Defence Responses in *Brassica nigra* and *B. napus* to the Fungal Pathogen *Leptosphaeria maculans*

Sofia Wretblad
Department of Plant Biology
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2002

Acta Universitatis Agriculturae Sueciae
Agraria 316

ISSN 1401-6249
ISBN 91-576-5843-9

Tryck: SLU Service/Repro, Uppsala 2002

“Grundforskning är vad jag gör när jag inte vet vad jag gör”
Wernher von Braun

Abstract

Wretblad, S. 2002. Defence responses in *Brassica nigra* and *B. napus* to the fungal pathogen *Leptosphaeria maculans*.
Doctor's dissertation.
ISSN 1401-6249, ISBN 91-576-5843-9

The fungal pathogen *Leptosphaeria maculans* causes blackleg disease on oilseed rape (*Brassica napus*). It attacks leaves, cotyledons, stems and pods, and is one of the main threats to oilseed rape production world wide. Field resistant cultivars are available, but the rapid evolution of new and more virulent *L. maculans* isolates demands a more efficient use of resistance sources. *Brassica* species containing the B-genome (i. e. *B. nigra, B. juncea* and *B. carinata*) are resistant to blackleg, and it is of interest to gain more knowledge about the underlying defence mechanisms. This thesis focuses on different aspects of B-genome derived resistance to *L. maculans*. At the genomic level, the location and organisation of loci conferring resistance to *L. maculans* in the B-genome was studied. At the metabolic level, the role of the glucosinolate-myrosinase system was studied, and at the sequence level a potential resistance gene was cloned and characterised.

The results from the mapping study showed that loci conferring blackleg resistance were maintained in a triplicated region, earlier designated B, on linkage groups 2, 5, and 8 of the B-genome. Cotyledon and adult-leaf resistance segregated as different loci in all three B-genome species, and may be governed by different mechanisms. The glucosinolate-myrosinase system is found mainly in cruciferous plants, and has been proposed as a mediator of defence against *L. maculans*. We studied resistance levels and glucosinolate profiles of 23 near isogenic lines derived from hybrids between *B. napus* and *B. nigra*, and found no correlation between resistance and glucosinolate content. The levels of indolylic glucosinolates were affected 5-8 days after inoculation with *L. maculans*, but it was observed in both resistant and susceptible plants. This response may represent a defence mechanism that the *Brassica* specialist *L. maculans* has evolved to overcome. Other components of the glucosinolate-myrosinase system were also studied in detail, and there were no indications that this system is responsible for blackleg resistance. Finally, a gene conferring resistance to *L. maculans (Lm1)* was cloned from *B. nigra*, using a PCR-based strategy. *B. napus* plants transformed with *Lm1* exhibited increased resistance to four different isolates of *L. maculans* in both cotyledons and adult leaves. *Lm1* encodes a novel protein with two putative transmembrane motifs. Related genes of unknown function were found in *Arabidopsis thaliana* and rice (*Oryza sativa*), and there was similarity to the symbiotic *nin* gene from *Lotus japonicus*. The exact function of *Lm1* remains to be elucidated.

Key words: blackleg, black mustard, B-genome, glucosinolate, *Lm1*, myrosinase, oilseed rape, *Phoma lingam*, resistance.

Author's address: Sofia Wretblad, Department of Plant Biology, Uppsala Genetics Centre, Swedish University of Agricultural Sciences, Box 7080, SE-750 07, Uppsala, Sweden. E-mail: Sofia.Wretblad@vbiol.slu.se

Svensk sammanfattning

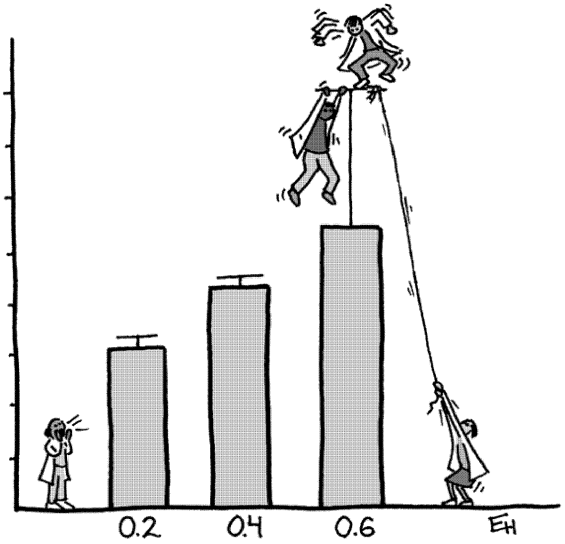
Raps *(Brassica napus)* odlas i stora delar av världen och är en av våra viktigaste grödor för produktion av vegetabilisk olja. Ett av de största problemen inom oljeväxtodlingen är svampsjukdomar, som kan leda till stora skördeförluster. Svampen *Leptosphaeria maculans* orsakar torröta och angriper blad, fröskidor och stjälkar. Sjukdomen har sedan 50-talet setts som ett allvarligt hot mot en lönsam rapsodling, men tack vare viss framgång i förädlingen av motståndskraftiga rapssorter har förlusterna begränsats. Svampen har dock visat sig ha mycket lätt för att anpassa sig till nya förhållanden, och nyligen har det rapporterats att den resistens som tidigare funnits inte längre är lika effektiv. Det är nu av yttersta vikt att undersöka de mekanismer som växten använder sig av för att försvara sig mot svampen, och på den vägen finna nya resistenskällor. I min forskning har jag framför allt studerat molekylära mekanismer i svartsenap *(Brassica nigra)*. Svartsenap är nära besläktad med raps, men innehåller det sk B-genomet och är helt resistent mot torröta. Jag har tittat på olika aspekter av denna resistens.

I en av studierna har vi undersökt var i B-genomet man hittar de gener som kodar för resistensegenskaperna. Detta kan vara intressant ur förädlingssynpunkt om man vill överföra endast de regioner där resistensgenerna är lokaliserade till en ny linje, och undvika andra, ofördelaktiga anlag. Det är även intressant ur grundforskningssynpunkt, eftersom placeringen av dessa gener kan ge information om hur de uppkommit. Vår forskning har visat att de olika resistensgenerna sitter nära varandra och har bevarats under evolutionen i en specific region som finns på tre olika ställen i B-genomet.

Den växtfamilj som både raps och svartsenap tillhör producerar en unik typ av försvarsmolekyler som kallas glukosinolater. Glukosinolaternas roll i försvar mot just torröta har länge debatterats, men då svartsenap innehåller höga halter av en glukosinolat (sinigrin) som saknas i raps, och som dessutom har hämmande inverkan på många svampar, har det föreslagits att det är denna som orsakar resistensen. Vi har studerat detta i återkorsade hybrider mellan raps och svartsenap och visat att halten sinigrin inte har något samband med resistensen. Däremot sker andra förändringar i glukosinolat-sammansättningen efter en påbörjad svampinfektion, men dessa sker både i mottagliga och resistenta växter. Av detta drar vi slutsatsen att *L. maculans*, som är specialist på glukosinolat-producerande växter, har anpassat sig till dessa förhållanden och kan motverka glukosinolaternas effekt. Då glukosinolater även kan ha negativa effekter på människor och djur har man haft som ett förädlingsmål att minska halterna av dessa ämnen i grödan. Det kan då vara viktigt att veta att torröte-resistensen inte försämras som ett resultat av detta. Däremot kan det eventuellt påverka motståndskraften mot mindre specialiserade svampar.

Slutligen har vi klonat och karaktäristerat en specifik gen *(Lm1)* från svartsenap som är viktig för växtens försvar mot torröta. Vi har överfört genen till raps och visat att den ger ökad resistens mot flera olika varianter av *L. maculans*, i olika vävnader i växten. Detta är en mycket värdefull egenskap om man vill utveckla sorter med hållbar resistens. Vi har undersökt genens DNA-sekvens och sett att den skiljer sig i strukturen från alla tidigare kända resistensgener. Däremot visade det sig finnas ett flertal ”släktingar” till denna gen i andra arter. De flesta av dessa har fortfarande okänd funktion, men en av dem är involverad i symbios. Detta är intressant, eftersom symbios och försvar egentligen har motsatt verkan, men uppenbarligen kan ha vissa gemensamma komponenter. Vi arbetar fortfarande med att undersöka vilken roll *Lm1* har i interaktionen mellan svartsenap och *L. maculans*.

Contents



Nice try people, but we're just going to have to repeat the experiment.

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to as their Roman numerals.

I. Dixelius C., and Wahlberg, S. (1999) Resistance to *Leptosphaeria maculans* is conserved in a specific region of the *Brassica* B genome. *Theoretical and Applied Genetics* 99, 368-372

II. 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

III. Andréasson E., Wretblad S., Granér G., Wu X., Zhang J., Dixelius C., Rask L., and Meijer J. (2001) The myrosinase-glucosinolate system in the interaction between *Leptosphaeria maculans* and *Brassica napus*. *Molecular Plant Pathology* 2, 281-286

IV. Wretblad S., Bohman S., and Dixelius C. (2002) The *Lm1* gene of *Brassica nigra* confers resistance to the blackleg fungus *Leptosphaeria maculans* (submitted)

Papers I, II, and III were reprinted with permission from the publishers.
Before 2000, the author's surname was Wahlberg.

Abbreviations

List of selected abbreviations commonly used in the text:

CC	coiled coil
ET	ethylene
HR	hypersensitive response
IAA	indole-3-acetic acid
JA	jasmonic acid
LRR	leucine rich repeat
LZ	leucine zipper
MBP	myrosinase binding protein
NBS	nucleotide binding site
PR	pathogenesis related
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
TIR	Toll/Interleukin receptor

Introduction

Plant diseases are a constant threat to agriculture, and throughout history they have been feared as much as human diseases and war. In early times, plant diseases were considered a punishment from God for peoples wrongs and sins, and they are mentioned in some of the oldest literature available (Old Testament, 750 B. C.). However, the first clues to the nature of plant pathology came around 300 B. C., when the Greek philosopher Teophrastus observed that plant diseases were more severe in the lowlands. Nobody believed, of course, that farmers in the lowlands sinned more than those on living on the hills, so he correctly blamed it on the rainy weather (but still argued that God was somehow behind it). This fatalistic view on plant diseases would sustain until the invention of the microscope in the mid-1600s, when people observed micro-organisms associated with diseased tissues. The real breakthrough for modern plant pathology came as a results of the Irish famine in the 1840s. The devastating epidemics of potato late blight stimulated interest in the cause and control of plant diseases. In 1861, the German scientist DeBary provided final proof that the fungus *Phytophtora infestans* was the cause of the late blight disease, and not just a product of it.

Crop protection is of tremendous importance in today's intensive agricultural systems. The development of agro-chemicals during the last 100 years has contributed significantly to decrease crop losses, but still one third of the potential world crop production each year is lost to diseases, insects and weeds (Agrios, 1997). Protective chemicals are effective, but a cause of much debate, as they are costly and can be harmful to the environment. An alternative to pesticides is to improve crop resistance to pathogens by breeding. For the past century, it has been known that some plants possess genetically inherited resistance mechanisms to combat pathogens, and breeders have utilised the natural variation to develop resistant cultivars by crossing. Recent advances in plant biotechnology have opened up further possibilities, as we are now able to transfer DNA between completely unrelated species. Moreover, gene technology and the use of model organisms have provided the tools to study the complex interactions between plants and pathogens at a molecular level. Understanding the mechanisms behind plant defence is crucial for the future development of crops with durable resistance to plant diseases.

This thesis deals with defence responses in *Brassica* species to the blackleg fungus *Leptosphaeria maculans*. Blackleg is becoming an increasing problem, since the existing resistance is on the verge of being broken down by the fungus. In the following introduction, I will shortly summarise the area of plant defence, focusing on plant resistance genes and on the glucosinolate-myrosinase system. I will also introduce the two participants in this plant-pathogen interaction.

An outline of plant defence systems

Plants are stationary organisms. They cannot move away from environmental stresses, and therefore need extremely efficient defence systems. During its lifecycle, a plant is exposed to a large number of potentially pathogenic organisms, but only a small fraction of these actually cause disease. Broadly, there are three reasons why a pathogen fails to invade the plant. Either, (1) the plant does not provide a suitable niche environment and is thus a non-host; or (2) the pathogen is unable to penetrate the preformed physical barriers or survive the toxic compounds produced by the plant; or (3) the plant recognises the invasion at an early stage and mounts specific defences designed to suppress the pathogen in question.

The main physical obstacle to pathogen attack is the waxy cuticular layer. If that is conquered, an array of toxic compounds await the pathogen. The basal chemical defence system of a plant is mainly composed of secondary metabolites with antimicrobial activity, like phytoalexins, saponins, cyanogenic glucosides or glucosinolates. The majority of these compounds are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways (reviewed in Dixon, 2001). They can either be preformed, or induced by pathogen attack or other environmental stresses.

Recognition

Any induced defence response depends on the ability of the plant to perceive the attack, recognise the invader and through signalling events activate the appropriate response. Recognition is based on pathogen-derived molecules, which can be either race-specific or general elicitors. General elicitors are often macro-molecules, like carbohydrates, glycoproteins or lipids, originating either from the pathogen itself or from the host plant as a result of attack (Benhamou, 1996). One example is the oligogalacturonides (OGAs), which are released from plant cell walls upon cell damage and have been shown to induce reactive oxygen species (ROS), phytoalexins, cell wall strengthening and expression of proteinase inhibitors (reviewed in Ridley *et al*, 2001).

The gene-for-gene concept

Race-specific recognition is governed by pair wise interactions between the products of plant resistance (*R*) genes and pathogen avirulence *(avr)* genes. This concept is termed the gene-for-gene hypothesis, and was proposed by Flor already in the 1940's (Flor, 1947). The function of a given *R*-gene is dependent on the genotype of the pathogen; that it carries the corresponding *avr*-gene. Conversely, a pathogen gene is called an *avr*-gene if its expression leads to specific recognition and triggers a defence response in the plant. When either of the genes are inactive or absent, the defence systems will remain inactive and

disease results. During the last decade, intense research has resulted in the identification of many *R*-genes from different plant species (reviewed in Jones, 2000). They encode resistance to a wide variety of pathogens, but their structures are to a large extent conserved. Details on the structure and function of *R*-genes are discussed separately.

Early events in plant defence

Following recognition, the earliest cellular responses are changes in ion fluxes, particularly calcium influx, which occur within minutes of elicitation (Jabs *et al.*, 1997). Subsequently, reactive oxygen species (ROS) are produced and mitogen-activated protein kinase pathways are activated (Ligterink *et al.*, 1997). The exact role of ROS is unclear, but they may be involved either in direct pathogen elimination, signal transmission or both. Within 15 minutes, transcripts encoding downstream signalling molecules appear. The activation of these defence genes results in cell-wall reinforcement by deposition of callose and lignin, and a hypersensitive response (HR) is induced to keep the infection localized. HR involves a form of programmed cell death and causes a lesion around the infection site (reviewed in Lam *et al.*, 2001). This effectively restricts the further spread of biotrophic pathogens. In addition to cell death, pathways are activated which lead to the production of various antimicrobial compounds, and the induction of pathogenesis-related (PR) proteins such as chitinases and glucanases. It is currently unclear whether resistance is due to the cell death *per se*, or if cell death is a consequence of the other numerous defence responses activated.

