Genetics and Comparative Mapping of Resistance to *Leptosphaeria maculans* in *Brassica napus*

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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Blackleg, caused by *Leptosphaeria maculans*, is a serious disease of *Brassica* species. This disease is most effectively and economically controlled by the use of resistant cultivars. Genetic analysis of resistance to *L. maculans* was conducted in the *B. napus* accessions, RB87-62 and DH88-752. The two resistant accessions and a susceptible *B. napus* cultivar Westar were crossed in a complete diallel and their progenies were evaluated for disease reaction by cotyledon and stem inoculation with *L. maculans*, isolate Pl86-12 (PG2). For crosses between Westar and either resistant parents, the segregation of F_2 populations fit 3:1 (resistant:susceptible) both for cotyledon and adult plant reaction, indicating single dominant gene models. Analysis of the relationship between the cotyledon and adult plant resistance showed that genes controlling these two traits were different but linked. These results were confirmed by segregation data from F_3 families. The segregation in the cross between two resistant accessions demonstrated that the pairs of resistance genes from the two different sources were non-allelic and independently assorting.

DH lines developed from single F_1 plants of the crosses between Westar and RB87-62, DH88-752 or Crésor (a *B. napus* cultivar), were employed for comparative mapping for cotyledon and adult plant resistance to *L. maculans*. The cotyledon and adult plant resistance genes were mapped 6.1 cM apart in RB87-62 and 10.0 cM apart in DH88-752

respectively. In Crésor, only the adult plant resistance gene was mapped. Although the resistance genes carried by the three accessions were derived from different sources, comparison of the arrangement and map distances of closely linked RAPD, AFLP and RFLP markers on the three resistance linkage maps demonstrated that there is a conserved chromosomal region that carries the resistance genes. The conserved regions covered 41.4 cM in RB87-62, 36.5 cM in DH88-752 and at least 32.0 cM in Crésor.

RAPD1, a RAPD marker, was closely linked with cotyledon and adult plant resistance in RB87-62 and DH88-752. For marker-assisted selection (MAS), RAPD1 was converted to a sequence characterized amplified region (SCAR) marker by sequencing the targeted RAPD products. The SCAR marker was tested in two doubled haploid (DH) populations developed from Westar × RB87-62 and Westar × DH88-752 and the F_2 plants developed from RB87-62 × DH88-752 and in all cases amplified the locus corresponding to RAPD1. This marker could provide an efficient and reliable screening tool for selection of blackleg resistant plants in breeding programs.

FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. Manuscripts follow the style recommended by Theoretical and Applied Genetics. Three manuscripts, each containing an abstract, an introduction, materials and methods, results and discussion, are presented. The manuscripts are preceded by a general introduction and literature review and followed by a general discussion and literature cited.

CHAPTER 1

GENERAL INTRODUCTION

Brassica species are widely cultivated as vegetable crops, condiments and fodder plants throughout the world, and the largest cultivation of these crops is for vegetable oil production (Downey and Röbbelen 1989). Due to their economic importance, many aspects of oilseed *Brassica* improvements, especially those related to modifications in fatty acid composition and reduction in levels of glucosinolates in the residual meal, have been achieved in the past decades. The first low erucic acid, low glucosinolate, *Brassica napus* cultivar, Tower, was released in 1974. The "double low" oil and meal product cultivars, with less than 2% erucic acid as percent of the total fatty acids and less than 30 μmoles of glucosinolates per gram oil-free meal (Downey and Rimmer 1993) were designated as canola. Canola is used for oilseed rape (*B. napus* and *B. rapa*) crops that produce the canola products. These substantive changes in the quality of seed oil and meal composition have prompted substantial increases in areas of cultivation in Australia and Canada (Downey and Rimmer 1993).

However, with the increased production of oilseed *Brassica* crops, especially oilseed rape, the build up of several diseases has caused significant yield losses and remains a constant threat to oilseed production (Rimmer and Buchwaldt 1995). One of these diseases, blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* (Tode: Fr.) Desm.), is a devastating disease of *Brassica* species in many areas of the world. Because of the inadequacy of control by cultural and

chemical means, the incorporation of blackleg resistance into oilseed lines with desirable agronomic quality is a major objective in breeding programs worldwide (Downey and Rimmer 1993). The resistant cultivar Jet Neuf was successfully introduced in 1977 (Gladders and Musa 1979), the genetic analysis of resistance in different researches demonstrated that resistance is a heritable and selectable character (Rimmer and van den Berg 1992). Thereafter, a large collection of different sources of resistance to this disease has been screened and characterized (Rimmer and van den Berg 1992; Downey and Rimmer 1993). Similarly, resistance to *L. maculans* in *B. juncea* has been well characterized and is generally considered to be more effective than resistance observed in *B. napus* (Roy 1978, 1984). Consequently, efforts have been made to transfer the B genome resistance into *B. napus* through interspecific hybridization (Sacristán and Gerdemann 1986).

L. maculans can infect all parts of susceptible plants throughout their life cycle. Two types of resistance, cotyledon or seedling resistance and adult plant resistance, referring to the plant growth stage when the resistance to *L. maculans* is expressed, have been considered the most important and have been characterized. Seedling infection can lead to a systemic, latent infection (Nathaniels and Taylor 1983). Adult plant infection (canker at crown or blackleg) is the most damaging symptom in terms of yield losses. However, the relationship between the degree of cotyledon lesion development and that of subsequent crown canker development has not been firmly established. Some reports have indicated that there is a correlation between cotyledon and adult plant resistance (Newman and Bailey 1987; McNabb et al. 1993; Bansal et al. 1994). Recent studies

suggested that cotyledon resistance and adult plant resistance could be two distinct traits and under different genetic control (Ferreira et al. 1995; Pang and Halloran 1996b; Ballinger and Salisbury 1996). Since genetic information on the relationship between cotyledon resistance and adult plant resistance is limited, the research presented here was carried out to determine the genetic control of resistance to *L. maculans* at cotyledon and adult plant stages respectively in crosses between a susceptible *B. napus* cultivar and two resistant accessions.

