Genes, Hormones and Signalling Pathways Implicated in Plant Defence to *Leptosphaeria maculans*

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Abstract

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Leptosphaeria maculans (anamorph: *Phoma lingam*) is a hemibiotrophic loculoascomycetous fungus which causes blackleg, a serious disease of *Brassica* oilseed crops. In order to examine the genetics of resistance to this disease and important signalling pathways, extensive studies on *L. maculans* interactions with Arabidopsis were carried out. Two resistance loci, $RLM1_{Col}$ and $RLM2_{Ler}$, have been identified due to transgressive segregation in F_2 progenies from the resistant accessions Col-0 and L*er*-0. $RLM1_{Col}$ encodes a TIR-NB-LRR resistance gene and confers specific resistance towards *L. maculans*, while a third *R*-gene *RLM3* confers resistance to *L. maculans*, *Alternaria brassicae*, *A. brassicicola* and *Botrytis cinerea*. *RLM1* is furthermore independent from signalling components, such as *SGT1*, previously associated to all TIR-NB-LRR resistance genes. In addition to these susceptible genotypes, EMS mutants (*lms1* to *lms11*) susceptible to the pathogen have been assessed in order to facilitate identification of the mechanisms required for resistance. The *lms5* mutant has been shown to be specifically susceptible to *L. maculans* and have altered auxin signalling. During a mapping approach *lms5* was found to most likely code for an F-box protein indicating involvement in protein turnover via the ubiquitine proteasome. This result together with mutants involved in protein degradation complexes indicates the importance of protein stability. In contrast to the other pathogens, the defence responses against *L. maculans* are independent of the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), while abscisic acid (ABA) and auxin, and the phytoalexin camalexin play more crucial roles. Resistance to *L. maculans* can furthermore be primed by ABA and β-aminobutyric acid (BABA) treatments. This priming results in increased callose deposition in a resistance gene-dependent manner. The callose deposition is furthermore regulated by *PR2*. Moreover comparative studies with *B. napus* have been undertaken, confirming that the Arabidopsis-*L. maculans* pathosystem can be used as a model for the *B. napus* - *L. maculans* interaction. Taken together, this work contributes to increase our knowledge about the Arabidopsis – *L. maculans* pathosystem.

Keywords: ABA, *Arabidopsis thaliana*, Auxin, Blackleg, Callose, Defence signalling, Hormones, *Phoma lingam, R*-genes

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Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their roman numerals:

I. **Staal, J., Kaliff, M., Bohman, S. and Dixelius, C.** (2006) Transgressive segregation reveals two *Arabidopsis* TIR-NB-LRR resistance genes effective against *Leptosphaeria maculans*, causal agent of blackleg disease. *The Plant Journal*, **46**, 218-230.

II. [§]**Kaliff, M.,** [§]**Staal, J., Myrenås, M. and Dixelius, C.** (2007) ABA is required for *Leptosphaeria maculans* resistance via *ABI1* and *ABI4* dependent signalling. *Molecular Plant-Microbe Interaction*, **20**, 335-345.

III. **Kaliff, M., Oide, S., Staal, J. and Dixelius, C.** (2007) An ABA dependent repression of beta-glucanase enhance callose deposition in the *Arabidopsis-Leptosphaeria maculans* pathosystem. (In manuscript)

IV. **Kaliff, M. and Dixelius, C.** (2007) *lms5*, a mutant with dual roles in auxin and *Arabidopsis – Leptosphaeria maculans* defence signalling. (In manuscript)

[§] indicates shared first authorship

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Additional manuscripts:
Staal, J., Kaliff, M., Dewaele, E., Persson, M. and Dixelius, C. (2007) Rapid identification of an Arabidopsis TIR-X alternative transcript involved in innate immunity against necrotrophic fungi. *The Plant Journal*, (Re-submitted)

Abbreviations

ABA – Abscisic acid
ACC – 1-Aminocyclopropane-1-carboxylic acid
ACO – ACC-oxidase
ACS – ACC synthase
ARF – Auxin responsive factor
Avr – Avirulence
BABA – ß-amino butyric acid
BTH – Benzothiadiazole
CC – Coiled coil
2,4-D – 2,4-dichlorophenoxyacetic acid
Dpi – Days post inoculation
EMS – Ethane methyl sulfonate
ET – Ethylene
GA – Gibberellic acid
HR – Hypersensitive response
Hpi – Hours post inoculation
INA – 2,6-dichloroisonicotinic acid
IAA – Indole-3-acetic acid
IR – Induced resistance
ISR – Induced systemic resistance
JA – Jasmonic acid
lms – *Leptosphaeria maculans* susceptible
LRR – Leucin-rich-repeat
MAMP – Microbe-associated molecular pattern
MAP – Mitogen activated protein
MeJA – Methyl jasmonate
1-NAA – 1-naphthaleneacetic acid
NADPH – Reduced form of nicotinamide adenine dinucleotide phosphate
NBS – Nucleotide binding site
NO – Nitric oxide
PAMP – Pathogen-associated molecular pattern
PCD – Programmed cell death
PG – Pathogenicity groups
PR – Pathogenesis related
PRR – Pattern recognition receptor
PTI – PAMP-triggered immunity
RIL – Recombinant inbreed line
R-gene – Resistance gene
RKL – Receptor-like kinase
ROS – Reactive oxygen species
SA – Salicylic acid
SAR –Systemic acquired resistance
SCF – Skp1/Cul1/F-box
TIR – Toll/interleukin-1 receptor
TTSS – Type III secretion system

Introduction

Plants are sessile organisms that need to adjust to their environment in order to survive. An ordinary plant in the wild is constantly challenged with different stresses. These stresses are either abiotic in the form of climate challenges (drought, wind or temperature) or biotic in the form of insect-pests and micro-organisms that may cause disease.

Each year more than 10% of global food production is lost due to diseases, making plant pathogens important for the world economy (Strange and Scott, 2005). Of all the plant pathogens, fungi probably cause the most damage (Maor and Shirasu, 2005). The increase of plant diseases is partly a result of the globalisation of crop production. For example, the introduction of a new crop species in a geographical area might introduce new diseases for that specific location. These new diseases could attack the old crops already grown in the primary area and thereby become a new threat to food production. In plant breeding one of the most difficult aspects is to produce crops that are pathogen resistant over a long time period. This task is a huge challenge since the pathogen populations change over space and time, and thus are difficult to control (Kover and Cacedo, 2001; McDonald and Linde, 2002; Strange and Scott, 2005). There are different theories for how to produce durable resistant cultivars, for example cultivars with more than one resistance gene (pyramiding), or multi-line varieties. Introducing resistance genes is time consuming using traditional breeding strategies, but the use of molecular methods, not least molecular markers linked to a desirable trait, can considerably facilitate and speed up the selection phase. Unfortunately the use of genetic modified crops (GMO) is prohibited or restricted by law in many countries restricting the use of these strategies (Strange and Scott, 2005).

This thesis has a focus on defence response studies in Arabidopsis against *Leptosphaeria maculans*, the causal agent of blackleg and stem canker on Brassica crops. In the following sections a brief summary of the current understanding of plant defence mechanisms with a slight focus towards components of importance to *L. maculans* will be presented.

Arabidopsis thaliana

Arabidopsis thaliana (hereafter Arabidopsis) is a dicotyledonous weed colonizing most parts of the world (Figure 1). It belongs to the crucifer family (*Brassicaceae*) and is closely related to Brassica species (Cavell *et al*., 1998; Alonso-Blanco and Koornneef, 2000). With a small size, high level of selfing and short lifecycle of six weeks Arabidopsis is easy to work with under laboratory conditions (Meinke *et al*., 1998; Alonso-Blanco and Koornneef, 2000). Another advantage with this model plant is all the natural variation present among different ecotypes (Alonso-Blanco and Koornneef, 2000; Koornneef *et al*., 2004; Holub, 2007; Shindo *et al*., 2007). An ecotype is an inbred germplasm collected at a certain location on a certain time, giving a germplasm adapted to a specific environment (Alonso-

Blanco and Koornneef, 2000). Today the term ecotype has been replaced by the term accession. Friedrich Laibach and co-workers started the collection work (Laibach, 1943) and the number of accessions has been increasing ever since. The most commonly used accessions in molecular genetic studies are Columbia (Col-0), Landsberg *erecta* (L*er*-0) and Wassilewskija (Ws-0).

Figure 1. The natural distribution (grey areas) of Arabidopsis (modified after Alonso-Blanco and Koornneef, 2000).

The first work where Arabidopsis was used was conducted within Friedrich Laibach's PhD project, published in 1907, where he determined the number of chromosome pairs to 5 (reviewed by Somerville and Koornneef, 2002). However, it was not until the 1950s that other researchers started to work more frequently with Arabidopsis, and it was as late as during the 1980s that Arabidopsis became accepted as a model organism by the entire plant research community. From the first work in 1907, knowledge about Arabidopsis has increased dramatically. One important hallmark was the presentation of the genome sequence (AGI, 2000; Wortman *et al*., 2003; Rensink and Buell, 2004). This made Arabidopsis the first plant with a completely sequenced genome, followed by rice in 2002 (Goff *et al*., 2002; Yu *et al*., 2002). The sequencing of Arabidopsis (Col) revealed a genome size of 119 Mb comprising approximately 27,000 genes (Table 1). 70% of the genome is estimated to be duplicated by two major duplication events (AGI, 2000; Blanc *et al*., 2000, 2003; Vision *et al*., 2000; Ziolkowski *et al*., 2003). The next major goal is to understand the functions of all proteins by 2010 (Somerville and Koornneef, 2002).

Table 1. Arabidopsis genome statistics.

No. of protein-coding genes	27, 384
Gene density (kb gene^{-1})	4.4
Average gene length (bp)	2, 195
No. genes involved in plant defence	2, 055

In addition to, all the above mentioned advantages Arabidopsis is easily transformed (Bechtold *et al*., 1993; Desfeux *et al.,* 2000), there is vast marker information, defined mutants can be ordered from stock centres, T-DNA insertion mutants are available in nearly all genes (Jander *et al*., 2002) and extensive gene expression data is assessable via softwares like Genevestigator (Zimmermann *et al*., 2004). The powerful Arabidopsis genetic system has been widely exploited to both identify resistance genes and genes taking part in defence signal transduction pathways and their regulation. All these topics have been extensively reviewed (Kunkel, 1996; Glazebrook, 2001; Holub, 2001; Hammond-Kosack and Parker, 2003). New advancements in the understanding of plant defence mechanisms do also include epigenetics (Stokes *et al*., 2002) and the impact of small non-coding RNAs (Katiyar-Agarwal *et al*., 2006).

Brassica species

Comparative genomic studies have shown that the tribe *Brassiceae* is the descendant of a common hexaploid ancestor with a genome similar to Arabidopsis (Figure 2). The three basic genomes of this ancestor diverged from each other shortly after divergence of the Arabidopsis and Brassica lineages ~20 million years ago (mya) (Yang *et al*., 1999). The rather recent split between the Arabidopsis and Brassica lineages provides extraordinary potential for genetic exploitations due to common genes and high (87%) sequence identity (Cavell *et al*., 1998). Further, phylogenetic analysis groups the Brassica species into the *nigra* and *rapa/oleracea* lineages, which diverged 8-10 mya (Warwich and Black, 1991; Lysak *et al*., 2005). Since then a range of rearrangements in the genomes have occurred (Sillito *et al*., 2000; Rana *et al*., 2004; Town *et al*., 2006; Schranz *et al*., 2007), leading to polyploidy, which has been important during evolution (Bancroft, 2001; Parkin *et al*., 2005).

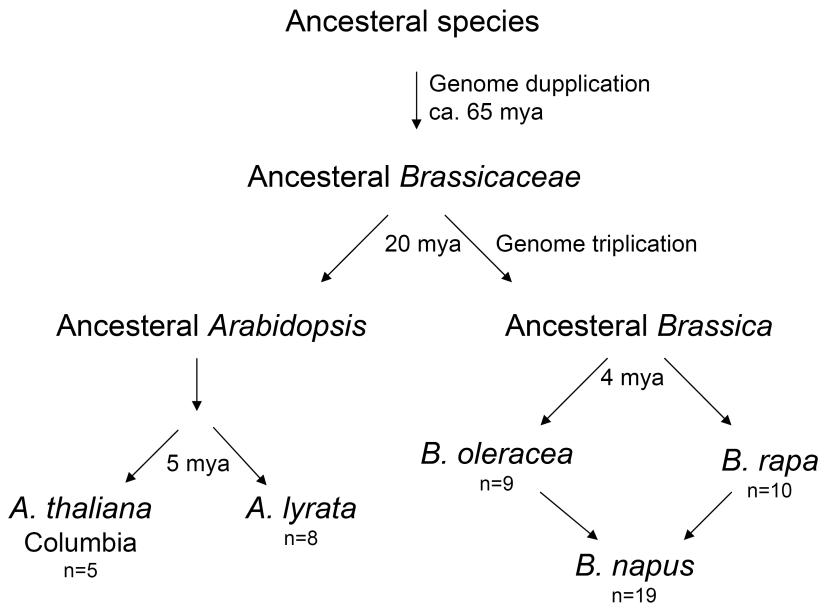

Figure 2. The Brassica genome evolution (modified after Rana *et al*., 2004; Koch *et al*., 2000).