Systemic acquired resistance

As a result of pathogen recognition, systemic acquired resistance (SAR) is also induced to protect distal parts of the plant. SAR refers to a distinct signal process which leads to long-lasting broad spectrum resistance which is effective against a range of pathogens and can last for several weeks. SAR is accompanied by local and systemic accumulation of salicylic acid (SA), and the expression of a set of specific SAR-genes (Uknes *et al.*, 1992; reviewed in Mauch-Mani and Metraux, 1998). SA and H_2O_2 are the only two molecules known, so far, to have a role in the onset and maintenance of SAR. However, grafting experiments using *nahG* transgenic plants which cannot accumulate SA, have shown that SA is necessary for the manifestation of SAR in systemic tissue, but not required at the site of initial infection (Vernooij *et al.*, 1994). At present the exact mechanisms behind SAR are unknown.

Defence signalling

Plant pathogens can be either biotrophic or necrotrophic. Biotrophs harvest nutrients from living cells, while necrotrophs kill the host and feed on its

contents, and the plant has to adapt its defence responses according to these fundamentally different strategies. To induce the appropriate responses for each pathogen, plants have evolved at least three different signalling pathways. Generally, the SA-dependent pathway regulates resistance to biotrophic pathogens, while the jasmonic acid (JA) and ethylene (ET) dependent pathways mainly regulate defence against necrotrophic pathogens (Thomma *et al.*, 1998). However, this kind of division is not definitive, as there is evidence for a role of SA in resistance to necrotrophs as well (Murphy *et al.*, 2000; Norman-Setterblad *et al.*, 2000). There is also extensive cross-talk between these different signal transduction networks (reviewed in Maleck and Dietrich, 1999). Many, but not all, JA/ET responses limit SA signalling and vice versa. Additionally, several lesion-mimic mutants display constitutively high expression of both PR-1, which is a marker for SA-dependent signalling, and of PDF1.2, which is a marker for JA/ET signalling (Glazebrook, 2001). This suggests that the pathways share common activating signals. JA/ET signalling also mediates induced systemic resistance (ISR). This phenomenon is similar to SAR, but is induced by selected strains of rhizosphere bacteria (reviewed in Pieterse *et al.*, 2001). Extensive research on defence signalling has unravelled very complex networks, and the use of *Arabidopsis* signalling mutants has been crucial for this work (reviewed in Glazebrook, 2001).

Effector molecules

A number of pathogenesis-related (PR) proteins that are induced as a result of defence signalling have been identified (reviewed in van Loon and van Strien, 1999). Presently, they are divided into fourteen families, although not all families have been found in all plant species examined. Different subsets of PR-proteins are activated by different pathways. As the name implies, PR-proteins were initially defined as proteins synthesized by the host only under pathological or related situations. They constitute a very heterogeneous group, and several of the members have so far unknown functions. One such example is PR-1, which is frequently used as a marker of SA dependent signalling but still has no defined function. Examples of proteins induced by JA/ET signalling are chitinases (PR3, PR4), defensins (PR12) and thionins (PR13). Over expression studies of these groups of proteins have shown that they directly effect resistance. Glucanases and chitinases are thought to exert their antimicrobial function by hydrolysing components of fungal cell walls. Other effectors that can be induced through specific recognition and signalling are toxic secondary metabolites like phytoalexins and glucosinolates. The role of glucosinolates in plant defence will be discussed separately.

Plant resistance genes

According to the gene-for-gene hypothesis, race specific resistance results from the direct or indirect interaction between a plant *R*-gene product, and its corresponding pathogen *avr*-gene. *Avr*-genes represent a structurally very heterologous group, which have nothing in common other than the fact that their presence triggers plant signals leading to race-specific resistance. In contrast, *R*-genes from diverse plant species with specificity for a wide variety of viral, bacterial or fungal pathogens often encode structurally similar proteins.

R-gene structures

Despite the wide range of pathogenicity molecules, *R*-genes encode only five classes of proteins divided on the basis of six known functional domains (reviewed in Bent, 1996; Dangl and Jones, 2001) (Fig. 1). The leucine-rich repeat (LRR) domain contains multiple serial repeats of leucines or other hydrophobic residues. LRRs are found in diverse eukaryotic proteins and function in protein-protein interactions, peptide-ligand binding and protein-carbohydrate interactions. LRR domains can be either cytoplasmic or extracytoplasmic, and are thought to play a significant role in the specificity of *R*-genes (Jones and Jones, 1997). Serine-threonine kinase domains are found also in several R-proteins where they are thought to modulate activation of signal transduction cascades through phosphorylation. Many resistance genes also encode nucleotide binding sites (NBS). Such domains occur in diverse proteins with ATP- or GTP-binding activity, suggesting that nucleotide triphosphate binding is essential for the function of these proteins. An interesting parallel to animal immunology is the presence of Toll-Interleukin Receptor (TIR) domains in some plant *R*-genes. Both the mammalian Interleukin-1 receptor (IL-1R) and the *Drosophila* Toll receptor trigger activation of a transcription factor (Kuno and Matsushima, 1994; Morisato and Anderson, 1995), and it is hypothesised that the related plant resistance genes work through a similar mechanism. The transcription factor activated by IL-1R stimulates production of active oxygen, and its activity is modulated by salicylic acid compounds, further connecting plant and animal responses (Bent, 1996). Additionally, the Toll protein interacts with the protein kinase Pelle, which has significant similarity to the *Pto* *R*-gene of tomato (Morisato and Anderson, 1995). Another *R*-gene domain is the leucine zipper (LZ), a consensus heptad repeat sequence which facilitates protein-protein interactions by promoting coiled-coil (CC) structures.

R-gene classes

The largest class of *R*-genes is the cytoplasmic NBS-LRR class. They seem to be specifically evolved as resistance genes and are highly adapted for this purpose. Their most striking feature is the variable number of carboxy-terminal LRRs, and

each protein of this class has a conserved NBS. The NBS-LRR class can be subdivided on the basis of N-terminal features; some have TIR domains while others have CC domains. The four remaining *R*-gene classes are more structurally diverse, and some members have even demonstrated functions in processes unrelated to plant defence. The *R*-gene class of serine/threonine kinases so far only consists of the tomato *Pto* gene (Martin *et al.*, 1993), which confers resistance to *Pseudomonas syringae*. Pto interacts with avrPto, but requires the presence of the NBS-LRR protein Prf for its function (Salmeron *et al.*, 1996). The *Xa21* gene, which confers resistance to *Xanthomonas oryzae* in rice, represents another class which encodes a transmembrane receptor carrying a large extracellular LRR domain and a cytoplasmic protein kinase domain (Song *et al.*, 1995). The *Cf-2, 4, 5* and *9* genes of tomato, mediating resistance to *Cladosporium fulvum* (de Wit and Joosten, 1999), have a structure similar to *Xa21*, but lack the kinase domain. It has been hypothesised that race-specific recognition resides within the LRR domain, but that LRR and kinase domains within a protein or from two different proteins often function together in signal transduction pathways (Dangl and Jones, 2001). Very recently, the first TIR-NBS-LRR gene containing a transcription factor domain was cloned (Deslandes *et al.*, 2002). It confers resistance to several strains of *Ralstonia solanacearum* in *A. thaliana* and was named *RRS1-R*. Other variants of the NBS-LRR class are *Ve1* and *Ve2* which confer resistance to *Verticillium dahlie* in tomato (Kawchuk *et al.*, 2001). They have a structure similar to the *Cf* genes, but has an N-terminal signals for receptor-mediated endocytosis.

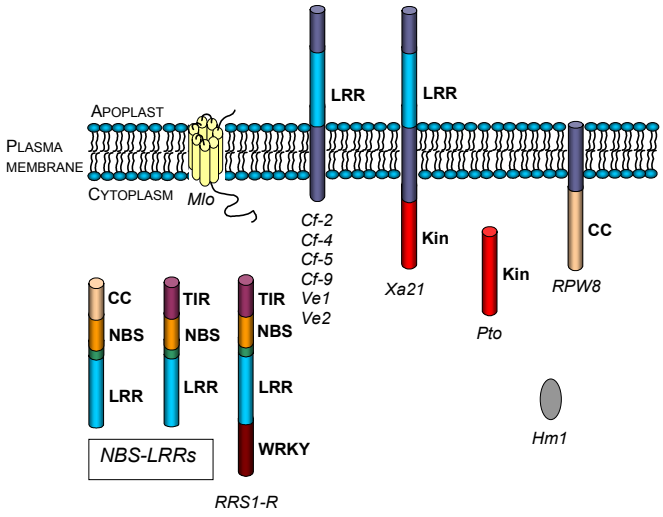


Figure 1. Schematic representation of the location and structure of the main classes of plant disease resistance proteins. CC = coiled coil, TIR = Toll/Interleukin receptor NBS = nucleotide binding site, LRR = leucine rich repeat, WRKY = WRKY transcription factor motif, Kin = kinase domain.

Non-typical R-genes

R-genes which lack the typical structural elements of the groupings described above have also been identified. The first *R*-gene to be cloned was *Hm1* from maize which bestows resistance to the fungus *Cochliobolus carbonum* (Johal and Briggs, 1992). This gene controls race specific resistance by encoding a reductase which inactivates the fungal HC toxin responsible for causing the disease. The recently cloned *RPW8* confers broad spectrum powdery mildew resistance in *Arabidopsis*. It encodes a putative membrane protein with no homology to other *R*-genes (Xiao *et al.*, 2001). In addition, the barley *Mlo* locus is involved in broad spectrum resistance to *Erisyphe graminis* and is predicted to be anchored in the membrane by at least six membrane-spanning helices (Büschges *et al.*, 1997).

Genomic organisation of *R*-genes

NBS-LRR sequences are relatively common in plant genomes. After completion of the *Arabidopsis* genome sequence, about 150 sequences with homology to this class were annotated (The *Arabidopsis* Genome Initiative, 2000). However, some of these appear to be non-functional pseudogenes. *R*-gene homologues are unevenly distributed in the genome, and often occur in clusters. The *Arabidopsis* genome has 46 singleton *R*-gene loci, 25 doublets, 7 loci with three copies and individual loci with four to nine NBS-LRR genes (Dangl and Jones, 2001). In spite of the high relative representation of these genes, this number is surprisingly small to mediate recognition of the wide variety of pathogens a plant may encounter. This may be explained by several different theories. Firstly, it is possible that many *R*-genes recognise more than one *avr*-gene. This has in fact been observed in several cases. For example, the tomato *Mi* gene confers both nematode and aphid resistance (Rossi *et al.*, 1998), *RPM1* recognises two non-homologous *avr*-genes (Bisgrove *et al.*, 1994), and alleles of the *RPP8/HRT* gene recognise an oomycete parasite and a virus (Cooley *et al.*, 2000). Secondly, it is possible that some ancient *R*-genes recognise very conserved pathogen molecules and thus perceive a broad range of pathogens. Thirdly, the clustered nature of many resistance loci suggests that one locus can evolve to generate an allelic series capable of recognising multiple *avr*-genes.

The guard hypothesis

The simplest interpretation of the gene-for-gene hypothesis would be direct interaction between the products of the *R*-gene and the *avr*-gene, which then activates downstream signalling. However, recent findings imply that this is not always the case (reviewed in Bonas and Lahaye, 2002). For example, the R-proteins of the Cf-family do not seem to interact directly with their corresponding avr-proteins. Furthermore, the kinase Pto needs the NBS-LRR protein Prf to mediate resistance, just as PBS1 and RPS5 function together. As an attempt to explain these findings, the “guard-hypothesis” has been put forward (van der

Biezen and Jones, 1998). In essence, it suggests that in addition to the gene-for-gene pair, guard proteins may exist that detect the coexistence of these two proteins in the cell. The guard protein may bind constitutively to the guardee, and detach only upon interaction with the avr-protein. Thereafter, it becomes active and triggers the defence. Alternatively, the guard only binds when there is an interaction between the R-avr proteins, and the binding activates signalling pathways. There are several possible variants to this hypothesis, but so far there is no experimental evidence to prove the guard-hypothesis. The exact mechanisms downstream of avr recognition are also unclear, although some important loci have been identified through mutant screens. Examples of such loci are *EDS1* and *NDR1* which are each required for the function of separate subsets of NBS-LRR genes (Aarts *et al.*, 1998).

The glucosinolate-myrosinase system

Glucosinolates are the main class of secondary metabolites in cruciferous crops, and they are thought to play a role in plant defence against insects, herbivores and certain microbial pathogens. Glucosinolates are almost exclusively found in the order *Capparales*, and constitute a group of around 115 different compounds. All are variants of a common basic structure, but fall into three different groups based on the nature of the side chain: aliphatic, aromatic or indolylic (reviewed in Chen and Andréasson., 2001; Mithen, 2001). Upon tissue disruption, glucosinolates come in contact with the enzyme myrosinase, which results in the release of numerous compounds with biological activities. Glucosinolate breakdown products are responsible for the characteristic taste and smell of many *Brassicaceae* crops, such as broccoli, cabbage, mustard and radish, and they have been shown to have anticarcinogenic effects (van Poppel *et al.*, 1999). However, there are also antinutritional aspects such as goitrogenic effects on livestock that feed on rapeseed meal (Griffiths *et al.*, 1998).

Despite the large number of existing glucosinolates, each plant species only contains a limited number of compounds, which gives rise to unique species-specific profiles. The major cruciferous crops contain a restricted range of less than a dozen different glucosinolates, while more than thirty have been identified in *A. thaliana* (Hogge *et al.*, 1988). The profile of each species is markedly influenced by developmental stage, tissue specificity and biological or physical stresses. Glucosinolates have been detected in all plant organs, but the capacity of de novo biosynthesis varies (Porter *et al.*, 1991). As many other secondary metabolites, glucosinolates are suggested to be actively transported (Lykkesfeldt and Lindberg-Møller, 1993), and independent studies have indicated long distance transport of glucosinolates from leaf tissue to seeds (Magrath, 1993; Kliebenstein *et al.*, 2001). Recent experiments using radio-labelled glucosinolates in *A. thaliana* have confirmed that this transport occurs via the phloem (Chen and Andréasson, 2001).

Structure and biosynthesis of glucosinolates

Glucosinolates are β-thioglucosides generated from the seven amino acids alanine, valine, leucine, isoleucine, phenylalanine, tyrosine or tryptophan (Fig. 2). The large variation arises from three types of side chain modifications. Firstly, many glucosinolates are chain-elongated forms of the original amino acid. Secondly, after elongation the amino acid may be further modified by, for example, introduction of sulphinyl groups, hydroxylation or methoxylation. Thirdly, relatively complex side chains occur in rare cases, such as glucosinolates containing a sinapoyl moiety. Of these in total 115 glucosinolates, approximately 50% are derived from chain-elongated methionine (aliphatic). About 10% are derived from tryptophan (indolylic) or phenylalanine and tyrosine (aromatic). Most of the remaining glucosinolates are derived from branch-chain amino acids, probably alanine or methionine. Table 1 shows examples of glucosinolates found in *Brassicaceae* crops.