The importance of *Brassica* crops has led to the need to improve their agronomic characteristics. This has prompted extensive investigation into the genome organization, inter- and intra- genomic relationships, and genome evolution between the economically important *Brassica* species. In the past decade, the molecular markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD), has been used to identify extensive molecular synteny among, and internal duplication within, the *Brassica* species (Song et al. 1991; Chyi et al. 1992; Lagercrantz and Lydiate 1995; Cheung et al. 1997; Slocum et al. 1990; Landry et al. 1991). This molecular elucidation combined with previous cytological evidence (Röbbelen 1960; Prakash and Hinata 1980) has led to the reaffirmation that diploid species of *Brassica* possess duplicated genomes, and that they may have evolved from an ancestral species with a lower basic chromosome number through the process of polyploidy and aneuploidy.

In recent years, a number of resistance genes (loci) from different sources conferring resistance to *L. maculans* have been characterized and corresponding resistance linkage

maps have been developed in *B. napus* (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997) and in the *Brassica* species containing the B genome (Plieske et al. 1998). However, most of these resistance linkage maps were constructed independently with different sets of probes, as well as different *Brassica* cultivars/lines, *L. maculans* isolates and methods of disease measurement. These differences have hindered the comparison of these linkage groups to identify molecular homology and colinearity in the region in which the resistance genes are located. Numerous studies of genetic mapping in *Brassica* species have shown that extensive conservation of linkage arrangements is common among *Brassica* species (Song et al. 1991; Chyi et al. 1992; Lagercrantz and Lydiate 1995; Cheung et al. 1997; Slocum et al. 1990; Landry et al. 1991).

Molecular markers, when combined with bulked segregant analysis (Michelmore et al. 1991), have been widely used for identifying markers associated with various disease resistance genes (Michelmore 1995; Young 1996; Mohan et al. 1997). More specific markers such as sequence characterized amplified region (SCAR) (Paran and Michelmore 1993) were developed for marker-assisted selection (MAS) for many disease resistance genes (Paran and Michelmore 1993; Adam-Blondon et al. 1994; Penner et al. 1995; Wechter and Dean 1998). Utilization of host resistance is the most effective method to control *L. maculans* and some common resistance sources are widely used in resistant cultivar breeding program. Therefore, developing molecular markers linked to resistance genes will greatly facilitate the process of breeding for resistance to this important disease.

The objectives of this study were:

1. To determine the inheritance of resistance to *L. maculans* in two *B. napus* accessions.
2. To utilize RAPD, AFLP and RFLP analysis to map the resistance gene(s) using double haploid (DH) lines.
3. To elucidate the homology of the regions carrying resistance to *L. maculans* in *B. napus* accessions through comparative mapping.
4. To develop sequence characterized amplified region (SCAR) markers for marker-assisted selection in resistance breeding program.

CHAPTER 2

LITERATURE REVIEW

2.1 The Host

2.1.1 History and economic importance

Historically, cultivation of *Brassica* species as a vegetable oil source for human consumption was concentrated in India and China (Downey and Rimmer 1993), and was recorded as long ago as 1500 BC (Prakash 1980). Since the Second World War, a dramatic increase in *Brassica* oilseed production has occurred worldwide. China, Europe, India and Canada are the major producers of oilseed *Brassica* crops (Kimber and McGregor 1995). One of the reasons for this increase is the favorably modified fatty acid composition in the oil and enhanced quality in the seed meal of canola. The present high interest in using rapeseed oil for industrial purposes, e.g. as a hydraulic fluid replacement or as an additive to diesel fuel, could result in expanded uses, hence more market opportunities (Downey and Rimmer 1993).

2.1.2 Genomic relationships of *Brassica* species

The genomic relationships among the common *Brassica* species have been established by cytological evidence (Morinaga 1934), and confirmed by the artificial synthesis of *Brassica napus* L. from crosses between the diploid species *Brassica rapa* L. and *Brassica oleracea* L. (U 1935). *B. rapa* (2n=20; AA), *Brassica nigra* L. (2n=16; BB), *B. oleracea* (2n=18; CC) are diploid species and *Brassica carinata* Braun (2n=34; BBCC), *Brassica juncea* Czern.

and Coss. (2n=36; AABB) and *B. napus* (2n=38; AACC) are amphidiploids resulting from interspecific hybridization between corresponding pairs of the primary diploid species. These relationships have been confirmed by the artificial resynthesis of the amphidiploid species by crosses between present representatives of the diploid species (Prakash and Hinata 1980; Attia and Röbbelen 1986). Genome organization and the extent of chromosomal rearrangement which occurred during the evolution of these species has been characterized by DNA-based marker analyses such as RFLP (McGrath and Quiros 1991; Chyi et al. 1992; Truco and Quiros 1994; Teutonico and Osborn 1994; Parkin et al. 1995; Sharpe et al. 1995; Cheung et al. 1997; Lagercrantz and Lydiate 1996).

