ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

AGRARIA 305

Molecular Studies of *Arabidopsis* and *Brassica* with Focus on Resistance to *Leptosphaeria maculans*

Svante Bohman

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

Molecular Studies of *Arabidopsis* and *Brassica* with Focus on Resistance to *Leptosphaeria maculans*

Svante Bohman
Department of Plant Biology
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2001

Acta Universitatis Agriculturae Sueciae
Agraria 305

ISSN 1401-6249
ISBN 91-576-5821-8

Tryck: SLU Service/Repro, Uppsala 2001

Abstract

Bohman, S. 2001. Molecular study of *Arabidopsis* and *Brassica* with focus on resistance to *Leptosphaeria maculans*. Doctors's dissertation.
ISSN: 1401-6249. ISBN 91-576-5821-8

Blackleg caused by *Leptosphaeria maculans* is a widespread fungal disease on *Brassica napus* (oilseed rape). In contrast, *Arabidopsis thaliana* and *B. nigra* are in general highly resistant. This study presents results from genomic interaction between the *A. thaliana* and *B. napus* genome with focus on *L. maculans* resistance. Identification and partial characterization of *A. thaliana* resistance in accessions, *L. maculans* susceptible mutants, and signaling pathways were also performed. Finally, a resistance gene to *L. maculans* from *B. nigra* was cloned and transferred to *B. napus*.

Chromosome counts and RFLP analyses of *A. thaliana* DNA content in *A. thaliana* (+) *B. napus* back-crossed progeny were performed. The results showed that in BC_2, originating from symmetric hybrids, the frequency of retained *A. thaliana* loci was reduced to 42%. The average chromosome number decreased from 48 in BC_1 to 39 in BC_2. These results can be compared with the asymmetric hybrid derived BC_1, that had 16% loci present and an average chromosome number of 38. Clearly, symmetric hybrid offspring retained most DNA as complete chromosomes whereas asymmetric hybrid offspring contained mostly DNA fragments. Pathogen screening of the hybrid offspring revealed both cotyledon and adult leaf resistance to *L. maculans*. The adult leaf resistance was localized to chromosome 3 from *A. thaliana*, on two areas on each side of the centromere.

A. thaliana resistance was examined in 171 accessions from 27 countries. Only four accessions displayed any susceptibility. To further explore the underlying causes of the resistance, a set of *L. maculans* disease susceptible mutants (*lms*) were isolated. Two of the mutants, *lms1* and *lms5* have been mapped to chromosome 2 and 1, respectively. The resistance was further analyzed with respect to defense signaling and effector molecules. The results indicated that resistance in *A. thaliana* against *L. maculans* depended on camalexin and is independent on salicylic acid, jasmonic acid or ethylene response. In contrast, *lms1* produced wild type level of camalexin and higher expression levels than wild type of *PR1*, and *PDF1.2*. The results suggest the possibility of at least two independent resistance factors in *A. thaliana*.

A gene conferring resistance to *L. maculans*, *Lm1*, was cloned from *B. nigra*. Sequence analysis revealed a novel protein with two putative trans-membrane motifs and homology to the nin protein of *Lotus japonicus* and to *A. thaliana* sequences of unknown function. The knowledge gained in *A. thaliana* and from *Lm1* will promote a further understanding of the mechanisms underlying resistance to *L. maculans*.

Key words: *Arabidopsis thaliana*, blackleg, *Brassica napus*, *Brassica nigra*, defense, intergenomic translocation, *Lm1*, *lms1*, *pad3*, *Phoma lingam*, proteomic analysis, somatic hybridization.

Author's adress: Svante Bohman, Department of Plant Biology, Box 7080, SLU, S-75007 Uppsala, Sweden. E-mail: Svante.Bohman@vbiol.slu.se

Contents

Introduction 7

The *Brassica* genus 7
B. napus is an important oil crop 8

Leptosphaeria maculans*, a severe pathogen on *B. napus 9
Infection cycle 10
Epidemiology 11
Induced defense responses in *B. napus* 12
Breeding for resistance in *B. napus* 12

***Arabidopsis thaliana* the model plant** 13
Resistance in *A. thaliana* to pathogens 14
Resistance genes and signaling in A. thaliana 14
Defense effector molecules 18

Aims of the present investigation 21

Present investigation-Results and discussion 22
Study on a genomic interaction between the *A. thaliana* and *B. napus* genome with focus on *L. maculans* resistance 22
Molecular pathways and genes involved in resistance to *L. maculans* 24

Conclusions 29

Future perspectives 30

References 31

Acknowledgements 39

Appendix

Papers I-IV

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

- Bohman, S, Forsberg, J., Glimelius, K., and Dixelius, C. 1999. Inheritance of *Arabidopsis* DNA in offspring from *Brassica napus* and *A. thaliana* somatic hybrids. Theor. Appl. Genet. 98:99-106.

- Bohman, S., Wang, M., and Dixelius, C. 2001. *Arabidopsis thaliana* derived resistance against *Leptosphaeria maculans* in a *Brassica napus* genomic background. Theor. Appl. Gen. (In press).

- Bohman, S., Thomma, B.P.H., Wang, M., and Dixelius, C. 2001. Resistance against *Leptosphaeria maculans* in *Arabidopsis thaliana* is multi-factorial. (Manuscript).

- Wretblad, S., Bohman, S., and Dixelius, C. 2001. The *Lm1* gene of *Brassica nigra* confers resistance to the blackleg fungus *Leptosphaeria maculans*. (Submitted).

Abbreviations

List of selected abbreviations used commonly in the text:

2-DE:	Two-Dimensional polyacrylamide gel Electrophoresis
Col-0	Columbia accession 0
EMS	Ethyl Methane Sulfonate
EST	Expressed Sequence Tag
ET	Ethylene
ha	Hectare
HR	Hypersensitive Response
JA	Jasmonic Acid
Ler-0	*Landsberg erecta* accession 0
NahG	Plant line expressing the enzyme salicylate dehydroxylase
pad	Phytoalexin deficient mutants
pv	Patovar
RFLP	Restriction Fragment Length Polymorphism
ROI	Reactive Oxygen Intermediates
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
Ws-0	Wassilewskija accession 0
wt	Wild type

Introduction

With time, the force of evolution changes all organisms. In crop plants, human breeding efforts have accelerated the speed in which these changes occur. The diversity in crops originating from the *Brassica* genus exemplifies this well. New techniques now allow gene transfer between sexually incompatible species; increasing our ability to modify our crops. The completion of the *Arabidopsis thaliana* genome sequence (The Arabidopsis genome initiative 2000) generates information of the regulation of important agricultural traits and a possibility to use newly discovered genes in future breeding of crops.

The *Brassica* genus

Domestication of the wild diploid species *B. rapa*, *B. nigra* and *B. oleracea* set the start of mankind's utilization in agriculture of crops originating from the *Brassica* genus (reviewed in Gómez-Campo and Prakash 1999). The *Brassica* plants had for humans, the advantage of multipurpose usage. Breeding efforts have now produced *Brassica* species specialized in crop production from all parts of the plant. The roots can be utilized in swede and turnip, the stems in kohlrabbi, the leaves in kale's and cabbages such as brussels sprouts, the inflorescences in broccoli and the seeds in oil and mustard production. Domestication of the first wild *Brassica* was probably by *B. rapa* which is mentioned in Sanskrit literature (1500 BC). By then the plant probably had been cultivated for a long time. *B. rapa* grows wild from west Mediterranean to central Asia. The widespread distribution area allowed domestication to occur on several places independently. *B. nigra* (black mustard) grows wild on an equivalent geographical area as *B. rapa*. The famous physician Hippocrates mentioned the species around 480 BC for its medical value. New results now support him since the glucosinolates, present in all *Brassica* species seem to have a possible anti-carcinogenic effect (Verhoeven et al. 1996; van Poppel et al. 1999). *B. oleracea* was probably the last one to be domesticated. It grows wild at the European Atlantic coast where domestication must have originated (Gómez-Campo and Prakash 1999). Presently *B. oleracea* grows wild on a larger area due to escapes from agriculture. Later on in history, cultivated forms were transferred east. Eventually, cultivation spread all the way to China. The first written description of *B. oleracea* cultivars was given by the Greek Teophrastus (370-285 BC). At that time, he could already identify stemkales and headed cabbages as separate cultivars. During cultivation of diploid *Brassica* species, the proximity in culturing of the different diploid species promoted intraspecific hybridization. This generated new amphidiploids through convergent alloploid evolution. The new species to originate from the process where *B. napus* (*B. oleracea* x *B. rapa*), *B. juncea* (*B. rapa* x *B. nigra*) and *B. carinata* (*B. nigra* x *B. oleracea*).

B. napus is an important oil crop

Characteristically for *Brassica* crops the *B. napus* species has produced two distinct crop products such as oil seed rape and swede (*B. napus* subspecies *oleifera* and *B. napus* subspecies *rapifera* respectively). The dominant crop of the two is by order of magnitude *B. napus* subspecies *oleifera*, hereafter referred to in the text as *B. napus,* or by its trivial name oilseed rape. The *B. napus* genome contains n=19 chromosomes, corresponding approximately to 1.2×10^9 bp of DNA, The genome is a combination of the parental genomes, *B. oleracea* (n=9) and *B. rapa* (n=10) (U 1935; Prakash et al. 1999). *B. napus* is considered to have the highest yield potential in favorable conditions of all *Brassica* oil crops (Mendham and Salisbury 1995). Winter and spring types exist. Winter varieties are mainly grown in Europe and China and spring varieties in areas such as Australia and Canada (Pouzet 1995). In Sweden, approximately 20,000 ha of winter oilseed rape and 13,000 ha of spring oilseed rape were cultivated in 2001 (Svensk Raps AB 2001).

With the increased production of oilseed rape, reports on insect induced damage and pathogen inflicting diseases have increased simultaneously. The cause and severity of disease varies over regions of the world and depend on; climate, type grown (winter or spring), and geographic spread of insects and pathogens. High levels of insecticides are necessary for ensuring high yields and good quality of oilseed rape (Ekbom 1995). Insects can feed on plants through phloem-feeding or chewing sometimes causing injury to the plant that are similar to pathogen induced damages. Defense induction in plants in response to insects and pathogens can follow similar pathways (Walling 2000). The secondary metabolite glucosinolates are examples of one of many molecules induced by both herbivores and pathogens (Rask et al. 2000). Evidence exist for defense mechanism trade-offs between pathogen induced resistance and resistance to insects. Induced cross-resistance can, however, also occur (Felton and Korth 2000). The different type of feeding elicit different type of plant response. Fungal diseases are the main cause of crop loss in oilseed rape (Rimmer and Buchwaldt 1995; Tewari and Mithen 1999) (Tab. 1). A range of at least 11 different viruses can also promote disease, but severe problems of infection are restricted to China. The most important virus disease is turnip mosaic virus. This virus has the ability to kill or stunt seedlings (Rimmer and Buchwaldt 1995). Some other bacterial and fungi diseases can occur but to a minor extent. Some examples of these are; *Xanthomonas campestris* pv. *campestris* (black rot), *Erwinia carotovora* (soft rot), *Pseudomonas syringae* pv. *maculicola* (bacterial leaf spot) and *Pernospora parasitica* (mildew) that infects primarily *B. oleracea.*

Table 1. Fungi causing the major diseases of oilseed rape

Name	Trivial name	Type	Severe symptoms
Albugo candida	White rust	Oomycete	Leaf covered with white chalk like blisters
Alternaria brassicae	Black spot	Ascomycete	Black spots on all above ground parts of the plant
Alternaria brassicicola	Black spot	Ascomycete	Same as *A. brassicae* but generally require higher temperature than *A. brassicae*
Leptosphaeria maculans	Black leg	Ascomycete	Blackened stem, stem cancer
Plasmodiophora brassicae	Clubroot	Uncertain Myxomycete (protozoan origin)	Stunted plants, galls on tap rot
Pyrenopezia brassicae[1]	Light leaf spot	Discomycete	White spore mass at the margin of expanding lesions
Sclerotinia sclerotiorum	Sclerotinia stem rot	Ascomycete	Blackened sclerotia
Verticillium longisporum[2]	Verticillium wilt	Deuteromycete	Brown girdling tap rot, damping off

[1] anamorph: *Cylidrosporum concentricum.* [2] former *V. dahliae*

Leptosphaeria maculans, a severe pathogen on oilseed rape

The loculoascomycetic fungi *Leptosphaeria maculans* (Desm) Ces. & de Not (anamorph: *Phoma lingam*) (Tode ex Fr.) (Desm.) belongs to the order *Pleiosporales* (Howlett et al. 2001). *L. maculans* is a facultative necrotroph which can cause seedling death, lodging (blackleg) and early senescence on oilseed rape. It is also able to infect *B. rapa* and *B. oleracea*. Isolates of *L. maculans* have been divided into virulent and avirulent types based on their reaction on oilseed rape (McGee and Petrie 1978). DNA restriction pattern analysis confirmed the division into two separate groups. The A and B group (Johnson and Lewis 1990). Koch et al. (1991), further divided the isolates into four pathogenicity groups (PG) based on disease reaction on three different *B. napus* cultivars. The PG1 group, characterized as avirulent on all cultivars tested is comparable to B group isolates. B group isolates generally form smaller and darker leaf spots than A group isolates (Ansan-Melayah et al. 1997). Only A group isolates are associated with occurrence of severe stem canker epidemics (Johnson and Lewis 1994). The B/PG1 isolates were formerly subdivided in three subclasses; NA1, 2, and 3 (Koch et al. 1991). Now, based on extensive biochemical characterization, it has been proposed that B/PG1 isolates belong to species outside the *L. maculans* species complex.