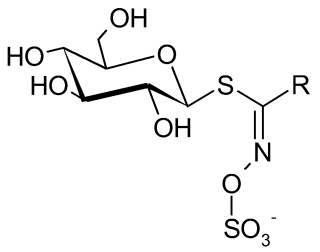


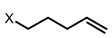
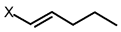
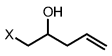
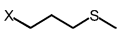
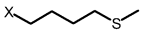
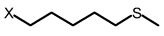
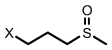
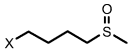
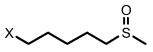
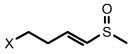
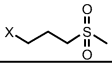
Figure 2. Basic structure of glucosinolates. R = side chain structure, see Table 1.
(Printed with permission from Bo Pontoppidan)

Biosynthesis

The proposed biosynthetic pathway of glucosinolates comprises side-chain elongation of amino acids, conversion of the amino acid into its corresponding aldoxime, and subsequently of the oxime into a basic glucosinolate structure, and lastly secondary chain-modifications (reviewed in Halkier, 1999). Three independent enzyme systems have been shown to catalyse the conversion of amino acids to aldoximes; i.e. cytochrome P450 dependent monooxygenases (Bennet *et al.*, 1997), flavin-containing monooxygenases (Oldfield *et al.*, 1999) and plasma membrane peroxidases (Ludwig-Müller *et al.*, 1990). The intermediate steps of glucosinolate biosynthesis are still somewhat speculative, while the final steps involving S-glycosylation (Reed *et al.*, 1993), (Gou and Poulton, 1994) and sulfation (Glendening and Poulton, 1988) are well elucidated. Recent research, complementing the purely biochemical approach with molecular genetics in *Arabidopsis,* have advanced the understanding of side chain elongation and modification. Aliphatic side chains of different *A. thaliana* ecotypes vary in length and modifications. Campos de Quiros *et al.* (2000) demonstrated that the difference in chain length was due to allelic variation at a

locus on chromosome 5, *GSL-ELONG*. Molecular analysis of this locus showed that it contained two adjacent genes, subsequently termed methylthioalkylmalate (*MAM*) synthases. Functional analysis has confirmed their role in chain elongation, since alterations of their expression alters side-chain lengths in methionine derived glucosinolates. Similarly, genetic analysis of *Arabidopsis* has been used to study genes involved in side chain modification. Two loci, *GSL-OHP* and *GSL-ALK* on chromosome 4, have been identified (Hall *et al.*, 2001). These findings open possibilities for genetic engineering of aliphatic glucosinolate profiles in *Brassica* crops. The indolylic glucosinolate pathways are more difficult to explore using these methods, as no natural variation exists in *Arabidopsis*.

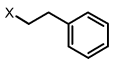
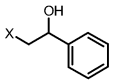
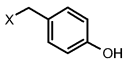
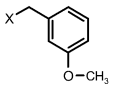
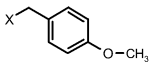
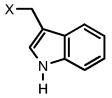
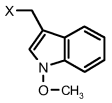
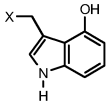
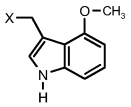
Table 1. Side chain structures of predominant glucosinolates in cultivated *Brassicaceae* (Mithen, 2001). X represents the general structure shown in Fig. 2.
(Printed with permission from Bo Pontoppidan)

Side chain structure	Trivial name	Semisystematic name
	Glucocapparin	Methylglucosinolate
	Sinigrin	2-Propenylglucosinolate / Allylglucosinolate
	Gluconapin	3-Butenylglucosinolate
	Glucobrassicanapin	4-Pentenylglucosinolate
	-	1-Pentenylglucosinolate
	Progoitrin	2(R)-Hydroxy-3-butenylglucosinolate
	Epiprogoitrin	2(S)-Hydroxy-3-butenylglucosinolate
	Napoleiferin	2-Hydroxy-4-pentenylglucosinolate
	-	1-Methylethylglucosinolate
	Glucococh learin	1-Methylpropylglucosinolate
	Glucocleomin	2-Hydroxy-2-methylpropylglucosinolate
	Glucoibeverin	3-(Methylthio)propylglucosinolate
	Glucoerucin	4-(Methylthio)butylglucosinolate
	Glucoberteroin	5-(Methylthio)pentylglucosinolate
	Glucoiberin	3-Methylsulfinylpropylglucosinolate
	Glucoraphanin	4-Methylsulfinylbutylglucosinolate
	Glucoalyssin	5-Methylsulfinylpentylglucosinolate
	Glucoraphenin	4-Methylsulfinyl-3-butenylglucosinolate
	Glucocheirolin	3-Methylsulfonylpropylglucosinolate

Degradation by myrosinase

Glucosinolates are only one part of a two-component pre-formed defence system, which by themselves have limited biological effects. The enzymatic breakdown into biologically active products is catalysed by thioglucosidases with the trivial name myrosinase (EC 3.2.3.1) (reviewed in Rask *et al.*, 2000). The aglucone formed by the enzymatic reaction spontaneously releases the sulphate moiety and rearranges to form either a thiocyanate, nitrile, epithionitrile or oxazolidine-thione depending on the nature of the glucosinolate and the reaction conditions (Fig. 3).

Table 1 continued.

Side chain structure	Trivial name	Semisystematic name
	Glucotropaeolin	Benzylglucosinolate
	Gluconasturtiin	2-Phenylethylglucosinolate
	Glucobararin	2(R)-Hydroxy-2-phenylethylglucosinolate
	Sinalbin	4-Hydroxybenzylglucosinolate
	-	2-Hydroxybenzylglucosinolate
	Glucolimnanthin	3-Methoxybenzylglucosinolate
	Glucoaubrietin	4-Methoxybenzylglucosinolate
	Glucobrassicin	Indol-3-ylmethylglucosinolate
	Neoglucobrassicin	1-Methoxyindol-3-ylmethylglucosinolate
	4-Hydroxyglucobrassicin	4-Hydroxyindol-3-ylmethylglucosinolate
	4-Methoxyglucobrassicin	4-Methoxyindol-3-ylmethylglucosinolate

The mustard-oil bomb

Myrosinases occur in specialised cells named "myrosin cells". The enzyme only comes into contact with the substrate when plant tissues are disrupted, as a result of wounding, insect- or pathogen attack. Myrosin cells were discovered already in 1884, as they were different in morphology and staining characteristics compared to neighbouring cells (Heinricher 1884). These cells are found in most tissues at a frequency of between 2% and 5%, and contain protein-rich granules (myrosin grains). The presence of myrosinases in these cells has been confirmed by immunohistochemical studies (Thangstad *et al.*, 1990; Höglund *et al.*, 1991), but myrosinase activity has been observed in non-specialised cells as well (Thangstad *et al.*, 1991). Myrosinase activity has been detected in all glucosinolate-containing plants and tissues investigated. Normally, the highest activity is found in seeds and seedlings. The location of glucosinolates is still unclear, but it is likely that they are stored in the cell vacoule. The only indication that glucosinolates may be concentrated in certain cell types comes from *A. thaliana*, where Koroleva *et al.* (2000) have described "S-cells" between the phloem and the endodermis in the flower stalk which are rich in sulphur and glucosinolates. The classical "mustard oil bomb" model states that myrosinase occurs in the cytoplasm and glucosinolates in the vacuole of the same cells, and detonation occurs when the cellular compartments are disrupted (Lüthy and Matile, 1984). This model still partly applies, but may have to be revised regarding the localisation of the different components.

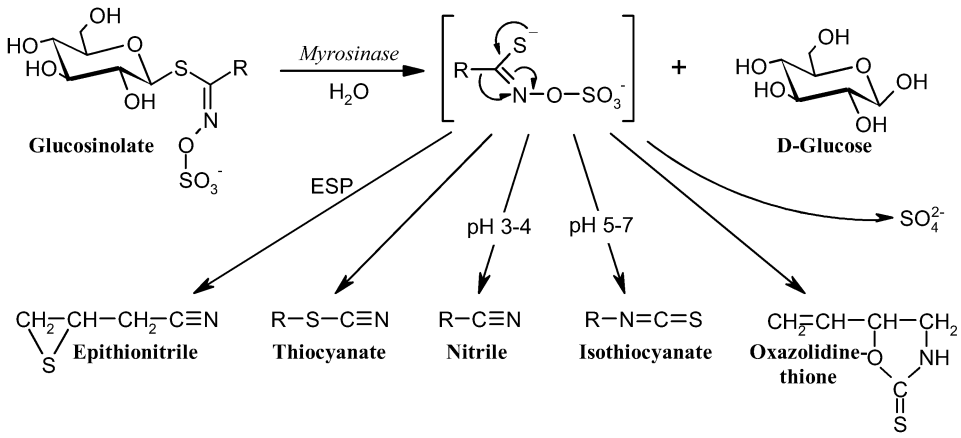


Figure 3. Myrosinase catalysed degradation of glucosinolates (reviewed in Rask *et al.*, 2000). (Printed with permission from Bo Pontoppidan)

Myrosinase and associated proteins

The myrosinase protein has a molecular mass of 59 kDa, is highly glycosylated and occurs in a dimeric form (Rask *et al.*, 2000). Myrosinase genes and cDNA clones have been isolated and characterised from several plant species, for

example *S. alba* (Xue *et al.*, 1992), *B. napus* (Falk *et al.*, 1992; Falk *et al.*, 1995), *B. campestris* (Machlin *et al.*, 1993) *and A. thaliana* (Chadchawan *et al.*, 1993). In *B. napus*, the genes coding for myrosinases have been divided into three subfamilies, MA, MB and MC, which contain five, ten to fifteen, and five genes, respectively. The MA and MC forms are exclusively expressed in the seed embryo, while the MB form is expressed in both embryonic and vegetative tissues throughout plant development (Lenman *et al.*, 1993). Some myrosinases are found in complexes with myrosinase binding proteins (MBPs) (Falk *et al.*, 1995) and myrosinase associated proteins (MyAPs) (Taipalensuu *et al.*, 1996). The exact function of these proteins remains unclear, but MBPs possess lectin activity, MyAPs have an esterase/lipase motif and they are both strongly wound-induced. A cofactor of myrosinase, the epithiospecifier protein (ESP) has also been identified (Tookey, 1973), which is thought to interact with myrosinase to produce epithionitriles as major hydrolysis products.

Potential functions of the glucosinolate-myrosinase system

Glucosinolate profiles do not only vary between species and genotypes, they also differ considerably between and even within individual plants, depending on developmental stage, tissue and photoperiod (Milford *et al.*, 1989; Clossais-Besnard and Larher, 1991; Rosa, 1997). The nutrient status of the plant also influences glucosinolate levels. Several studies have shown that increased sulphur availability leads to increased glucosinolate content (Mailer, 1989; Kaur *et al.*, 1990) and that the balance between nitrogen and sulphur is crucial (Zhao *et al.*, 1994). In addition, abiotic stresses such as drought (Bouchereau *et al.*, 1996) is another influencing factor. Finally, biotic stresses caused by insect or pathogen attack impose drastic changes on the glucosinolate profile (discussed separately). The responsiveness of the glucosinolate-myrosinase systems to several different environmental factors has brought forward different theories regarding its potential roles in the plant.

Brassica species require high amounts of sulphur, and it has been proposed that glucosinolates may act as a sulphur storage pool which can be mobilised through hydrolysis by myrosinase (Schnug and Heneklaus, 1993). This model has, however, been dismissed due to results showing that sulphate is the main storage compound in vegetative tissue (Zhao *et al.*, 1999). A possible role for indolylic glucosinolates could be as precursors for the plant hormone indole-3-acetic acid (IAA), as the hydrolysis product of glucobrassicin, indole-3-acetonitrile, is an immediate IAA precursor (Searle *et al.*, 1982). In addition, recent results demonstrated that a glucosinolate-derived isothiocyanate in *Raphanus sativu*s is involved in regulation of phototropism (Hasegawa *et al.*, 2000). Nevertheless, the most accepted theory to date is that of the glucosinolate-myrosinase system as a defence against insects, herbivores and pathogens (Bennett and Wallsgrove, 1994).

The glucosinolate-myrosinase system in plant defence

Insects and herbivores

Glucosinolates and their breakdown products clearly play a role in the interactions between plants and insects (Chew, 1988). However, this defence seems mainly to be effective against generalist insects (as well as against birds, molluscs and slugs), which are not adapted to glucosinolate containing hosts (Blau *et al.*, 1978; Giamoustaris and Mithen, 1995; Bodnaryk, 1997). The insecticidal activity of glucosinolate breakdown products is thought to arise from inhibition of the Krebs cycle (Pracros and Gourdoux, 1992). In contrast, insects specialised on *Brassicaceae* hosts utilise specific glucosinolate profiles to localise their hosts, as isothiocyanates attract and stimulate feeding and egg laying of these insects. Several studies have correlated increased insect damage by specialists with increasing glucosinolate content (Giamoustaris and Mithen, 1995; Siemens and Mitchell-Olds, 1996; Lambdon *et al.*, 1999). These interactions are very specific, and minor alterations in glucosinolate chain structure can have major effects on insect behaviour.

Microbial pathogens

The role of glucosinolates in defence against pathogens is less clear. Pathogen challenge can induce changes in the levels and relative amounts of individual glucosinolates (Doughty *et al.*, 1991; Doughty *et al.*, 1996). In addtition, *in vitro* studies have shown that isothiocyanates and other glucosinolate degradation products are toxic to fungi and bacteria (Greenhalgh and Mitchell, 1976; Mithen *et al.*, 1986; Milford *et al.*, 1989; Sarwar *et al.*, 1998). However, very few *in vivo* studies have been able to correlate glucosinolates to pathogen resistance. Tierens *et al.* (2001) investigated the effect of glucosinolate deficiency in *A. thaliana* on seven fungal and bacterial pathogens, and observed that only one of these (*Fusarium oxysporum*) was more aggressive on the low-glucosinolate mutants than on wild-type plants. Thus, it is possible that the impact of the glucosinolate-myrosinase system differs significantly between different pathogens. In analogy to insect pests, certain pathogens may have evolved to be able to tolerate and detoxify glucosinolates. For example, a putative cyanide hydratase gene for degradation of nitriles was recently cloned from *L. maculans* (Sexton and Howlett, 2000). Nevertheless, crucifers have long been used as green manure and biofumigation crops, as isothiocyanates are toxic to many soil-borne pathogens of other crops. Another indication of a defence related role for the glucosinolate-myrosinase system is its responsiveness to signalling molecules involved in defence pathways. The levels of certain glucosinolates are induced in response to SA, JA and wounding (Kiddle *et al.*, 1994; Doughty *et al.*, 1995). In addition, both MBP and myrosinase levels are influenced by these compounds (Taipalensuu *et al.*, 1997a; Taipalensuu *et al.*, 1997b).

The *Brassica* species

Several of our economically important oilseed and vegetable crops, like oilseed rape, turnip rape, cabbage, kale, broccoli, cauliflower and oilseed mustards, belong to the *Brassica* genus. Their ancestors have been utilised since the start of agriculture due to their wide applications. The leaves, stems, roots, and buds are edible and the seeds can be used for oil extraction or as a condiment. The *Brassica* genus includes the cultivated diploid species *B. rapa* (turnip rape, formerly *B. campestris*), *B. nigra* (black mustard) and *B. oleracea* (cabbage) and the amphidiploid species *B. juncea* (Indian mustard), *B. napus* (oilseed rape, swede) and *B. carinata* (Abyssinian mustard).