There are today three diploid Brassica species with the genomes denoted AA corresponding to *B. rapa*, BB = *B. nigra* and CC =*B. oleracea*. These species are the sources to new amphidiploid species containing different genome combinations (Figure 3). The relationship between these species was established by cytological studies in the 1930's (U, 1935). Genome sequence initiatives are today ongoing on *B. rapa* and *B. oleracea* genomes (Yang *et al*., 2006; http://brassica.bbsrc.ac.uk).

The genus *Brassica* comprises a large variety of important horticultural and agricultural crops. For example, several of our important cole crops like white cabbage, broccoli, and cauliflower all belonging to the *B. oleracea* species complex (Snogerup *et al*., 1990), and *B. napus* and *B. rapa* are important oil producing crops. *B. napus* includes two different subspecies, swede (*B. napus* subspecies *brassica*) and oilseed rape (*B. napus* subspecies *oleifera*) which is the most predominant form. Brassica crops have been cultivated at least since 1500 BC, according to notions in the Sanskrit literature, and *B. napus* was first cultivated in southern Europe (Doweny and Röbbelen, 1989). Today crops from the *Brassica* genus are cultivated all over the world. In Sweden, the main Brassica crop is *B. napus* of canola quality (low erucic acid and glucosinolate content) present as spring or winter types. In 2006, 48,300 ha were used for winter oilseed rape compared to 35,400 ha of the spring type in Sweden (Svensk Raps AB, 2007). Both the spring and winter types of *B. rapa* (turnip rape) are also grown in Sweden but to a lesser extent than oilseed rape. Oil from these two Brassica species is used as cocking oil, lubricant, chemical products and biofuel while the residues can be used as a protein rich oil seed cakes for animal feed (Bell, 1995; Körbitz, 1995; Sonntag, 1995).

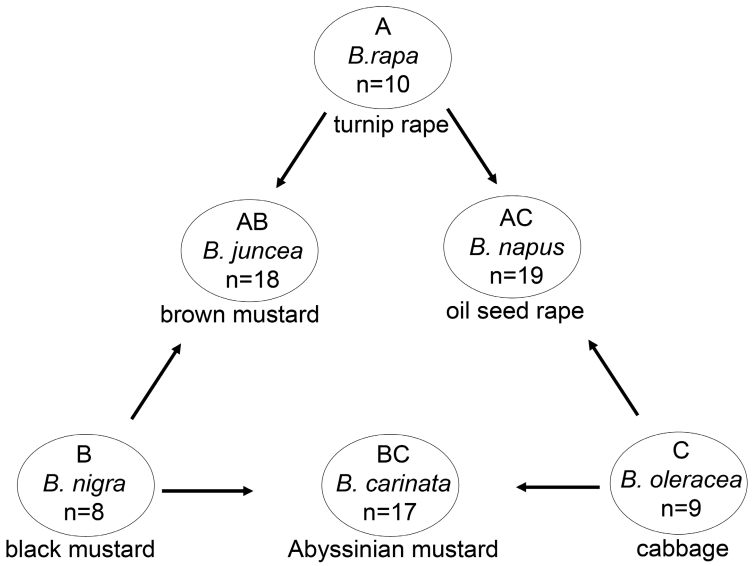

Figure 3. Genomic relationships among the Brassica species as suggested by U (1935).

In defence breeding, the diploid genomes valuable genetic resources are particularly important, carrying different traits which can be transferred and combined through various breeding strategies to a chosen recipient genotype. One nice example is the recent transfer of *L. maculans* resistance from a French forage *B. rapa* variety to resynthesized *B. napus* (Leflon *et al*., 2007). With increasing acreages of Brassica crops, not least as a result of the growing demand for biofuel, results in higher risks of disease problems. Today, different fungi constitute the main pathogens on Brassica oil crops (Table 2). Most of these fungi can also infect Arabidopsis.

Table 2. Fungal diseases on Brassica crop plants.

Causing agent	Disease	Class	Symptoms	Present in Europe
Albugo candida	White rust	Oomycete	Leaf covered with white blisters	Minor role in Europe
Alternaria brassicae	Black spot	Ascomycete	Black spots on all above ground parts of plants	Yes
Alternaria brassicicola	Black spot	Ascomycete	Same as *A. brassicae*	Yes
Botryotina fuckeliana (anamorph *Botrytis cinerea*)	Grey mould	Ascomycete	Leafs turn yellow and start to wilt	Common, but rarely serious damage
Fusarium oxysporum	Fusarium wilt	Ascomycete	Resemble those of Verticillium wilt	Not reported
Hyaloperonospora parasitica (erlier Peronosphora parasitica)	Downy mildew	Oomycete	Clorotic and necrotic lesions surrounded by sporangiiophores	Yes
Leptosphaeria maculans (anamorph *Phoma lingam*)	Blackleg	Ascomycete	Blackened stem, stem canker	Yes, minor disease in Sweden
Mycosphaerella brassicicola (anamorph *Astromella brassicae*)	Ring rot	Ascomycete	Broun lesions on infected leaf	Yes, rare in Sweden
Mycosphaerella capsellae (anamorph *Pseudocercosporella capsellae*)	White leaf spot	Ascomycete	White or verry light lesions on the leaf	Rare in Europe, not reported in Sweden
Plasmodiophora brassicae	Clubroot	Uncertain Myxomycete (protozoan origin)	Stunted plants, galls on tap rot	Yes
Pyrenopeziza brassicae (anamorph *Cylindrosporium concentrium*)	Light leaf spot	Discomycete	White spore mass at the margin of expanding lesions	Yes, minor disease in Sweden
Sclerotinia sclerotiorum	Sclerotinia stem rot	Ascomycete	Blackened sclerotia	Yes
Verticillium longisporum	Verticillium wilt	Deuteromycete	Brown girdling tap rot, damping of	Yes, most important disease in Sweden

Plant pathogens

The definition of plant pathogens varies depending on source. Generally, organisms that cause infectious plant diseases are defined as pathogens and that includes fungi, ooomycetes, bacteria, viruses, protozoa, and nematodes. Exceptions are insect pests, mites and vertebrates that cause damage to plants. Pathogens can also be divided into three different categories, based on their lifestyle (Agrios, 1997). There are biotrophs which require living host tissue in order to survive and reproduce. It is therefore important for biotrophs to avoid killing their host. The host on the other side can respond by a hypersensitive response (HR) reaction to fend off the pathogen by nutrient deficiency. If the pathogen is an obligate biotroph it cannot be cultured on synthetic media plates in a laboratory environment. The opposite of the biotrophs are the necrotrophs which

live on dead material. These pathogens use toxins and other compounds to kill the host tissue. In this case the plant cannot defend itself by HR, since such a response only promotes fungal growth. Instead, the plant has to use other defence mechanisms. The necrotrophic fungi can usually grow on media plates without any problem. The last category is a mix of the two other since a hemibiotroph organism can live both as a biotroph as well as a necrotroph. Usually hemibiotrophs infect the plant as a biotroph and switch over to a necrotropic lifestyle at a later stage during the infection. These pathogens are generally easy to handle but might need to be boosted by some living plant material once in a while.

Plant defence and defence mechanisms

It is estimated that the fungal kingdom harbours 1.5 million species, of which only a small fraction of 75,000-100,000 species have been identified (Hawksworth, 2001). Approximately 10,000 fungal species are considered to be plant pathogenic (Agrios, 1997). Nevertheless, it is rather rare to observe diseased plants in nature. The explanation is that plants, just like animals, have defence systems that help them to recognise foreign invaders (Figure 4).

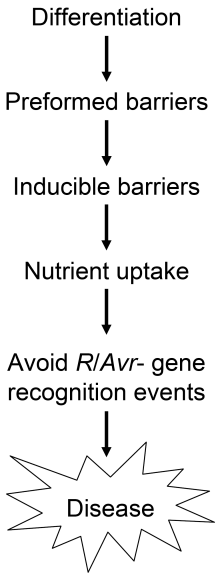

Figure 4. Steps in the defence of a plant that need to be overcome by the pathogen in order to cause disease. For more detailed information, see Thordal-Christensen (2003).

Multiple defence systems are known to protect plants from pathogens. The best studied are; resistance-gene mediated resistance, basal defence, non-host resistance, systemic acquired resistance (SAR) and various induced resistance. The literature on these topics is vast. In the following section, some hallmarks and recent discoveries will be described.

Pathogen effectors

The recognition in a plant that an invader is present is based on secretion of pathogen effectors. The term effector now includes all earlier used terms or factors such as avirulence, virulence, elicitor, toxin *etc* (Kamoun, 2007). A neutral term is useful since several effectors can have dual and conflicting functions depending on the circumstances (Espinosa and Alfano, 2004).

Some effectors are macro-molecules like carbohydrates, glycoproteins or lipids and originate from the pathogen itself or from the plant cells as a result of the attack (Blein *et al*., 2002; Chisholm *et al*., 2006; Garcia-Brugger *et al*., 2006). Whereas other effectors consist of peptides or proteins secreted by the pathogens (Chisholm *et al*., 2006; Eulgem and Somssich, 2007). One of the most well studied effectors in plant immunity is flagellin, a peptide deriving from the flagellum of *Pseudomonas syringae* (Felix *et al*., 1999). The discovery of recognition of bacterial flagellin by a receptor-like kinase (RLK) transmembrane receptor in plants generated a new view of plant innate immunity that in parts are very similar to animal immune systems (Gómez-Gómes and Boller, 2002; Ausubel, 2005).

Non-self molecules like flagellin are an example of a pathogen-associated molecular pattern (PAMP), which are specific effectors. Since these molecular patterns also exist in neutral and non-pathogenic microbes, the term microbe-associated molecular pattern (MAMP) is more accurate in a general context. A range of terms is used to describe these recognition events, for comprehensive terminology, see Mackey and McFall (2006) and Bittel and Robatzek (2007). PAMPs are recognised by cell-surface receptors in the plant, the RLKs, generally denoted pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI), recently reviewed by Chisholm *et al*. (2006) and Jones and Dangl (2006).

Plant resistance genes

The specific plant defence system is governed by resistance (*R*) genes encoding proteins that recognise avirulence (Avr) proteins secreted by the pathogen. Each *R*-gene recognises only a few Avr-proteins (Jones and Jones, 1996; Dangl and Jones, 2001; Nimchuk *et al*., 2003; McHale *et al*., 2006). The theory of the gene-for-gene relationship was established by Flor 1942 and was based on interaction studies between flax and *Melampsora lini,* reviewed by Flor (1971). This tight interaction between pairs of genes mimics the antibody-antigen relationship in the animal systems and is named the ligand-receptor concept (Gabriel and Rolfe, 1990). A direct R-Avr interaction has been demonstrated in very few cases (Jia *et al*., 2000; Deslandes *et al*., 2003; Dodds *et al*., 2006; Ellis *et al*., 2007). Concurrently with accumulating data on resistance and avirulence genes the guard hypothesis was proposed (van der Biezen and Jones 1998). This theory suggested an indirect recognition between the pathogen effectors and the R-protein. In other words, the R-protein detects the virulence-promoting activity of altered host-targets, rather than the Avr-protein itself (see reviews by Van der Hoorn *et al*., 2002; Innes, 2004). The Arabidopsis *R*-genes *RPM1* and *RPS2* are nice examples for this model. Both of these *R*-genes respond to *P. syringae* effectors that are

exported to the interior of cells by the bacterial Type III secretion system (TTSS). These effectors do not bind to RPM1 and RPS2, rather they target a novel, membrane associated protein called RIN4 (gardee) that physically co-localizes with RPM1 and RPS2 (Mackey *et al*., 2002; Mackey *et al*., 2003; Axtell and Staskawicz, 2003; Kim *et al*., 2005). Other examples of the guard hypothesis are the Arabidopsis RPS5 protein (Shao *et al*., 2003) and the tomato Cf-2 protein (Krüger *et al*., 2002; Rooney *et al*., 2005).