2.2 The pathogen

2.2.1 Biology and taxonomy

Leptosphaeria maculans (Desm.) Ces. & de Not. is a hemibiotrophic and pseudothecial loculoascomycete, with heterothallic, bipolar (1 gene, 2 alleles) mating control (Venn 1979; Cargeeg and Thurling 1980). *L. maculans* produces pseudothecia containing cylindrical bitunicate asci, each ascus has eight spindle shaped, haploid ascospores. *Phoma lingam* (Tode ex Fr.) Desm. is the imperfect state of *L. maculans*. A notable characteristic in the dispersal of *P. lingam* is asexual spores produced in pycnidia. Large numbers of single-celled, hyaline, cylindrical conidia are exuded from pycnidia in a pinkish matrix and dispersed by rain splash (Boerema et al. 1981; Williams 1992).

L. maculans overseasons on infected crop residue and seed (Petrie 1978; Gladders and Musa 1980). Oilseed rape can be infected initially by either ascospores or pycnidiospores as

early as the time of seedling emergence with spores present on seed or deposited by wind or rain. Secondary infection can be caused by pycnidiospores (Krüger 1983; Gladders and Musa 1980).

2.2.2 Pathogenicity

Isolates of *L. maculans* may be consistently divided into aggressive or non-aggressive groups. Unsuccessful crosses and different patterns of molecular analyses between the two groups suggested that they might actually represent different species (Badawy et al. 1991; Delwiche 1980; Koch et al. 1991; Mengistu et al. 1991; Meyer et al. 1992; Schäfer et al. 1992; Plummer et al. 1994). Within the aggressive types of *L. maculans*, evidence for pathogenic specialization has been reported by Koch et al. (1991). Based on the disease reaction of isolates on cotyledons of three *B. napus* cvs. Westar, Glacier, and Quinta, three pathogenicity groups (PGs) could be identified. They were designated as PG2, PG3, and PG4 respectively. Nonaggressive isolates were designated as PG1. Though isolates of PG3 and PG4 predominate in Australia and Europe, PG2 isolates are common in western Canada (Williams 1992). In nature, high variabilities of virulence within the population of *L. maculans* may exist. Within four PGs, 19 subgroups have been reported on a set of resistant cultivars (Kuswinanti et al. 1995).

2.3 Disease and occurrence

L. maculans can infect many members of the Cruciferae including the six economically important *Brassica* species, *B. rapa*, *B. nigra*; *B. oleracea*, *B. napus*, *B. juncea*, and *B.*

carinata (Williams 1974). Cotyledons, leaves, stems, crown, roots and pods of these species can be attacked by virulent isolates, causing seedling damping-off, stem canker, leaf lesions, shriveled pods and seeds, girdled stem crown (blackleg) as common symptoms (Gabrielson 1983; Petrie 1979).

Losses in cruciferous crops due to blackleg have been documented for over 100 years (Henderson 1918). This disease is now epidemic in most rapeseed producing regions in Australia, Europe and western Canada (Salisbury et al. 1995; West et al. 1999; Gugel and Petrie 1992). In the early 1970s, rapid expansion of cultivation of *B. napus* was followed by blackleg epidemics in France and Britain (Alabouvette and Brunin 1970; Cook and Evans 1978). In eastern England, stem cankers affected 84% and 77% of plants in pre-harvest samples in 1995 and1996 respectively (Gladders et al. 1998). In Australia where rapeseed production began in the mid 1960s, severe blackleg epidemics posed a major threat to the industry. As a result, the area sown to rapeseed plummeted and did not increase significantly for a decade (Salisbury et al.1995). In western Canada, aggressive isolates of *L. maculans* were first discovered in Saskatchewan in 1975, in Alberta in 1983, and in Manitoba in 1984 (Gugel and Petrie 1992), and blackleg outbreaks in most rapeseed growing regions have occurred. Overall, there is ample evidence that blackleg can severely limit rapeseed production in these countries (Salisbury et al. 1995; West et al. 1999; Gugel and Petrie 1992).

Several strategies, such as crop rotation, stubble management, chemical control, sanitation and the cultivation of resistant cultivars, have been advised for blackleg control (Cook and Evans 1978; Petrie 1986; Alabouvette and Brunin 1970; Roy 1978). Among these options,

cultivation of resistant cultivars is the most effective and the least costly method to achieve sustainable control of this important disease (Rimmer and van den Berg 1992; Gugel and Petrie 1992).

2.4 Aspects of *Brassica* resistance

The incorporation of blackleg resistance into *B. napus* lines with desirable agronomic qualities is a major objective of oilseed rape breeding programs in western Canada, Australia and Europe where the disease has seriously caused yield losses and remains a constant threat to oilseed rape production (Downey and Rimmer 1993). Due to inadequacy of control of the disease by cultural management (rotation, stubble treatment) and chemical practices (foliar spray, seed treatment), the use of resistant cultivars has become the most promising strategy for the sustainable management of blackleg (Rimmer and van den Berg 1992; Gugel and Petrie 1992).

2.4.1 Resistance sources

From studies on the evaluation of resistance in *B. napus* to *L. maculans*, a large number of resistant accessions has been reported (Rimmer and van den Berg 1992; Downey and Rimmer 1993). The major sources of resistance used in breeding programs throughout the world have been European cultivars such as Major, Jet Neuf, Cresus, etc. and Japanese cultivars such as Chisaya, Chikuzen, Norin 20 and Mutu (Rimmer and van den Berg 1992; Salisbury et al. 1995).