The *B. napus* species is divided into two different crops; oilseed rape (subspecies *oleifera*) and swedes (subspecies *rapifera*), the former greatly dominating in cultivation area and economical importance. It is one of the most important sources of edible vegetable oil, and can be grown world wide in spring or winter forms. The main areas of oilseed rape production are Europe, Canada, Australia, India and China. In Sweden, approximately 35 000 ha of oilseed rape was planted in the autumn of 2001. Fungal diseases are the main cause of crop loss in oilseed rape cultivation, the most important diseases being white rust (*Albugo candida*), black spot (*Alternaria brassicae* or *A. brassicola*), blackleg (*L. maculans*), clubroot (*Plasmodiophora brassicae*), Sclerotinia stem rot (*Sclerotinia sclerotiorum*), light leaf spot (*Pyrenopeziza brassicae*) and Verticillium wilt (*Verticillium longisporum*) (Rimmer and Buchwaldt, 1995; Steventon *et al.*, 2002). Insect pests are also a limiting factor in oilseed rape production, while virus- and bacterial diseases occur to a minor extent. *Brassica rapa* is known as turnip rape, and is the non-bulbing form of the true turnip. It is the most cold-hardy of the *Brassica* oilseeds, which makes it suitable for cultivation in northerly latitudes. *B. juncea*, on the other hand, is well adapted to drier conditions. It is grown as an oilseed crop in India and China, but has otherwise mainly been grown for condiment purposes. However, interest in *B. juncea* as an oilseed crop has recently developed in Australia, where it is regarded as more drought-tolerant alternative to *B. napus*. *B. nigra*, black mustard, was historically grown along with white mustard (*S. alba*) as a source of condiment for the spice trade. However, since the 1950's it has been superseded by *B. juncea*, the main reason being its pod characteristics, which render it unsuitable for mechanical harvesting (Hemingway, 1995). Nowadays, only very small amounts of *B. nigra* are traded on the world market, but it is still used as a source in the breeding of *Brassica* crops. It carries desirable traits such as heat and drought tolerance and resistance to the blackleg disease.

The *Brassica* genomes

The relationship between the *Brassica* species was established by cytological studies in the 1930's (Morinaga, 1934; U, 1935). The three diploid species each represent a parental genome of the amphidiploid species (Fig. 4). *B. rapa* contains the A genome (n=10), *B. nigra* contains the B-genome (n=8) and *B. oleracea* contains the C genome (n=9), and these are combined in *B. juncea* (A+B genomes, n=18), *B. napus* (A+C genomes, n=19) and *B. carinata* (B+C genomes, n=17). These relationships have later been confirmed by morphological, biochemical and RFLP studies (Mithen *et al.*, 1984; Takahata and Hinata, 1986; Song *et al.*, 1988). Amphidiploid species have also been resynthesized by interspecific crossing of the parental genotypes (Olsson and Ellerström, 1960) and by protoplast fusions (Glimelius, 1999).

RFLP linkage maps and comparative maps have generated a great deal of information on the organization of the *Brassica* genomes. The three genomes are partially homologous. They presumably derive from a common ancestor, although it has been suggested that the A and C genomes originated from a single lineage whereas the B genome represents an individual lineage. A general property of all *Brassica* genomes is the high degree of duplication, and the amphidiploid genomes in particular are highly complex. Linkage maps have been constructed for *B. napus, B. nigra, B. oleracea, B. rapa* and *B. juncea* (reviewed in Quiros, 1999). In a study of a highly polymorphic cross of *B. nigra*, it was observed that almost the entire *B. nigra* genome could be assigned into groups of triplicated collinear chromosomal fragments (Lagercrantz and Lydiate, 1996). From these data, it was hypothesised that the B-genome descended from a hexaploid ancestor.

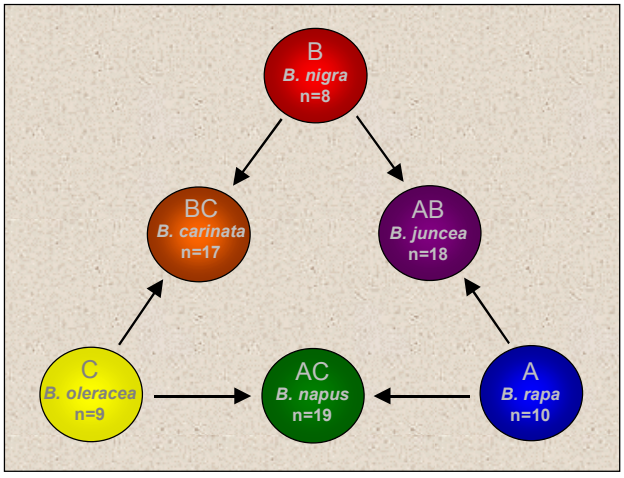


Figure 4. Genomic relationships between the *Brassica* crop species, arranged according to U (1935).

Breeding for resistance

Two major advances in oilseed rape breeding led to the present demand for rapeseed products; the reduction of the two antinutritional factors erucic acid and glucosinolates, combined in so called double-low varieties. However, as the Bronowski cultivar has been the single source for the low glucosinolate trait, all current cultivars are closely related to each other. This genetic uniformity has increased the crops vulnerability to blackleg and other diseases. Breeding for resistance is a constantly ongoing process, as many pathogens readily adapt to new cultivars and break their resistance. The disease pressure is, to some extent, specific for each geographic area, but some diseases are problematic worldwide. Separate breeding programs have been devoted to these pathogens (reviewed in Becker *et al.*, 1999). *Sclerotinia* is a difficult challenge for breeders, as no genetic source of resistance has yet been identified. Instead, strategies involving apetalous cultivars have been tried, to eliminate the senescing petals as growing ground for ascospores. Furthermore, the introduction of a wheat gene coding for oxalate oxidase has been used to inhibit the action of the phytotoxin oxalic acid which is produced by the fungus (Thompson *et al.*, 1993). White rust (*A. candida*) is a serious problem mainly for *B. rapa* and *B. juncea*, while most *B. napus* cultivars are resistant. For this disease, the existing variation has allowed selection for tolerance, but not durable resistance. *L. maculans* is another example where breeders have previously been relatively successful, but are now faced with the fact that the fungus has bettered them.

L. maculans resistance sources

Within the *B. napus* gene pool, there is some natural variation in blackleg field resistance. Since the introduction of the field resistant winter cultivar Jet Neuf in 1977, blackleg disease has been effectively controlled in Europe. Jet Neuf is the result of *B. napus* selection, and it is still the most important resistance source in conventional breeding for the European market. The situation in Canada and Australia, where mainly spring cultivars are grown, has been more problematic. About ten years ago, however, Australian breeders started utilising material of Asian origin. This material is also derived from *B. napus* selection, and exhibits strong resistance already at the seedling stage (cultivars Maluka, Shiralee etc.). These cultivars have been used in Canada as well, but they are not effective against the more virulent blackleg isolates found in Europe. A high degree of resistance to blackleg in all plant parts is found in the B-genome containing species *B. nigra*, *B. juncea* and *B. carinata* (Sacristan and Gerdemann, 1986; Sjödin and Glimelius, 1988; Rimmer and van der Berg, 1992). Effort has been put into transferring this resistance to oilseed rape, but so far it has been difficult to obtain stable resistance. Recently, a new cultivar was released in Australia (Surpass 400), which contains resistance derived from the A-genome of *B. rapa* ssp. *sylvestris*. It is possible that future research will focus even more on the A- and C-genomes as resistance sources, since they are more easily incorporated in the *B. napus* background than material from the B-genome (Pers. comm. Ingrid

Happstadius, Svalöf Weibull). Screening for resistance in wild crucifers, both closely and distantly related to *B. napus*, has also been initiated to widen the pool of potential resistance donors (Séguin-Swartz *et al.*, 2000). Alarming reports describing isolates virulent on *B. juncea* and *B. nigra* have now come from both Australia and France (Purwantara *et al.*, 1998; Somda *et al.*, 1999; Brun *et al.*, 2001). This has made the issue of developing new resistance sources even more urgent. Biotechnological approaches may be helpful as an alternative or complement to traditional breeding techniques. For example, promising results have been obtained by over expression of a tomato chitinase gene (Grison *et al.*, 1996), a pea defence gene (Wang *et al.*, 1999) and the *R*-gene *Cf9* (Hennin *et al.*, 2001). Specific resistance genes in existing cultivars of *B. napus* have been studied on the genetic level (Ansan-Melayah *et al.*, 1998; Balesdent *et al.*, 2001), and hopefully more molecular data will follow. With a fungus as variable as *L. maculans*, gene pyramiding in the production of new cultivars might be helpful in obtaining a more durable resistance. The combination of quantitative and specific resistance traits in one cultivar, as well as "multi-lines" comprising a mix of individuals with different resistance sources is currently being discussed. Most of the ongoing breeding efforts are carried out in commercial companies, and much of the progress in this field is therefore confidential.

Defence responses to *L. maculans*

Resistance to *L. maculans* is usually categorised as adult stage resistance or seedling resistance. Adult stage resistance is generally considered to be multigenic with a strong influence of environmental conditions, while seedling resistance is often described as a single-gene trait (Ferreira *et al.*, 1995; Meyerhofer *et al.*, 1997; Ansan-Melayah *et al.*, 1998; Pilet *et al.*, 1998). Although some genetic data regarding *L. maculans* resistance is available, the mechanisms behind the resistance are still to a large extent unknown. Several studies have reported on hypersensitive responses such as necrosis of guard cells, callose- and lignin-deposition (Hammond and Lewis, 1986; Chen and Howlett, 1996; Roussel *et al.*, 1999). Induction of phytoalexins has also been observed, but it could not be correlated to resistance (Rouxel *et al.*, 1991). Furthermore, the induction of pathogenesis-related proteins after *L. maculans* inoculation has been reported (Rasmussen *et al.*, 1992; Dixelius, 1994) as well as the induction of previously uncharacterised proteins (Lamkadmi *et al.*, 1996; Brownfield and Howlett, 2001). An EST screen performed on *L. maculans* inoculated *B. napus* leaves yielded more than 50 ESTs that could be linked to defence, of which a large proportion were of unknown function (Fristensky *et al.*, 1999). This may reflect a complex defence against *L. maculans*, possibly involving, so far, unexplored pathways. Results from *A. thaliana* have suggested that *L. maculans* resistance is independent of salicylic acid, jasmonic acid and ethylene responses (Bohman, 2001).

The fungal pathogen *Leptosphaeria maculans*

More than 200 years ago, Tode first described the saprophytic organism *Sphearia lingam*, which he found on dead red cabbage stems. In 1849, the fungus was reclassified into the genus *Phoma*. *Phoma lingam* (Tode ex Fr.) Desm. has since then been known as the causal agent of blackleg, dry rot and canker diseases in cruciferous crops. As recently as 1957, its sexual stage was found and confirmed as being *Leptosphaeria maculans* (Desm.) Ces & De Not (reviewed in Williams, 1992). *L. maculans* is a loculoascomycete belonging to the order *Pleiosporales*.

Blackleg disease

L. maculans can attack stems, leaves, cotyledons, pods and seeds of both *B. napus, B. rapa* and *B. oleracea*. The characteristic symptoms are leaf lesions, usually greyish in colour with visible pycnidia, and stem cankers in the later stages (Fig. 5). The major yield loss is due to lodging as a result of basal, girdling cankers. Cankers also restrict the flow of moisture and nutrients up to the ripening seeds, causing premature ripening and shriveled seeds and pods.

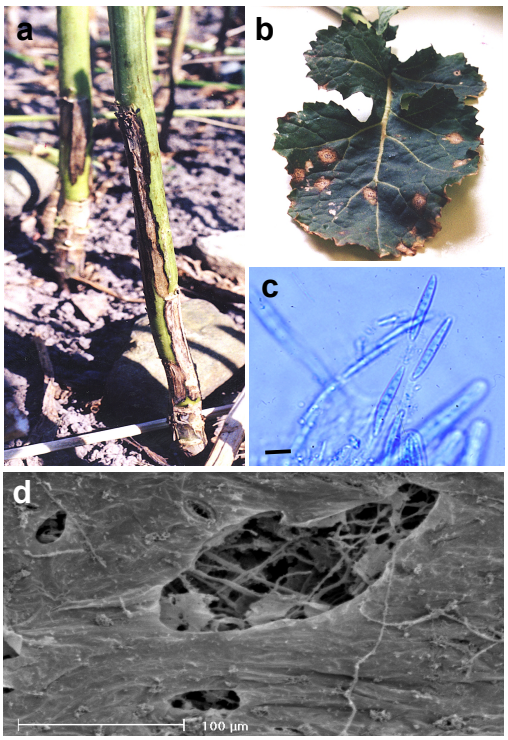


Figure 5. (a) stem infections and (b) leaf spots, caused by *L. maculans* on oilseed rape. (c) *L. maculans* ascospores found in pseudothecia. Bar represents 16 µm. (d) scanning electron microscopy of *L. maculans* hyphae growing in a *B. napus* leaf.

Disease cycle

Primary infection is mainly initiated by airborne sexual ascospores (Fig. 5c), which are released from pseudothecia on infected crop debris. The disease can also arise from infected seed. Seedlings are infected by invasion of cotyledons, while younger leaves are invaded through stomata or wounds. *L. maculans* is a facultative necrotroph, that during the initial phase of infection grows in a biotrophic manner. However, behind the hyphal front the fungus becomes necrotrophic and produces asexual pycnidiospores in the dead tissue (Hammond *et al.*, 1985). Pycnidiospores can cause significant secondary spreading when dispersed to neighbouring plants, but do not germinate as efficiently as ascospores (Wood and Barbetti, 1977). After initial infection, the fungal hyphae grow intercellularly and in xylem vessels down the petiole (Fig. 5d). This phase is basically symptomless, but when the fungus finally invades and kills the stem cortex a black stem canker is produced. After harvest, pycnidiospores can colonize remaining stem tissue saprophytically, and dormant mycelium survives between seasons. When conditions are optimal, pseudothecia are formed, which release ascospores for a new round of infection (Fig. 6).

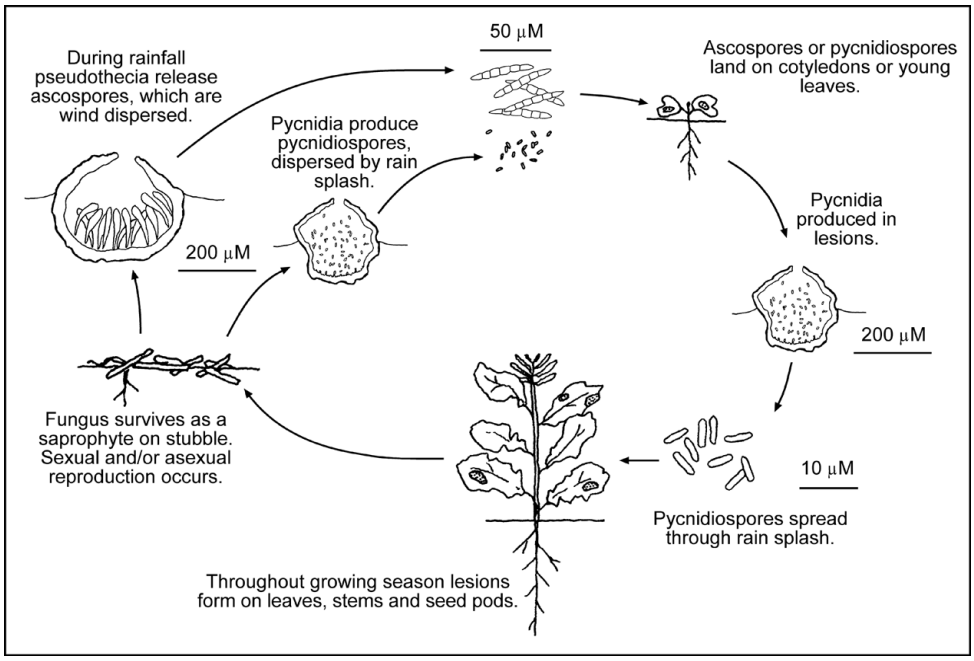


Figure 6. The life cycle of *L. maculans* on *B. napus* (reviewed in Howlett *et al*., 2001).
(Printed with permission from B. Howlett)

Epidemiology

Blackleg has been a serious problem for *Brassica* oilseed production mainly in Europe, Australia and Canada (reviewed in West *et al.*, 2001). Epidemic outbreaks, causing yield losses of up to 80%, have occurred in France during the 1950s and in Australia during the 1970s. Improved control strategies, such as crop rotation, stubble destruction, fungicide treatment and varieties with better resistance have since limited the damage caused by blackleg. At present, the fungus is endemic in most oilseed rape growing areas. The severity of the disease varies between seasons, regions and crops, and although harvest losses can reach 50%, it is normally limited to less than 10% by the use of pesticides. However, the finding of new, highly virulent strains greatly increases the risk of future epidemics. In Sweden, severe epidemics have not been reported, and it has been assumed that only avirulent isolates are present. However, during the last few years, some basal stem cankers have been observed and the presence of virulent isolates has been confirmed in Sweden (Kuusk *et al.*, 2002).