During the 1990s, a significant breakthrough was made for our present understanding of plant defence mechanisms. The identification of a range of plant resistance genes (Hammond-Kosack and Jones, 1996, 1997; Ellis *et al*., 2000; Belkhadir *et al*., 2004; Martin *et al*., 2005), in combination with our increasing genome data, has led to our gaining insight into the distribution and evolutionary aspects of these genes (Holub, 2001; Meyers *et al*., 2003, 2005a, 2005b; McDowell, 2004; Tiffin and Moeller, 2006; Staal and Dixelius, 2007). Collectively, the plant resistance genes can be divided into different classes based on their encoding protein motifs (Figure 5). The largest class of R-proteins includes those that contain a nucleotide binding site (NBS) and leucine-rich-repeat (LRR) domains. In Arabidopsis, the NB-LRR class comprises 149 genes divided into different groups. These groups consists of 92 genes which include a Toll/interleukin-1 receptor domain, *i.e*. TIR-NB-LRR encoding proteins, 51 genes that encode coiled-coil-NB-LRR (CC-NB-LRR) proteins, 6 where the N-terminal is lost and 58 genes lacking the LRR domain (Dangl and Jones, 2001; Meyers *et al*., 2002; Nimchuk *et al*., 2003; McHale *et al*., 2006). Interestingly, the large TIR-NB-LRR class has not been found in any monocotyledonous plant species yet. One important notion is that R-proteins can both reside completely intracellular, but also have plasma membrane spanning regions and expose domains outside the cell wall. Pathogen effectors can target these genes directly or indirectly independent of cellular location.

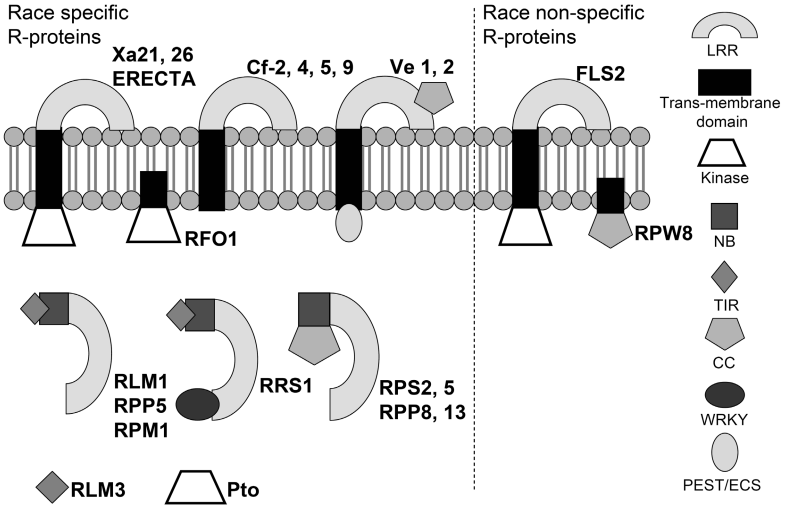

Figure 5. Different classes of known disease resistance proteins. The R-proteins can be membrane bound as well as cytosolic localised. For a more detailed explanation of the different *R*-gene classes, see Hammond-Kosack and Parker (2003).

Taken together, today we can visualise two distinct routes in the immune system. Either the presence of the slowly evolving PAMPs, like flagellin that interact with transmembrane pattern recognition receptors (PRRs), or interactions with NB-LRR proteins that largely take place inside the cell. The outcome, for the PAMP-triggered immunity, effector-triggered susceptibility and effector-triggered immunity, which together influence the levels of effective resistance over time, has been coined the zigzag model (Figure 6).

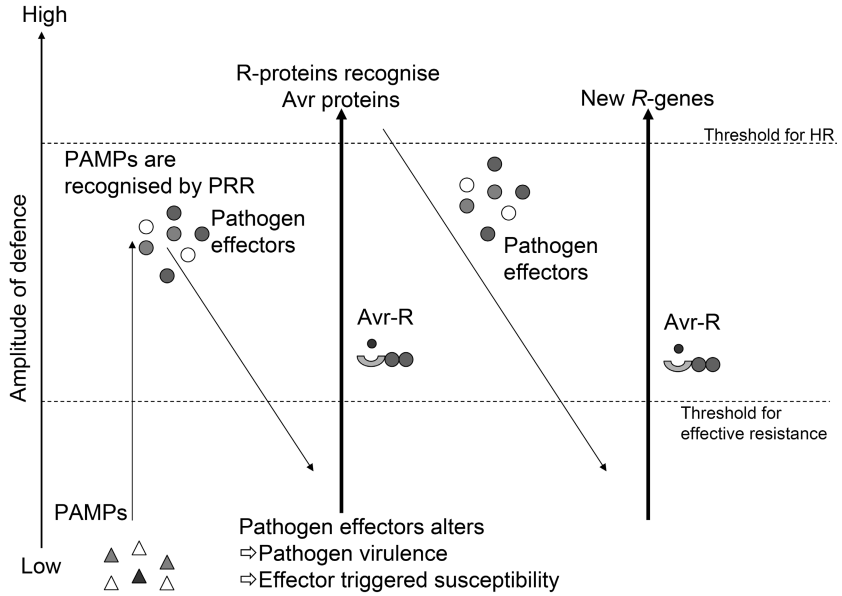

Figure 6. The Zigzag model of the quantitative output of the plant immune system. For more detailed information see Jones and Dangl, 2006. (Printed with permission from J. Jones).

The downstream events particularly triggered by PAMPs comprise MAP kinase signalling followed by activation of transcription factors particularly WRKYs (Eulgem and Somssich, 2007). WRKYs are also important in specific systems. In barley, WRKY proteins repress PAMP-triggered defence and in the presence of MLA (powdery mildew resistance locus), this R-protein interferes with the WRKY repressor function, suggesting a link between pattern recognition receptors and R-protein triggered immunity (Shen *et al*., 2007). Further discussions on these topics can be found in Bent and Mackey (2007).

Downstream events and induced resistance

Recognition of a pathogen results in a rapid activation of a range of cellular defence responses. *R*-gene-mediated resistance is often accompanied by an oxidative burst via rapid production of reactive oxygen species (ROS). ROS together with nitric oxide (NO) contributes to the defence by exhibiting

antimicrobal effects and by effecting cell walls by cross-linking and cellular protector genes (reviewed in Lamb and Dixon, 1997; Wendehenne *et al*., 2004). Another result of the NADPH-dependent oxidative burst is the special form of programmed cell death (PCD) that leads to HR. A hypersensitive response is common against avirulent pathogens in hosts with matching resistance genes and results in cell death at the site of infection to control the pathogen growth. ROS has also been shown to play a crucial role as a convergence regulator between biotic and abiotic stress responses (reviewed in Fujita *et al*., 2006).

ROS production initiates salicylic acid (SA) dependent signalling, leading to pathogenesis related (PR) proteins and systemic acquired resistance (SAR) induction (Ryals *et al*., 1996; Lamb and Dixon, 1997; Feys and Parker, 2000; Nimchuk *et al*., 2003; Grant and Lamb, 2006). SAR can also be activated without previous ROS production. SAR induces alertness in the whole plant against attacks. This preparedness (priming) remains in the plant for up to a week. Induced systemic resistance (ISR) is another system that can be activated in a plant (van Loon *et al*., 1998; Pieterse *et al*., 2002; Ton and Mauch-Mani, 2004; Grant and Lamb, 2006). ISR functions similarly to SAR but is less broad spectrum and independent of PR proteins and is induced by jasmonic acid (JA) and ethylene (ET) dependent signalling. Both SAR and ISR are well studied and there are excellent reviews that describe both systems (Ryals *et al*., 1996; van Loon *et al*., 1998; Durrant and Dong, 2004; Conrath, 2006; Grant and Lamb, 2006). Several recent studies report different compounds that can prime plants and thereby prepare them to defend against pathogen attacks. The priming process corresponds to ISR but it is not dependent on JA or ET (Ton and Mauch-Mani, 2004; Ton *et al*., 2005; Conrath *et al*., 2006; Beckers and Conrath, 2007). All of these three defence systems subsequently induce callose deposition, lignin content, proteases and PR proteins.

Two compounds known to prime for resistance are β-amino butyric acid (BABA) and abscisic acid (Ton and Mauch-Mani, 2004; Ton *et al*., 2005; Conrath *et al*., 2006; **II**). This specific priming leads to induced resistance that is SA dependent or independent depending on the pathogen system, but it has hitherto been shown to be camalexin independent (Ton and Mauch-Mani, 2004). BABA-induced resistance (BABA-IR) is also effective against abiotic stresses, such as drought and salinity (Cohen, 2002). A general role in primed plants is that defence systems are not activated before the plant encounter stress, instead the plant is in an alerted state which makes it possible to respond to the stress stimuli faster (Sticher *et al*., 1997; Mauch-Mani and Métraux, 1998, Pieterse *et al*., 1998; Zimmerli *et al*., 2000; Ton *et al*., 2002).

Taken together, the molecular mechanisms underlying activation of plant defence responses are exceedingly complex. With steadily increasing data, not least those originating from microarray analyses, showing extensive cross-talk between earlier denoted abiotic and biotic pathways (Schenk *et al*., 2000; Nemhauser *et al*., 2006). The most well-studied defence interactions derive from hormones such as ethylene (ET), jasmonic acid (JA) and salicylic acid (SA). But over recent years it has been established that abscisic acid (ABA), auxin and lately gibberellic acid (GA), that earlier had been restricted to basal plant physiological processes, also are important when plants are exposed to stress, makings the signalling web even more complex.

Hormones

A plant hormone is generally described as a signalling molecule produced at a specific location. They occur in very low concentrations, and cause altered processes in target cells at other locations. Phytophormones or plant growth regulators are other names used in the literature.

During the past decade, much progress has been made towards understanding the mechanisms underlying plant hormone reception, activity, biosynthesis and signalling events. Plant hormones have been extensively studied since the mid 19th century and will not be reviewed in detail in this thesis. The following sections will instead highlight links between hormones and plant defence to pathogens.

The involvement in defence interactions by hormones is often divided into responses against necrotrophic or biotrophic pathogens (Flors *et al*., 2005; Glazebrook, 2005). To divide the defence response into these two categories is oversimplified, since exceptions to these groups start to emerge. However, it facilitates the picture of the different signal transduction events. The hormone-mediated signalling is exceedingly complex and can work both in synergistic or antagonistic manners (Dong, 1998; Fey and Parker, 2000; Kunkel and Brooks, 2002; Andersson *et al*., 2004). It is also known that many pathogens can produce plant hormones themselves and thereby alter the host responses (Dörffling *et al*., 1984; Crocoll *et al*., 1991; Murphy *et al*., 1997; Maor *et al*., 2004).

Salicylic acid

Salicylic acid is a ubiquitous hormone significant for plant growth and development as well as plant defence against biotrophic pathogens (Gaffney *et al*., 1993; Delaney *et al*., 1995; Hayat and Ahmad, 2007). Salicylic acid is biosynthesized from the amino acid phenylalanine and is a phenolic compound (reviewed in Hayat and Ahmad, 2007). SA has two functional analogues, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BHT) that are commonly used in various bioassays (Métraux *et al*., 1991; Görlach *et al*., 1996). Salicylic acid can be inactivated in transgenic *nahG* plants by the bacterial enzyme salicylic hydroxylase (*nahG*) that converts SA to catechol (Gaffney *et al*., 1993). However, caution should be taken since side effects, such as inappropriate production of H_2O_2 can occur in *nahG* plants and mutants in the SA biosynthetic pathway like *sid2* should if possible be used (van Wees and Glazebrook, 2003).

There is no known receptor identified for SA yet, but there are two important genes, non-expressor of PR genes 1 (*NPR1*) and enhanced disease susceptibility 1 (*EDS1*) that define the downstream response (Cao *et al*., 1997; Ryals *et al*., 1997; Century *et al*., 1998; Glazebrook, 2001). *NPR1* and other SA-response genes are regulated by transcription factors of the TGA (contain basic leucine zipper motif) family (Zhang *et al*., 1999) and cytosolic NPR1 plays a crucial role in the SA-JA interaction (Spoel *et al*., 2003). The most common marker for SA-mediated gene expression is *PR1* (Uknes *et al*., 1992), but it also activates *PR2* (Lawton *et al*., 1995). SA is an important part of SAR, as it is reported to be involved both in local as well as long distance signalling (Durner *et al*., 1997). However, even if

SA is important for the signalling it is not SA that is the signalling molecule (Vernooij *et al*., 1994; Park *et al*., 2007) The resistance induced by SA is often due to an increased SA sensitivity rather than elevated biosynthesis (Yu *et al*., 1997).