In contrast to *B. napus*, *B. juncea*, *B. carinata* and *B. nigra* exhibit a high level of

resistance to *L. maculans*, although some susceptible cultivars within these species have been reported (Sjödin and Glimelius 1988; Roy 1978; Sawatsky 1989; Gugel et al. 1990; Keri 1991). The infection response on seedlings of most cultivars of *B. juncea* to *L. maculans* is associated with a hypersensitive reaction (Roy 1978). The resistance genes in these species were assumed to be located on the B genome, common to *B. nigra*, *B. juncea* and *B. carinata*, but absent in *B. napus* (Roy 1978; Sacristán and Gerdemann 1986; Sjödin and Glimelius 1988). Chèvre et al. (1996) reported that resistance at the cotyledon stage was correlated with the presence of chromosome 4 of *B. nigra* in *B. napus-B. nigra* addition lines. In *B. juncea*, chromosome B8 has been shown to carry a resistance gene (Chèvre et al. 1997). These results suggest that there are different loci in the B genome which confer resistance to *L. maculans*. To utilize the well-characterized resistance to *L. maculans* in the B genome of *Brassica*, intensive efforts have been made to transfer the resistance (especially from *B. juncea*) into *B. napus* through interspecific hybridization. Some *B. napus* selections with *B. juncea* type resistance following interspecific hybridization between *B. napus* and *B. juncea* have been achieved, such as Onap ^{JP} (Roy 1978, 1984), though no cytological evidence has been presented whether the resistance has been introgressed into the A or C genome, or whether Onap ^{JP} is aneuploid.

2.4.2 Cotyledon and adult plant resistance

Although *L. maculans* can infect various parts of the plant at different plant growth stages, cotyledon resistance and adult plant resistance have been characterized (Cargeeg and Thurling 1980; Rimmer and van den Berg 1992). The phenotype of adult plant infection

(crown canker), measured in the stem at plant maturity, is the most damaging symptom related to yield loss. Cotyledon infection can result in systemic infection, and efforts have been made to use cotyledon resistance for screening or predicting adult plant resistance.

There have been reports showing that there was no correlation between cotyledon resistance and adult plant resistance in *B. napus*, *B. juncea, and B. nigra.* (Roy 1984; Badawy et al. 1991; Zhu et al. 1993; Pang 1992; Salisbury et al. 1995; Chèvre et al. 1996). Chèvre et al. (1996) reported that there was no correlation between cotyledon resistance and the development of stem canker in *B. napus-B. nigra* addition lines under field conditions. Salisbury et al. (1995) reported that in *B. juncea*, cotyledon resistance and adult plant resistance were distinct characters. This conclusion was supported by the evidence that the apparent lack of symptoms on cotyledons and leaves was often associated with subsequent extensive colonization of the tap root or basal stem tissue in *B. juncea* (Gugel et al. 1990; Keri 1991). Dixelius and Wahlberg (1999) found cotyledon resistance and adult-leaf resistance controlled by different loci in the three B genome species, *B. juncea*, *B. nigra* and *B. oleracea.*

However, Chèvre et al. (1997) reported that cotyledon tests were an efficient method to select for the plants carrying the *B. juncea* resistance. Kutcher et al. (1993) reported that in most combinations of isolates of *L. maculans* and accessions of *Brassica* spp., a resistant cotyledon reaction was associated with a resistant stem reaction. However, in some accessions, some isolates incited a resistant cotyledon reaction, but incited a susceptible or moderately resistant stem reaction, and within combinations resulting in a susceptible cotyledon reaction, some accessions showed a resistant stem reaction. Bansal et al. (1994)

used disease severity data of 33 DH lines (resistance was developed from *B. napus* cvs. Shiralee and Maluka) to study the correlation between cotyledon and adult plant resistance under greenhouse conditions. For most lines, disease severity data were highly correlated, but a few DH lines gave a susceptible reaction on cotyledons and a moderately resistant reaction at the adult plant stage. Adult plant resistance to *L. maculans* is the major component of resistance required in oilseed *Brassica* breeding program. Considering the importance of the disease and the standard practice of breeding for resistance, information on the genetics of resistance to *L. maculans* in different *Brassica* species is rather limited. Comparison between inheritance studies on the relationship of cotyledon resistance and adult plant resistance are generally complicated by differences in the types of inoculum, method of inoculation, the number of isolates involved, the developmental stage of the plants and the methods of evaluation used. There is a pressing need to clarify the relationship between cotyledon resistance and adult plant resistanc.

2.4.3 Genetics of resistance

The effectiveness of selection in *B. napus* for cotyledon resistance to *L. maculans* suggested high heritability (Ballinger and Salisbury 1989). This was consistent with the results of Williams and Delwiche (1979) and Delwiche (1980). From genetic studies, Delwiche (1980) reported that cotyledon resistance in a French winter breeding line was controlled by a single dominant gene (*Lm1*), and a second dominant gene (*Lm2*) conferred resistance in another line. Tests for independence of these two genes indicated linkage. Sawatsky (1989) reported that the cotyledon resistance in two French *B. napus* lines (R8314

and R8317) was controlled by a single recessive gene and adult plant resistance was conferred by two dominant genes, designated *Bl-1* and *Bl-2*.

Inheritance of adult plant resistance in some *B. napus* accessions was considered to be partial, with no expression at the cotyledon stage. Resistance in Jet Neuf, a *B. napus* cv., used as a source of resistance in Australia (Salisbury et al. 1995), was partial, nonspecific and polygenically controlled. Thompson (1983) also reported that adult plant resistance to *L. maculans* in some *B. napus* resistance sources was polygenically inherited. In recent years, several genomic regions have been found to be associated with resistance to *L. maculans* in oilseed rape and quantitative trait loci (QTL) models have been proposed and mapped in *Brassica* species (Dion et al. 1995; Ferreira et al. 1995; Pilet et al. 1998).