Biology of *L. maculans*

Taxonomy

The taxonomy of this fungus is confusing, as *L. maculans* appears to comprise several related and morphologically similar species. Historically, *L. maculans* isolates have been divided into two different groups. Strains that cause stem cankers have been named aggressive, virulent, tox^{+} or A-group, while strains causing milder symptoms have been named non-aggressive, avirulent, tox^{-} or B-group. The B-group (also called PG1) has been divided into the subclasses NA1, NA2 and NA3. It has recently been proposed that the B-group consists of species outside the *L. maculans* complex, and NA1 has been renamed *L. biglobosa* (Shoemaker and Brun, 2001). Isolates of the A-group are further subdivided into pathogenicity groups PG2, PG3, and PG4, based on the disease reactions of the cultivars Westar, Quinta and Glacier (Koch *et al.*, 1991). The distinction between these groupings has also been confirmed by methods like isozyme studies, rDNA analysis, RAPD and AFLP analysis (reviewed in Howlett *et al.*, 2001). In Europe, most *L. maculans* isolates belong to PG3 or PG4, Canada mainly has PG2 isolates, while isolates belonging to both PG2, 3 and 4 have been observed in Australia (Purwantara *et al.*, 2000). In addition, isolates attacking *B. juncea* are found almost exclusively in Australia.

Genome analysis

L. maculans has a genome size of about 34 Mb and approximately 16 chromosomes (Cozijnsen *et al.*, 2000). The genome also contains two linear plasmids encoding their own replication machinery, which are still of unknown function (Lim and Howlett, 1994). Two genetic maps based on AFLP markers have been developed for *L. maculans* (Cozijnsen *et al.*, 2000), (Pongam *et al.*, 1998). Several *avr*-loci have been described, but as yet none have been cloned.

AvrLm1 and *AvrLm2* are closely linked and confer avirulence to multiple *B. napus* cultivars, while *AvrLm4* mediated avirulence is specific to the Jet Neuf resistance (Ansan-Melayah *et al.*, 1995; Ansan-Melayah *et al.*, 1998; Balesdent *et al.*, 2001). The fungal gene *alm1* appears to be involved in the gene-for-gene interaction with the resistance loci *LEM1,* identified in *B. napus* cv. Major (Pongam *et al.*, 1998). Eleven protein-encoding genes have been cloned from *L. maculans*, of which five appear relevant for virulence (reviewed in Howlett *et al.*, 2001). Two of these are ABC-transporters which may be important for active transport of toxins or other virulence factors. Two genes are cellulases, possibly for degradation of plant cell walls, and yet another encodes a cyanide hydratase gene, which could be important for detoxification of plant defence compounds. *L. maculans* is transformable, and reporter systems such as β-glucoronidase (GUS) (Chen and Sèguin-Swartz, 1997) and green-flourscent protein (GFP) (Sexton and Howlett, 2001) have been successfully applied.

Phytotoxins

The A-group of *L. maculans* is known to produce several phytotoxins. Although the fungus is host-selective, the first toxin to be isolated was sirodesmin PL, which is toxic to a broad range of organisms (Férézou *et al.*, 1977). A host-selective toxin, phomalide, was later found to be produced during a short window between 30-60 h of culture (Pedras *et al.*, 1993). The short production time is believed to be due to inhibition of phomalide biosynthesis by sirodesmin (Pedras and Biesenthal, 1998). Application of phomalide causes lesions on *B. napus* leaves, while *B. juncea* is much less affected.

Aims of the study

Blackleg resistance is a very important trait in the breeding of *Brassica* oilseed crops, because disease outbreaks can be devastating. The general aim of this study has been to clarify the mechanisms involved in defence against *L. maculans*, with focus on the resistance derived from the *Brassica* B-genome. The long-term goal is to improve the understanding of this complex plant-pathogen interaction in order to find ways of controlling the disease.

The specific aims of the study were to:

- Map resistance loci in the *Brassica* B-genome by using RFLP markers on material derived from asymmetric somatic hybrids.

- Study the role of the glucosinolate-myrosinase system in *L. maculans* resistance.

- Clone and characterise sequences in the B-genome involved in defence responses to *L. maculans.*

Results and discussion

The present study describes different aspects of resistance to *L. maculans*, mainly with focus on *Brassica* B-genome derived resistance. At the genomic level, the location and organisation of loci conferring resistance to *L. maculans* in the B-genome was studied. At the metabolic level, the role of the glucosinolate-myrosinase system was studied, and at the sequence level a potential resistance gene was cloned and characterised.

Resistance to *L. maculans* is conserved in a specific region of the *Brassica* B genome (I)

The location of resistance loci in the B-genome was studied using backcrossed offspring from asymmetric hybrids between *B. napus* and the three B-genome species *B. nigra*, *B. juncea* and *B. carinata*. Asymmetric hybrids only contain limited parts of the donor genome, either as whole chromosomes or as fragments. The presence of RFLP markers positioned on the B-genome was analysed, and the offspring was screened for *L. maculans* resistance. Both cotyledon and adult leaf resistance was assayed in individuals from the first, second and third back-cross generations of all three hybrid categories. 46 RFLP markers detecting 85 loci were used in the investigation. In total, four co-segregating markers for cotyledon and adult-leaf resistance were found. In the material derived from the *B. napus – B. carinata* hybrids, two cotyledon resistance loci were found on linkage groups 5 and 8, and one adult-leaf locus was found on linkage group 2. In the material with additional *B. juncea* DNA, a cotyledon resistance locus was localised to linkage group 5 and two adult-leaf loci to linkage groups 2 and 8. Finally, in the *B. nigra* derived material, a cotyledon resistance loci was found on linkage group 2 and two adult-leaf loci on linkage groups 5 and 8. Thus, all resistance loci were found to map to linkage groups 2, 5 or 8 and more specifically to a triplicated region in the B-genome designated B (Lagercrantz and Lydiate, 1996). In accordance with this, stem resistance to *L. maculans* has also been shown to map to three different linkage groups in the B-genome (Struss *et al.*, 1996). Unfortunately, the maps generated by Struss *et al.* (1996) and by Lagercrantz and Lydiate (1996) have not been integrated, making direct comparisons difficult. In our study cotyledon and adult-leaf resistance cosegregated as different loci in all three species. This is in agreement with previous observations in *B. juncea* (Salisbury and Ballinger, 1993), and suggests that resistance in these two tissues is governed by different genes, and possibly by different mechanisms. Plant resistance genes often occur in complexes or clusters encoding similar sequences (Botella *et al.*, 1997). This could potentially

increase their flexibility and diversity by facilitating genetic rearrangements. The fact that all resistance loci detected in this study were localised to a specific region fits nicely with this model. However, whether their organisation really is a result of gene duplications or conversions can only be settled through DNA sequence analysis. In paper **IV**, a resistance gene mapping to this region was sequenced. Apparently this gene has several closely related sequences in the *Brassica* genomes, although it is not yet clear if they are localised to the same region.

The role of the glucosinolate-myrosinase system in the defence against *L. maculans* (II, III)

Several studies suggesting involvement of the glucosinolate-myrosinase system in defence against fungal pathogens have been presented, for example *Peronospora parasitica* (Greenhalgh and Mitchell, 1976), and *Alternaria* spp. (Milford *et al.*, 1989). High glucosinolate levels have also been correlated to decreased lesion size in *L. maculans* infections (Mithen *et al.*, 1987), but the relevance of the glucosinolate-myrosinase system in blackleg disease is far from clear. We wanted to study the dynamics of different components of this system during *L. maculans* attack in more detail, with the aim of clarifying the role of the glucosinolate-myrosinase system in the *Brassica – L. maculans* interaction.

B-genome derived resistance to L. maculans in near isogenic B. napus lines is independent of glucosinolate profile (II)

B-genome containing species produce large amounts of the aliphatic glucosinolate sinigrin. *In vitro* assays have shown that sinigrin degradation products are highly toxic to *L. maculans* cultures (Mithen *et al.*, 1986), prompting the speculation that this glucosinolate could be a B-genome derived resistance factor. In paper **II**, we utilised near-isogenic hybrid lines between *B. napus* and *B. nigra* to study glucosinolate content in relation to *L. maculans* resistance. Adult leaves of twenty-three lines were analysed prior to inoculation, and disease symptoms were rated 15 days post inoculation. We found no significant correlation between glucosinolate content and resistance to *L. maculans*. We were also interested in monitoring any changes in the plant glucosinolate profiles that might be induced during the course of infection. Therefore, two representative lines, L_1 and L_2, were chosen along with the two parental materials *B. napus* and *B. nigra* for further investigation. L_1 had a glucosinolate profile similar to *B. napus* and was susceptible to *L. maculans*, while L_2 was resistant to *L. maculans* and had an elevated sinigrin content, although the overall indole glucosinolate profile was similar to that of *B. napus*. The glucosinolate profiles were analysed at time points ranging from 4 h to 8 days after inoculation. Interestingly, we observed increased levels of the indole glucosinolate 4-methoxy-glucobrassicin 5-8 days post inoculation in plants that had been treated with fungal spores. Control plants inoculated with water did not show this

response. The change in profile was clearly related to the presence of the fungus, but unrelated to resistance, as the response was seen both in susceptible and resistant plants. The glucosinolate-myrosinase system is influenced by SA and JA signals in the plant, as well as by wounding. The wound response is quite immediate and was detected in both control (water) and spore inoculated material 4 h post inoculation. In contrast, the elevated levels of 4-methoxy-glucobrassicin were only observed in later stages of the infection, when symptoms started to develop on susceptible plants. It could thus be a response coupled to the fungal transition from biotrophy to necrotrophy. It may also be a result of SA or JA induction after infection. Artificial application of SA and methyl jasmonate to oilseed rape seedlings have been shown to induce increased levels of aromatic and indole glucosinolates, respectively, 3-7 days after treatment (Wallsgrove *et al.*, 1995). Indolyl glucosinolates may function as precursors for both IAA (Ludwig-Müller and Hilgenberg, 1988) and phytoalexins (Monde *et al.*, 1994), and the changed profile may have a secondary, down-stream function beyond the glucosinolate-myrosinase system. Obviously, although elicited by the fungus, these responses have no apparent effect on *L. maculans* virulence.

Proteins involved in glucosinolate breakdown are also crucial for the function of the glucosinolate-myrosinase system. We monitored the expression of MBP during infection, but observed no differences between susceptible and resistant material. However, genomic analysis of the near-isogenic lines indicated that a sequence coding for MBP from *B. nigra* had been transferred along with *L. maculans* resistance to the L_2 line. Whether the transfer of MBP is merely a coincidence or actually relevant for resistance remains unclear. It is noteworthy, though, that in our screen for resistance genes in paper **IV**, one of the selected sequences was homologous to MBP. MBP transcript sizes in **I** were similar in all four materials tested, and sequence analysis is necessary to conclude if the *B. nigra* derived MBP has properties different from the *B. napus* form. Using RFLP and phenotypic markers, we concluded that a region from linkage group 1 (Struss *et al.*, 1996) of the B-genome had been transferred to the resistant L_2 line. Again, it should be noted that the position on the map of Lagercrantz and Lydiate (1996) has not been elucidated. The transferred region contains loci for both *L. maculans* resistance and sinigrin. Thus, correlations between sinigrin and resistance observed in other studies may have been a result of a genetic linkage rather than a resistance function of sinigrin.

The myrosinase-glucosinolate system in the interaction between L. maculans and B. napus (III)

As previously mentioned, the glucosinolate-myrosinase system has multiple components, and merely studying glucosinolates is not sufficient. In paper **III**, we aimed to get a more complete picture of the whole system in relation to *L. maculans* defence. As much of the work on myrosinase and its associated proteins has been carried out in *B. napus* (reviewed in Rask *et al.*, 2000), we chose to compare cotyledon responses of one resistant *B. napus* cultivar (Maluka)

and one susceptible cultivar (Westar) after *L. maculans* challenge. This study is the first to address effects on multiple components of the glucosinolate-myrosinase system after *L. maculans* infection, including concentrations of individual glucosinolates, product formation, myrosinase isoform distribution and activity, and levels of MBP. The glucosinolate profiles of Maluka and Westar were similar, with the exception that Maluka contained neoglucobrassicin, which was not present in Westar. However, testing another resistant cultivar (Quantum) excluded neoglucobrassicin as a resistance factor. There was also a small reduction in the glucobrassicin and neoglucobrassicin content of the inoculated, resistant material. The myrosinase activity was generally higher in Maluka than in Westar, but both species showed an age-dependent pattern which was unaffected by the pathogen. To ensure that there were no specific, local differences in the myrosinase distribution which would not be reflected in a general activity assay, immunohistochemical studies were carried out. No local induction of myrosinase was observed at the infection sites, and myrosinase was exclusively found in the myrosin cells of both Maluka and Westar. Myrosinase isoforms were further tested by RT-PCR, showing that approximately 50% of the transcripts coded for MB1, 20% for MB2 and 30% for MC2 in both cultivars. The degradation product profiles and the MBP expression was also similar in both control and inoculated plants of both cultivars. Consequently, we could draw the conclusion that there were no differences in any components of the myrosinase complex that could be directly related to *L. maculans* resistance. However, as some differences on the glucosinolate level were observed, we undertook a sulphur-starvation strategy to deplete Maluka and Westar plants of glucosinolates before fungal challenge. It was clear that although no glucosinolates were present, resistance levels were still the same in both cultivars.

The results from both **II** and **III** indicated that the glucosinolate-myrosinase system is not a major determinant of blackleg resistance. Although changes in the glucosinolate profiles occur as a result of fungal inoculation, it did not affect the growth of the fungus. It is possible that *L. maculans*, which is a specialised *Brassica* pathogen, has evolved to overcome the toxicity of glucosinolate breakdown products. To date, there is no clear evidence that *L. maculans* possesses glucosinolate detoxification systems, but the fungus has been shown to detoxify related compounds, such as phytoalexins. In addition, *L. maculans* produces ABC transporters (Condie and Taylor, 1999) and a putative cyanide hydratase (Sexton and Howlett, 2000) which could be relevant in this context. In **III**, decreased levels of certain glucosinolates were observed in resistant material after inoculation. This could possibly suggest active utilisation of glucosinolates by the fungus. However, our studies on plants depleted of glucosinolates show that although *L. maculans* tolerates the glucosinolate-myrosinase system, it is not dependent on these compounds for virulence. It should be noted that the investigations in **II** and **III** were performed on adult leaves and cotyledons, respectively, and that different fungal isolates and plant materials were used.