NA1 isolates have been reclassified as *L. biglobosa* (Shoemaker and Brun 2001). NA2 isolates are most closely linked to *Phoma wasabie* (Reddy et al. 1999) and NA3 currently contain only one isolate, PHW126 (Koch et al. 1991; Howlett et al. 2001). The interaction between *L. maculans* A group isolates (hereafter referred to as *L. maculans* only) and *B. napus* have been further characterized in respect to gene-for-gene interactions. The first avirulence gene (*avrlm1*) described by Ansan-Melayah et al. (1995) was identified in a cross between PG3 isolates that are avirulent on *B. napus* cv. Quinta and a PG4 isolate virulent on Quinta. Subsequently Pongam et al. (1998), Ansan-Melayah et al. (1998) and Balesdent et al. (2001) further identified the avirulent genes, *alm1*, *avr2* and *avrlm4*, respectively.

L. maculans have a genome size of about 34 Mb and approximately 16 chromosomes (Howlett 1997; Cozijnsen et al. 2000). Some of the chromosomes are of a dispensable B type (Leclair et al. 1996). The genomic content also contain two linear plasmids, 9 and 10 kb, respectively. The plasmids seem to encode their own replication machinery. No function of the plasmids has been revealed (Lim and Howlett 1994). Eleven protein coding genes have been identified so far in the *L. maculans* genome. This includes five with a possible roles in infection, such as, ABC transporters, cellulases and endopolygalacturonases (reviewed in Howlett et al. 2001). Additionally, 120 ESTs have been isolated from growing hyphae. Besides proteins directly involved in pathogenicity, the fungus also produces several phytotoxins. The first one isolated was sirodesmin (Férézou et al. 1977) which exist in six similar forms. Another toxin, phomalide, is produced before sirodesmin production starts during fungal growth, but then the start of sirodesmin production inhibits phomalide synthesis (Pedras and Biesenthal 1998).

Infection cycle

Disease caused by *L. maculans* is initiated when sexual ascospores or asexual pycnidiospore land on a susceptible oilseed rape plant (Howlett et al. 2001). When the ascospore or pycnidiospore germinates, hyphae infect the plant through stomata openings or through wounds. The initial invasion occurs in a biotrophic mode but behind the hyphal front the fungus become necrotrophic. Hyphae invade vascular tissue and spread through the petiole down to the stem. This eventually causing blackleg symptoms, and in worst cases lodging (Hammond and Lewis 1986). The lifecycle is completed when new ascospores are generated in the necrotrophic regions caused by the fungus (Fig. 1). Further details on the nature of *L. maculans* invasion into *B. napus* can hopefully be given by studying in vivo the growth of green fluorescent protein tagged isolates of *L. maculans* (Sexton and Howlett 2001).

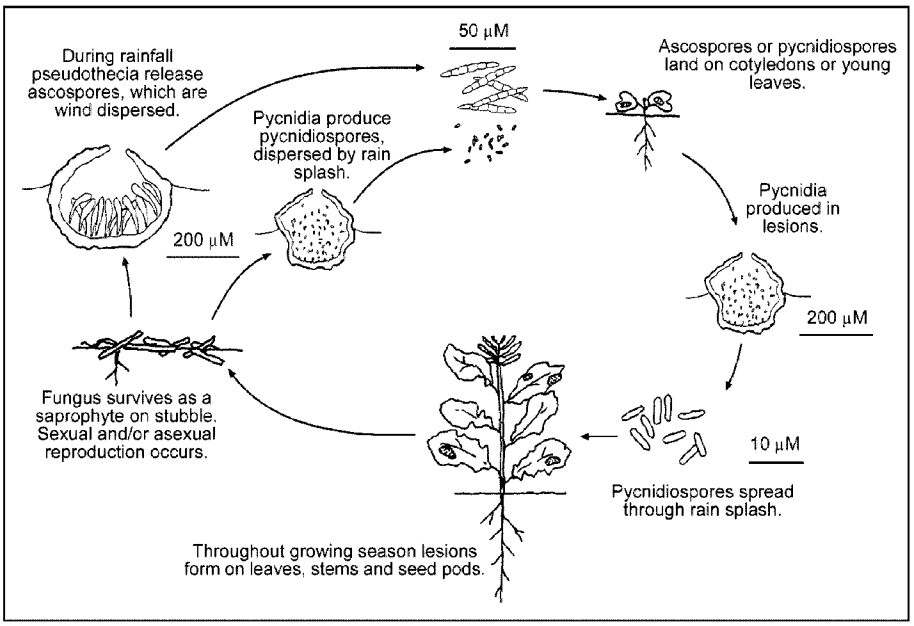

Figure 1. Life cycle of *Leptosphaeria maculans* on *Brassica napus* (reproduced with permission from Howlett et al. 2001).

Epidemiology

The fungus can cause severe disease on oilseed rape in temperate regions of the world such as Canada, Australia, and Europe, but not in China where no A group isolates have been identified. The disease is usually monocyclic and epidemics are generally initiated by airborne ascospores. Infection can also arise from infected seed, stubble, and rain splashed conidia (West et al. 2001). In Sweden, *L. maculans* has not been regarded as a major problem in oilseed rape production. Most damage have been attributed to Verticillium wilt (Svennson and Lerenius 1987), and *Sclerotinia sclerotiorum* (Wallenhammar and Sjöberg 2001). Plants can however be co-infected by *L. maculans* and *V. longisporum* making analysis of crop loss due to the specific pathogen difficult (Ndimande 1976). Recent analysis of *L. maculans* isolates in southern Sweden have showed that A group isolates (PG3 and PG4) dominated *L. maculans* induced stem lesions of oilseed rape (Kuusk 2000). The possibility of a climate change in Sweden due to global warming could increase the problems caused by *L. maculans* since severe epidemics can occur in England or France with just slightly warmer weather conditions.

Induced defense responses in *B. napus*

Only partial resistance exist in the important oilseed crops, *B. napus*, *B. rapa*, and in the different cabbage crops (*B. oleracea*) (reviwed in Howlett et al. 2001; West et al. 2001). Not much is presently known about the underlying molecular mechanisms that make plants resistant to *L. maculans*. Several studies have been performed of the response in *B. napus* to *L. maculans* inoculations. Hammond and Lewis (1986) reported on deposit of lignin-like structures and that calcium accumulates. Additionally, Roussel et al. (1999) showed that avirulent isolates induce HR and that the lumen of vessels in the HR area was occluded by a fibrillar-like material. Further experiments have showed that pathogenesis related proteins PR accumulates differently in resistant and susceptible plants (Dixelius 1994). One of these PR proteins is chitinase (Rasmussen et al. 1992). Fristensky et al. (1999), isolated 277 EST clones in *L. maculans* inoculated *B. napus* leaf and 20 % of the ESTs could be linked to defense. The defense related clones were divided in ten defense gene families and three resistance gene families. The most prevalent defense transcript was a 10 kDa Cxc750 gene with unknown function. On the protein level, Lamkadmi et al. (1996) found nine induced proteins on day one after inoculation and characterized a 23 kDA protein that accumulated between day one to four in the necrosis zone.

Breeding for resistance in *B. napus*

Resistance in many existing winter *B. napus* cultivars depend most extensively on genetic material from the Jet Neuf cv. that contain polygenic resistance (Pilet et al. 1998a; Pilet et al. 1998b; Pilet et al. 2001). Specific resistance genes from *B. napus* also exist in research material (Ansan-Melayah et al. 1997a; Ansan-Melayah et al. 1998; Balesdent et al. 2001). Resistance exists further in the related species *B. nigra*, *B. rapa*, *B. juncea* (Roy 1984; Sjödin and Glimelius 1988; Rimmer and van den Berg 1992), and *A. thaliana* (Brun and Tribodet 1995; Chen and Séguin-Swartz 1996); **II**; **III**). However, isolates virulent on *B. juncea* have been reported in Australia (Purwantara et al. 1998) and France (Somda et al. 1999; Brun et al. 2000). There have also been reports of *L. maculans* isolates virulent on *B. nigra* derived resistance (Brun et al. 2001). Utilizing resistance from distantly related genomes confer the co-transfer of many unwanted traits. Low frequency of recombination in interspecific crosses makes breeding for stable integrated resistance difficult (Chevre et al. 1997). This is also exemplified in **II**, where resistance was transferred with *A. thaliana* chromosome 3 but no recombination into the *B. napus* chromosomes could be identified. To avoid the transfer of unwanted traits, *Agrobacterium* mediated transfer of candidate resistance genes have been used. Overexpression of a tomato chitinase (Grison et al. 1996), a pea defense gene (Wang et al. 1999), a resistance gene to *Cladiosporum fulvum* (*Cf9*) (Hennin et al. 2001), and a resistance protein in *B. nigra* (**IV**) have all showed promising results of improved resistance. The susceptible *B. napus*

produces endogenous chitinases and other defense genes, however, host-fungus systems co-evolve, resulting in breakdown of indigenous plant defenses through adaptation by the fungus. Transformation of related chitinases, defensins and thionins still reactive against the specific pathogen can therefore have an affect.

Arabidopsis thaliana the model plant

A. thaliana is a small insignificant weed belonging to the *Brassicaceae* family. It is estimated that the *Brassica* and *Arabidopsis* lineage diverged 12.2-19.2 million years ago (Cavell et al. 1998). The *Brassica* and *A. thaliana* genomes exhibit extensive homology to each other but the *Brassica* diploid genomes contain roughly three copies of each *A. thaliana* segment (reviewed in (Bancroft 2001). Triplication seemed to be followed by extensive deletions, and significant changes in genome microstructure have occurred since the *Brassica and Arabidopis* lineage diverged. In contrast to the *Brassiceae* tribe, no crop plants emerged from the *A. thaliana* tribe (*Sisymbrieae*). Early recognition of its virtues in plant research such as; a short generation time, small size, prodigious seed set, small genome, and low percent of repetitive DNA, generated its present position as a model plant (reviewed in Meinke et al. 1998). The almost finished sequence of the *A. thaliana* genome (The Arabidopsis genome initiative 2000), together with the numerous mutants generated (Nottingham Arabidopsis Stock Centre 2001), make it ideal for plant research.