Pang and Halloran (1996a) indicated that the expression of crown-canker development in the F_2 and backcross progeny of Maluka × Niklas possessed both a major and minor gene component. Therefore, the authors proposed that the resistance in this material was a quantitative trait (Pang and Holloran 1996b). Pilet et al. (1998) identified several QTLs that were associated with adult plant resistance in DH lines derived from the cross of Darmor × Yudal.

Resistance in *B. juncea* has been reported to be controlled by major gene(s) (Roy 1978,1984), and by two genes interacting in a dominant recessive epistatic action (Keri et al. 1997). Three chromosomes have been found associated with the resistance in *B. nigra*, and subsequent genetic analysis showed that the inheritance of resistance was controlled by polygenic factors (Zhu et al. 1993). The presence of two independent resistance genes in *B. juncea* was supported by data from addition line analysis (Struss et al. 1996). The result

showed that at least two dominant genes confer resistance and that one gene is sufficient to confer the same level of resistance as in the donor species. Plieske et al. (1998) investigated the inheritance of resistance in three *B. napus* recombinant lines containing resistance derived from either *B. nigra*, *B. juncea* or *B. carinata*. Adult plant resistance in these B genome resistant sources is controlled by single dominant gene respectively. Pang and Halloran (1996c) studied genetic control of adult plant resistance in R13 (resistance was derived from *B. juncea*). Segregation ratios from F_3 families indicated that resistance was controlled by three genes. Dixelius (1999) analyzed the inheritance of resistance to *L. maculans* in near-isogenic lines derived from asymmetric somatic hybrids between *B. napus* + *B. nigra* and *B. napus* + *B. juncea* respectively. One single dominant allele controled the resistance in the *B. napus-B. juncea* line, whereas two independent dominant loci were found in the *B. napus-B. nigra* line.

2.5 Interspecific transfer of the B genome resistance into *B. napus*

It is well established that resistance to *L. maculans* in *B. juncea* is more complete than that in *B. napus* (Roy 1984; Sacristán and Gerdeman 1986), and consequently, efforts have been made to transfer the *B. juncea* and *B. nigra* resistance to *B. napus* through interspecific hybridization (Roy 1978, 1984; Sacristán and Gerdeman 1986; Struss et al. 1991) and somatic hybridization (Sjödin and Glimelius 1989a). Roy (1978, 1984) produced homozygous resistant lines with regular meiotic behavior similar to that of oilseed rape *B. napus* after several backcrosses to oilseed rape of the F_1 of *B. napus-B. juncea* hybrids followed by selfing. Transfer of the B genome resistance into *B. napus* was also made

through somatic hybridization. Somatic hybridization has been used to improve disease resistance, through transfer of resistance to crops from related, mostly wild species (Dixelius and Glimelius 1995). Fertile interspecific asymmetric hybrids between *B. napus* and *B. nigra* and *B. napus* and *B. juncea* were made and used in an extensive backcrossing program to produce near-isogenic lines containing resistance to *L. maculans* in *B. napus* (Sjödin and Glimelius 1989a, 1989b).

Evidence for highly homologous and duplicated chromosome segments, and extensive rearrangements in the *Brassica* genomes is abundant (McGrath and Quiros 1991; Chyi et al. 1992; Teutonico and Osborn 1994; Parkin et al. 1995; Sharpe et al. 1995; Cheung et al. 1997; Lagercrantz and Lydiate 1996). This high degree of homology and duplication with intra- and inter- genomic conservation of linkage blocks may permit homologous recombination among *Brassica* species.

There are two, possibly three, chromosomes carrying resistance genes to *L. maculans* in *B. juncea*. If this resistance is introgressed into *B. napus* by homologous recombination, the assumption is that there are two or three regions in *B. napus* genome which are homologous to corresponding regions carrying blackleg resistance in *B. juncea* genome. This hypothesis has been supported by the results of Struss et al. (1995), who demonstrated the occurrence of intergenomic translocation in *B. napus-B. nigra* addition plants and the integration of *B. nigra* chromatin into *B. napus* using RFLP and RAPD markers. Chèvre et al. (1997) observed that two B genome resistance genes introgressed into *B. napus* were independent. Therefore, it is assumed that the B genome resistance could be incorporated into different regions in *B. napus*. Barret et al. (1998) used RAPD specific markers from the donor species

(*B. juncea*) as probe and localized them on the genetic map of the recipient (*B. napus*).

2.6 *Brassica-Leptosphaeria maculans* pathosystem

Plant-pathogen interactions are often determined by pairs of complementary genes of the host and pathogen. This gene-for-gene hypothesis (Flor 1971) has either been suggested or established in a number of pathosystems. Recently, direct interaction between the protein encoded by the *avrPto* gene of *Pseudomonas syringae* pv. *tomato* and the protein encoded by the *Pto* gene of tomato was demonstrated using the yeast two-hybrid system (Tang et al. 1996).

