dharam.

This work would have been hard to complete without: Dr Jeppe, always being a friend and having time for a beer. I will never forget when you tricked to give me a ride to the airport at 3am. Tamara, thanks for always having time for research and personal discussions and for always being there when I needed you to. Nurun, I appreciate you for your friendship and for believing in me as a scientist and a person. I am also grateful for your threats and for kicking my butt when I (probably) needed it.

Ljiljana, no words would be enough. Thanks for every support, including endless scoring of leaves, roots, labeling of al-foil and tubes, for statistical and scientific discussions, but most of all for your endless belief in me and all support given.

Appreciation to my family's support.
We have been through a lot and I would not have made this without you. Stoffe och pappa tack för att ni alltid trott på mig och för att ni alltid ställer upp för mig. Mamma för att alltid tro på det goda hos människor och för din kämpande natur. Utan er och ert stöd hade jag aldrig klarat av detta. Mamma- du lämnade oss förtidigt men i mitt hjärta finns du för alltid, jag kommer också alltid att kämpa till det sista andetaget.

Živeli!