Resistance in *A. thaliana* to pathogens

About 26,000 genes from 11,000 families are estimated to be present in the *A. thaliana* genome. Of the 26,000 genes, 2,055 are denoted as involved in plant defense (The Arabidopsis genome initiative 2000). The vast number of genes involved point out the complexity inherent in plant defense. Many complex defense systems had to evolve to withstand the vast array of plant pathogens and insects. The simultaneous evolution of plants and plant defenses forced a co-evolution in the pests and pathogens creating much of the world's biodiversity (reviewed in Rausher 2001). Generally, the defense is up to its task and it is often mentioned in the literature that most plants are resistant to most pathogens. If walking in nature or indeed managing the garden plot it is impossible not to have noticed frequent insect caused damage or pathogen infections. One has to keep in mind then that although a plant is resistant to most pathogens, numerous pathogens and pests have been specialized to infect a specific plant species or a group of related species.

The classes of organisms causing damage and disease in *A. thaliana* are the same as for all plants and indeed even humans. Insects, (the pests) and bacteria, fungi, protozoan and viruses (the pathogens). Interestingly, it has been shown that human opportunistic pathogenic bacteria can live and multiply in a

defense compromised *A. thaliana* mutant (Plotnikova et al. 2000). Plant pathogens can in a simplified manner be classified as either biotrophs or necrotrophs (Agrios 1997; Dangl and Jones 2001). Biotrophs live on and penetrate living tissue and demand a living host to complete its life cycle. Necrotrophs kill the host usually by toxins and live on the dead material. The division is not so clear cut considering the fact that many biotrophs eventually can inflict necrotrophic regions of the plant. This sometimes occurs as a side effect of nutrient depletion. Necrotrophs, on the other hand, can have an initial biotrophic growth phase (no damage can be seen on plants) but eventually necrotrophic areas on the plant develop.

Resistance genes and signaling in A. thaliana

The inducible plant defenses initiate when the plant recognizes the pathogen. The invasion strategy of the pathogen can therefore determine how the plant will sense the pathogen (Fig. 2). The biotrophs try to invade the plant, and harvest nutrition without alerting the plant defense. Many effector molecules, known as virulence genes, are exported during the invasion to modulate the plant response (Grant and Mansfield 1999; Dangl and Jones 2001). To counteract the biotrophic strategy, plants have evolved a number of resistance (*R*) genes that somehow recognize the presence of the pathogen or its effector molecules. If a virulence gene is recognized by a *R*-gene and the effect is resistance, the virulence gene is denoted as an avirulence (*avr*) gene (Flor 1971). If both a *R*-gene and its corresponding *avr* gene are present, the result is resistance. However, if either the *R*-gene or the *avr*-gene is missing, the pathogen can infect the plant and disease can progress without any outward response. *R*-genes in *A. thaliana* have been isolated against all types of pathogens. Despite their wide range of action the *R*-genes can be divided based on the presence of domains in four different classes (more classes have been identified in other plants but they carry similar domains) of proteins, encoding six known functional domains. The different domains are; leucine rich repeats (LRR), coiled coil (CC), nucleotide binding domain (NB), kinase, and toll and interleukin 1 receptor domain (tir) (reviewed in Dangl and Jones 2001). LRR and CC domains are involved in protein-protein interaction, peptide ligand binding, and protein carbohydrate interactions. The LRRs are hypothesized to directly recognize avr molecules but evidence for this is scarce. The NB and the kinase domains are involved in regulation. NB is regulated by ATP or GTP binding and kinase by phosphorylation. The toll and interleukin-1 receptor domain is homologous to an intracellular signaling domain in *toll* and *IL1* genes in *Drosophila* and mammals respectively. The pathogens that damage the plant directly through production of toxins and cell wall degrading enzymes can be recognized (apart from *R*-genes) by endogenous signal particles generated from wounded plant tissue. Examples of such signal molecules are oligogalacturonides (Benhamou et al. 1990; Coté and Hahn 1994; Leon et al. 2001) and cutin monomers (Schweizer et al. 1996).

When *R*-genes or endogenous elicitors recognize the pathogen, the appropriate signals must be sent and responses initiated. Detectable early responses are ion channel fluxes and generation of reactive oxygen intermediates (ROI). A few genes have so far been identified by mutations as necessary for mediating *R*-gene downstream signaling. Some of these are; NDR1 (Century et al. 1997), PBS2 (Warren et al. 1999), EDS1 (Falk et al. 1999), and PAD4 (Jirage et al. 1999) (Tab. 2). Different groups of *R*-genes seem to depend on different downstream signaling genes (reviewed in Glazebrook 2001). The response to avirulent biotrophic pathogens is often a hypersensitive response (HR). The HR is characterized by a programmed cell death with components conserved in animals and plants. The HR is also characterized by induction of defense responses that serve to confine and inhibit the pathogen such as callose, ligning and ROI deposition (reviewed in Lam et al. 2001). HR is generally coupled to functional *R*-genes but it is not always a requirement for resistance (Clough et al. 2000). Indeed, in one necrotrophic interaction invasion of the plant could be aided by induction of HR (Govrin and Levine 2000).

Table 2. Characterized defense mutants in *A. thaliana* mentioned in this thesis

Mutant	Phenotype	Gene product, homology	Reference
coi1-1	JA insensitive	LRRs and an F-Box	Xie et al. 1998
eds1	Enhanced disease susceptible to virulent P. syringae. Block together with *pad4* the response to a specific set of *R-genes*	Catalytic site of lipases	Glazebrook et al. 1996; Falk et al. 1999
ein2-1	Ethylene insensitive	metal-ion transporters	Alonso et al. 1999
dnd1	Deficient in HR, elevated expression of *PR* genes, dwarfy	Cyclic nucleotide-gated ion channel	Clough et al. 2000
gsm1-1	Metabolic block in aliphatic glucosinolate biosynthesis	Not cloned	Haughn et al. 1991
NahG	Degrades SA	Transgene, salicylate hydroxylase	Gaffney et al. 1993
ndr1	Non specific disease resistance	Putative transmembrane domains	Century et al. 1997
opr3	Defective in JA biosynthesis	isozyme of 12-oxophytodienoate reductase	Stintzi and Browse 2000
pad1	Phytoalexin deficient (reduced camalexin production), also involved in SA and JA dependent responses	Not cloned	Glazebrook et al. 1997; Thomma et al. 1999b
pad2	Reduced camalexin production	Not cloned	Glazebrook et al. 1997
pad3	Camalexin null mutant	P450 gene	Zhou et al. 1999
pad4	Reduced camalexin production, also involved in SA and specific R-gene dependent responses	triacyl glycerol lipases	Zhou et al. 1998; Jirage et al. 1999; Rustérucci et al. 2001
pbs2	Blocks together with ndr1 in the response to a specific set of *R-genes*	Not cloned	Warren et al. 1999

In the downstream signaling response to pathogens, two distinct albeit interacting signaling pathways have been identified that are activated in response to specific type of pathogens. Generally, the SA dependent pathway regulates resistance to biotrophic and biotrophic like pathogens, such as mildew fungi and certain bacteria e.g. *Pseudomonas* (Delaney et al. 1994). The SA pathway also regulate the systemic acquired resistance (SAR) that is a plant response generating a long lasting systemic increase in general resistance (Uknes et al. 1992; Hunt et al. 1996) The other pathway is the JA/ET dependent pathway. This pathway seems to regulate resistance to necrotrophic pathogens (Thomma et al. 1998), although some evidence also exists on a role for SA in resistance to necrotrophs (Murphy et al. 2000; Norman-Setterblad et al. 2000). JA and ET dependent resistance pathways do not always act in conjunction with another. Norman-Setterblad et al. (2000), showed that although the necrotrophic bacteria *Erwinia carotovara* and its culture filtrate stimulated induction of several defense genes in concert, the vegetative storage protein (Atvsp), a JA responsive protein was further induced in the ethylene responsive mutants *etr1-1* and *ein2*. Additionally activation of *Atvsp* by JA was reduced if ET was added simultaneously. JA and ET can also mediate resistance to different necrotrophic pathogens. The JA responsive mutant *coi1-1* gave enhanced susceptibility to both *A. brassicicola* and *Botrytis cinerea* (Thomma et al. 1998) whereas the ET responsive mutant *ein2-1* gave increased susceptibility only to *B. cinerea* (Thomma et al. 1999b).

JA/ET are also involved in the phenomena of induced systemic resistance (ISR). ISR is similar to SAR but are induced by soilborn *Rhizobacteria* (reviwed in Pieterse et al. 2001). Recent experiments with a mutant defective in JA synthesis (*opr3*) have showed that JA in itself is not necessary for some of the JA pathway dependent responses. Precursors in JA synthesis, the cyclopentenones, could fulfill some mechanisms previously attributed to JA (Stintzi et al. 2001). Additionally, methyl-JA could also be the diffusible intercellular signal transducer, mediating intra- and interplant JA response (Thomma et al. 2000; Seo et al. 2001). JA and ET also affect other important plant regulations, such as root growth for JA (Creelman and Mullet 1997), and ripening for ethylene (Abeles et al. 1992). Considerable cross talk exists between the JA/ET and SA dependent pathways. Some JA/ET responses can limit SA responses and vice versa. In contrast, ISR and SAR can by simultaneous activation, generate higher resistance than by SAR activation alone. This suggests that not all JA dependent responses are inhibited by SA (van Wees et al. 2000). A number of genes have now been identified that operate in SA, JA and ET dependent signaling pathways. Other isolated mutants affecting disease resistance still need further characterization to clarify their exact role in defense (reviewed in Glazebrook 2001).

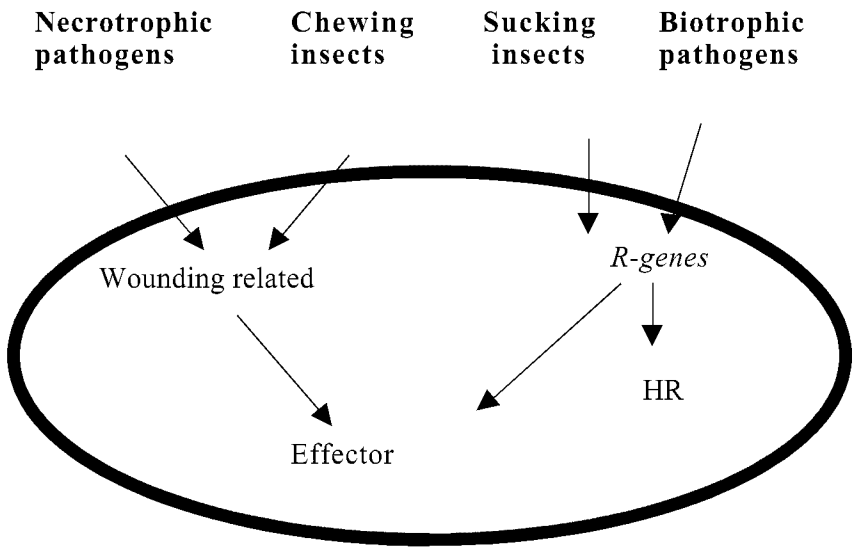

Figure 2. Simplified model of plant-pathogens and the plant response

Defense effector molecules

How intricate and interesting recognition and signaling pathways might be, it is the induction of downstream molecules that mediate the resistance. *A. thaliana* display a number of classes of defense related effector molecules. Most of these are shared with other plants. The pathogenesis related (PR) proteins were initially identified as response proteins induced in SAR (reviewed in van Loon and van Strien 1999). Now PR proteins also include proteins that are expressed in response to JA/ET dependent signals. The PR proteins constitute presently fourteen protein families, PR1 to PR14. Evidence indicates that PR1, PR-2 and PR5 are SA dependent and required for increased protection against the biotrophic fungus *P. parasitica* (Clarke et al. 1998). Some of the PR proteins are associated with known resistance effects. In others, the mechanisms generating the pathogen inhibiting effect are still not known. PR1 is one such protein with unknown mechanisms (van Loon and van Strien 1999). Nevertheless, the clear SA dependent response of PR1 leads it to be a commonly used marker of SA response induction (Rogers and Ausubel 1997). The most studied PR proteins expressed after JA and ET productions are the defensins (PR12), the thionins (PR13) and the chitinases (PR3-4). Several reports exist that have described a direct effect on increased resistance due to overexpression of defensins (Terras et al. 1995), thionins (Epple et al.