In the *Brassica-Leptosphaeria* pathosystem, a single gene controlling resistance to *L. maculans* has been reported and mapped in a number of resistance sources at the molecular level (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997). Analysis of virulence in some *L. maculans* isolates has demonstrated that single genes in the pathogen control cultivar avirulence/virulence. An avirulence gene (*AvrLm1*) in *L. maculans* isolate a.2 (PG3), controlling cultivar specificity on a *B. napus* cv. Quinta has been reported (Ansan-Melayah et al. 1995). *LEM1*, a single gene controlling resistance to *L. maculans* PHW1245 (PG2) in a *B. napus* cv. Major was mapped based on the cotyledon reactions in a DH population (Ferreira et al. 1995). The corresponding avirulence gene, a*lm1* in PHW1245 isolate was identified and mapped using AFLP markers (Pongam et al. 1998). In another study, Ansan-Melayah et al. (1998) reported that an avirulence gene, *AvrLm2,* conferred cotyledon incompatibility when *B. napus* cv. Glacier was inoculated with PHW1245. These studies imply that a gene-for-gene relationship is present in the *Brassica-L. maculans* pathosystem.

2.7 Molecular mapping of resistance genes in *Brassica* species to *L. maculans*

Developing disease resistance is one of the major components in most plant breeding programs. The desirable resistance (R) genes are generally incorporated into adapted cultivars through hybidization followed by resistance screening and selection of their progenies. Direct screening of resistance through pathogen inoculation and field testing may not be desirable for a number of reasons, such as: lack of routine screening methods and informative races of pathogen for discriminating new resistance genes, quarantine, host escapes, or field inoculations which may not permit the precise identification of a specific gene or gene combination because mixtures from endemic races may occur. Meanwhile, genetic complexities of numerous host-pathogen relationships make disease screenings more difficult to conduct because of incomplete expression of the resistance, aggressiveness of the pathogen, or sensitivity of disease reaction to environmental conditions. Some screenings are time consuming or can be conducted only at particular locations, times of year, or stages of plant development. Therefore, development of indirect screening is an attractive strategy for plant breeders to improve simultaneously a multitude of complex traits. Molecular markers allow all regions of the genome to be exploited for linkage to any traits of interest. The key advantages of molecular markers are that they allow breeders to avoid expensive, distracting, or generation-delayed tests, their independence from environmental effects, and the ability to test plants at any stage of growth. This has resulted in the identification of an increasing number of molecular markers linked with resistance genes using a variety of approaches (Michelmore 1995; Young 1996; Mohan et al. 1997).

2.7.1 Molecular techniques

The direct detection and exploitation of DNA sequence polymorphism represents one of the most significant recent developments in molecular biology. These technologies include RFLP, RAPD, AFLP, etc. Polymorphisms detected with RFLP and AFLP reflect restriction site variation, RAPD polymorphisms result from DNA sequence variation at primer binding sites and from DNA length differences between primer binding sites. This is also true for AFLP. Bulked segregant analysis (BSA) (Michelmore et al. 1991) was developed as a method for rapidly identifying markers linked with any specific gene, two bulked DNA samples can be generated from a segregating population. Each pool or bulk contains individuals that are identical for a particular trait such as disease resistant or susceptible but randomized for unlinked loci. In major staple food crops such as rice, wheat, maize, soybean, potato and barley, as well as in sorghum, tomato, pea, alfalfa, and others, molecular markers linked with important traits including disease resistance have been developed (Michelmore 1995; Young 1996; Mohan et al. 1996).

2.7.2 Mapping populations

Different segregating populations such as doubled haploid (DH) lines, near-isogenic lines (NIL), recombinant inbred lines (RIL), F_2 segregating populations, etc. are commonly used for the identification of markers linked with disease resistance genes. Techniques such as microspore culture of F_1 plants can be used to develop a completely homozygous plant population. The use of homozygous plant lines and pathogen isolates, whenever available, can simplify and facilitate the understanding of the genetic mechanisms involved in host-

pathogen relationships.

The advantages proposed for producing double haploid plants compared to obtaining homozygous lines by inbreeding are: homozygosity is reached in one generation following hybridization as compared to five or six generations of conventional inbreeding; the doubled haploid plants are completely homozygous at all loci, where some residual heterozygosity will persist in breeding lines even after five or six generations of inbreeding; recessive genes that may be masked by the presence of a dominant allele in diploid heterozygous breeding lines are uncovered and will be expressed in double haploid plants.

In *Brassica* species, DH plants are usually developed through tissue culture of male gametes or microspore culture. The procedure used to produce DH plants involves the selection of buds containing late uninucleate to early binucleate microspores which have not undergone first pollen mitosis (Pechan and Keller 1988). At this stage, microspores have the ability to switch from gametophytic to sporophytic control by undergoing symmetric instead of asymmetric division which occurs in vivo to promote gametogenesis (Zaki and Dickinson 1990). Buds are macerated to release microspores that can be characterized cytologically and then cultured in a liquid medium to recover haploid embryos. Haploid plants are then regenerated from these embryos and chromosomes are doubled by submerging the plant roots in an antimicrotubule agent such as colchicine. A completely homozygous diploid plant will be obtained. Factors that affect DH plant production include the genotypes and growth conditions of the donor plant, culture medium and culture environment.

The ability to develop fertile plants from haploid microspore in some *Brassica* species has had a major impact on genetic studies and plant breeding. In the *Brassica-L. maculans*

pathosystem, DH lines can be used to assess the host-pathogen interaction phenotype under different screening conditions, some of which are destructive, and plant developmental stages. This allows the comparison of results under different experimental conditions and testing of disease reaction to the pathogen in each line can be repeated. Therefore, DH populations have been widely used in resistance gene(s) mapping of various diseases in *Brassica* species (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997; Pilet et al. 1998).