Jasmonic acid

Jasmonic acid is mainly involved in wound and necrotrophic defence responses (Thomma *et al*., 1998), but is also required for stamen and pollen development (Feys *et al*., 1994). Jasmonic acid is synthesised from linolenic acid by the octadecanoid pathway (Doares *et al*., 1995; Turner *et al*., 2002). JA can be converted to different esters, including methyl jasmonate (MeJA), which often is used to induce JA responses (Feys *et al*., 1994). Perception of JA is reported to be dependent on coronatine-insensitive1 (*COI1*) or jasmonate resistant1 (*JAR1*) (Xie *et al*., 1998). Recently COI1, which is an F-box protein, has been suggested to function as the JA receptor (Chen *et al*., 2007; Farmer, 2007). JA responses are inclined to be post-translational regulated via E3 ubiqutin ligase, since SCF^{COI1} (Skp1/Cul1/F-box) is involved in targeting proteins for degradation via the proteasome (Turner *et al*., 2002; Stone and Callis, 2007). JA-mediated signalling results in different responses depending on if it is a wound reaction or a pathogen response (Chini *et al*., 2007). A transcription factor (MYC2) regulates these JA dependent events (Anderson *et al*., 2004; Chini *et al*., 2007; Thines *et al*., 2007) resulting in production of the storage protein VSP2 (Benedetti *et al*., 1995) or defensin PDF1.2 (Penninckx *et al*., 1998), respectively.

Ethylene

Ethylene is involved in a range of developmental processes such as root production, flowering, senescence and fruit ripening as well as abiotic and biotic stress responses (reviewed in Abeles *et al*., 1992; Johnson and Ecker, 1998). Ethylene can affect degrees of susceptibility and the severity of disease symptoms besides taking part in signalling events (Bent *et al*., 1992). In biotic stress it has been shown that ET particularly is involved in defence against necrotrophic pathogens (Glazebrook, 2005). Ethylene is considered to function in the same defence signalling pathway as JA (reviewed in Wang *et al*., 2002; Broekaert *et al*., 2006) and the defensin *PDF1.2* is commonly used as the marker gene to detect gene activation in this route (Penninckx *et al*., 1998). JA and ET also co-regulate the pathogenesis-related (PR) genes *PR3* and *PR4* (Penninckx *et al*., 1998). 1-Aminocyclopropane-1-carboxylic acid (ACC) takes part in the last step in the biosynthetic pathway (reviewed in Yang and Hoffman, 1984) and is frequently used instead of ET which is a gas (C_2H_4) and difficult to handle in assays as the inducer of the ET pathway. The production of ET starts with methionine and two important enzymes are ACS (ACC synthase) and ACO (ACC oxidase). ACS is the enzyme that produces ACC, and ACO converts ACC to ET (reviewed in Wang *et al*., 2002). There are five known ET receptors in Arabidopsis, ETR1, ETR2, ERS1, ERS2 and EIN4 (Chang *et al*., 1993; Hua *et al*., 1995; Hua and Meyerowitz, 1998; Sakai *et al*., 1998). These receptors can further be divided in

two categories, those that have a receiver domain (ETR1, ETR2 and EIN4) and those without (ERS1 and ERS2), reviewed in Wang *et al*. (2002). The downstream signalling has four main steps controlled by CTR1, EIN2, EIN3 and ERF, from where the signal is further transduced (Chang and Shockey, 1999; Stepanova and Ecker, 2000). This regulation also involves protein degradation by the SCF^{CUL3} complex (Yoshida *et al*., 2006). In Arabidopsis natural variation in ethylene sensitivity exists. For example, Ws-0 is more sensitive than most other accessions (Roman *et al*., 1995).

Abscisic acid

ABA has many functions in plant development (seed and bud dormancy, stomata closure) and has for a long time been known as a major hormone in abiotic stress responses (reviewed in Leung and Girandat, 1998; Rock, 2000). During recent years ABA has also been found to be involved in plant-pathogen interactions (Ton and Mauch-Mani, 2004). The effect of ABA has been reported to both induce susceptibility, as in the case of *Pseudomonas syringae* (Truman *et al*., 2006) as well as to induce or prime for resistance, as in the case of *L. maculans*, *Alternaria brassicicola* and *Plectosphaerella cucumerina* (**II**; Ton and Mauch-Mani, 2004). ABA is an isoprenoid plant hormone produced from zeaxanthin via a well known pathway involving ABA1, ABA2 and ABA3 (reviewed in Finkelstein and Rock, 2002; Schwartz *et al*., 2003; Nambara and Marion-Poll, 2005) and can be produced as different isomers. The isomer (+) ABA is however the only active form. The endogenous levels are regulated by the precise balance between biosynthesis and catabolism, involving cytochrome P450. In 2007 the membrane bound ABA receptor GCR2 was identified by Liu and co-workers. GCR2s roll as a receptor has however been questioned (Gao *et al*., 2007; Johnston *et al*., 2007). ABA signalling involves G proteins, protein phosphatases (PP2C, ABI1 and ABI2) and protein kinases as well as Ca^{2+} which is an important second messenger in ABA signalling (reviewed in Leung and Griandat, 1998; Finkelstein and Rock, 2002).

Auxin

Auxin is one of the major plant hormones regulating many development processes and the signalling pathway is thoroughly investigated. There are excellent reviews written about auxin function and signalling for those interested in more details (Davies, 1995; Kepinski and Leyser, 2002; Kepinski and Leyser, 2005; Woodward and Bartel, 2005; Leyser, 2006; Quint and Gray, 2006). There are three commonly used auxins, the natural indole-3-acetic acid (IAA) and the two synthetic 1-naphthaleneacetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (reviewed in Teale *et al*., 2006). IAA is synthesised from tryptophan using cytochrome P450 or via a tryptophan independent pathway (Ljung, 2002; Cohen *et al*., 2003). Rather recently the F-box protein TIR1 was shown to be the auxin receptor (Kepinski and Leyser, 2005; Dharmasiri *et al*., 2005a). At the same time three additional members of the TIR1 family, AFB1-AFB3, were found to function as auxin receptors (Dharmasiri *et al*., 2005b). AFB genes encode F-box

proteins that assemble into the SCF complex and are partially redundant with TIR1 in mediating auxin responses. This strengthens the already established theory that auxin signalling influences protein turnover via the SCF complex (Gray *et al*., 1999; Gray *et al*., 2001; Quint and Gray, 2006). This response is also dependent on ASK1 and COP9 proteins, which can bind to the same SCF complex (Schwechheimer *et al*., 2001; Thomann *et al*., 2005). Auxin signalling involves three major gene families *Aux/IAA*, *GH3* and *SAUR* (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). These proteins are synthesised within minutes after auxin treatment, without the need of any protein translation, defining them as primary auxin response genes (Hagen and Guilfoyle, 2002). Among the major gene families, the *Aux/IAA* family is the best characterised (Liscum and Reed, 2002). This signalling involves the auxin responsive factor (ARF) which can form heterodimers with the Aux/IAA domains in auxin responsive genes and thereby regulate the transcription of the genes (Kim *et al*., 1997; Guilfoyle and Hagen, 2007). The Aux/IAA proteins are short lived and the half-life is decreased even more by the presence of auxin (Zenser *et al*., 2001). However, the degradation rates vary among different Aux/IAA family members (Dreher *et al*., 2006). Auxin has recently been found to be involved in plant defence signalling, and the effect on the resistance depends on the pathosystem studied (**IV**, Navarro *et al*., 2006; Robert-Seilaniantz *et al*., 2007).

Interactions between hormones

All hormones are interacting with each other in a complex manner to facilitate the most effective response in the plant. Most of these opposing hormone activities are regulated via modulation of SCF complexes and protein degradation (Rogg and Bartel, 2001; Thomann *et al*., 2005; Dreher and Callis, 2007). A simplified overview of how the hormones regulate each other can be seen in Figure 7. One of the best studied antagonistic relationships is the one between JA and SA (Devoto and Turner, 2003; Beckers and Spoel, 2006). It is also known that ABA down-regulates all of the previously mentioned hormones and ET represses ABA while SA up-regulates ABA production (Anderson *et al*., 2004; Robert-Seilaniantz *et al*., 2007). Auxin on the other hand, up-regulates ET and probably JA, while SA is down-regulated (Robert-Seilaniantz *et al*., 2007). If plant defence is simplified down to the two pathways that lead to biotroph or necrotroph resistance, the following hormone interactions can be depictured. In resistance against biotropic pathogens increased SA levels increase the resistance. At the same time JA/ET and auxin are down-regulated as these hormones increases the susceptibility towards biotrophs. For a necrotropic pathogen it is the opposite situation. The resistance is increased by JA/ET and auxin, which levels are increased, while SA increases susceptibility.

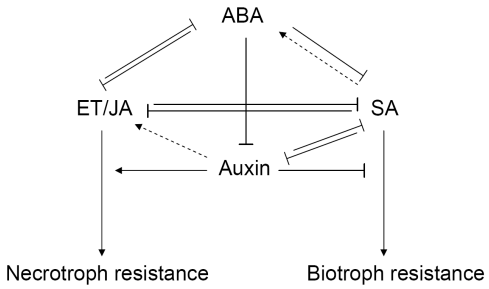

Figure 7. Schematic overview of cross-talk between hormones in plant defence.

Secondary metabolites of importance in plant defence

Secondary metabolites are organic compounds that are not directly involved in growth and development (Taiz and Zeiger, 1998). Unlike primary metabolites, secondary metabolites have a restricted distribution in the plant kingdom and a particular secondary metabolite is often only found in one taxonomically related group. The function or importance of these compounds to the organism is usually of an ecological nature as they are used in defence (toxins, callose, phytoalexins, cutin, waxes, *etc*), for interspecies competition, and to facilitate the reproductive processes (colouring agents, attractive smells, *etc*). There are several secondary metabolites involved in plant defence. In the following sections callose and camalexin will be discussed. These metabolites are involved in defence against several necrotrophic pathogens including *L. maculans* (Bohman *et al*., 2004; **II**; **III**).

Callose

The first line in plant defence is the physical barrier constituted by the waxy cuticular layer and the cell wall (Thordal-Christensen, 2003; Chassot *et al*., 2007). This barrier can be reinforced by lignin and other compounds like callose, at pathogen penetration sites or invasion attempts. Callose is a β-1,3-linked glucan that can be produced in the cell wall in response to wounding or pathogen attack (Worrall *et al*., 1992). In addition to defence mechanisms, callose is also involved in pollen development particularly important in the pollen tube cell wall where it can induce male sterility (Worrall *et al*., 1992; Flors *et al*., 2005). Callose can also be found in abscission zones, dormant phloem and plasmodesmata (Stone and Clark, 1992). Callose depositions can be induced both by biotic and abiotic stress. Generally when challenge by biotic cues the glucan polymer forms papillae between the plasma membrane and the cell wall to prevent penetration of the pathogen by local reinforcement of the cell wall at the site of penetration attempt (Zimmerli *et al*., 2000; Donofrio and Delaney, 2001; Ton and Mauch-Mani, 2004). Callose deposition is dependent of the glucan synthase like 5 (*GSL5*) also known as powdery mildew resistance 4 (*PMR4*) gene (Østergaard *et al*., 2002; Jacobs *et al*., 2003; Nishimura *et al*., 2003), and is induced by ABA and BABA-IR (Ton and Mauch-Mani, 2004; Ton *et al*., 2005; **II**).

Camalexin

If the plant cell wall is penetrated by a pathogen there is an arsenal of antimicrobal compounds called phytoalexins that can defend against the pathogen. Phytoalexins are low-molecular-weight compounds with antimicrobial activity (Paxton, 1981). Among these antimicrobal compounds there are both specific as well as broad-spectrum compounds (Thomma *et al*., 1999). Different plant species produce different phytoalexins, but plants within a given family often produce phytoalexins of the same chemical class (Smith, 1996; Hammerschmidt, 1999). Phytoalexins in species within *Brassicaceae* consist of an indole ring with a sulphur-containing moiety (Pedras *et al*., 1997). *B. napus* produces brassinin, cyclobrassinin, 1-methoxybrassinin and spirobrassinin (Figure 8) while the major phytoalexin in Arabidopsis is camalexin (Pedras *et al*., 1998; Pedras *et al*., 2000).

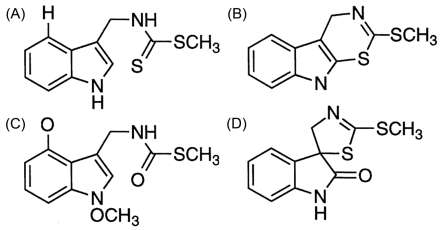

Figure 8. Structures of phytoalexins present in *B. napus*.
(A) Brassinin,
(B) Cyclobrassinin,
(C) 1-methoxybrassinin,
(D) Spirobrassinin

Camalexin is produced from tryptophan, via cytochrome P450 monooxygenases (Figure 9), followed by a few unknown biosynthetic steps which subsequently lead to the final step which is catalyzed by PAD3 also named CYP71B15 (Glawischnig *et al*., 2004; Glawischnig, 2007; Schuhegger *et al*., 2006, 2007). Phytoalexins are only produced as a response to a stimuli or a signal (Smith, 1996). In Arabidopsis the level of camalexin content is genotype-dependent (Kagan and Hammerschmidt, 2002; Denby *et al.,* 2004; Schuhegger *et al.,* 2006). Thus, this variation must be considered when working with mutants that have different genetic backgrounds, as it can affect the degree of susceptibility towards a pathogen.