1997), and chitinase (Grison et al. 1996). Of these, the inducible defensin PDF1.2 have extensively been used as a marker of JA/ET dependent signaling (Penninckx et al. 1998; Thomma and Broekaert 1998; Thomma et al. 1998); (Petersen et al. 2000). Today, global gene induction experiments comparing SA, JA ET or pathogen induced gene expression have revealed a high number of genes that are either induced or repressed (Schenk et al. 2000). Several of these genes probably do not mediate any significant increase in resistance but could be side effects of resistance mediated events. Others could constitute new and so far uncharacterized resistance mediators.

Apart from PR proteins *A. thaliana* also produce a number of low molecular weight secondary metabolites involved in defense. The majority of secondary metabolites are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways (reviewed in Dixon 2001). The substances generally have a broad toxicity to pathogens. Efficiency of the substances can be dependent on whether or not a pathogen has the enzymatic machinery to detoxify the metabolite. The secondary metabolites can be subdivided into two categoris, phytoalexins (compounds that are synthesized de novo) and phytoanticipins (preformed substances). In *A. thaliana*, the major phytoalexin is camalexin (Tsuji et al. 1992). A number of mutants deficient in phytoalexin production (*pad*) were subsequently identified (Glazebrook and Ausubel 1994). No change in resistance from wt could be observed after inoculation of the *pad* mutants with avirulent *P. syringae*. When challenged with virulent strains, *pad1* and *pad2*, but not *pad3* allowed higher growth of the bacteria. Characterization of the *pad* mutants showed that *pad1* and *pad2* were not completely interrupted in camalexin production. This indicates that the *pad1* and *pad2* mutations had interrupted defense signals regulating more than camalexin. Rogers et al. (1996), further showed that camalexin acts on the membrane integrity of *P. syringae* but that *in planta* camalexin levels were below the toxicity threshold level for *P. syringae*. Experiments with downy mildew showed that the *pad2* and *pad3* mutants gave slightly decreased resistance and that the *pad4* mutant allowed significantly more mildew growth (Glazebrook et al. 1997). Pad4 is also involved in downstream signaling of specific *R*-genes, and is together with the signaling protein EDS1, involved in upstream regulation of SA dependent signaling (Zhou et al. 1998; Feys et al. 2001; Rustérucci et al. 2001). The effects of camalexin on pathogens were further studied by Pedras et al. (1998). They showed that also the *Brassica* fungal pathogens, *L. maculans*, and *A. brassicae* and the phytopathogenic bacteria *Pseudomonas cichorii, Erwinia carotovora* and *Xanthomonas campestris* were unable to detoxify camalexin. Later studies showed that detoxifying mechanisms of camalexin do exist in the phytopathogen, *Rhizoctonia solani*, which can transform camalexin into significantly less toxic metabolites (Soledade et al. 2000). The *pad3* mutant has been cloned and the mutated gene has been identified as a *P450* gene probably directly involved in camalexin synthesis (Zhou et al. 1999). So far then, *pad3* is the only true

camalexin null mutant isolated. Disruption of camalexin production seems to have the most serious effects in resistance to fungal pathogens. Apart from slight decrease in *Peronospora* resistance, camalexin deficiency also gives enhanced susceptibility to *A. brassicicola* (Thomma et al. 1999b) and *L. maculans* (**III**).

Another major class of defense related secondary metabolites in *A. thaliana* are the glucosinolates. Glucosinolates are present in the whole *Brassicaceae* family and consist of sulfur containing glucosides. Upon tissue damage glucosinolates are catalyzed by endogenous thioglucosides (myrosinases) into glucose and potentially toxic breakdown products. In *A. thaliana*, a total of 23 glucosinolates have been detected (Hogge et al. 1988). The glucosinolates can be both preformed and induced by stress. Therefore they can be classified as both phytoalexins and phytoanticipins. Their role in defense is not completely clear. It is possible they also have a role as storage compounds (Andreasson 2000; Rask et al. 2000). Development of clubroot (*P. brassicae*) symptoms have been hypothesized to be caused by breakdown of indole glucosinolates releasing large amounts of auxin responsible for the clubroot symptoms (Butcher et al. 1974). In agreement with this statement, free auxin and indole-3-acetonitrile (IAN) increased in infected *A. thaliana* wt and developing clubroot symptoms. Free IAA and IAN were reduced in some glucosinolate-affected mutants but not in others. The mutants with reduced level did show reduced clubroot symptoms whereas the other mutants did not (Ludwig-Müller et al. 1999). Further characterization of the mutants and their specific impact on IAA and glucosinolate production need to be performed before the IAA, glucosinolate and clubroot connection is verified. Nevertheless, glucosinolates are toxic to a range of pathogens *in vitro* and several experiments have indicated an antimicrobial role *in vivo*. Analyses of antimicrobial activity in crude extract of *A. thaliana* leaf identified 4-methylsulphhinylbutyl isothiocyanate (ITC) as a major phytoanticipin. ITC is the breakdown product from the main aliphatic glucosinolate, 4-methylsulphhinylbutyl glucosinolate (4-MSBGS). It had an inhibitory effects on a range of pathogens *in vitro* (Tierens et al. 2001). Experiments utilizing the *gsm1-1* mutant (Haughn et al. 1991) largely deficient in ITC production showed that *Fusarium oxysporum* induced slightly albeit significantly increased leaf damage. For *A. brassicicola, E. carotovara, P. parasitica, B. cinera P. syringae* and *Plectosphaerella cucumerina* no change to wt was observed (Tierens et al. 2001). The glucosinolates can also be induced by pathogens. Brader et al. (2001), revealed a specific increase in 3-indolyl methylglucosinolate (IGS), an aromatic glucosinolate, after challenge with culture filtrate from *E. carotovara*. The induction was JA but not SA or ET dependent as *coi1* did not induce IGS but *NahG* responded as wt and *ein2-1* produced even higher levels. 4-MSBGS was not significantly effected in *coi1-1* indicating a different regulation between induced and preformed glucosinolates. IGS as ITC is toxic to *E. carotovara in vitro*, but it remains to

show if IGS restrict *E. carotovara in vivo*. Nevertheless, induced and preformed glucosinolates play a role in *A. thaliana* defense against certain pathogens. In contrast, neither preformed nor induced glucosinolates have been linked to *L. maculans* resistance in *B. napus*. (Wretblad and Dixelius 2000; Andreasson et al. 2001). It could be speculated that the capacity of *L. maculans* to metabolize certain glucosinolates is a key advantage as a *Brassica* pathogen (Pedras et al. 2000).

Aim of the present investigation

The general aims of this study was to analyze the model plant *A. thaliana* and the crop plant *B. napus* on intergenomic interactions and on resistance mechanisms to *L. maculans*. *A. thaliana* is one of the most studied plants so far in science. The explorations of *A. thaliana* have generated knowledge applicable in all plants. *B. napus* is an important oilseed crop and one of the closest crop-relatives to *A. thaliana*. Results in others and ours study on *A. thaliana* can therefore be of use in future *B. napus* breeding efforts.

The specific aims of this study was to:

- Analyze traits and intergenomic interaction between *A. thaliana* and *B. napus* in offspring of *A. thaliana* (+) *B. napus* somatic hybrids.

- Analyze the function and genomic position of *A. thaliana* derived resistance against *L. maculans* in a *B. napus* genomic background.

- Identify and understand resistance resources and pathways in *L. maculans* resistant *A. thaliana* and *B. nigra*

Present investigation-Results and discussion

In the present study, the model plant *A. thaliana* has been analyzed both with respect to genomic interchange with *B. napus* and as a potential resistance resource to the fungal pathogen *L. maculans*. Resistance towards *L. maculans* was also analyzed in *B. nigra*.

Study on a genomic interaction between the *A. thaliana* and *B. napus* genome with focus on *L. maculans* resistance

Symmetric and asymmetric somatic hybridization are two techniques to generate either a complete fusion of two genomes or fusion of one genome with parts of the other genome (reviewed in Glimelius 1999). Large amount of genomic DNA or alternatively organelle DNA can be transferred by this method between sexually incompatible species. The advantage with symmetric hybridization is that all the traits present in the donor species are theoretically transferred to the recipient species, normally as intact chromosomes. By asymmetric hybridization only parts of the donor genome is transferred, either as whole chromosomes or chromosome fragments. This is advantageous in breeding because unwanted traits are either not transferred or can be removed faster by back-crossing in combination with efficient selection. Today, transformation by *Agrobacterium* is the method of choice for transferring single traits. Nevertheless, somatic hybridization can be an efficient technique when the purpose is to transfer complex traits encoded by uncharacterized genes. An examples of this is the construction of CMS systems (Pelletier et al. 1983) or transfer of fungal resistance (**III**).

Inheritance of *Arabidopsis* DNA in offspring from *Brassica napus and A. thaliana* somatic hybrids (**I**)

In **I,** inheritance of *A. thaliana* DNA in offspring from symmetric and asymmetric *A. thaliana* (+) *B. napus* somatic hybrids were compared. The amount of remaining *A. thaliana* DNA were detected by chromosome counts and RFLP markers. When back-crossing plants from the symmetric hybrid group to *B. napus*, the BC_1 offspring contained DNA from all five *A. thaliana* chromosomes indicating that normal division of *A. thaliana* chromosomes occurred during the meiosis. In BC_1 gametes on the other hand, a varying number of *A. thaliana* chromosomes ranging from one to five should form. In BC_2 therefore, reduced frequency of retained *A. thaliana* loci could be expected. RFLP analysis showed that the *A. thaliana* loci were reduced by 58% not far from a theoretically calculated average value of 50%. In comparison, in the asymmetric hybrid offspring (BC_1) 84% of *A. thaliana* loci analyzed were absent. Generally, after back-crossing, symmetric hybrid offspring tend to retain most DNA as complete chromosomes. Whereas, asymmetric hybrid offspring contained DNA fragments that could give increased likelihood of recombination and promote the reduction of donor

DNA after fewer back-crosses. The materials generated by back-crossing symmetric and asymmetric hybrids constitute a library of *A. thaliana* DNA in *B. napus*, ready to use for breeding purposes and studies on specific gene functions.

*Arabidopsis thaliana derived resistance against Leptosphaeria maculans in a Brassica napus genomic background (**II**)*

The *A. thaliana* accessions Col-0 and Ler-0 used as donors in somatic hybridization to *B. napus* are resistant to *L. maculans* (**III**). The molecular mechanisms behind the resistance are presently unknown in *A. thaliana* and other resistant species. In *A. thaliana*, previous work on *L. maculans* resistance have been restricted to observations that the resistance existed and could be coupled to HR like symptoms (Brun and Tribodet 1995; Chen and Seguin-Swartz 1996; Chen and Seguin-Swartz 1999). In **II,** it is shown that the resistance in *A. thaliana* towards *L. maculans* can be transferred to *B. napus* through somatic hybridization. The resistance from *A. thaliana* could as in the *Brassica* genus be divided in cotyledon and adult resistance (Dixelius 1999). Initially, both cotyledon and adult leaf resistance were transferred to *B. napus*. After one back-cross to *B. napus* cotyledon resistance segregated from adult leaf resistance and was lost in the following generations of adult leaf resistant plants. Poor seed set and complete to semi male sterility in the first generations hybrids rendered mapping of cotyledon resistance difficult. In adult leaf resistance plants molecular characterization showed that complete transfer of *A. thaliana* chromosome 3 produced a resistant phenotype to *B. napus*. Further characterizations of back-crossed and subsequently selfed asymmetric hybrids, containing parts of chromosome 3, identified two parts on each side of the centromere which co-segregated with resistance. The transferred regions, less than ten percent of chromosome 3, contain approximately 675 genes. In the regions a number of defense related genes are present. Notably, a large P450 cluster that includes a *P450* gene (*pad3*) involved in one of the steps in production of the phytoalexin camalexin. Other defense-associated genes in the area were ABC-type transport like proteins, proline rich proteins, disease resistance like proteins and *EDS-1*.