Thus, the results from **II** and **III** cannot be easily compared. Resistance mechanisms may differ between tissues and isolates, and yet conditions differing from those used in these studies may give other results. However, the results from **II** and **III**, in combination with results from other groups (Mithen and Magrath, 1992; Giamoustaris and Mithen, 1997; Sexton *et al.*, 1999), strongly suggest that *L. maculans* resistance is determined by factors other than the glucosinolate-myrosinase system.

The *Lm1* gene of *B. nigra* confers resistance to the blackleg fungus *L. maculans* (IV)

Although genetic evidence suggests the involvement of gene-for-gene resistance mechanisms in the *Brassica – L. maculans* interaction (Ansan-Melayah *et al.*, 1995; Ansan-Melayah *et al.*, 1998; Balesdent *et al.*, 2001), no *R*-genes from this system have been cloned to date. We utilised sequences from the previously characterised *R*-genes *Hm1* and *Pto* in a PCR-based screen to identify possible *R*-gene candidates in *B. nigra*. The screen resulted in five different sequences; three unknown, one clone with homology to MBP and one clone with limited (23%) homology to *Pto*. These were subsequently transferred to susceptible *B. napus* plants by *Agrobacterium* transformation. After initial resistance tests, on of the unknown sequences (*Lm1)* was chosen for further analysis.

Transgenic plants carrying the *Lm1* cDNA behind a constitutive promoter showed significantly improved resistance to *L. maculans*, both in the cotyledons and the adult leaves. Fungal isolates representing PG2, PG3 and PG4 all gave similar results, indicating that *Lm1* confers broad spectrum resistance in different plant tissues. Due to the large variation in the *L. maculans* population, broad spectrum resistance is a valuable trait in the breeding of blackleg resistant varieties. Although the transgenic plants did not show complete resistance, as compared to *B. nigra*, the disease symptoms were reduced and delayed, which could be crucial under field conditions. We observed no increased resistance to other fungal pathogens tested, indicating that the mechanism is specific to *L. maculans*.

Genetic mapping was carried out, to confirm the presence and localisation of *Lm1* in the B-genome. *Lm1* cosegregated with markers on linkage groups 3 and 8, both of which have previously been linked to *L. maculans* resistance. The position on linkage group 3 corresponded to a loci for stem resistance (Struss et al., unpublished), and the position on linkage group 8 corresponded to the adult leaf resistance in *B. nigra* and to cotyledon resistance in *B. juncea* mapped in paper **I**. It was obvious from Southern blots that multiple sequences with high similarity to *Lm1* were present in all *Brassica* genomes tested, *B. napus* included. The presence of a "gene family" of *Lm1*-like proteins was also confirmed when a BLAST search against sequence data bases was performed. Several DNA sequences of unknown function on *A. thaliana* chromosomes I, IV and V, as well

as a sequence from rice (*Oryza sativa*), showed very high (above 90%) homology to *Lm1*. The presence of similar sequences in both di- and monocots suggests a wide distribution in the plant kingdom. We have picked up three genomic clones from *B. nigra* with different degrees of *Lm1* homology, but at present they have not been sequenced or mapped to the B-genome. At the amino acid level, approximately 15 proteins with significant homology to the putative amino acid sequence of Lm1 were identified. Most of these were also from *A. thaliana* and of unknown function. However, an interesting exception was the nin (nodule inception) protein of *Lotus japonicus*, which is involved in the symbiotic interaction with the bacterium *Mesorhizobium loti* (Shauser *et al.*, 1999). This finding supports the speculations about a common origin of plant defence and symbiotic mechanisms (Baron and Zambryski, 1995; Parniske, 2000). Expression studies on *Lm1* revealed no induction by pathogen challenge, but rather a constitutive expression in *B. nigra*.

The role of *Lm1* in plant defence is still unknown, and can only be speculated upon. None of the typical *R*-gene motifs were present, placing *Lm1* in the group of non-typical *R*-genes, together with, for example, *RPW8* and *Mlo*. In the N-terminal part of the protein, two probable membrane spanning regions are located. This part of the sequence bears a similarity to other membrane anchored proteins from a variety of organisms, but is not present in the highly homologous sequences from *A. thaliana, O. sativa and L. japonicus*. It is possible that the region between the two transmembrane domains is a sensory domain located outside the membrane, while the remaining part is a cytoplasmic effector domain. The fact that resistance in the transgenic plants was not complete probably reflects the complex, multifactorial nature of resistance to *L. maculans*. The presence of *Lm1* alone is not sufficient for complete resistance in the genomic background of *B. napus*, perhaps due to the lack of interacting proteins with sufficient compatibility. *Lm1* does not appear to be involved in race-specific recognition, since several isolates were affected. However, the possibility of a direct interaction with a pathogen *avr*-product still exists, if this product is of a general nature and present in all *L. maculans* isolates. Another possible model is the role of *Lm1* as a co-receptor, or guard, necessary to transmit downstream signals leading to defence responses upon *R-avr* interaction. This would, however, require the presence of a matching *R*-gene in *B. napus*. An alternative function of *Lm1* is in the basal defence, although it seems less likely since the resistance appears to be *L. maculans* specific.

Conclusions

- *L. maculans* resistance loci in the *Brassica* B-genome are preserved in the triplicated B region on linkage groups 2, 5 and 8. Cotyledon and adult leaf resistance segregated as different loci in all three B-genome species, suggesting that they are mediated by different mechanisms.

- The glucosinolate-myrosinase system is not a major determinant in resistance to *L. maculans*. This conclusion could be drawn from two separate investigations using different plant material, tissues and fungal isolates. Fungal challenge did, however, induce changes in the glucosinolate profile. These changes may result from a basal plant defence response which the *Brassica* specialist *L. maculans* has evolved to overcome. Studies on plants depleted of glucosinolates further showed that the fungus does not benefit from or require glucosinolates for virulence.

- The first gene conferring resistance to *L. maculans* was cloned from *B. nigra*. *Lm1* encodes a possible membrane bound protein, with homology to several related genes widely spread across the plant kingdom. Interestingly, one of these genes is involved in symbiotic interactions. Transgenic *B. napus* plants carrying *Lm1* cDNA exhibited improved resistance to a broad range of *L. maculans* isolates both on cotyledons and adult leaves. The exact function of *Lm1* remains to be elucidated.

Future perspectives

With the emerging threat of blackleg epidemics, as a result of fungal adaptation, a better understanding of the *Brassica* - *L. maculans* interaction is necessary. Hopefully, this thesis has contributed something to this understanding, but it is obvious that much work remains.

In my opinion, our work and other investigations on the glucosinolate-myrosinase system have, together, shown quite clearly that it is not involved in *L. maculans* defence. Thus, I see no reason for further exploration of this system as a source of blackleg resistance. However, at a more basic level, we still have many unanswered questions. For example, it would be interesting to learn more about the interplay between the glucosinolate-myrosinase system and other pathways. Do the changed levels of indolylic compounds in inoculated plants reflect a metabolic shunt leading to increased levels of phytoalexins or IAA? If so, what recognition events trigger this response and against what pathogens is it effective?

Resistance loci showed preservation with respect to location within a specific region in the B-genome, but are the sequences themselves related? Sequence data on the genes responsible for adult-leaf and cotyledon resistance would be very informative regarding the evolution and function of these genes. The process between mapping a locus and cloning a gene can be long and laborious, but the *Lm1* sequence could be useful as a template in the cloning procedure, if there indeed is sequence similarity between these different loci.

Nevertheless, the functional analysis of *Lm1* is of the highest priority in our future work. Sequence analysis of the genomic clones is necessary to confirm the composition of the cDNA sequence, and also gives a possibility to go further with promoter studies. Reporter gene constructs can be made to study the distribution and expression pattern of *Lm1*. It would also be relevant to transform *B. napus* with *Lm1* behind its endogenous promoter, as opposed to a constitutive promoter. The genomic *Lm1*-like sequences from *B. nigra* will be interesting to compare, and possibly to map to the B-genome. Studying the expression of other defence-related genes in relation to *Lm1* may be informative, and studies on the protein level are necessary for further functional analyses. Much of the protein work relies on the possibility to over-express and raise antibodies against Lm1. Unfortunately, we have been unsuccessful in using *E. coli* for this purpose, but we plan to try eukaryotic expression systems instead. Screens for interacting proteins of both fungal and plant origin are crucial for understanding the role of *Lm1* in plant defence, and work in a yeast-two-hybrid system with an *A. thaliana* library has been initiated. The presence of *Lm1*-like sequences in *A. thaliana* opens up further possibilities, such as gene knockouts and mutant studies. However, the fact that many similar sequences exist in the genome can be problematic and has already caused difficulties in our expression analyses and *in situ* studies. Testing yet other pathogens on *Lm1* transformants is also needed to confirm if *Lm1* is species specific or has a more basal function. Last, but not least, it would be very interesting to test the performance of *Lm1*-plants under field conditions.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B., Daniels, M. J., Parker, J. E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two R gene-mediated signalling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 95, 10306-10311.

Agrios, G. N. (1997). *Plant Pathology*, 4 edn. San Diego, CA, USA: Academic Press.

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

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

Balesdent, M. H., Attard, A., Ansan-Melayah, D., Deourme, R., Renard, M., Rouxel, T. (2001). Genetic control and host range of avirulens towards *Brassica napus* cvs. Quinta and JetNeuf in *Leptosphaeria maculans*. *Phytopathol.* 91, 70-76.

Baron, C., Zambryski, P. C. (1995). The plant response in pathogenesis, symbiosis and wounding: Variations on a common theme? *Ann. Rev. Genetics* 29, 107-129.

Becker, H. C., Löptien, H., Röbbelen, G. (1999). Breeding: An overview. In *Biology of Brassica Coenospecies*, pp. 413-460. Edited by C. Gomez-Campo. Amsterdam: Elsevier Science B. V.

Benhamou, N. (1996). Elicitor-induced plant defence pathways. *Trends Plant Sci.* 1, 233-240.

Bennet, R. N., Kiddle, G., Wallsgrove, R. M. (1997). Involvement of cytochrome P450 in glucosinolate biosynthesis in white mustard. *Plant Physiol.* 114, 1283-1291.

Bennett, R. N., Wallsgrove, R. M. (1994). Tansley Review No. 72: Secondary metabolites in plant defence mechanisms. *New Phytol.* 127, 617-633.

Bent, A. F. (1996). Plant disease resistance genes: Fuction meets structure. *Plant Cell* 8, 1757-1771.

Bisgrove, S. R., Simonich, M. T., Smith, N. M., Sattler, N. M and Innes, R. W. (1994). A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. *Plant Cell* 6, 927-933.

Blau, P. A., Feeny, P., Contard, L., Robson, D. S. (1978). Allylglucosinolate and herbivorous caterpillers: a contrast in toxicity and tolerance. *Science* 200, 1296-1298.

Bodnaryk, R. P. (1997). Will low-glucosinolate cultivars of *Brassica juncea* and *Sinapis alba* be vulnerable to insect pests? *Can. J. Plant Sci.* 77, 283-287.

Bohman (2001). *Molecular studies of Arabidopsis and Brassica with focus on resistance to Leptosphaeria maculans.* Doctoral thesis, Swedish University of Agricultural Sciences.

Bonas, U., Lahaye, T. (2002). Plant disease resistance triggered by pathogen derived molecules: refined models of specific recognition. *Curr. Opin. Microbiol.* 5, 44-50.

Botella, M. A., Coleman, M. J., Hughes, D. E., Nishimura, M. T., Jones, J. D. G., Somerville, S. C. (1997). Map positions of 47 *Arabidopsis* sequences with sequence similarity to disease resistance genes. *Plant J.* 12, 1197-1211.

Bouchereau, A., Clossais-Besnard, N., Bensaoud, A., Leport, L., Renard, M. (1996). Water stress effects on rape seed quality. *Eur. J. Agron.* 5, 19-30.

Brownfield, L., Howlett, B. (2001). An abundant 39 kDa protein from *Brassica napus* (canola) id present in the apoplast of leaves infected with the fungus *Leptosphaeria maculans*. *Austr. Plant Pathol.* 30, 7-9.

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

Büschges, R. K., Hollricher, R., Panstruga, G., Simons, M., Wolter, A., Frijters, R., van Daelen, T., vander Lee, P., Diergaarde, J., Groendijk, S., Töpfsch, P., Vos, F., Salamini, P., Schulze-Lefert (1997). The barley *Mlo* gene: A novel control element of plant pathogen resistance. *Cell* 88, 695-705.

Campos de Quiros, H., Magrath, R., McCallum, D., Kroymann, J., Scnabelrauch, D., Mitchell-Olds, T., Mithen, R. (2000). Alpha-keto acid elongation and glucosinolate biosynthesis in *Arabidopsis thaliana*. *Theor. Appl. Genet.* 101, 429-437.

Chadchawan, S., Bishop, J., Thangstad, O. P., Bones, A. M., Mitchell-Olds, T., Bradley, D. (1993). *Arabidopsis* cDNA sequence encoding myrosinase. *Plant Physiol.* 103, 671-672.

Chen, C. Y., Howlett, B. J. (1996). Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves of Indian mustard (*Brassica juncea*) inoculated with avirulent isolates of *Leptosphaeria maculans*. *Physiol. Mol. Plant Pathol.* 48, 73-81.

Chen, C. Y., Séguin-Swartz, G. (1997). The use of a beta-glucoronidase-marked isolate of *Leptosphaeria maculans* to study the reaction of crucifers to the blackleg fungus. *Can. J. Plant. Pathol.* 19, 327-330.

Chen, S., Andreasson, E. (2001). Update on glucosinolate metabolism and transport. *Plant Physiol. Biochem.* 39, 743-758.

Chew, F. S. (1988). Biological effects of glucosinolates. In *Biologically active natural product: Potential use in agriculture*, pp. 155-181. Edited by H. G. Cutler: American Chemical Society.

Clossais-Besnard, N., Larher, F. (1991). Physiological role of glucosinolates in *Brassica napus*. Concentration and distribution pattern of glucosinolates among plant organs during a complete life cycle. *J. Sci. Food Agric.* 56, 25-38.

Condie, J., Taylor, J. (1999). ABC transporters from *Leptosphaeria maculans*: Potential involvement in toxin secretion and phytoalexin resistance. In *9th International Congress of Molecular Plant-Microbe Interactions*. Amsterdam

Cooley, M. B., Pathorana, S., Wu, H. J., Kachroo, P., Klessig, D. F. (2000). Members of the Arabidopsis *HRT/RPP8* family of genes confer resistance to both viral and oomycete pathogens. *Plant Cell* 12, 663-676.

Cozijnsen, A., Popa, K. M., Purwantara, A., Rolls, B. D., Howlett, B. J. (2000). Genome analysis of the plant pathogenic ascomycete *Leptosphaeria maculans*; mapping mating type and host specificity. *Mol. Plant Pathol.* 1, 293-302.

Dangl, J. L., Jones, D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826-833.

de Wit, P., Joosten, M. (1999). Avirulence and resistance genes in the *Cladosporium fulvum*-tomato interaction. *Curr. Opin. Microbiol.* 2, 368-373.

Deslandes, L., Olivier, J., Theulières F., Hirsch J., Feng, D. X., Bittner-Eddy, P., Beynon, J., Marco, Y. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci.* 99, 2404-2409.