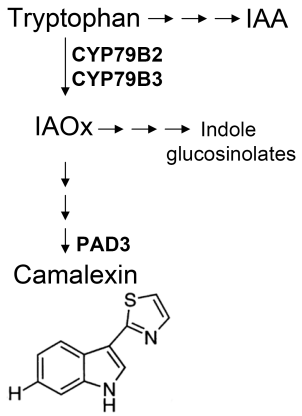

Figure 9. Schematic overview of camalexin synthesis.

The fungal pathogen *L. maculans*

The hemibiotrophic fungus *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.) the casual agent of blackleg (Figure 10) was first described in 1849 as *Phoma lingam,* the asexual stage of the pathogen. In 1863 the name changed to *Leptosphaeria maculans* (Desm.) Ces. & de Not. The sexual stage was first identified 1957 in a field in New Zealand (Williams, 1992) and since then the prevalence has increased. *L. maculans* is an ascomycete belonging to the class Dothideomycetes (Loculoascomycetes), order Pleosporales. Other important plant pathogens such as *Alternaria*, *Botrytis* and *Mycosphaerella* can also be found within Pleosporales (Rouxel and Balesdent, 2005).

In Europe *L. maculans* often co-infects *B. napus* together with *L. biglobosa* (West *et al*., 2002; Fitt *et al*., 2006a). Previously *L. biglobosa* and *L. maculans* were divided into separate groups, B- and A-group, respectively (Koch *et al*., 1991; William and Fitt, 1999). These two groups are now regarded as two different species as they differ morphologically, molecularly and show no evidence of sexual mating (Mendes-Pereira *et al*., 2003; Fitt *et al*., 2006b). There has been many different grouping and classifications within the *L. maculans* species during the last 15 years. The most common ones are based on pathogenicity groups (PG), how virulent an isolate is on different host genotypes, and phytotoxin production Tox+ and Tox0 (West *et al*., 2001, 2002). Today there is a new classification which is based on avirulence genes (*Avr* gene) present in individual isolate (Balesdent *et al*., 2005). This new classification showed that there are more simple races in Canada and Europe (fewer virulence genes) compared to Australia. In Europe there are only eight different races present of which two are of major prevalence. In Australian isolates, on the other hand, the races comprise many diverse and complex *Avr* combinations. This is most likely as a result of the high recombination frequency.

Epidemiology and disease cycle

L. maculans strictly infects crucifers and mainly *Brassica* crops (West *et al*., 2001; Fitt *et al*., 2006a, 2006b). Epidemics of blackleg are most severe in Australia, where only *L. maculans* occurs so far. In Canada and Western Europe where both *L. maculans* and *L. biglobosa* are present the damage varies between region and year. Where the disease occur total destruction of the seedlings is rare even if yield losses at harvest usually are 10% and can reach 30-50% due to lodging of the plants.

The primary inoculum of *L. maculans* is ascospores, which can be viable for about 6 weeks on infested stubble (Howlett *et al*., 2001; West *et al*., 2001; Rouxel and Balesdent, 2005). The ascospores are released from pseudothecia located on stubble and plant debris throughout the growing season, causing leaf and steam lesions (Figure 10). In these lesions pycnidia with pycnidiospores are produced. These spores, a second inoculum, spread in the field by rain splash. Secondary infection is of less importance in Canada and Europe. Following leaf infection *L. maculans* colonises the intercellular space between mesophyll cells and then grow down into the xylem via the petiole. This intercellular systemic phase is biotrophic

and visual symptoms less obvious. In the next stage (the necrotrophic) the fungus invades and kills the cells of the stem cortex, resulting in a blackened canker (Figure 10). The very last stage in the lifecycle is a saprophytic stage on the stubble where sexual reproduction occurs (Figure 10). During the sexual stage *L. maculans* has a high degree of sexual recombination and outcrossing, involving two different mating type loci (Barrins *et al*., 2002; Cozijnsen and Howlett, 2003).

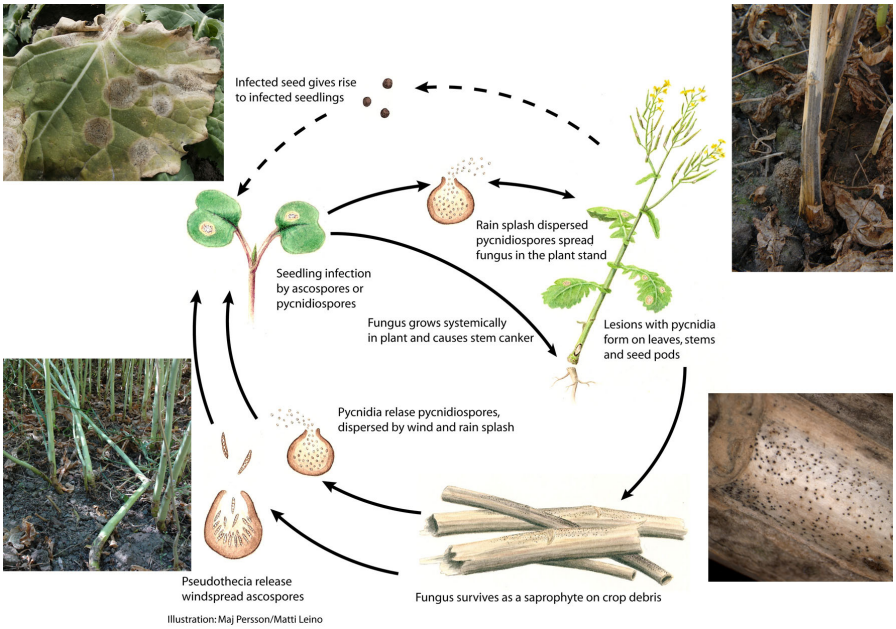

Figure 10. Life cycle of *L. maculans*.
L. maculans is spread either via wind (ascospores) or rain splash (ascospores and pycnidiospores) and grows initially as a biotroph before switching to a necrotrophic phase later in its infection cycle to generate pycnidia. The inserted pictures show leaf lesions (top left), blackleg (top right), lodging (bottom left), pycnidia (bottom right). Printed with permission from Gunilla Berg, the Swedish Board of Agriculture (SJV).

Genome information

The *L. maculans* haploid genome of the French isolate v23.1.1 is about to be sequenced in collaboration between Genescope and INRA. The genome size is estimated to about 34 Mb and predicted to encode 10,000 genes (Plummer and Howlett, 1995; Cozijnsen *et al*., 2000; Howlett, 2004). These genes are distributed over 15-16 chromosomes including a non-Mendelian-transmitted mini-chromosome. *L. maculans* shows a high degree of genome plasticity by having changes in chromosome length (Plummer and Howlett, 1995). These changes occur during meiosis by unequal pairing of chromosomes and might be relevant for the fungus in its ability to respond to selection pressure.

Resistance against *L. maculans*

There are two kinds of resistance against *L. maculans*. A qualitative resistance based on gene-for-gene resistance which is effective throughout the plants entire life (Ansan-Melayah *et al*., 1998; Delourme *et al*., 2004). This resistance has a high potential for being overcome due to the fungus sexual recombination, effective spore dispersal and large population size (Howlett *et al*., 2001; McDonald and Linde, 2002). The Brassica B genome carries a resistance gene, a homolog to an Arabidopsis gene, against *L. maculans* conferring resistance in all stages of the plant. This gene confers a hypersensitive type of resistance (Rimmer and Buchwaldt, 1995; Saal and Struss, 2005). The other type is a quantitative resistance which is active in the adult plant. This resistance is dependent on many different genetic factors such as phytoalexin, callose and defence-related proteins and are therefore less easy to overcome by the fungus (Hammond *et al*., 1985; Rasmussen *et al*., 1992 a, 1992b; Chen and Howlett, 1996; Chen and Seguin-Swarts, 1997, 1999; Roussel *et al*., 1999; Brownfield and Howlett, 2001).

Beside the qualitative and quantitative resistance, there is a resistance called field resistance. In field resistance all components are influenced by agronomic practices and environmental factors, besides the genetic factors in the pathogen and host (Pilet *et al*., 1998; West *et al*., 2001; Fitt *et al*., 2006a).

Fungal *Avr* genes comprise a range of heterogenous sequences, lacking common signature domains as encoded by plant resistance genes (Laugé and de Wit, 1998; Kamoun, 2007). The cloning of *AvrLm1* and *AvrLm6* makes *L. maculans* the sixth fungal species in which an *Avr* gene has been characterised (Gout *et al*., 2006; Fudal *et al*., 2007). Genetic mapping of the *Avr* genes in *L. maculans* has shown that there are at least four unlinked regions of these genes in the genome. The mapping has also identified two *Avr* gene clusters (*AvrLm1-AvrLm2-AvrLm6* and *AvrLm3-AvrLm4-AvrLm7*) making *L. maculans* the first filamentous fungi with such genome organisation (Delourme *et al*., 2004). However, how *AvrLm* genes interact with plant host genes is still unknown and no structures supporting transport of effectors to intercellular spaces are known.

The Arabidopsis-*L. maculans* pathosystem

Transgressive segregation in crosses between the Arabidopsis accessions L*er*-0 and Col-0 revealed two evolutionary connected resistance loci, $RLM1_{\mathrm{Col}}$ and $RLM2_{\mathrm{L}er}$ (**I**). The $RLM1_{\mathrm{Col}}$ locus comprises a cluster of homologous TIR-NB-LRR (Toll/interleukin-1 receptor – nucleotide binding – leucine rich repeat) encoding genes, where At1g64070 plays a major role in *L. maculans* resistance. The TIR-NB-LRR (TNL) family of plant disease resistance genes consists of protein domains of pre-eukaryotic evolutionary origin, which are of central importance for cell death regulation and innate immunity in both plants and animals, indicating conserved mechanisms (Staal and Dixelius, 2007).

One feature of the Arabidopsis-*L. maculans* pathosystem is the large extent of naturally occurring resistance (Bohman *et al*., 2004). In a large screening comprising 168 accessions, only one An-1, was found to be susceptible. This

information was utilised to identify the *RLM3*$_{Col}$ gene encoding a putative TIR-X protein (Staal *et al*., 2007).

The wealth of characterized Arabidopsis mutants linked with genetic and molecular analysis has furthermore showed that the resistance is independent of the common defence pathways involving salicylic acid, jasmonic acid and ethylene (Bohman *et al*., 2004). Our pathosystem has also shown independence of the *R*-gene signalling components *NDR1*, *EDS1*, *PAD4* and *SGT1b*, in contrast to *RAR1* and *HSP90*, which are essential (Bohman *et al*., 2004; **II**). The role of camalexin, callose deposition and the impact of ABA and auxin signalling are further elaborated on in this thesis.

Knowledge of plant defence has grown dramatically the last 15 years and has become exceedingly complex. One very well studied pathosystem is the Arabidopsis-*Pseudomonas syringae* interaction. When comparing *L. maculans* with *P. syringae* "a rule of thumb" is that the two pathogens induce responses in an opposite manner. One example is the effect of the two hormones auxin and ABA. In the Arabidopsis-*L. maculans* pathosystem these two hormones confers resistance, while in the Arabidopsis-*P. syringae* pathosystem they confer susceptibility (**II**; **IV**; de Torres-Zabala *et al*., 2007; Robert-Seilaniantz *et al*., 2007).

Aims of the study

The overall aim was to use the model organism Arabidopsis in order to identify genes involved in resistance and defence signalling against *L. maculans*. By using the Arabidopsis-*L. maculans* pathosystem components important for the defence signalling have been characterised. In a long-term perspective, the information gained in the Arabidopsis system will be evaluated in the Brassica system, and hopefully become valuable tools in breeding strategies for the improvement of resistance to this plant pathogen.

The specific aims of this study were;

- Cloning of resistance genes in Arabidopsis responsible for resistance to *L. maculans*.
- Study the impact on plant hormones in defence responses and their cross-talk.
- Study priming and its mechanisms within the Arabidopsis-*L. maculans* pathosystem.
- Characterisation of the *lms5* mutant.