Protein 2DE analysis was performed to get a better understanding of which proteins actually were expressed after infection. The material chosen for analysis were BC_1F_4 plants resistant against *L. maculans,* which was compared to susceptible *B. napus*. The BC_1F_4 plants were generated after one back-cross followed by four selfings of the original symmetric hybrid (*A. thaliana* (+) *B. napus*). The BC_1F_4 generation contained the complete chromosome 3 in all plants and in 40% of the plants, chromosome 1. The co-transfer of chromosome 1 could be a result of increased male fertility generated by genes on chromosome 1 as it did not co-segregate with resistance in previous generations. Interestingly, very few proteins were found to be differently expresses on 2-DE protein gels after *L. maculans* inoculation despite presence

of an entire chromosome of *A. thaliana*. Out of the approximate 600 visible proteins on a gel, only six extra proteins were identified in the resistant BC_1F_4. Fourteen proteins were additionally expressed in susceptible *B. napus*. Four of the proteins expressed in BC_1F_4 could be identified by fractionation and analyzing on a Q-tof MS. The proteins showed a 100% homology to the *A. thaliana* proteins glycolate oxidase, photosystem 1 subunit 2 precursor, chloroplast ribosomal L1 like protein, and a polyubiqitinase. The group of identified proteins all have high expression levels and are involved in basic cell maintenance. Some of the proteins could also be linked to a general stress response. Ubiquitinase is involved in protein degradation, and over-expression in yeast have shown elevated resistance to some abiotic stresses and increased susceptibility to others such as cadmium (Chen and Piper 1995). Ubiquitination is also linked to JA signaling. The JA responsive mutant *coi1* encode F box proteins with homology to other F box proteins. These proteins function as receptors that selectively recruit repressor proteins into a complex promoting ubiquitination and subsequent removal of the repressors (Xie et al. 1998). Glycolate oxidase are down regulated by inoculation with *A. brassicicola* (Schenk et al. 2000). Increased intensity of many protein spots on the 2DE gels indicated that many proteins were induced or repressed after induction with *L. maculans*. Problems associated with the 2DE technique hinder reliable evaluation of too small changes. When induction of proteins was evaluated between inoculated and uninouculated susceptible *B. napus,* nine different spots were identified in infected leaf 24 h after inoculation with *L. maculans* (Lamkadmi et al. 1996). This result closely correlates to the numbers (14) seen in differently expressed susceptible *B. napus* compared with resistant BC_1F_4 (**II**). This above numbers could be further compared to the 277 ESTs that were induced after *L. maculans* inoculation (Fristensky et al. 1999). The low amount of changes seen on the 2DE gels compared to EST analyses can, however, be explained by lack of detection by proteins expressed in low amounts. Also, induction of RNA expression does not always lead to protein expression.

Molecular pathways and genes involved in resistance to *L. maculans*

Resistance against *Leptosphaeria maculans* in *Arabidopsis thaliana* is multi-factorial (**III**)

The success in the transfer *A. thaliana* resistance from both *A. thaliana* accessions Col-0 and Ler-0 (**II**) prompted us to start a detailed study on mechanisms of *A. thaliana* resistance with the aim to understand *A. thaliana* derived resistance in *B. napus*. A survey on the response to *L. maculans* on a large group of *A. thaliana* accessions representing 27 countries was conducted. Resistance in *A. thaliana* was generally the rule in most accessions. Only five out of 171 accessions showed any symptoms at all. One of the five accessions wilted spontaneously shortly after disease symptoms could be registered. The remaining four showed moderate symptoms. No accession

could be found that showed susceptibility as severe as by *B. napus*. This information indicates that resistance in *A. thaliana* could possibly be of the non-host type. To understand the underlying molecular mechanisms of the resistance, a set of EMS mutated Ler-0 seeds were scored for *L. maculans* susceptibility. The screening procedure aimed at identifying mutants with a similar susceptible phenotype displayed by *B. napus*. Twelve plants were identified and renamed *L. maculans* susceptible (*lms*) 1-12. The identified mutants could be divided in three groups based on response to isolates from the four different pathogenicity groups of *L. maculans* present. In the first group, *lms1-4,* showed a systemic spread of the infection resulting in wilting of several leaves. Two of the isolates, *lms1* and *lms2,* were susceptible to all isolates tested, whereas *lms3* and *lms4* showed susceptible reactions primarily towards PG2 isolates. Group three, *lms5* to *lms9,* showed no systemic spread and reacted primarily to PG2 isolates. In the last group only moderate symptoms were visible after inoculation and mostly by PG2 isolates. *lms1* and *lms5* were chosen as representatives of group one and two and analyzed further for chromosomal map position. Mapping with CAPs markers (Konieczny and Ausubel 1993) positioned *lms1* on chromosome 2 and *lms5* on the southern end of chromosome 1. No complete linkage to either mutant was, however, found. Control experiment on a cross between Ler-0 and Col-0 showed that susceptible plants could be generated in F_2 (4.4%). These plants were reconfirmed as susceptible in F_3. The control experiment pointed to an incompatibility in resistance between Col-0 and Ler-0. The incompatibility could be explained if a system in which two different resistance alleles exist in the accessions, perhaps involved in recognition, of the fungus.

Necrotrophic pathogens, such as *A. brassicicola* activate JA/ET dependent resistance while biotrophic pathogens such as *P. parasitica* activate SA mediated resistance (Thomma et al. 1998). *L. maculans* is considered as a facultative necrotoph since the fungus initially grows inter-cellularly without causing cell death, but later promotes necrosis and can live saprophytically on dead plant material (Hammond and Lewis 1986). The response to *L. maculans* in *A. thaliana* could for this reason, in theory, be dependent either on SA, JA/ET or both. To answer this question, a set of mutants and a transgene previously characterized as involved in defense interactions in *A. thaliana* such as SA, JA, ET and camalexin signaling were included in the study (**III**). The aim was to analyze which, if any, of previously characterized defense signaling pathways were activated, and necessary for resistance to *L. maculans*. Since previous characterizations by other pathogens had been performed, results from the analysis could be used to directly compare *L. maculans* resistance to other characterized pathogens. The data in (**III**) indicated that neither JA, ET, or SA signaling pathways are absolutely required for resistance. In contrast, the phytoalexin camalexin is necessary for complete resistance. The camalexin null mutant *pad3* that showed susceptibility is mutated in a *P450* gene (Zhou et al. 1999). Camalexin is also produced by *Camelina sativa* and production

of camalexin has been transferred from *C. sativa* to *B. oleracea* by somatic hybridization, which resulted in resistance to *A. brassicicola* (Sigareva and Earle 1999). Interestingly, *pad3* is located in the area of chromosome 3 that was transferred in both symmetric and asymmetric hybrid resistant offspring (**I**; **II**). Therefore were the resistant BC_1F_4 generation analyzed for presence of camalexin after inoculation with *L. maculans*. No production of camalexin could however be recorded. A possible explanation for this could be lack of substrates in the biosynthetic pathway of camalexin. It can not be ruled out by the present investigation that new or modified phytoalexins active against *L. maculans* could have formed, generating new resistance. Interestingly, *lms1* produced wt, or higher, levels of camalexin after *L. maculans* inoculation. Therefore other factors in conjunction to camalexin seem to be responsible for resistance. To further explore the nature of resistance, RNA expression of the defense genes *PR1* and *PDF1.2* were analyzed in susceptible and resistant mutants as well as in resistant wt accessions. *PR1* was used as a marker for SA induced pathway and *PDF1.2* as a marker for ET/JA induced pathways. The results showed that both PR1 and PDF1.2 expression could be induced by *L. maculans*. Additionally, expression of neither *PR1* nor *PDF1.2* at least at the transcriptional level were necessary for resistance since in *lms1* both *PR1* and *PDF1.2* were produced at a higher levels than in wt (**III**).

We have further isolated a T-DNA mutant susceptible to *L. maculans* in *A. thaliana* (unpublished data). Preliminary analysis indicates that a Rac GTPase activating (*RacGap*) gene on chromosome 2 has been knocked out. The location of the *RacGap* mutant is not in or around the putative chromosomal position of *lms1*. RacGaps activate Rac proteins that are members of a group of small GTP binding proteins (Winge et al. 2000). The Rac proteins are involved in many cellular mechanisms such as biogenesis of legume root nodules (Cheon et al. 1993) and regulation of cell death (Kawasaki et al. 1999 #395]. A possible cause of the T-DNA generated disruption of RacGap could therefore be a malfunction of the HR following pathogen attack. The *dnd1* mutant also lacks a proper HR (Yu et al. 1998) but, in contrast to our T-DNA mutant (Fig. 3), increased susceptibility to *L. maculans* is not shown (**III**). *dnd1* do however produce elevated levels of PR proteins and have a dwarfy phenotype (Yu et al. 1998). Both are qualities with a potential to disturb the interaction between *A, thaliana* and *L. maculans*.

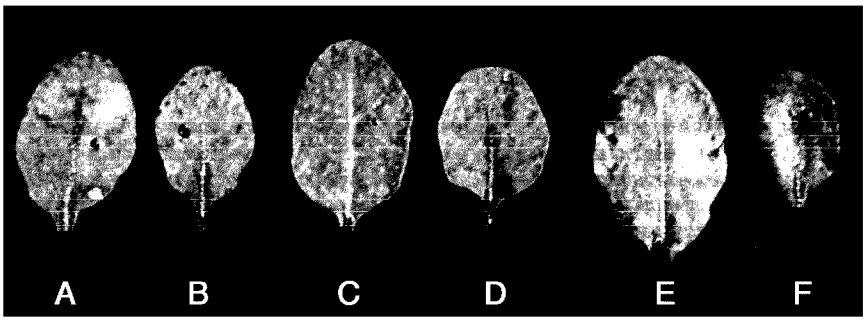

Figure 3. *A. thaliana* Ws-2 leaves inoculated with *L. maculans*. (A and B), Wt. (C and D), T-DNA mutant complemented with DNA covering the region of T-DNA insertion. (E and F), T-DNA mutant interrupted in a putative *RacGap* gene.

The requirement for camalexin group *L. maculans* with the necrotrophic *A. brassicicola*. *A. brassicicola* is a necrotrophic pathogen attacking virtually all *Brassica* species (Tewari and Mithen 1999). The resistance in *A. thaliana* differs however from *L. maculans* in that A. *brassicicola* require a functioning JA pathway (Thomma et al. 1998). Resistance to *L. maculans* seems to be independent of this pathway. However, different JA pathway mutants have been used in the analysis, *jar1-1* for *L. maculans* and *coi1* for *A. brassicicola*. It can not be ruled out that *jar1-1* and *coi1* affect JA signaling differently. The data generated by resistance screening, camalexin analysis and RNA expression experiments on analyzed wt and mutants have been put together in a model of *A. thaliana* responses to *L. maculans* (Fig. 4). Generally, the model show that resistance to *L. maculans* in *A. thaliana* is different from the well studied systems of *P. syringae* and mildews in an independence of SA signaling and SA dependent effectors even though *L. maculans* inoculation stimulates the SA pathway. The resistance is independent also of JA/ET dependent pathways indicating that *A. thaliana* posses a strong defense against *L. maculans*. Additionally, camalexin is an important effector molecule against *L. maculans* but it is not the only one as *lms1* produces at least wt levels. The requirement of a *RacGap* gene possibly involved in hypersensitive cell death adds a level of complexity to the resistance. Another indication of HR involvement comes from heterologous expression of the *Cf9* resistance gene from tomato in *B. napus* (Hennin et al. 2001) If *L. maculans* was applied on an Avr9-mediated HR site, disease development of *L. maculans* was delayed. Finally, to understand the nature of *L. maculans* resistance it is necessary to remember that the fungus has both a biotrophic and necrotrophic growth phase. Resistance mediators from the plant can therefore be stimulated and directed to both.