2.7.3 Mapping resistance genes to *L. maculans* in *B. napus*

Diseases can be controlled with single genes or quantitative resistance sources. Combining the resistance into adapted cultivars is a major challenge for plant breeders. Therefore, efforts to develop molecular markers tightly linked with resistance from different sources have been undertaken. Ferreira et al. (1995) used a DH population developed from the cross of Major × Stellar to map the resistance to *L. maculans* from Major. A single major locus (*LEM1*) was identified on linkage group six based on cotyledon reaction inoculated with PHW1245 (a PG 2 isolate). This locus, in addition to four other genomic regions, was also identified by interval mapping using quantitative measurements of the cotyledon- and stem- inoculated plant reactions. Moreover, two genomic regions, different from those identified in cotyledon and stem inoculations, were associated with field resistance tested in western Canada. This result suggests that different resistance genes may be functioning at different plant developmental stages or under different environmental conditions, or may reflect differences in virulence of the field pathogen population. Another study using a DH population derived

from the cross of Crésor × Westar has mapped the resistance to *L. maculans* under field conditions in western Canada (Dion et al. 1995). A chromosomal region was responsible for controlling adult plant resistance in all four year-site tests. Subsequently, a major gene (*LmFr1*) was identified on a RFLP linkage map by QTL analysis. A second QTL, responsible for a small proportion of the variation of disease resistance, was detected in one of the four year-site assays. A DH population was also used for the molecular analysis of resistance *to L. maculans* in a *B. napus* cv. Shiralee (Mayerhofer et al. 1997). Based on inoculation with a mixture of *L. maculans* isolates from western Canada in greenhouse tests, a single major locus (*LmR1*) controlling cotyledon resistance was mapped using RAPD and RFLP markers. Interestingly, there is evidence showing that these two resistance loci, *LmFr1* in Crésor and *LmR1* in Shiralee, were also located on linkage group six (Parkin IAP and Lydiate DJ personal communication). This emphasizes the importance of linkage group six (LG6) in *B. napus* as a major location carrying resistance genes to *L. maculans*.

2.7.4 Mapping resistance genes in the B genome of *Brassica to L. maculans*

In contrast to *B. napus*, resistance to *L. maculans* in *Brassica* species with the B genome is expressed as a hypersensitive response and is effective throughout the plant life cycle (Roy 1984; Rimmer and van den Berg 1992). Addition lines in *Brassica* species have been used as tools to identify chromosomes carrying specific genes involved in agronomic traits such as fatty acid composition and glucosinolate content. In an attempt to characterize which chromosomes in the B genome carry resistance genes to *L. maculans*, a set of *B. nigra-B. napus* addition lines was developed by Zhu et al. (1993). They reported that two

chromosomes carried resistance at the cotyledon stage and one at the adult plant stage. In another study using a different set of *B. napus-B. nigra* addition lines, Chèvre et al.(1996) reported that chromosome 4 was found to carry resistance to *L. maculans* in *B. nigra*.

Chèvre et al. (1997) showed that the resistance in a *B. napus-B. juncea* recombinant line is under monogenic control and highly effective under field conditions. Three RAPD markers linked to the introgression were identified and the resistance gene was located on the chromosome B8 of *B. juncea* instead of on chromosome 4 as identified in *B. nigra* (Chèvre et al. 1996). Two hypotheses have been proposed for this difference: either the *B. nigra* chromosome 4 segment, carrying the resistance gene, had been translocated to chromosome 8 in the B genome of *B. juncea*, or different resistance genes are carried by two different chromosomes. Furthermore, Plieske et al. (1998) reported that the resistance derived from the three B genome species showed the similar levels of resistance. The similar arrangements and distances of closely linked RFLP markers linked with the resistance genes demonstrated that there is a conserved chromosomal region in which the resistance genes in the three B genome species are located. To identify the location of cotyledon and adult-leaf resistance in the three B genome species, Dixelius and Wahlberg (1999) used backcross generations of asymmetric somatic hybrids between *B. nigra*, *B. juncea* or *B. carinata* and *B. napus* to analyze the relationship of the presence of B genome markers and resistance to *L. maculans*. RFLP analysis identified four markers cosegregating with resistance that were mapped to different loci on linkage group 2, 5 and 8 of the *Brassica* B genome. Because in these different studies, the linkage groups were constructed and designated independently with different sets of markers, it is impossible to determine the relationship of these linkage

groups in which the resistance genes were identified.

2.7.5 Pyramiding resistance (R) genes

Monogenic race-specific resistance genes are attractive to plant breeders because they are easy to incorporate into susceptible materials through backcrossing. However, this kind of resistance is generally not durable with highly variable plant pathogens. Using MAS to pyramid resistance genes for which some knowledge of their complementary gene action is available may make major gene resistance more durable. Durability of resistance has been increased in several crops by incorporating diverse major resistance genes. For example, pyramiding race-specific genes in wheat has been highly effective in controlling stem rust (*Puccinia graminis* f. sp. *tritici*) for 40 years in Canada and north-central USA (Schafer and Roelfs 1985).

Gene pyramiding requires incorporation of several different resistance genes into a single cultivar. Due to the race specificity of R genes, plant populations have to be inoculated systematically with different races of pathogen to ensure appropriate gene combinations are being selected. With MAS, R gene segregation can be followed by the presence of markers closely linked with the R genes. Hence, R genes from diverse sources can be incorporated into a single genotype. Pyramiding of bacterial blight resistance genes *Xa1*, *Xa3*, *Xa4*, *Xa5* and *Xa10* in different combinations using molecular markers in rice has recently been reported (Yoshimura et al. 1995).

There is some evidence for the loss of field resistance in Australia to *L. maculans* with canola cultivars such as Maluka and Eureka after years of cultivation (Salisbury et al. 1995).