Results

Identification of *R*-genes involved in defence against *L. maculans*

Crosses between Col-0 and L*er*-0 showed transgressive segregation, indicating two dominant *R*-genes (**I**). In order to identify the loci responsible for the segregation Col-4 x L*er*-0 recombinant inbreed lines (RIL) from Lister and Dean (1993) were used. Another set of RILs (L*er*-2 x Cvi-1) was further used to confirm the genomic localisation on chromosome 1. Using T-DNA knockout lines (salk_014088 and salk_014096) the gene At1g64070 was identified as *RLM1*. This gene is present in Col-0 but absent in L*er*-0. L*er*-0 on the other hand harbours *RLM2* (At4g17800–At4g24140), a paralogue to *RLM1*, which is absent in Col-0. From a cross between Col-0 and L*er*-0, a plant lacking both *RLM1* and *RLM2* was identified in F_2. This genotype was selfed and this highly susceptible line was hereafter denoted $rlm1_{Ler}rlm2_{Col}$. During the complementation of $rlm1_{Ler}rlm2_{Col}$ with the genomic Col-0 sequence of At1g64070, three T_1 lines showed weak lesions phenotype indicating that an additional gene is involved in the resistance. In the $RLM1_{Col}$ locus three potential *R*-genes are present (At1g63750, At1g63880, At1g63870). Studies revealed that At1g63880 has a minor contribution to the resistance response governed by this locus. To further understand the role of this particular gene family, a 26-mer RNA interference (RNAi) construct was designed to specifically target TIR-NB-LRR genes within the $RLM1_{Col}$ locus. Twelve susceptible plants were found when 79 T1 RNAi plants of the L*er*-0 background were evaluated. The appearance of the first necrotic lesion varied from 5 to 17 days post-inoculation. These results support our hypothesis that the resistance is dose-dependent and dependent on genes structurally related to those found in $RLM1_{Col}$.

In order to identify additional resistance genes the susceptible accession An-1 was crossed with Col-0 followed by a case-control bulk segregate comparison strategy. Resistant respectively susceptible F_3 plants from Col-0 x An-1 crosses were pooled separately. This identified only four genes (At4g13100, At4g16990, At4g19530 and At4g23290) with over 2-fold differential expression (Staal *et al*., 2007). Using genetic mapping and RT-PCR we could identify At4g16990, an *RPP5*-like gene, as the *R*-gene responsible for the resistance. These results were confirmed using T-DNA knockouts (gabi_235E02, gabi_491E04, salk_146865, salk_048620 and salk_067449). The gene At4g16990 was named *RLM3* and was found to be absent in An-1 ($rlm3_{An}$) due to a large indel (insertion/deletion) polymorphism. *RLM3* codes for a TIR-X putative protein and therefore it belongs to the small group of *R*-genes that has lost additional common motifs. The entire *RLM3* work is presented outside the thesis but is commented here since it brings in valuable information.

Pathogen specificity

The identified *R*-genes were evaluated for there specificity against different pathogens. *Leptosphaeria maculans*-susceptible ($rlm1_{Ler}rlm2_{Col}$) plants and T-DNA mutants in *RLM1* candidate genes were tested against *Botrytis cinerea* and

Alternaria brassicicola without displaying any susceptibility, suggesting that *RLM1* is a *L. maculans*-specific resistance gene (**I**).

An-1 shows enhanced susceptibility to a range of pathogens, such as *B. cinerea*, *A. brassicicola* and *A. brassicae.* Therefore, to assess whether *RLM3* affected resistance against these pathogens, the susceptible and resistant F_3 lines, from a Col-0 x An-1 cross, were evaluated to clarify their responses. All *L. maculans* susceptible lines showed susceptibility to these three pathogens (Staal *et al*., 2007). Further, T-DNA mutants in *RLM3* were also found to be susceptible to these pathogens. In order to be able to compare *RLM3* with other known *R*-genes a quantitative evaluation of different *Avr* genes in a *P. syringae* DC3000 background was performed. The effect of *P. syringae* was assessed on Col-0, An-1 and the *RLM3* T-DNA mutants. This investigation revealed that the introduced *Avr* genes resulted in a reduced susceptibility in all genotypes analysed, further highlighting the opposite responses between *P. syringae* and *L. maculans*.

Pathogen specificity was also assessed in the *lms5* mutant. The fungal pathogens used were *A. brassicicola*, *B. cinerea*, *Hyaloperonospera parasitica* (Noco2) and *Verticillium longisporum*. These analyses showed that *lms5* has the same resistant phenotype as L*er*-0 (**IV**), making *lms5* different from *lms1* since *lms1* shows susceptibility towards *A. brassicicola, B. cinerea* and *V. longisporum* (Bohman *et al*., 2004; Staal, 2006). We also evaluated susceptibility towards the bacterial pathogen *P. syringae*. The results showed significantly lower bacterial content in *lms5* compared to L*er*-0 in the *P. syringae* strain DC3000. This observation is in agreement with previous findings on *RLM3* mutants and the contrasting responses induced by *P. syringae* compared to *L. maculans* (**IV**; Robert-Seilaniantz *et al*., 2007).

Priming and callose deposition

Synthesis of 1,3-ß-glucans (callose) are known to be involved in defence responses and has been found to be induced by the *Brassica napus–L. maculans* resistance genes *LepR1* and *LepR2* (Yu *et al*., 2005). This led us to examine the importance of callose and its *R*-gene dependency in Arabidopsis. All our comparisons of callose deposition are made 2 days post-inoculation on aniline blue stained leaves. In the case of *RLM1*, a comparison between (*RLM1*$_{Col}$)*pad3-1* and *rlm1*$_{Ler}$*pad3-1* revealed that *RLM1*, like the *B. napus LepR* genes, are required for efficient callose deposition in response to *L. maculans* infection (**I**). Furthermore, both the callose synthase mutant *pmr4-1* and the papilla mutant *pen1* were found to be susceptible phenotypes to *L. maculans*. Callose content has also been assessed in the *lms1* and *lms5* mutants. The results showed increased callose content in *lms5,* while *lms1* displayed the same level as the wild-type L*er*-0 (**IV**). *rar1-13* was also included in this study. This mutant showed significantly lower callose content compared to L*er*-0 indicating that *rar1-13* and *lms5* are not affecting the same signalling pathway. These results further indicate that *rar1* is located downstream of *RLM1* and *RLM2* (**I**).

Increased callose deposition has been found to be a result of ABA- and BABA-IR (Ton and Munch-Mani, 2004). In order to investigate if this priming is effective in our system susceptible and resistant Arabidopsis plants were pre-treated with ABA or BABA 2 days pre-inoculation. Priming caused a significant

induction of resistance in some susceptible Arabidopsis mutants (*aba1-3*, *pad3-1*, *pmr4-1*, *esa1* and *pen1*), while others (*abi1-1* and *lms1*) were unaffected (**II**). The genotypes $rlm1_{Ler}pad3$ and $rlm1_{Ler}rlm2_{Col}$ both displayed partial restoration of resistance after ABA treatment, which demonstrates that ABA acts as a signal downstream of $RLM1_{Col}$.

We also analysed the callose content in the pre-treated plants in order to evaluate the effect on callose deposition of priming. This analysis revealed a correlation between induced resistances and altered, *i.e*. higher callose deposition (**II**). The analysis of callose depositions in *pad3* (in Col background, containing $RLM1_{Col}$) and $rlm1_{Ler}pad3$ after pre-treatment revealed that callose deposition is enhanced in *pad3* plants, whereas callose deposition in $rlm1_{Ler}$ plants is not affected by pre-treatments. The dependency of $RLM1_{Col}$ for callose deposition shows that ABA and BABA enhances callose deposition downstream of *RLM1*.

The induction by ABA and BABA in the Arabidopsis - *L. maculans* interaction is a result of a local deactivation of PR2, a β-1,3-glucanase, a protein which normally degrades callose (**III**). This down-regulation of *PR2* is most likely located upstream but independent of *ABI4*, while *ABI1* is required for the signalling. BABA pre-treated material exhibits a local repression of *PR2* expression at the inoculation site as seen after ABA pre-treatment. The reduction of *PR2* expression in ABA pre-treated plants inoculated with SA suggests that ABA repress *PR2* expression downstream of SA. These data support our hypothesis that *PR2* is locally repressed at sites of callose deposition, which implies that callose depositions are at least in part regulated by turnover rather than biosynthesis. The conclusion drawn from these results is that PR2 has callase functions which affect callose deposition.

The importance of camalexin

As mentioned previously camalexin levels are accession dependent. *L. maculans* induces approximately 30% of the camalexin levels in the L*er*-0 accession compared to Col-0, 48 h after inoculation (**I**). A similar difference in camalexin induction levels between L*er*-0 and Col-0 has previously been observed in response to *A. brassicicola* (Kagan and Hammerschmidt, 2002). During the identification of *RLM1,* a large variation in camalexin induction among the susceptible Col-0 x L*er*-0 plants was discovered (**I**). The variation in disease symptoms of plants with non-functional *RLM1* and *RLM2* alleles can partially be explained by camalexin induction, as the susceptible RILs displayed trends of a negative correlation between camalexin induction and the level of susceptibility. This difference in camalexin induction between Col-0 and L*er*-0 could explain the difficulties in identifying susceptible mutants displaying a clear disease phenotype in the Col-0 background. In order to avoid these problems, mutants in Col-0 background with weakly susceptible phenotypes were crossed with *pad3* in order to eliminate the influence of camalexin. Although camalexin induction is important for resistance preliminary results indicate that T-DNA knockout mutants in *CYP71A13*, a gene upstream *pad3* in the camalexin synthesis (Nafisi *et al*., 2007), are resistant against *L. maculans* (M. Kaliff unpublished).

Characterization of *lms5*

The characterization of the EMS mutant *lms5* in L*er*-0 background was performed to get an insight in its function in resistance. During culturing on MS-1 media, an increase in lateral root growth was observed in the *lms5* mutant. Due to the extensive root growth, a root assay on auxin was performed. In this assay the *lms5* mutant showed significantly less sensitivity compared to L*er*-0 and *lms1* (**IV**). This observation was followed up by measurement of the endogenous hormone levels of IAA and ABA. In the *lms5* mutant the endogenous IAA level is significantly lower compared to wild-type (L*er*-0). The IAA content is increased by wounding, with a peak 6 hpi (hours post inoculation). The IAA levels decrease faster in inoculated material compared to the wounding control. In the *lms5* mutant the IAA level is significantly higher in the inoculated material 6 hpi compared to wild-type. The ABA content on the other hand decreases following wounding and shows a cyclic pattern with a decrease at 6 and 48 hpi. This cyclic pattern is more pronounced in the susceptible *lms1* and *lms5* mutants compared to L*er*-0, and in the *lms5* mutant the endogenous ABA level is significantly lower compared to L*er*-0. These data might be correlated with the stronger induction of callose found in these mutants. The IAA results indicate that the *lms5* mutants IAA/AUX signalling is interrupted. In normal AUX/IAA signalling, the AUX/IAA proteins are rapidly degraded resulting in downstream gene expression leading to a negative feedback loop that decreases the endogenous IAA level. Due to the interrupted signalling this rapid negative feedback loop is absent in *lms5*, resulting in increased auxin levels after *L. maculans* challenge.

The *lms5* mutant has been mapped using SNP and CAPS markers developed from the Monsanto Col-0 and L*er* polymorphism data (http://www.arabidopsis.org/Cereon/index.jsp). These markers were used on a Col-0 x *lms5* mapping population that had been evaluated with SNP markers for the *RLM1* locus to avoid interference with *RLM1* in the subsequent analysis. By using SNPs and CAPs markers, a 100 kbp area on chromosome 1 was identified. The most interesting candidate genes in this area are an F-box (At1g53550), HSP17.6 (At1g53540) and a 26s proteasome subunit (At1g53750). T-DNA knockouts in each gene have been tested on root-assay as well as susceptibility towards *L. maculans*. Preliminary sequence data reveal two mutations in the promoter of At1g53550 and one amino acid substitution in At1g53540. These two genes are now used for complementation of *lms5*.

Involvement of ubiquitin-proteasome complex components

Several results indicate that the ubiquitin-proteasome complex is involved in resistance signalling against *L. maculans*. The first indication was revealed during the analysis of *RLM1* and *RLM2* loci since we found a requirement for *RAR1* in the resistance response (**I**). On the other hand, neither the *RAR1*-associated *SGT1b* mutation nor the *SGT1b*-like gene *SGT1a* exhibited any visible influence on *L. maculans* resistance. Screening of the T-DNA mutants in an *HSP90* chaperone (*athsp90.1-1* and *athsp90.1-2*), involved in *RAR1*/*R*-gene activity, showed on the other hand, that *HSP90.1* possesses a moderate influence on *L. maculans* resistance. Results from the different studies point to the involvement of the

ubiqutin-proteasome complex in the resistance signalling. This knowledge prompted us to analyse which of the complexes most affects the susceptibility. In order to do so defined mutants in different complexes components (*CUL3a-1*, *CUL3b-1*, *ASK1-1*, *AXR6-1* (also known as *CUL1*), *AXR6-2* and *SUR2-1*) were assessed (**IV**). Only *ask1-1*, *axr6-1* and *axr6-2* were susceptible, while *cul3a-1*, *cul3b-1*, and *sur2-1* were resistant. To further investigate the role of ubiquitin-proteasome complexes a collection of T-DNA knockout mutants are under evaluation. Preliminary results show that mutants in *COP9*, *CUL4* and *RBX1* are resistant towards *L. maculans* further supporting the hypothesis that the auxin associated SCF complex is specifically involved in resistance signalling and that particularly the protein recognition parts in the complex are of crucial importance, as the *CUL1* is the cullin associated to the auxin complex and together with *ASK1* and an F-box are responsible for protein recognition.