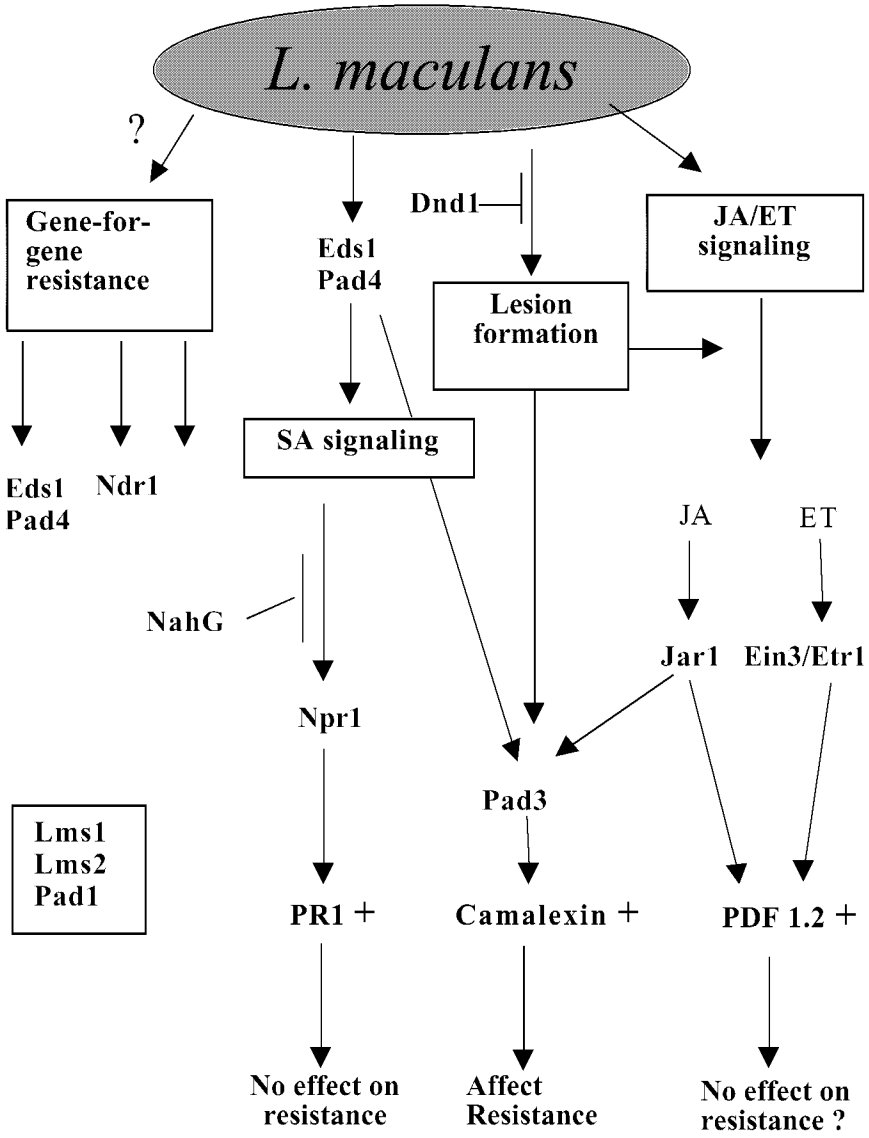

Figure 4. A model describing signaling pathways and effector molecules tested for interaction between *L. maculans* and *A. thaliana*. Mutants have been tested for all proteins in the model. No affect on resistance were seen in the mutated proteins colored red. In contrast mutations in the green colored protein gave a susceptible response. (+) denote induced expression after fungal inoculations. The mutants listed in the box could not be put into any signal pathway. ?, denote possible but unknown pathway Many additional proteins and mutants are known in the pathways described above but have not been tested in the *L. maculans-A. thaliana* interaction.

The Lm1 gene of Brassica nigra confers resistance to the blackleg fungus Leptosphaeria maculans (**IV**)

B. nigra cultivars constitute a source of resistance to *L. maculans*. We initiated a screen for resistance genes towards *L. maculans* (**IV**). Several cDNA clones were picked up and analyzed for expression after inoculation. One cDNA clone, named *L. maculans* 1 (*Lm1*), was transformed to *B. napus*. Transformants were analyzed for increased resistance to *L. maculans*. Disease symptoms decreased on average 45 to 58% depending on isolate analyzed in transformed plants. *Lm1* constitute the first cloned resistance gene in *B. nigra*. The mechanisms of *Lm1* resistance are unknown, the only indication is a putative homology to a nodulin inception factor factor (*nin*) in *Lotus japonicas* and presence of two transmembrane domains. Close homologues to *Lm1* exist in *A. thaliana*, whereas in *B. napus,* homology seems to be restricted to the 3' end, indicating a possible function of resistance in the 5′ end.

Conclusions

- Back-crosses of *A. thaliana* (+) *B. napus* symmetric and asymmetric hybrids generated a pool of *A. thaliana* DNA in the *B. napus* genome. Back-crossing of symmetric hybrids generated initially, a transfer of predominantly complete chromosomes whereas asymmetric hybrids generate smaller fragments that could potentially mediate faster integration of desired traits and a more rapid removal of unwanted DNA than in the symmetric hybrids.

- In both symmetric and asymmetric hybrids *A. thaliana* derived *L. maculans* resistance were transferred to *B. napus*. The transferred reistance could be divided into cotyledon and adult leaf resistance. The symmetric hybrids generated a stable adult leaf resistant line in BC_1F_4 containing chromosome 3 that co-segregated with resistance. In the asymmetric hybrids, two areas on either side of the centromere of chromosome 3 co-segregated with resistance.

- Analysis of the molecular base for resistance in *A. thaliana* showed a dependency of camalexin and an independence of a functioning SA, JA/ET pathways. Additionally, at least two independent *L. maculans* susceptible mutants have been isolated and characterized.

- Resistance towards *L. maculans* in *B. nigra* has been analyzed and a resistance gene, *Lm1* have been isolated. Transformation of *Lm1* into *B. napus* gives significantly higher levels of resistance to *L. maculans*.

Future perspectives

The understanding of molecular mechanisms of resistance towards *L. maculans* in *A. thaliana* and *B. nigra* are still far from complete. In *A. thaliana* further characterization and cloning of *lms1* need to be achieved. Cloning of *lms1* could reveal new signaling pathways and effector molecules necessary for *L. maculans* resistance since *lms1* seems functional in SA as well as JA/ET dependent responses. More extensive analyses of the levels of camalexin, PR1 and PDF1.2 will be performed in *lms1* and wt to investigate if the trends of increased expression are statistically significant. Induction of PR1 with INA and PDF1.2 with paraquat will also be performed to clarify whether increased expression is due to enhanced fungal colonization in *lms1* or if it is a inherent process caused by the malfunction of *lms1*.

Further study on the nature of the *A. thaliana* derived resistance in resistant symmetric and asymmetric hybrid offspring is also necessary. One question to ask is if the transfer of multiple *P450* could have generated new phytoalexins active against *L. maculans*. Plants are unique in their high production of secondary metabolites. Evolution of the large *P450* gene family, approximately 286 in *A. thaliana* versus 94 in *Drosophila* and 3 in yeast, have been one important factor in generating this metabolic complexity (The Arabidopsis genome initiative 2000). Analysis by metabolic profiling of the resistant BC_1F_4 versus susceptible *B. napus* could shed some light over this question. HPLC analysis on the phytoalexin profile in *B. napus* compared to resistant BC_1F_4 after fungal inoculation could reveal which, if any, new phytoalexins that have formed. Recognition of the pathogen and exact timing of the defense responses are other highly interesting areas. The time of defense response initiation is crucial for resistance in many pathogens systems. Precise timing of resistance responses requires further clarification. One interesting phenomena is the induction of resistance genes at 11 days post inoculation in *A. thaliana* (unpublished results). What is happening at day 11 that need a restart of the resistance machinery? On the other hand, the involvement of a *RacGAP* gene possibly in HR, indicates the importance of very early responses. Analysis of disturbances of the HR in the *RacGap* T-DNA mutant is presently ongoing.

In *B. nigra* resistance, *Lm1* is the first cloned resistance gene. The mechanisms of *Lm1* resistance are however so far unknown, the only indication is a putative homology to nin factors. To clarify the role of *Lm1* several strategies could be envisioned such as determining possible anti-fungal effects *in vitro* and identification of putative protein interactions. The presence of homologous proteins in *A. thaliana* should also be further studied. Searching for knockouts in available collections could reveal if the *A. thaliana* homologues also are involved in resistance.

References

Abeles, F.B., Morgan, P.W. and Salvet, M.E.J. (1992). *Ethylene in Plant Biology*. Academic press, San Diego.

Agrios, G.N. (1997). Genetics of Plant Disease. In: *Plant Pathology*. Academic Press, San Diego, pp 115-142.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284:** 2148-2152.

Andreasson, E. (2000). Structural and functional studies of the myrosinase-glucosinolate system in *Arabidopsis thaliana* and *Brassica napus*. Thesis, Agraria 208, Dep. of Plant Biology, SLU. Uppsala.

Andreasson, E., Wretblad, S., Granér, G., Wu, X., Zhang, J.M., Dixelius, C., Rask, L. and Meijer, J. (2001). The myrosinase-glucosinolate system in the interaction between *Leptosphaeria maculans* and *Brassica napus*. Molecular. Plant Pathol. **In press**.

Ansan-Melayah, D., Balesdent, M.H., Buée, M. and Rouxel, T. (1995). Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathology **85:** 1525-1529.

Ansan-Melayah, D., Balesdent, M.H., Delourme, R., Pilet, M.L., Tanguy, X., Renard, M. and Rouxel, T. (1997a). Gene-for-gene interactions in the *Leptosphaeria maculans-Brassica napus* pathosystem. Acta Horticulturae **459:** 329-334.

Ansan-Melayah, D., Rouxel, T., Bertrandy, J., Letarnec, B., Mendes-Pereira, E. and Balesdent, M.H. (1997). Field efficiency of *Brassica napus* specific resistance correlates with *Leptosphaeria maculans* population structure. European Journal of Plant Pathology **103:** 835-841.

Ansan-Melayah, D., Balesdent, M.H., Delourme, R., Pilet, M.L., Tanguy, X., Renard, M. and Rouxel, T. (1998). Genes for race-specific resistance against blackleg disease in *Brassica napus* L. Plant Breeding **117:** 373-378.

Balesdent, M.H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M. and Rouxel, T. (2001). Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. Phytopathology **91:** 70-76.

Bancroft, I. (2001). Duplicate and diverge: the evolution of plant genome microstructure. Trends in Genetics **17:** 89-93.

Benhamou, N., Chamberland, H. and F.J., P. (1990). Implications of pectic components in cell surface interactions between tomato root cells and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Plant Physiology **92:** 995-1003.

Brader, G., Tas, E. and Palva, E.T. (2001). Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen *Erwinia carotovora*. Plant Physiology **126:** 849-860.

Brun, H. and Tribodet, M. (1995). Pathogenicity of *Leptosphaeria maculans* isolates on one ecotype of *Arabidopsis thaliana*. Cruciferae Newsletter **17:** 74-75.

Brun, H., Levivier, S., Somda, I., Ruer, D., Renard, M. and Chèvre, A.M. (2000). A field method for evaluating the potential durability of new resistance sources: Application to the *Leptosphaeria maculans-Brassica napus* pathosystem. Phytopathology **90:** 961-966.