Dixelius, C. (1994). Presence of the pathogenesis-related proteins 2, Q and S in stressed *B. napus* and *B. nigra* plantlets. *Physiol. Mol. Plant Pathol.* 44, 1-8.

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

Doughty, K., Kiddle, G., Pye, B., Wallsgrove, R., Pickett, J. (1995). Selective induction of glucosinolates in oilseed rape leaves by methyl jasmonate. *Phytochem.* 38, 347-350.

Doughty, K. J., Porter, A. J. R., Mortin, A. M., Kiddle, G., Bock, C. H., Wallsgrove, R. (1991). Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.)leaves. II. Response to infection by *Alternaria brassicae* (Berk.) Sacc. *Ann. Appl. Biol.* 118, 469-477.

Doughty, K. J., Blight, M. M., Bock, C. H., Fieldsend, J. K., Pickett, J. A. (1996). Release of alkenyl isothiocyanates and other volatiles from *Brassica rapa* seedlings during infection by *Alternaria brassicae*. *Phytochem.* 43, 371-374.

Falk, A., Xue, J., Lenman, M., Rask, L. (1992). Sequence of cDNA clone encoding the enzyme myrosinase and expression of myrosinase in different tissues of *Brassica napus*. *Plant Sci.* 83, 181-186.

Falk, A., Taipalensuu, J., Ek, B., Lenman, M., Rask, L. (1995). Characterization of a myrosinase binding protein. *Planta* 195, 387-395.

Falk, A., Ek, B. and L. Rask (1995). Characterization of a new myrosinase in *Brassica napus*. *Plant Mol. Biol.* 27, 863-874.

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

Ferreira, M. E., Rimmer, S. R., Williams, P. H., Osborn, T. C. (1995). Mapping loci controlling *Brassica napus* resistance to *Leptosphaeria maculans* under different screening conditions. *Phytopathol.* 85, 213-217.

Flor, H. H. (1947). Host-parasite interactions in flax rust - Its genetics and other implications. *Phytopathol.* 45, 680-685.

Fristensky, B., Balcerzak, M., Daifen, H., Zhang, P. (1999). Expressed sequence tags from the defense response of *Brassica napus* L. to *Leptosphaeria maculans*. *Mol. Plant Pathol. On-line*, http://www.bsp.org.uk/mppol/1999/0301fristensky.

Giamoustaris, A., Mithen, R. (1997). Glucosinolates and disease resistance in oilseed rape (*Brassica napus* ssp. *oleifera*). *Plant Pathol.* 46, 271-275.

Giamoustaris, A., Mithen, R. A. (1995). The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126, 347-363.

Glazebrook, J. (2001). Genes controlling expression of defense responses in *Arabidopsis* - 2001 status. *Curr. Opin. Plant Biol.* 4, 301-308.

Glendening, T. M., Poulton, J. E. (1988). Glucosinolate biosynthesis. Sulfation of desulfobenzylglucosinolate by cell-free extracts of cress (*Lepidium sativum* L.). *Plant Physiol.* 86, 319-321.

Glimelius, K. (1999). Somatic hybridization. In *Biology of Brassica Coenospecies*, pp. 107-148. Edited by C. Gomez-Campo. Amsterdam: Elsevier Science B. V.

Gou, L., Poulton, J. E. (1994). Partial purification and characterization of *Arabidopsis thaliana* UDPG:thiohydroximate glucosyltransferase. *Phytochem.* 36, 1133-1138.

Greenhalgh, J. R., Mitchell, N. D. (1976). The involvment of flavour volatiles in the resistance to downey mildew of wild and cultivated forms of *Brassica oleracea*. *New Phytol.* 77, 391-398.

Griffiths, D. W., Birch, A. N. E., Hillman, J. R. (1998). Antinutritional compounds in the *Brassicaceae*. Analysis, biosynthesis, chemistry and dietary effects. *Hort. Sci. Biotechnol.* 73, 1-18.

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

Halkier, B. A. (1999). Glucosinolates. In *Naturally occuring glycosides: chemistry, distribution and biological properties*, pp. 193-223. Edited by R. Ikan. London: John Wiley and Sons Ltd.

Hall, C., McCallum, D., Prescott, A., Mithen, R. (2001). Biochemical genetics of glucosinolate modification in *Arabidopsis* and *Brassica*. *Theor. Appl. Genet.* 102, 369-374.

Hammond, K. E., Lewis, B. G., Musa, T. M. (1985). A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathol.* 34, 557-565.

Hammond, K. E., Lewis, B. G. (1986). Ultrastructural studies of the limitations of lesions caused by *Leptosphaeria maculans* in stems of *B. napus* var. *oleifera*. *Physiol. Mol. Plant Pathol.* 28, 251-265.

Hasegawa, T., Yamada, K., Kosemura, S., Yamamura, S., Hasegawa, K. (2000). Phototropic stimulation induces the conversion of glucosinolate to phototropism-regulating substances of radish hypocotyls. *Phytochem.* 54, 275-279.

Heinricher, E. (1884). Über Eiweistoffe führende Idioblasten bei einigen Cruciferen. *Ber. Dtsch. Bot. Ges.* II, 463-466.

Hemingway, J. S. (1995). The mustard species: Condiment and food ingredient use and potential as oilseed crops. In *Brassica Oilseeds*, pp. 373-383. Edited by D. Kimber, McGregor, D. I. Wallingford: CAB International.

Hennin, C., Höfte, M., Diedrichsen, E. (2001). Functional expression of *Cf9* and *Avr9* genes in *Brassica napus* induces enhanced resistance to *Leptosphaeria maculans*. *Mol. Plant-Microbe Interact.* 14, 1075-1085.

Hogge, L. R., Reed, D. W., Underhill, E. W. & G. W. Haughn (1988). HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography/mass spectrometry. *J. Chrom. Sci.* 26, 551-556.

Howlett, B., Idnurm, A., Soledade-Pedras, M. (2001). *Leptosphaeria maculans,* the causal agent of blackleg disease of Brassicas. *Fungal Genet. Biol.* 33, 1-14.

Höglund, A.-S., Lenman, M., Falk, A., Rask, L. (1991). Distribution of myrosinase in rapeseed tissues. *Plant Physiol.* 95, 213-221.

Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., Sceels, D. (1997). Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc. Natl. Acad. Sci.* 94, 4800-4805.

Johal, G. S., Briggs, S. P. (1992). Reductase activity encoded by the *Hm1* disease resistance gene in maize. *Science* 258, 985-987.

Jones, D. A., and Jones, J. D. G. (1997). The role of leucine rich repeat proteins in plant defences. *Adv. Bot. Res.* 24, 89-167.

Jones, D. A. (2000). Resistance genes and resistance protein function. In *Molecular Plant Pathology. Ann. Plant Rev.*, pp. 108-143. Edited by M. Dickinson, Beynon, J. Sheffield: Sheffield Academic Press.

Kaur, S., Gupta, S. K., Sukhija, P. S., Munshi, S. K. (1990). Accumulation of glucosinolates in developing mustard (*Brassica juncea* L.) seeds in response to sulphur application. *Plant Sci.* 66, 181-184.

Kawchuk, L. M., Hachey, J., Lunch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., Prüfer, D. (2001). Tomato *Ve* disease resistance genes encode cell suface-like receptors. *Proc. Natl. Acad. Sci.* 98, 6511-6515.

Kiddle, G., Doughty, K., Wallsgrove, R. (1994). Salicylic acid-induced accumulation of glucosinolates in oilseed rape (*Brassica napus* L.) leaves. *J. Exp. Bot.* 45, 1343-1346.

Kliebenstein, D., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., Mitchell-Olds, T. (2001). Genetic control of natural varaition in *Arabidopsis* glucosinolate accumulation. *Plant Physiol.* 126, 811-825.

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

Koroleva, O. A., Davies, A., Deeken, R., Thorpe, M. R., Tomos, A. D., Hedrich, R. (2000). Identification of a new glucosinolate-rich cell type in *Arabidopsis* flower stalk. *Plant Physiol.* 124, 599-608.

Kuno, K., Matsushima, K. (1994). The IL-1 receptor signalling pathway. *J. Leukocyte Biol.* 56, 542-547.

Kuusk, A.-K., Happstadius, I., Zheu, L., Steventon, L. A., Giese, H., Dixelius, C. (2002). Presence of *Leptosphaeria maculans* groups A and B isolates in Sweden. *J. Phytopathol.* (accepted)

Lagercrantz, U., Lydiate, D. J. (1996). Comparative mapping in *Brassica*. *Genetics* 144, 1903-1910.

Lam, E., Kato, N., Lawton, M. (2001). Programmed cell death, mitochondia and the plant hypersensitive response. *Nature* 411, 848-853.
Lambdon, P. W., Hassall, M., Mithen, R. F. (1999). Feeding preferences of wood pigeons and flea beetles for oilseed rape and turnip rape. *Ann. Appl. Biol.* 133, 313-328.
Lamkadmi, Z., Esnaoult, M. A., Normand, M. E. (1996). Characterization of a 23 kDa polypeptide induced by *Phoma lingam* in *Brassica napus* leaves. *Plant Physiol. Biochem.* 34, 589-598.
Lenman, M., Falk, A., Rödin, J., Höglund, A. S., Ek, B., Rask, L. (1993). Differential expression of myrosinase gene families. *Plant Physiol.* 103, 703-711.
Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H., Scheel. D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. *Science* 276, 2054-2057.
Lim, L., Howlett, B. J. (1994). Linear plasmids, pLM9 and pLM10, can be isolated from the phytopathogenic ascomycete *Leptosphaeria maculans* by pulsed-field gel electrophoresis. *Curr. Genetics* 26, 276-280.
Ludwig-Müller, J., Hilgenberg, W. (1988). A plasma membrane-bound enzyme oxidizes L-tryptophan to indole-3-acetaldoxime. *Physiol. Plant.* 74, 240-250.
Ludwig-Müller, J., Rausch, T., Lang, S., Hilgenberg, W. (1990). Plasma membrane bound high plant isoenzymes convert tryptophane to indole-3-acetaldoxime. *Phytochem.* 29, 1397-1400.
Lykkesfeldt, J., Lindberg-Møller, B. (1993). Synthesis of benzylglucosinolate in *Tropaeolum majus* L.- Isothiocyanates as potent enzyme inhibitors. *Plant Physiol.* 102, 609-613.
Lüthy, B., Matile, P. (1984). The mustard oil bomb: rectified analysis of the subcellular organization of the myrosinase system. *Biochemie und Physiologie der Pflanzen* 179, 5-12.
Machlin, S., Mitchell-Olds, T., Bradley, D. (1993). Sequence of a *Brassica campestris* myrosinase gene. *Plant Physiol.* 102, 1359-1360.
Magrath, R. R. M. (1993). Maternal effects on the expression of individual aliphatic glucosinolates in seeds and seedlings of *Brassica napus*. *Plant Breed.* 111, 249-252.
Mailer, R. J. (1989). Effects of applied sulphur on glucosinolate and oil concentrations in the seeds of rape (*Brassica napus* L.) and turnip rape (*Brassica rapa* L. var. *silvestris* (Lam.) Briggs). *Austr. J. Exp. Agr.* 40, 617-624.
Maleck, K., Dietrich, R. A. (1999). Defense on multiple fronts: how do plants cope with diverse enemies. *Trends Plant Sci.* 4, 215-219.
Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., Tanksley, S. D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262, 1432-1436.
Mauch-Mani, B., Metraux, J. P. (1998). Salicylic acid and systemic acquired resistance to pathogen attack. *Annals of Botany* 82, 535-540.
Meyerhofer, R., Bansal, V. K., Thiagarajah, M. R., Strimgam, G. R., Good, A. G. (1997). Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. *Genome* 40, 294-301.
Milford, G. F. J., Fieldsend, J. K., Porter, A. J. R., Rawlinson, C. J., Evans, E. J., Bilsborrow, P. (1989). Changes in glucosinolate concentrations during the vegetative growth of single and double low cultivars of winter oilseed rape. *Asp. Appl. Biol.* 23, 83-90.
Mithen, R. F., Lewis, B. G., Heany, R. K., Fenwick, G. R. (1984). Glucosinolates of wild and cultivated *Brassica* species. *Phytochem.* 26, 1969-1973.
Mithen, R. F., Lewis, B. G., Fenwick, G. R. (1986). In vitro activity of glucosinolates and their products against *Leptosphaeria maculans*. *Trans. Brit. Mycol. Soc.* 87, 433-440.
Mithen, R. F., Lewis, B. G., Fenwick, G. R., Heaney, R. K. (1987). Resistance of *Brassica* species to *Leptosphaeria maculans*. *Trans. Brit. Mycol. Soc.* 88, 525-531.

Mithen, R. F., Magrath, R. (1992). Glucosinolates and resistance to *Leptosphaeria maculans* in wild and cultivated *Brassica* species. *Plant Breed.* 108, 60-68.
Mithen, R. F. (2001). Glucosinolates and their degradation products. *Adv. Bot. Res.* 35, 213-262.
Monde, K., Tagasugi, M., Ohnishi, T. (1994). Studies on stress metabolites. 19. Biosynthesis of cruciferous phytoalexins. *J. Am. Chem. Soc.* 116, 6650-6657.
Morinaga, T. (1934). Interspecific hybridization in *Brassica*. VI. The cytology of F1 hybrids of *B. juncea* and *B. nigra*. *Cytologia* 6, 62-67.
Morisato, D., Anderson, K. V. (1995). The signalling pathways that establish dorsal-ventral pattern of the *Drosophila* embryo. *Ann. Rev. Genet.* 29, 371-399.
Murphy, A. M., Holcombe, L. J., Carr, J. P. (2000). Characteristics of salicylic acid induced delay in disease caused by a necrotrophic fungal pathogen in tobacco. *Physiol. Mol. Plant Pathol.* 57, 47-54.
Norman-Setterblad, C., Vidal, S., Palva, E. T. (2000). Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell-wall degrading enzymes from *Erwinia carotovora*. *Mol. Plant-Microbe Interact.* 13, 430-438.
Oldfield, M. F., Bennett, R. N., Kiddle, G., Wallsgrove, R. M., Botting, N. P. (1999). Biochemical characterization of an aldoxime-forming flavoprotein involved in 2-phenylethylglucosinolate biosynthesis in *Brassica* species. *Plant Physiol. Biochem.* 37, 99-108.
Olsson, G., Ellerström, S. (1960). Species crosses within the genus *Brassica*: II. Artificial *Brassica napus* L. *Hereditas* 46, 351-386.
Parniske, M. (2000). Intracellular accomodation of microbes by plants: a common developmental program for symbiosis and disease? *Curr. Opin. Plant Biol.* 3, 320-328.
Pedras, M. S. C., Taylor, J. L., Nakashima, T. T. (1993). A novel chemical signal from the blackleg fungus: Beyond phytotoxins and phytoalexins. *J. Org. Chem.* 58, 4778-4780.
Pedras, M. S. C., Biesenthal, C. J. (1998). Production of the host-selective toxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. *Can. J. Microbiol.* 44, 547-553.
Pieterse, C. M. V., van Pelt, J. A., van Wees, S. C. M., Ton, J., Leon-Kloosterziel, K. M., Keurentjes, J. J. B., Varhagen, B. W. M., Nkoester, M., van der Sluis, I., Bakker, P. A H. M., van Loon, L. C. (2001). Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. *Eur. J. Plant Pathol.* 107, 51-61.
Pilet, M. L., Delourme, R., Foisset, N., Renard, M. (1998). 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.). *Theor. Appl. Genet.* 96, 23-30.
Pongam, P. Osborn, T. C., Williams, P. H. (1998). Genetic analysis and identification of amplified fragment length polymorphic markers linked to the *alm1* avirulence gene of *Leptophaeria maculans*. *Phytopathol.* 88, 1068-1072.
Porter, A. J. R., Morton, A. M., Kiddle, G., Doughty, K. J. and R. J. Wallsgrove (1991). Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.) leaves. I. Effect of leaf age and position. *Ann. Appl. Biol.* 118, 461-467.
Pracros, P. M. R., Gourdoux, L. (1992). Metabolic alterations induced by ingested glucosinolates in yellow mealworm larvae (*Tenebrio molitor* L.). *Animal Biol.* 1, 77-83.
Purwantara, A., Salisbury, P. A., Burton, W. A., Howlett, B. J. (1998). Reaction of *Brassica juncea* (Indian mustard) lines to Australian isolates of *Leptosphaeria maculans* under glasshouse and filed conditions. *Eur. J. Plant Pathol.* 104, 895-902.
Purwantara, A., Barrins, J. M., Cozijnsen, A. J., Ades, P. K., Howlett, B. J. (2000). Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. *Mycol. Res.* 104, 772-781.
Quiros, C. F. (1999). Genome structure and mapping. In *Biology of Brassica Coenospecies*, pp. 217-245. Edited by C. Gomez-Campo. Amsterdam: Elsevier Science B. V.