Comparative studies in *B. napus*

In order to analyse if ABA- and BABA-IR are effective in *B. napus*, comparative studies were made. In agreement with our observations in Arabidopsis we could observe an increased resistance in ABA and BABA pre treated *B. napus* cv. Hanna as well as *B. napus* cv Hanna *nahG* genotype (**II**). As a control the resistant *B. napus* cv. Surpass 400 was used (Li and Cowling, 2003). Besides susceptibility studies, the callose content was assessed. In agreement with our observations on Arabidopsis, resistant *B. napus* cv. Surpass 400 exhibited enhanced levels of callose deposition after pathogen challenge, when compared to the susceptible cv. Hanna upon fungal inoculation (**II**). Staining of leaves inoculated with water showed no callose deposition, demonstrating that the callose observed was exclusively pathogen-induced. However, when ABA and BABA pre-treated cv. Hanna or cv. Hanna *nahG* genotype were analyzed, the callose levels increased to levels comparable to Surpass 400.

Defence studies in *B. napus*

Previous studies on progeny from asymmetric somatic hybrids between Arabidopsis and *B. napus* suggested that two regions on the Arabidopsis chromosome 3 are linked to *L. maculans* resistance (Bohman *et al*., 2002). In order to develop this further we have used a specially designed addition line, *B. napus* cv. Hanna+At3 (Carlsson, 2007). This genotype originates from a chromosome doubled haploid line, resulting in a stable line of *B. napus* cv. Hanna containing the whole Arabidopsis chromosome 3.

The Hanna+At3 plants were assessed against different fungal pathogens, known to infect *B. napus*. When the plants were challenged with *L. maculans* we could see a clear reduction in susceptibility compared to the susceptible wild-type cv. Hanna (Figure 11). This reduced susceptibility could also be seen if the plants were challenged with *A. brassciicola*, *A. brassicae* and *B. cinerea* (M. Kaliff unpublished results).

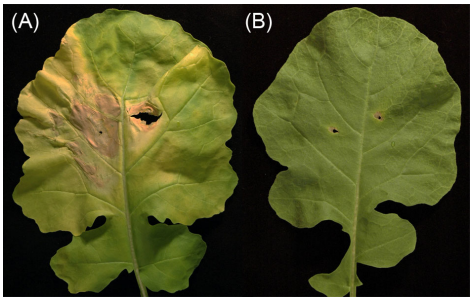

Figure 11. *L. maculans* infected *B. napus*. *B. napus* cv. Hanna (A) is susceptible to *L. maculans*, but introducing Arabidopsis chromosome 3 (cv. Hanna+At3) confers reduced susceptibility (B).

A microarray study comprising *B. napus* cv. Hanna and cv. Hanna+At3 has been performed on CATMA arrays in order to identify genes involved in the resistance response (Karlsson, 2006). These results are now being confirmed and candidate genes are under evaluation.

Conclusions and discussion

Several of the projects include physical mapping of resistance genes. This approach is complicated by the influence of camalexin (**I**). Most of the mapping populations consist of crossings between Col and L*er*, accessions where different levels of camalexin are induced. The higher level of camalexin in the Col-0 background can suppress weakly susceptible phenotypes. Due to this suppression T-DNA knockout mutants in candidate genes can be screened as resistant, which complicates the mapping procedure. Except for camalexin content, the impact of *R*-gene redundancy can in some cases obstruct the analysis. This redundancy can consist of either a loci containing several *R*-genes as in the case of *RLM1* (**I**) or redundancy of genes located in different positions in the genome due to gene duplication.

The identification of *RLM3* was based on a combination of genetics and microarray analysis (Staal *et al*., 2007), which is not the most common method for gene identification. In order to succeed with this method the material was grown randomly in order to even out all genetic differences except the one responsible for the resistance. A microarray based method can be a quick method to identify the region were the gene of interest is located. However, in order to be successful the mapping population needs to have few genetic differences besides the one responsible for the trait that is to be mapped. If there are too many differences there will be difficulties in identifying which differently expressed gene that is responsible for the trait of interest.

As mentioned earlier, there are several different classes of *R*-genes encoding different protein motifs. Perhaps the most striking in our work is the identification of a small group to which *RLM3* belongs. Beside the structural differences between *RLM1* and *RLM3* there is a difference in pathogen specificity (**I**; Staal *et al*., 2007). In the case of *RLM3* we have identified a broad spectrum *R*-gene effective against several fungal pathogens (Staal *et al*., 2007). By using specific *R*-

genes in our studies we can investigate how the different signalling components are linked together in different signalling pathways.

In our studies with ABA and BABA (**II**) we concluded that the β-glucan callose is a factor that contributes to resistance against *L. maculans*. However, other factors are of significance as the susceptible mutant *lms5* displays elevated levels of callose compared to resistant L*er*-0 (**IV**). During our studies on callose (**II**) we confirmed the *R*-gene dependency in callose deposition in Arabidopsis previous identified by Yu and co-workers (2005) in *B. napus.* The role of *PR2* as a regulator in callose deposition has also been investigated (**III**). *PR2* is regulating callose deposition by degradation of callose. This degradation is a local response at the infection site and is further activated by SA, which activates *PR2* expression. On the other hand, when callose has been deposited at the infection site, it is stable for at least 5 days (**II**). Indicating that *PR2* only affects callose before it has been deposited.

The *lms5* mutant displaying altered auxin responses and specific altered susceptibility towards *L.* maculans is intriguing, calling for an in depth analysis of the mutated gene (**IV**). The characterisation of *lms5* has been a challenge as the weakly spotted phenotype is readily masked by the high camalexin content in all mutants in Col-0 background. A further complication has been the mutations localisation close to *RLM1*, which has hampered the mapping work. In order to avoid *RLM1* to influence the *lms5* identification we had to wait with the fine mapping until *RLM1* was identified and could be excluded from the *lms5* mapping population. The candidate genes from the fine mapping indicate that the *lms5* mutant has altered protein stability or protein degradation. This subject is complex with many different factors influencing the signalling. In order to conclude which degradation complex that is affected in *lms5* mutants involved in different complexes has been assessed. The knowledge that protein turnover is involved in many developmental processes as well as the auxin resistant phenotype of *lms5* led us to assess a wide range of defined mutants (Table 3). Due to the auxin resistant phenotype of *lms5* we analysed the auxin receptors (TIR, AFB1 and AFB3) by qPCR, but no differences compared to L*er*-0 could be detected. The fact that there are no differences in the auxin receptors, compared to wild-type, indicates that the altered auxin phenotype seen in *lms5* is due to alterations elsewhere in the auxin signalling. These results, together with the screening results, lead us to believe that *lms5* has a changed auxin phenotype as a result of an alteration in protein degradation. This alteration is most likely linked to the SCF complex and more specifically to the recognition of proteins targeted for degradation. At this point the candidate genes from the identified chromosomal region are evaluated. The most promising candidate is an F-box protein. There are approximately 700 F-box proteins in Arabidopsis (Gange *et al*., 2002) which have among other things ubiquitin-protein ligase activity. Further supporting the hypothesis that *lms5* has an alteration linked to the SCF complex. The work on *lms5* will continue and include complementation, protein interaction and localisation studies besides influences on defence signalling.

Table 3. Arabidopsis genotypes and their response towards *L. maculans*.
Several genotypes can be included under more than one category. Due to space limitation they are only included once.
All genotypes are in Col-0 background unless stated otherwise.

Defence/signalling categories	*L. maculans* response	Defence/signalling categories	*L. maculans* response	Defence/signalling categories	*L. maculans* response
ABA		*pad3-1*	S	**JA**	
aba1-3[1]	S	*cyp71A13*	r*	*coi1-16*	R
aba2-1	R	*cyp79B2*	U	*coi1-16* x *ein2*	R
aba2 x *pad3*	S>pad3	*cyp79B3*	U	*coi1-16* x *ein2* x *NahG*	R
aba3-1	R	*cyp79B2* x *cyp79B3*	U	*coi1-16* x *NahG*	R
aba3 x *pad3*	S>pad3	*ups1-1*	S	*jar1-1*	R
abi1-1[1]	S				
abi1-1R1	S	**Cell death**		**Ligning/Cell wall**	
abi1-1R2	R	*acd1-20*	S	*irx4*[1]	S
abi1-1R3	R	*dnd1*	R	*irx4 x lms5*	S
abi1-1R4	R	*ran1-1*	S		
abi1-1R5	R	*rcd1*	R	**non-coding RNA**	
abi1-1R4 x *abi2-1R6*	S	*vad1-1*	S	*35S::miRNA393*	R
abi1-1R7	R			*dcl1 (caf-1)*	U
abi2-1[1]	R	**Cytokinin**		*dcl2*	U
abi3-1[1]	R	*ckh1-1*[1]	s	*dcl3*	U
abi4-1	R	*ckh2-1*[1]	R	*dcl-4*	U
abi4 x *pad3*	S>pad3				
abi5-1[2]	R	**Defence-related**		**Oxidative stress/ Second messenger**	
gcr2-1	R	*bik1*	r	*nia1nia2*	S
gcr2-2	R	*bos1*	s	*Atnos1*	R
gcr2-3	R	*bos2*	R	*AtRbohD*	S
lms1 x *abi1-1*[1]	S	*bos3*	S	*AtRbohF*	S
ost2	S	*ibs2*	R	*AtRbohDF*	S
		lms1[1]	S	$rlm1_{Ler}rlm2_{Col}$ x *nia1nia2*	R
Auxin		*lms2*[1]	S	$rlm1_{Ler}rlm2_{Col}$ x *RbohF*	R (F_1)
35S::AFB1	R	*lms3*[1]	S	*vtc1-1*	R
ain1-1[3] (*ein5*)	S	*lms4*[1]	S		
air1-8	U	*lms5*[1]	S	**Penetration**	
aux1-7	S	*lms6*[1]	S	*pen1-1*	s
aux1-7 x *axr4-2*	U	*lms7*[1]	S	*pen2-1*	s
aux1-7 x *ein2*	S	*lms8*[1]	S	*pen2-1* x *sag101-2*	s
aux40	R	*lms9*[1]	S	*pen2* x *eds1*	s*
axr1-12	S	*lms10*[1]	S	*pen2-1* x *pad4-1*	s*
axr2	R	*lms11*[1]	S	*pen2-1* x *pad4-1* x *sag101-1*	s*
axr3-1	U	*lms1* x *lms5*	S		
axr4-1	S	*mos2*	R	**Protein stabilisation/ SCF**	
axr4-2	U	*mos5*	s	*ask1-1*	S
eir1-1 (pin2)	R	*mos5* x *snc1* x *npr1*	S	*axr6-1* (*cul1*)	S
pin1-2	R	*ndr1-1*	R	*axr6-2*	S
pin1-5	R	*nho1*	s	*cop9*	R*
sar1-1	R*	*npr1-1*	R	*cul3a*	R
sar3-1	R*	*npr1-2*	R	*cul3b*	R
sur1(rty1-1)	R	*npr1-3*	R	*cul4*	R
tir1-1	R	*pmr2-1*	s	*eta3*	R
tir3-101 (big)	R	*pad3* x *lms5*	S	*eta3* x *tir1*	R
		sni1	R	*AtHSP90.1-1*	S
Brassenosteroid				*AtHSP90.1-2*	S
bak1-3	r*	**ET**		*rar1-13*[1]	S
bak1-4	r*	*ctr1-1*	R	*rar1-13* x *lms5* [1]	S
		ein2	R	*rar1-21*	S
Callose		*ein2* x *NahG*	R	*rbx*	R*
pmr4-1	S	*ein3-1*	R	*sgt1a-1*[2]	R
pmr4 x *pad3*	S>pad3	*ein5*	r	*Sgt1b*	R
		ein5 x *pad3*	S>pad3	*Sgt1b* x *rar1-21*	R
Camalexin		*eto1-1*	R	*sur2-1*	R
esa1	S	*etr1-1*	R	*pad3* x *hsp90*	S>pad3
pad1	s	*ran1-1*	s	*pad3* x *rar1*	S>pad3
pad2-1	R			*pad3* x *eta3*	S=pad3

cont.

Table 3. Cont.