Brun, H., Ruer, D., Levivier, S., Somda, I., Renard, M. and Chèvre, A.M. (2001). Presence in *Leptosphaeria maculans* populations of isolates virulent on resistance introgressed into *Brassica napus* from the *B. nigra* B genome. Plant Pathology **50:** 69-74.

Butcher, D.N., El-Tigani, S. and Ingram, D.S. (1974). The role of indole glucosinolates in the clubroot disease of the *Cruciferae*. Plant Pathology **4**: 127-141

Cavell, A., Lydiate, D., Parkin, I., Dean, C. and Trick, M.A. (1998). A 30 centimorgan segment of *Arabidopsis thaliana* chromosome 4 has six collinear homologues within the *Brassica napus* genome. Genome **41:** 62-69.

Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E. and Staskawicz, B.J. (1997). NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278:** 1963-1965.

Chen, C.Y. and Séguin-Swartz, G. (1996). Hypersensitive response of *Arabidopsis thaliana* to an isolate of *Phoma lingam* virulent to oilsed and turnip rape. Cruciferae Newsletter **18:** 106-107.

Chen, C.Y. and Seguin-Swartz, G. (1999). Reaction of wild crucifers to *Leptosphaeria maculans*, the causal agent of blackleg of crucifers. Canadian Journal of Plant Pathology **21:** 361-367.

Chen, Y. and Piper, P.W. (1995). Consequences of the overexpression of ubiquitin in yeast: elevated tolerances of osmostress, ethanol and canavanine, yet reduced tolerances of cadmium, arsenite and paromomycin. Biochimica et biophysica acta **1268:** 59-64.

Cheon, C.I.L., N G; Siddique, A B; Bal, A K; Verma, D P (1993). Roles of plant homologs of Rab1p and Rab7p in the biogenesis of the peribacteroid membrane, a subcellular compartment formed de novo during root nodule symbiosis. The EMBO Journal **12:** 4125-4135.

Chèvre, A.M., Barret, P., Eber, F., Dupuy, P., Brun, H., Tanguy, X. and Renard, M. (1997). Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 1. Identification of molecular markers, chromosomal and genomic origin of the introgression. Theoretical and Applied Genetics **95:** 1104-1111.

Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X. (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. Plant Cell **10:** 557-569.

Clough, S.J., Fengler, K.A., Yu, I.C., Lippok, B., Smith, R.K., Jr. and Bent, A.F. (2000). The Arabidopsis *dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proceedings National Academy of Sciences U.S.A. **97:** 9323-9328.

Coté, F. and Hahn, M.G. (1994). Oligosacharins: structures and signal transduction. Plant Molecular Biology **26:** 1375-1411.

Cozijnsen, A.J., Popa, K.M., Rolls, B.D., Purwantara, A. and Howlett, B.J. (2000). Genome analysis of the plant pathogenic fungus *Leptosphaeria maculans*. Molecular Plant Pathology **1:** 293-302.

Creelman, R.A. and Mullet, J.E. (1997). Biosynthesisand action of jasmonates in plants. Annual Review of Plant Physiology and Plant Molecular Biology **48:** 355-381.

Dangl, J.L. and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature **411:** 826-33.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science 266: 1247-1250.

Dixelius, C. (1994). Presence of the pathogenesis-related proteins 2, Q and S in stressed *Brassica napus* and *B. nigra* plantlets. Physiological and Molecular Plant Pathology **44:** 1-8.

Dixelius, C. (1999). Inheritance of the resistance to *Leptosphaeria maculans* of *Brassica nigra* and *B. juncea* in near-isogenic lines of *B. napus*. Plant Breeding **118:** 151-156.

Dixon, R.A. (2001). Natural products and plant disease resistance. Nature **411:** 843-847.

Ekbom, B. (1995). Insect Pests. In: *Brassica Oilseeds*, (eds.) D.S. Kimber, and D.I. Mcgregor, CAB International, Wallingford pp 141-152.

Epple, P., Apel, K. and Bohlmann, H. (1997). Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*. Plant Cell **9:** 509-520.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J. and Parker, J.E. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proceedings National Academy of Sciences U.S.A. **96:** 3292-3297.

Felton, G.W. and Korth, K. (2000). Trade-offs between pathogen and herbivore resistance. Current Opinion in Plant Biology **3:** 309-314.

Férézou, J.P., Riche, C., Quesneau-Thierry, A., Pascard-Billy, C., Barbier, M., Bousquet, J.F. and Boudart, G. (1977). Structures de deux toxines isolées des cultures du champignon *Phoma lingam* Tode: La sirodesmine PL et la desacetylsirodesmin PL. Journal de Chim. **1:** 327-334.

Feys, B.J., Moisan, L.J., Newman, M.A. and Parker, J.E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO Journal **20:** 5400-5411.

Flor, H.H. (1971). Current status of the gene-for gene concept. Annual Review of Phytopathology **9:** 275-296.

Fristensky, B., Balcerzak, M., He, D.-F. and Zhang, P. (1999). Expressed sequence tags from the defense response of *Brassica napus* to *Leptosphaeria maculans*. Molecular Plant Pathology Online http://www.bspp.org.uk/mppol/1999/0301FRISTENSKY.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754-756.

Glazebrook, J. and Ausubel, F.M. (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proceedings National Academy of Sciences U.S.A.**91:** 8955-8959.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143:** 973-82.

Glazebrook, J., Zooki, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmits, R. and Ausubel, F.M. (1997). Phytoalexin-deficient mutants of Arabidopsis reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics **146:** 381-392.

Glazebrook, J. (2001). Genes controlling expression of defense responses in Arabidopsis--2001 status. Current Opinion in Plant Biology 4: 301-308.

Glimelius, K. (1999). Somatic hybridization. In: *Biology of Brassica coenospecies*. (ed.) C. Gómez-Campo. Elsevier, Amsterdam, pp 107-148.

Gómez-Campo, C. and Prakash, S. (1999). Origin and Domestication. In: *Biology of Brassica coenospecies*. (ed.) C. Gómez-Campo. Amsterdam, Elsevier, pp 33-58.

Govrin, E.M. and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Current Biology **10:** 751-757.

Grant, M. and Mansfield, J. (1999). Early events in host-pathogen interactions. Current Opinion in Plant Biology **2:** 312-319.

Grison, R., Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J.J. and Toppan, A. (1996). Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nature Biotechnology **14:** 643-646.

Hammond, K.E., and Lewis, B.G. (1986). Ultrastructural studies of the limitation of lesions caused by *Leptosphaeria maculans* in stems of *Brassica napus* var. *oleifera*. Physiological and Molecular Plant Pathology **28:** 251-265.

Haughn, G.W., Davin, L., Giblin, M. and Underhill, E.W. (1991). Biochemical genetics of pant secondary metabolites in *Arabidopsis thaliana*. Plant Physiology **97:** 217-226.

Hennin, C., Hofte, M. and Diederichsen, E. (2001). Functional expression of *Cf9* and *Avr9* genes in *Brassica napus* induces enhanced resistance to *Leptosphaeria maculans*. Molecular Plant-Microbe Interactions **14:** 1075-85.

Hogge, L.R., Reed, D.W., Underhill, E.W. and Haughn, G.W. (1988). HPLC Separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography/mass spectrometry. Journal of Chromatographic Science **26:** 551-556.

Howlett, B.J. (1997). Genome analysis of the fungal plant pathogen, *Leptosphaeria maculans* using pulsed field gel electrophoresis. Electrophoresis **18:** 1544-7.

Howlett, B.J., Idnurm, A. and Pedras, M.S. (2001). *Leptosphaeria maculans*, the causal agent of blackleg disease of brassicas. Fungal Genetics and Biology **33:** 1-14.

Hunt, M.D., Neuenschwander, U.H., Delaney, T.P., Weymann, K.B., Friedrich, L.B., Lawton, K.A., Steiner, H.Y. and Ryals, J.A. (1996). Recent advances in systemic acquired resistance research, a review. Gene **179:** 89-95.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999). *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proceedings National Academy of Sciences U.S.A. **96:** 13583-13588.

Johnson, R.D. and Lewis, B.G. (1990). DNA polymorphism in *Leptosphaeria maculans*. Physiological and Molecular Plant Pathology **37:** 417-424.

Johnson, R.D. and Lewis, B.G. (1994). Variation in host range, systemic infection and epidemiology of *Leptosphaeria maculans*. Plant Pathology **43:** 269-277.

Kawasaki, T.H., K; Ono, E; Hatakeyama, S; Iwano, M; Satoh, H; Shimamoto, K (1999). The small GTP-binding protein rac is a regulator of cell death in plants. Proceedings of the National Academy of Sciences of the U.S.A. **96:** 10922-10926.

Koch, E., Song, K., Osborn, T.C. and Williams, P.H. (1991). Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Molecular Plant-Microbe Interactions **4:** 341-349.

Konieczny, A. and Ausubel, F.M. (1993). A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant Journal **4:** 403-410.

Kuusk, A.K. (2000). A field and laboratory study of *Leptosphaeria maculans* in Sweden year 2000. Examensarbete, Inst. F. ekologi och växtproduktionslära / Växtbiologi, SLU, Uppsala.

Lam, E., Kato, N. and Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response. Nature **411:** 848-853.

Lamkadmi, Z., Esnault, M.A. and Normand, M.E. (1996). Characterization of a 23 kDa polypeptide induced by *Phoma lingam* in *Brassica napus* leaves. Plant Physiological Biochemistry **34:** 589-598.

Leclair, S., Ansan-Melayah, D., Rouxel, T. and Balesdent, M. (1996). Meiotic behaviour of the minichromosome in the phytopathogenic ascomycete *Leptosphaeria maculans*. Current Genetics **30:** 541-548.

Leon, J., Rojo, E. and Sanchez-Serrano, J.J. (2001). Wound signalling in plants. Journal of Experimental Botany **52:** 1-9.

Lim, L. and Howlett, B.J. (1994). Linear plasmids, pLm9 and pLm10, can be isolated from the phytopathogenic ascomycete *Leptosphaeria maculans* by pulsed-field gel electrophoresis. Current Genetics **26:** 276-80.

Ludwig-Müller, J., Pieper, K., Ruppel, M., Cohen, J.D., Epstein, E., Kiddle, G. and Bennett, R. (1999). Indole glucosinolate and auxin biosynthesis in *Arabidopsis thaliana* (L.) Heynh. glucosinolate mutants and the development of clubroot disease. Planta **208:** 409-419.

McGee, D.C. and Petrie, G.A. (1978). Variability of *Leptosphaeria maculnas* in relation to blackleg of oilseed rape. Phytopathology **68:** 625-630.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998). *Arabidopsis thaliana*: a model plant for genome analysis. Science **282:** 662-682.

Mendham, N.J. and Salisbury, P.A. (1995). Physiology: Crop development, growth and yield. In: *Brassica Oilseeds*. (eds.) D.S. Kimber and D.I. Mcgregor. CAB International, Wallingford, pp 11-64.

Murphy, A.M., Holcombe, L.J. and Carr, J.P. (2000). Characteristics of salicylic acid-induced delay in disease caused by a necrotrophic fungal pathogen in tobacco. Physiological and Molecular Plant Pathology **57:** 47-54.

Ndimande, B. (1976). Studies on *Phoma Lingam* (Tode ex Fr.) Desm. and the dry rot on oil seed rape, *Brassica napus* (l.) var. oleifera metzger. Thesis, Plant Pathology and Entomology, SLU. Uppsala

Norman-Setterblad, C., Vidal, S. and Palva, E.T. (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia carotovora*. Molecular Plant-Microbe Interactions **13:** 430-438.

Nottingham Arabidopsis Stock Centre (2001). http://nasc.nott.ac.uk/, Plant Science Division, School of Biosciences, University of Nottingham.

Pedras, M.S.C. and Biesenthal, C.J. (1998). Production of the host-selective phytotoxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. Canadian Journal of Microbiology **44:** 547-553.

Pedras, M.S.C., Khan, A.Q. and Taylor, J.L. (1998). The phytoalexin camalexin is not metabolized by *Phoma lingam*, *Alternaria brassicae*, or phytopathogenic bacteria. Plant Science **139:** 1-8.