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

Rasmussen, U., Bojsen, K., Collinge, D. (1992). Cloning and characterization of a pathogen-induced chitinase in *Brassica napus*. *Plant Mol. Biol.* 20, 277-287.

Reed, D. W., Davin, L., Jain, J. C., Deluca, V., Nelson, L., Underhill, E. W. (1993). Purification and properties of UDP-glucose:thiohydroximate glucosyltransferase from *Brassica napus* L. seedlings. *Arch. Biochem. Biophys.* 305, 526-532.

Ridley, B. L., O'Neill, M. A., Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signalling. *Phytochem.* 57, 929-967.

Rimmer, S. R., van den Berg, C. G. J. (1992). Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans*. *Can. J. Plant Pathol.* 14, 56-66.

Rimmer, S. R., Buchwaldt, L. (1995). Diseases. In *Brassica Oilseeds*. Edited by D. Kimber, McGregor, D. I. Wallingford: CAB International.

Rosa, E. A. S. (1997). Daily variation in glucosinolate concentrations in the leaves and roots of cabbage seedlings in two constant temperature regimes. *J Sci Food Agric* 73, 364-368.

Rossi, M., Goggin, F., Milligan, S. B., Kaloshian, I., Ullman, D. E., Williamson, V. M. (1998). The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci. USA* 95, 9570-9754.

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

Rouxel, T., Kollmann, A., Boulidard, L. and R. Mithen (1991). Abiotic elicitation of indole phytoalexins and resistance to *Leptosphaeria maculans* within *Brassicaceae*. *Planta* 184, 271-278.

Sacristan, M. D., Gerdemann, M. (1986). Different behaviour of *Brassica juncea* and *B. carinata* as sources of *Phoma lingam* resistance in experiments of interspecific transfer to *B. napus*. *Z. Pflanzenzüchtg.* 97, 304-314.

Salisbury, P., Ballinger, D. (1993). Race variability in *Leptosphaeria maculans*. *Blackleg Newsletter* 1, 3-4.

Salmeron, J. M., Oldroyd, G. E. D., Rommens, C. M. T., Scofield, S. R., Kim, H. S., Lavelle, D. T., Dahlbeck, D., Staskawicz, B. (1996). Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* 86, 123-133.

Sarwar, M., Kirkegaard, J. A., Wong, P. T. W., Desmarchelier, J. M. (1998). Biofumigation potential of brassicas. III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil* 201, 103-112.

Schnug, E., Haneklaus, S. (1993). Physiological backgrounds of different sulphur utilization in *Brassica napus* varieties. *Asp. Appl. Biol.* 34, 235-242.

Searle, L. M., Chamberlain, K., Rausch, T., Butcher, D. N. (1982). The conversion of 3-indolylacteonitrile by myrosinase, and its relevance to the clubroot disease of the Cruciferae. *J. Exp. Bot.* 33, 935-942.

Séguin-Swartz, G., Chen, C. Y., Gugel, R. K. (2000). Blackleg resistance: Where and how? *Blackleg News* 6, 9-12.

Sexton, A. C., Kirkegaard, J. A., Howlett, B. J. (1999). Glucosinolates in *Brassica juncea* and resistance to Australian isolates of *Leptosphaeria maculans*, the blackleg fungus. *Austr. Plant Pathol.* 28, 95-102.

Sexton, A. C., Howlett, B. J. (2000). Characterisation of a cyanide hydratase gene in the phytopathogenic fungus *Leptosphaeria maculans*. *Mol. Gen. Genet.* 263, 463-470.

Sexton, A. C., Howlett, B. J. (2001). Green flourescent protein as a reporter in the *Brassica* - *Leptosphaeria maculans* interaction. *Physiol. Mol. Plant Pathol.* 58, 13-21.

Shauser, L., Roussis, A., Stiller, J., Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402, 191-195.

Shoemaker, R. A., Brun, H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Can. J. Bot.* 79, 412-419.

Siemens, D. H., Mitchell-Olds, T. (1996). Glucosinolates and herbivory by specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): consequences of concentration and induced resistance. *Env. Entomol.* 25, 1344-1353.

Sjödin, C., Glimelius, K. (1988). Screening for resistance to blackleg, *Phoma lingam* (Tode ex Fr.)Desm. within *Brassicaceae*. *J. Phytopathol.* 123, 322-332.

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

Song, K. M., Osbourn, T. C., Williams, P. H. (1988). *Brassica* taxonomy based on nuclear restriction fragment polymorphisms (RFLPs). 1. Genome evolution of diploid and amphidiploid species. *Theor. Appl. Genet.* 75, 789-794.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. Z., Zhu, L. H., Fauquet, C., Ronald, P. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. *Science* 270, 1804-1806.

Steventon, L. A., Fahleson, J., Hu, Q., Dixelius, C. (2002). Identification of the causal agent of Verticillium wilt of winter oilseed rape in Sweden as *V. longisporum*. *Mycol. Res.* (accepted)

Struss, D., Quiros, C. F., Plieske, J., Röbbelen, G. (1996). Construction of *Brassica* B genome synteny groups based on chromosomes extracted from three different sources by phenotypic, isozyme and molecular markers. *Theor. Appl. Genet.* 93, 1026-1032.

Taipalensuu, J., Eriksson, S., Rask, L. (1997a). The myrosinase binding protein from *Brassica napus* seeds possesses lectin activity and has a highly similar vegetatively expressed wound-inducible counterpart. *Eur. J. Biochem.* 250, 680-688.

Taipalensuu, J., Andreasson, E., Eriksson, S., Rask, L. (1997b). Regulation of the wound-induced myrosinase-associated protein transcript in *Brassica napus* plants. *Eur. J. Biochem.* 247, 963-971.

Taipalensuu, J., Falk, A., Rask, L. (1996). A wound- and methyl jasmonate-inducible transcript coding for a myrosinase-associated protein with similarities to an early nodulin. *Plant Physiol.* 110, 483-491.

Takahata, Y., Hinata, K. (1986). A consideration of the species relationships in subtribe *Brassicinae (Cruciferae)* in view of cluster analysis of morphological characters. *Pl. Sp. Biol.* 1, 79-88.

Thangstad, O. P., Iversen, T. H., Slupphaug, G., Bones, A. (1990). Immunocytochemical localization of myrosinase in *Brassica napus* L. *Planta* 180, 245-248.

Thangstad, O. P., Evjen, K., Bones, A. (1991). Immunogold-EM localisation of myrosinase in *Brassicaceae*. *Protoplasma* 161, 85-93.

The *Arabidopsis* Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408, 796-813.

Thomma, B. P. H. J., Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogensang, R., Cammue, B. P. A., Broekaert, W. (1998). Separate jasmonate-dependent and salicylate-dependent defense response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci.* 95, 15197-15111.

Thompson, C., Dunwell, J. M., Johnstone, C. E., Lay, V., Ray, J., Scmidt, M., Watson, H., Nisbet, G. (1993). Degradation of oxalic acid by transgenic canola plants expressing oxalate oxidase. In *Proceedings of the Eighth Crucifer Genetics Workshop*. Satkatoon, Canada.

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

Tookey, H. L. (1973). Crambe thioglucoside glucohydrolase: Separation of a protein required for epithiobutane formation. *Can. J. Biochem.* 51, 1654-1660.
U, N. (1935). Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japan. J. Bot.* 7, 389-452.
Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward. E., Ryals, J. (1992). Acquired resistance in *Arabidopsis*. *Plant Cell* 4, 645-656.
Wallsgrove, R. M., Bennett, R. N., Doughty, K. J., Schrijvers, S., Kiddle, G. (1995). Glucosinolate metabolism in diseased plants. *Aspects Appl. Biol.* 42, 251-256.
van der Biezen, E. A., Jones, J. D. G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23, 454-456.
van Loon, L. C., van Strien, E. A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55, 85-97.
van Poppel, G., Verhoeven, D. T. H., Verhagen, H., Goldbohm, R. A. (1999). *Brassica* vegetables and cancer prevention - epidemiology and mechanisms. *Adv. Nutr. Cancer 2* 472, 159-168.
Wang, Y., Nowak, G., Culley, D., Hadwiger, L. A., Fristensky, B. (1999). Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). *Mol. Plant-Microbe Interact.* 12, 410-418.
Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz Jawhar, R., Ward, E., Uknes, S., Kessman, H., Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6, 959-965.
West, J. S., Kharbanda, P. D., Barbetti, M. J., Fitt, B. D. L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol* 50, 10-27.
Williams, P. H. (1992). Biology of *Leptosphaeria maculans. Can. J. Plant Pathol.* 14, 30-35
Wood, P., Barbetti, M. J. A. (1977). A study in the inoculation of rape seedlings with ascospores and pycnidiospores of the blackleg disease causal agent, *Leptopshaeria maculans*. *J. Aust. Inst. Agric. Sci.* 43, 43.
Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., Turner, J. (2001). Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. *Science* 291, 118-120.
Xue, J., Lenman, M., Falk, A., Rask, L. (1992). The glucosinolate-degrading enzyme myrosinase in *Brassicaceae* is encoded by a gene family. *Plant Mol. Biol.* 18, 387-398.
Zhao, F., Evans, E. J., Bilsborrow, P. E. and J. K. Syers (1994). Influence of nitrogen and sulphur on the glucosinolate profile of rapeseed (*Brassica napus* L). *J Sci Food Agric* 64, 295-304.
Zhao, F. J., Blake-Kalff, M. M. A., Riley, N., Hawkesford, M. J., McGrath, S. P. (1999). Sulphur utilisation efficiency in oilseed rape. In *Proceedings of 10th International Rapeseed Congress*. Canberra.

Acknowledgements

And now to the most important section. The only part everybody reads. Who cares if you forget a reference or two? Acknowledgements, on the other hand, is serious stuff! Honestly, making a thesis is not so hard if you are surrounded by the right people. I have definitely been lucky.

First of all, sincere thanks to my excellent supervisor Christina. It has been a pleasure working with you – always supportive, encouraging and enthusiastic. Always hard working, but never too busy to spare a moment. Thank you!

To my bosses Kristina G and Per B, thanks for creating a friendly and professional atmosphere at the department!

I especially want to thank all past and present members of the "Dixan group". Gunilla has been an incredible help with practical things from plants to test tubes. Svante - thanks for good company and collaboration (I wish I had taken your advice on limiting the calory intake during thesis writing…). Lisa, this place just isn't the same since you left! I miss our giggles in and and outside the office. Einar, I miss your helpful attitude and happy mood. Janne… what can I say? It's a privilege to be around when you spread your wisdom, no matter what the subject is. Qiong, Patrick, Lecong – thanks for giving our group an international touch! Jens and Anna, it's nice to know someone will care for my clones in the future! And Linda, my two-times summer student – thanks for your great work on the lambda library!

Special thanks also to the rest of the Plant Breeding section. To Ingrid, Gun and Maj-Britt – thanks for all your help and experience and for making the labwork run smoothly. Birgitta, ordning och reda, always with a smile on your face. Marie, thanks for being an inspiration in the teaching duties. To Matti, Marita, Carina, Berit, Anki, Vasilios, Oksana, Jenny and all others for fun company.

And to the people who left – Lili, sunshine, you moved too far away! Miss you terribly. Mia, luckily still in town, thanks for sharing your office and thoughts with me for a couple of years. To Maria L for creative spexing in the Plant Breeding group. Bo, thank you for providing the glucosinolate figures for my thesis. To Erik A for nice collaboration. To Susanna E, Karin F, Ulrika R and Malin E for always taking the time for some girl-talk.

To all other friends in near and far corridors at the Plant Biology Department – you made the last few years enjoyable! I would like to mention you all by name, but I won't.

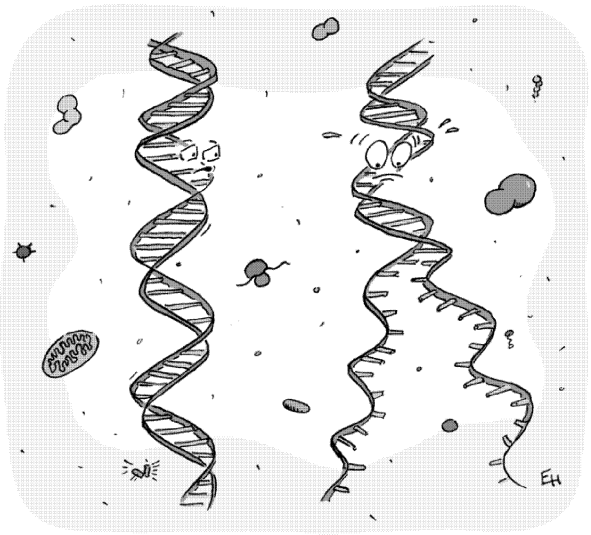
Thanks to Richard Hopkins for language corrections, and to Ed Himelblau for allowing me to illustrate my thesis with his cute cartoons. Ulla P, thanks for your never ending optimism and help with in situ experiments.

To Anders E and Anna R at the Micro…something department. It's nice having friends on the other side. To my dear friend Gabriella, come back soon! Meanwhile Lotta and I will save a seat for you at the biotech-babe lunches.

Tack till tjejligan Ulrika, Malin och Pernilla. Er vänskap är guld värd.
Jag vill också tacka de stolta kvinnorna i LaCappella för avkopplande skönsång, gärna på arbetstid. Och tack till Erik och Jenny för trevliga semestrar i snö och sol!

Mamma och pappa, tack för att ni alltid finns till hands när jag behöver er (och annars också, för den delen!). Tack för att ni uppmuntrat mina forskarstudier och muntrat upp mig när det behövts. Mina syskon Anders och Susanna, tack för att ni finns och visar att det alltid finns två sätt att se på saker och ting. Tack till mina svärföräldrar Anna och Hans, för att dörren till ert hem i Valbo alltid står öppen (och gör den inte det finns ju nyckeln i blomkrukan!). Och slutligen min älskade Per. Thanks for showing me... äh, du vet vad jag menar! Tack för all din support – både på det tekniska och känslomässiga planet. Du betyder allt.

Kram från *Sofia*



Psst, Bob...you're unzipped.