Defence/signalling categories	*L. maculans* response	Defence/signalling categories	*L. maculans* response	Defence/signalling categories	*L. maculans* response
***R*-gene**		*lms5* x $rlm1_{Ler}rlm2_{Col}$ x *pad3*	S	*rps101c*	R
At1g63880	s	*pad3* x *At1g63880*	*S>pad3*	*snc1*	r
eds1-1[2]	R	*pad4-1*	*R*		
GABI235E02	S	*pad4-1* x *sag101-1*	*R*	**SA**	
GABI491E04	S	*pbs3*	R	*NahG*	R
LCN 4-5	s	*pbs1-2*	R	*NahG* x *ein2*	R
LCN 4-6	S	$rlm1_{Ler}rlm2_{Col}$	S	*sid2*	R
LCN 4-6 x *lms5*	S	$rlm1_{Ler}$ x *pad3-1*	S	*sid2* x *ein2*	R
LCN 4-6 x $rlm1_{Ler}rlm2_{Col}$	S	$rlm1_{Ler}rlm2_{Col}$ x An-1	R		
lms5 x $rlm1_{Ler}rlm2_{Col}$	S	$rlm1_{Ler}rlm2_{Col}$ x *LerWs*	S		

1= L*er*-0 2= Ws-0 3= C24
* = Preliminary result U = Under investigation
R = Resistant r = Moderate resistant
S = Susceptible s = Moderate susceptible

Source: Bohman *et al*., 2004; **I**; **II**, **IV**, J. Staal, M. Kaliff, M. Persson and C. Dixelius, unpublished.

In the comparative studies of *B. napus* we could confirm that the Arabidopsis-*L. maculans* pathosystem can be used as a model for the *B. napus* - *L. maculans* interaction (**II**). The use of the knowledge received from the more investigated Arabidopsis can be used in resistance breeding programs to accelerate the progress.

PAD3 the last enzyme in the camalexin synthesise pathway is located on chromosome 3 in the Arabidopsis genome, but the introduction of *PAD3* into *B. napus* is not enough to introduce camalexin production. We have however not studied if the introduction of Arabidopsis chromosome 3 alters the composition of other phytoalexins already present in *B. napus* (Pedras *et al*., 2007). Since the *B. napus* type phytoalexins are closely related to camalexin it is possible that the introduction of *PAD3* alters the entire phytoalexin composition. Either the relationship of the already present phytoalexins is altered, or the introduction of *PAD3* enables new types of phytoalexins to be produced. This possible alteration might be one of the reasons that the cv. Hanna+At3 has a reduced susceptibility towards *L. maculans*.

Defence signalling is a complex network of different components, with new components added all the time. If we compile the information obtained during the years we visualise different parallel signalling pathways (Figure 12), all important for the defence against *L. maculans*. The work done within this thesis has particularly resulted in an increased knowledge of how the phytohormones ABA and auxin are implicated in the defence, as well as the identification of *L. maculans* specific resistance genes. Despite all the facts we have generated on this pathosystem, there are still plenty of unsolved questions, not least on the gene regulation and gene function levels.

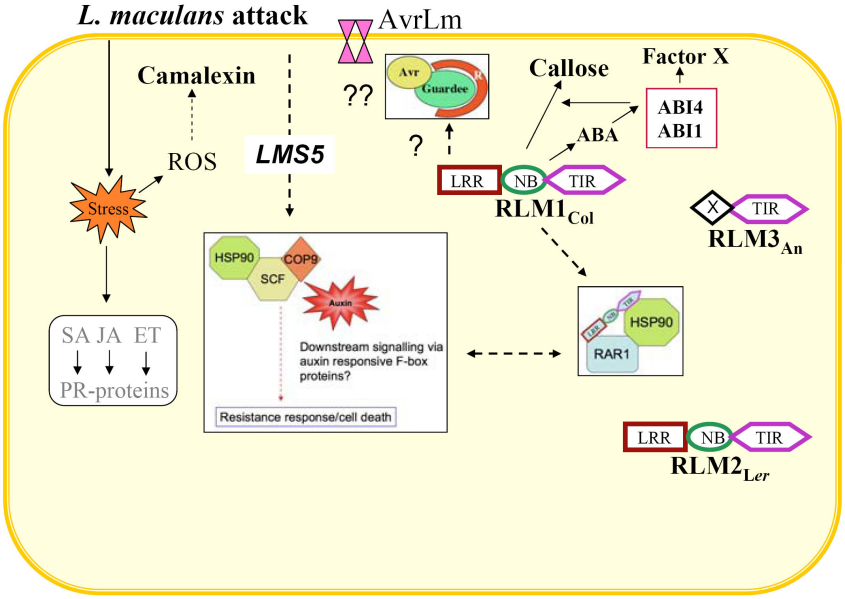

Figure 12. Overview of the defence signalling pathways against *L. maculans*. Resistance relies on several independent responses – an *R*-gene dependent resistance and the secondary metabolites camalexin and callose. ABA is required for efficient R-gene induced callose but also induces a callose independent response via an unknown factor. The common pathogen response hormones SA, JA and ET influence disease development but do not determene resistance. Auxin on the other hand has been shown to influence resistance, most likely via protein turnover.

Svensk sammanfattning

Denna avhandling beskriver interaktionerna mellan en svamppatogen, torröta (*Leptosphaeria maculans*), och dess värdväxter. Den viktigaste värdväxten, raps (*Brassica napus*), är en gröda vars produkter används till matlagning, foder, biobränsle och smörjmedel inom industrin. Under 2006 såddes 83700 ha raps (Svensk raps AB, 2007). Ett stort problem är att många sjukdomar, framför allt svampar, angriper raps vilket har en negativ inverkan på avkastningen. För att förhindra detta försöker man framställa resistenta sorter genom förädling och för att göra detta krävs att man har kunskap om hur växten försvarar sig mot sjukdomar.

För att studera torröta har vi använt oss av en släkting till raps. Denna släkting, *Arabidopsis thaliana* eller backtrav som den heter på svenska, är ett litet ogräs som ofta används inom växtforskningen. Backtrav är vad som brukar kallas en modellorganism, vilket betyder att växten används i studier för att forskare ska förstå hur andra växter eller organismer fungerar. Anledningarna till att man använder Arabidopsis som modellorganism är, dels att växten är liten och har en kort generationstid (tid från frö till frö). Under tidens lopp har mycket kunskap

samlats om denna växt i form av t.ex. sekvenserat genom (dvs. man har kunskap om hur alla gener ser ut) och det finns tillgång till olika mutanter.
Torröta orsakas av en svamp och anses i Sverige vara en mindre allvarlig rapssjukdom men det förutspås att den kommer att bli vanligare i och med att klimatet blir varmare. I övriga världen orsakar torröta årligen skördeförluster på 10-30 % och i Australien är skördeförlusten upp till 50% vissa år. Sjukdomen yttrar sig först i form av fläckar på bladen och därefter sprids den till stjälken som försvagas vilket leder till stjälkbrott (Figur 10). Svampen lever både på levande och död vävnad och i dess livscykel (Figur 10) finns både asexuell och sexuell förökning vilket gör att svampen förändras genetiskt med tiden och kan på så sätt bryta resistensen hos de olika värdväxterna.

Växters försvar mot sjukdomar påminner till stor del om människors ärvda immunförsvar. Det första steget i försvaret är igenkänning av pathogenen (sjukdomsaltraren), detta sker oftast genom att så kallade R-proteiner (försvarsproteiner) hos växten binder till ett specifikt Avr-protein (Avirulens protein) hos patogenen. Dessa Avr-proteiner är oftast nödvändiga för att patogenen ska kunna infektera växten. När sedan växten har känt igen patogenen startar flera olika försvarsreaktioner som i slutändan begränsar spridningen av sjukdomen. Detta försvar ser olika ut beroende på vad det är för kategori av patogen. Patogener kan förenklat delas in i tre olika grupper; biotrofer (lever av levande växtmaterial), nekrotrofer (lever av dött växtmaterial) och hemibiotrofer (kan leva både som biotrof och som nekrotrof). Generellt kan man säga att en växt som angrips av en patogen med en biotrof livsstil försvarar sig genom att döda vävnaden runt angreppsområdet och på så sätt svälta ut patogenen. Denna typ av försvar kallas för HR (Hypersensitivity Respons eller översatt överkänslighets respons) och den kan även förekomma mot andra typer av patogener. Mot nekrotrofer fungerar det inte att döda vävnaden eftersom det istället gynnar patogenen. I dessa lägen använder sig växten istället bland annat av giftiga ämnen som exempelvis camalexin (ett antimikrobiellt ämne) eller barriärer som kallos (en poly-glukan) vilket svampar inte kan passera samt olika försvarsproteiner och syreradikaler som dödar patogenen. Utöver detta aktiveras olika hormoner i växten som i sin tur påverkar olika signalvägar som leder till att växten kan försvara sig mot angreppet. Det hela försvåras dock av att patogenerna har olika metoder för att undvika upptäckt eller påverka växten att starta fel typ av försvarsreaktioner.

Jag har undersökt olika komponenter som är viktiga i växters försvarssignalering mot torröta. Som en del har vi identifierat försvarsgener vilka är nödvändiga för att växten ska kunna identifiera svampen och starta lämpliga försvarsmekanismer. Den andra delen av arbetet har bestått av att undersöka växthormonernas inverkan på försvaret mot torröta.

De identifierade försvarsgenerna finns i olika accessioner hos Arabidopsis (en accession är en genotyp av Arabidopsis som är isolerad från en specifik plats). Den ena genen vi identifierat saknas hos An-1 och är orsaken till att denna accession är mottaglig mot torröta, till skillnad från normalfallet där Arabidopsis är resistent. Detta är den enda av 168 undersökta accessionen som är mottaglig mot torröta. Denna gen identifierades med hjälp av en mikroarray studie (mikroarray är en metod där man kan jämföra genuttrycket i två olika prov genom att låta dessa binda till en genspecifik sekvens på ett glas) där genuttrycksnivåerna mellan mottagliga individer jämfördes med dem som finns i resistenta individer. I

detta mikroarray försök detekterades 4 gener med olika uttrycksnivåer i de resistenta och mottagliga växterna. Dessa genkandidater undersöktes närmare och vi kunde konstatera att det var en gen på Arabidopsis kromosom 4 (At4g16990) som orsakar mottagligheten i An-1 acessionen. Denna gen är uppbyggd som en klassisk resistens gen som döptes till *RLM3* (Resistence to *L. maculans* 3). Den andra resistensgenen vi har identifierat finns i accessionen Col-0 men saknas i L*er*-0 och ger upphov till mottagliga plantor om dessa accessioner korsas med varandra. Även denna gen, som är belägen på kromosom 1 (At1g64070), är en klassisk resistensgen som vi har döpt till *RLM1* (Resistence to *L. maculans* 1).

I studierna om vilka växthormoner som har betydelse för försvaret har framförallt två hormoner, ABA och auxin, visat sig vara viktiga. Hormonet ABA är sedan tidigare känt som ett viktigt hormon vid tork- och saltstress, men har på senare år visat sig vara involverat i försvarssignalering mot patogener. Det finns flera studier som visar att man kan förbehandla (genom vattning) växten med ABA för att på så sätt förbereda (priming) växten inför ett kommande patogen angrepp. Denna beredskap hos växten gör att den snabbare kan försvara sig mot angreppen. Våra studier visar att denna beredskap kräver närvaro av specifika försvarsgener så som *RLM1* och att beredskapen leder till ökad deposition av kallos. Vi har även visat att denna beredskap också fungerar i raps och resulterar i betydligt lägre sjukdom i de förbehandlade individerna. Utöver ABA fungerar även BABA (som inte produceras av växten själv) för induktion av denna beredskap. Det andra hormonet, auxin, är ett av de mest studerade växthormonerna och har stor betydelse för växtens utveckling. Först på senare tid har det visat sig involverat i försvarssignalering. Troligen är det inte auxinet som sådant som är viktigt utan snarare auxinets effekt på proteinstabilitet och nedbrytning av proteiner som är viktigt. I våra studier ser vi att mutanter som har olika steg i protein-stabiliserings/-degraderings kedjan utslagen blir mottagliga mot torröta.

Utöver hormonstudierna och identifieringen av resistensgener har även en mutant, *lms5*, (*L. maculans* suceptible 5) karaktäriserats. Det visade sig att *lms5* är specifikt mottaglig mot torröta, vilket gör att mutanten kan avslöja viktig information om försvarssignaleringen mot patogenen. Studierna har även visat att *lms5* är okänslig mot auxin (mutanten kan inte känna av närvaro av auxin på samma sätt som vildtypen gör). Genom användandet av markörer (med känd lokalisering i Arabidopsis genomet) har mutationen identifierats till ett område på kromosom 1 som innehåller 30 gener. Utav dessa har vi tre kandidatgener som för närvarande undersöks närmare. Detta arbete kommer att pågå tills genen har blivit identifierad och därefter kommer olika studier av genen i fråga att genomföras.

Sammanfattningsvis kan vi konstatera att denna studie har bidragit med att identifiera nya komponenter som är viktiga i växtens försvarssignalering mot torröta.

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