Pedras, M.S.C., Okanga, F.I., Zaharia, I.L. and Khan, A.Q. (2000). Phytoalexins from crucifers: Synthesis, biosynthesis, and biotransformation. Phytochemistry **53:** 161-176.

Pelletier, G., Primard, C., Vedel, F., Chetrit, P., Remy, R., Rouselle and Renard, M. (1983). Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Molecular and General Genetics **191:** 244-250.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10:** 2103-2113.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B. and Mundy, J. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103:** 1111-1120.

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Leon-Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P.A.H.M. and Van Loon, L.C. (2001). Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. European Journal of Plant Pathology **107:** 51-61.

Pilet, M.L., Delourme, R., Foisset, N. and Renard, M. (1998a). Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in winter rapeseed (*Brassica napus* L.). Theoretical and Applied Genetics **96:** 23-30.

Pilet, M.L., Delourme, R., Foisset, N. and Renard, M. (1998b). Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). Theoretical and Applied Genetics **97:** 398-406.

Pilet, M.L., Duplan, G., Archipiano, M., Barret, P., Baron, C., Horvais, R., Tanguy, X., Lucas, M.O., Renard, M. and Delourme, R. (2001). Stability of QTL for field resistance to blackleg across two genetic backgrounds in oilseed rape. Crop Science **41:** 197-205.

Plotnikova, J.M., Rahme, L.G. and Ausubel, F.M. (2000). Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in Arabidopsis. Plant Physiology **124:** 1766-1774.

Pongam, P., Osborn, T.C. and Williams, P.H. (1998). Genetic analysis and identification of amplified fragment length polymorphism markers linked to the *alm1* avirulence gene of *Leptosphaeria maculans*. Phytopathology **88:** 1068-1072.

Pouzet, A. (1995). Agronomy. In: *Brassica Oilseeds*. (eds.). D.S. Kimber and D.I. Mcgregor. CAB international, Wallingford, pp 65-92.

Prakash, S., Takahata, Y., Kirti, P.B. and Chopra, V.L. (1999). Cytogenetics. In: *Biology of Brassica coenospecies*. (ed.) C. Gómez-Campo. Elsevier, Amsterdam, pp 59-106.

Purwantara, A., Salisbury, P.A., Burton, W.A. and Howlett (1998). Reaction of *Brassica juncea* (Indian mustard) lines to Australian isolates of *Leptosphaeria maculans* under glasshouse and field conditions. European Journal of Plant Pathology **104:** 895-902.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in *Brassicaceae.* Plant Molecular Biology **42:** 93-113.

Rasmussen, U., Giese, H. and Mikkelsen, J.D. (1992). Induction and purification of chitinase in *Brassica napus* L. ssp. *oleifera* infected with *Phoma lingam*. Planta **187:** 328-334.

Rausher, M.D. (2001). Co-evolution and plant resistance to natural enemies. Nature **411:** 857-864.

Reddy, P.V., Patel, R. and White, J.F.J. (1999). Phylogenetic and developmental evidence supporting reclassification of cruciferous pathogens *Phoma lingam* and *Phoma wasbie* in *Plenodomus.* Canadian Journal of Botany **76:** 1916-1922.

Rimmer, S.R. and Buchwaldt, L. (1995). Diseases. In: *Brassica Oilseeds.* (eds.) D.S. Kimber and D.I. Mcgregor. CAB International, Wallingford, pp 65-92.

Rimmer, S.R. and Van den Berg, C.G.J. (1992). Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans.* Canadian Journal of Plant Pathology **14:** 56-66.

Rogers, E.E. and Ausubel, F.M. (1997). Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in PR-1 gene expression. Plant Cell **9:** 305-316.

Rogers, E.E., Glazebrook, J. and Ausubel, F.M. (1996). Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in Arabidopsis-pathogen interactions. Molecular Plant-Microbe Interactions **9:** 748-757.

Roussel, S., Nicole, M., Lopez, F., Ricci, P., Geiger, J.-P., Renard, M. and Brun, H. (1999). *Leptosphaeria maculans* and cryptogein induce similar vascular responses in tissues undergoing the hypersensitive reaction in *Brassica napus.* Plant Science **144:** 17-28.

Roy, N.N. (1984). Interspecific transfer of *Brassica juncea* - type high blackleg resistance to *Brassica napus.* Euphytica **33:** 295-303.

Rustérucci, C., Aviv, D.H., Holt, B.F.I., Dangl, J.L. and Parker, J.E. (2001). The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in Arabidopsis. Plant Cell **13:** 2211-2224.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings National Academy of Sciences U.S.A **97:** 11655-11660.

Schweizer, P., Felix, G., Buchala, A., Müller, C. and Métraux, J.-P. (1996). Perception of free cutin monomers by plant cells. Plant Journal **10:** 331-341.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S. and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proceedings National Academy of Sciences U.S.A **98:** 4788-4793.

Sexton, A.C. and Howlett, B.J. (2001). Green fluorescent protein as a reporter in the *Brassica-Leptosphaeria maculans* interaction. Physiological and Molecular Plant Pathology **58:** 13-21.

Shoemaker, R.A. and Brun, H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans.* Canadian Journal of Botany **79:** 412-419.

Sigareva, M.A. and Earle, E.D. (1999). Camalexin induction in intertribal somatic hybrids between *Camelina sativa* and rapid-cycling *Brassica oleracea.* Theoretical and Applied Genetics **98:** 164-170.

Sjödin, C. and Glimelius, K. (1988). Screening for resistanace to *Phoma lingam* (Tode ex Fr.)Desm. within *Brassicaceae.* Journal of Plant Phytopathology 123: 322-332

Soledade, M., Pedras, C. and Khan, A.Q. (2000). Biotransformation of hte phytoalexin camalexin by the phytopathogen *Rhizoctonia solani*. Phytochemistry **53:** 56-69.

Somda, I., Delourme, R., Renard, M. and Brun, H. (1999). Pathogenicity of *Leptosphaeria maculans* isolates on a *Brassica napus-B. juncea* recombinant line. Phytopathology **89:** 169-175.

Stintzi, A. and Browse, J. (2000). The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proceedings National Academy of Sciences U.S.A **97:** 10625-10630.

Stintzi, A., Weber, H., Reymond, P., Browse, J. and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proceedings National Academy of Sciences U.S.A **98:** 12837-12842

Svennson, C. and Lerenius, C. (1987). An investigation on the effect of Verticillium wilt (*Verticillium dahliae* Kleb.) on oilseed rape. International organization for Biological control Bulletin **10:** 30-34.

Svensk Raps AB (2001). Arealer av oljeväxter och lin 2001. Länsvis samt totalt. http://www.svenskraps.se/oljevaxt/arealer_oljevaxter.asp.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, A.P.B. and Brokaert, F.W. (1995). Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 7**:** 573-588.

Tewari, J.P. and Mithen, R.F. (1999). Diseases. In: *Biology of Brassica coenospecies*. (ed.) C. Gómez-Campo. Elsevier Amsterdam, pp. 309-411.

The Arabidopsis genome initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796-815.

Thomma, B.P.H.J. and Broekaert, W.F. (1998). Tissue-specific expression of plant defensin genes *PDF2.1* and *PDF2.2* in *Arabidopsis thaliana*. Plant Physiology and Biochemistry **36:** 533-537.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proceedings National Academy of Sciences U.S.A **95:** 15107-15111.

Thomma, B.P., Eggermont, K., Tierens, K.F. and Broekaert, W.F. (1999a). Requirement of a functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiology **121:** 1093-102.

Thomma, B.P.H.J., Nelissen, I., Eggermont, K. and Broekaert, W.F. (1999b). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant Journal **19:** 163-171.

Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F. and Cammue, B.P.A. (2000). Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonate. Plant Physiology and Biochemistry **38:** 421-427.

Tierens, K.F., Thomma, B.P., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B.P. and Broekaert, W.F. (2001). Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. Plant Physiology **125:** 1688-1699.

Tsuji, J., Jackson, E.P., Douglas, A.G., Hammerscmidt, R. and Somerville, S.C. (1992). Phytoalexin Accumulation in *Arabidopsis thaliana* during the Hypersensitive Reaction to *Pseudomonas syringae* pv *syringae*. Plant Physiology **98:** 1304-1309.

U, N. (1935). Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Japan Journal of Botany **7:** 389-452.

Uknes, S., Mauch Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell. **4:** 645-656.

van Loon, L.C. and van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology **55:** 85-97.

van Poppel, G., Verhoeven, D.T., Verhagen, H. and Goldbohm, R.A. (1999). Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Advances in Experimental and Medical Biology **472:** 159-68.

van Wees, S.C., de Swart, E.A., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proceedings National Academy of Sciences U.S.A **97:** 8711-8716.

Verhoeven, D.T., Goldbohm, R.A., van Poppel, G., Verhagen, H. and van den Brandt, P.A. (1996). Epidemiological studies on brassica vegetables and cancer risk. Cancer epidemiology, biomarkers and prevention **5:** 733-748.

Wallenhammar, A. and Sjöberg, A. (2001). Agartest – ett enkelt hjälpmedel vid riskbedömning av bomullsmögel i oljeväxter. Svensk Frötidning **3:** 8-9.

Walling, L.L. (2000). The myriad plant responses to herbivores. Journal of Plant Growth Regulation **19:** 195-216.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999). Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans)* disease in transgenic canola (*Brassica napus*). Molecular Plant-Microbe Interactions **12:** 410-418.

Warren, R.F., Merritt, P.M., Holub, E. and Innes, R.W. (1999). Identification of three putative signal transduction genes involved in *R* gene-specified disease resistance in Arabidopsis. Genetics **152:** 401-412.

West, J.S., Kharbanda, P.D., Barbetti, M.J. and Fitt, B.D.L. (2001). Epidemiology and management of *Leptosphaeria maculans* (*phoma* stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathology **50:** 10-27.

Winge, P., Brembu, T., Kristensen, R. and Bones, A.M. (2000). Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. Genetics **156:** 1959-1971.

Wretblad, S. and Dixelius, C. (2000). B-genome derived resistance to *Leptosphaeria maculans* in near isogenic *Brassica napus* lines is independent of glucosinolate profile. Physiologia Plantarum **110:** 461-468.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998). *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091-1094.

Yu, I.C., Parker, J. and Bent, A.F. (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis *dnd1* mutant. Proceedings National Academy of Sciences U.S.A **95:** 7819-7824.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F. and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell **10:** 1021-1030.

Zhou, N., Tootle, T.L. and Glazebrook, J. (1999). Arabidopsis *PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. Plant Cell **11:** 2419-2428.

Acknowledgements

I would like to express my gratitude to everyone that has contributed to this thesis.

First and foremost to my supervisor, Christina Dixelius, for your complete and continuous support.

To the present and past members of Christina's research group. Gunilla Swärdh for your good mode, that always cheered me up. Sofia Wretblad, for good company, especially on our research travels. Lisa Butterworth, thanks for liven up the atmosphere at work and for good Salsa company. Janne Fahlesson, for good advises on scientific writing. Qiong Hu, Lecong Zhou, and Patrick Okori for good company, at any strange time in the lab.

To my Professor Kristina Glimelius, for help and support, always when I asked for it, and to all present and past members of the Glimelius lab for making the lab such a good place to work. Marie, thank you for being such a good room mate during these years.

All members of the research school on Plant and Forest Biotechnology, for providing an invaluable network, and sharing many good times all over Sweden. Good waffles! Thank you Marianne for organizing it all.

To all, present and past members, of the Genetic centre for providing such a good atmosphere. Special thanks to the sport group for helping me keep fit enough to finish this thesis and to the F.S.E people for helping me forget it. Many thanks also, to the foreign squad, Gosia, Mathieu, and Vasili for giving a good perspective on the Swedish peculiarities.

Many thanks, finally to my friends and family, for inspiring me to start and to finish this thesis.