The loss of resistance may be due to the changing virulence of pathogen populations, but this has not been tested. Stable blackleg resistance is essential to ensure the future of the canola industry in western Canada, Australia and Europe. Combinations of different resistance genes to blackleg may improve the effectiveness and durability of the resistance.

2.7.6 QTL analysis in resistance to *L. maculans* in *Brassica*

Most important agronomic traits such as yield, plant height and days to flowering are controlled by a number of genes. The number of genes and their interactive effects controlling the expression of quantitative traits are poorly understood. Molecular markers that exploit the entire genome of interest provide the opportunity to analyze the Mendelian factors determining quantitative traits. Genomic regions that contribute to quantitative traits can then be identified using regression analysis, interval mapping, or both. Examples of quantitative resistance mapping have been described in various plant species (Michelmore 1995; Young 1996; Mohan et al. 1997).

Resistance can be inherited as a quantitative trait that is controlled by quantitative trait loci (QTL). Examples of the identification of discrete Mendelian loci that, acting together, contribute to economically important disease resistance, are blast resistance in rice, late blight resistance in potato, grey leaf spot resistance in maize, cyst nematode resistance in soybean and stripe-rust resistance in barley (Young 1996).

Pilet et al. (1998) used a DH population from the cross of Darmor × Yudal to identify QTL associated with field resistance to *L. maculans* in France by measuring the mean disease index and the percentage of lost plants in two years. Resistance in Darmor is derived from

B. napus cv. Jet Neuf that shows partial and nonspecific resistance, and has been widely cultivated in western and eastern Europe for 10 years (Hammond and Lewis 1987). A total of 13 genomic regions distributed across 10 of the 19 linkage groups were associated with field resistance in either of two years or both. Among them, four QTL had major additive effects during the two years. When field resistance was evaluated in western Canada, mapping studies indicated that only two genomic regions were associated with field resistance in Major (Ferreira et al. 1995), and one major locus (*LmFr1*) and a second environmentally dependent minor region were identified in Crésor (Dion et al. 1997). This may be due to differences in virulence of pathogen populations of *L. maculans* in Canada and France or difference in the sources of resistance. Isolates in western Canada are predominantly PG2 and French isolates are mainly PG3 and PG4 (Mengistu et al. 1991). This difference is also reflected by the fact that Crésor, resistant to *L. maculans* in western Canada, is very susceptible under field conditions in France (Pilet et al. 1998).

Quantitative traits appear to have the most potential for MAS, because conventional selection for these complex traits with low to moderate heritability is difficult and costly due in part to confounding environmental effects. Resistance to some other important diseases in *Brassica* species such as *Sclerotinia sclerotiorum* rot and *Rizoctonia solani* root rot, are believed to be under QTL control. The occurrence and severity of these disease are strongly influenced by the environment.

2.7.7 Development of specific markers for marker-assisted selection (MAS)

More specific markers such as sequence characterized amplified region (SCAR) offer

additional refinements over RAPD markers since they allow multiplex analysis and improve reproducibility associated with RAPD markers. Generally, these markers produce a single polymorphic band that is more reproducible across labs, easier to score, and applicable for use with low-quality DNA obtained through simplified DNA extraction procedures. Compared with other specific markers, SCAR markers are more frequently used, because specific primers can be easily designed by sequencing RAPD, AFLP and RFLP products.

Numerous SCAR markers have been developed for MAS for many disease resistance genes, e.g. downy mildew resistance gene in lettuce (Paran and Michelmore 1993), nematode resistance gene in tomato (Williamson et al. 1994), barley stem rust resistance gene *Rpg1* (Penner et al. 1995), and anthracnose resistance gene in common bean (Adam-Blondon et al. 1994). To date, no SCAR markers have been reported for MAS of resistance to *L. maculans* in *Brassica* species.

2.7.8 Other disease resistance genes mapped in *Brassica* species

2.7.8.1 White rust

Molecular mapping of resistance to white rust, caused by *Albugo candida*, has been conducted in the DH population derived from the cross of Major × Stellar. A single locus, *ACA1*, conferring resistance to an isolate of *A. candida* from *B. carinata*, was mapped on linkage group 9 of a *B. napus* RFLP genetic map (Ferreira et al. 1995). Two independent studies on resistance to *A. candida* race 2 in a canola-quality *B. juncea* breeding line mapped a single locus on a RAPD linkage map (Prabhu et al. 1998) and on a RFLP linkage map (Cheung et al. 1998) respectively.

2.7.8.2 Black rot

Molecular mapping of resistance to black rot, caused by *Xanthomonas campestris* pv. *campestris* was conducted in *B. oleracea* (Camargo et al. 1995). Two genomic regions on linkage group 1 and 9 were associated with both young and adult plant resistance. Two additional QTL on linkage group 2 that were involved in young plant resistance were also detected. The result was consistent with previous genetic studies showing resistance to black rot as a quantitative trait in *B. oleracea* (Sharma et al. 1972; Williams 1980).

2.7.8.3 Light leaf spot

Characterization and localization of QTL for light leaf spot resistance (*Pyrenopeziza brassicae*) was conducted in a DH population developed from Darmor× Yudal. Ten genomic regions associated with resistance on leaves and on stems were distributed on 8 of the 19 linkage groups of the genetic map (Pilet et al. 1998). Interestingly, two regions, located on DY6 and DY10, were considered to be associated with the resistance to *L. maculans* as well. Common QTL for different resistance genes have been reported in various diseases (Young 1996). These results imply that multiple disease resistance (MDR) QTL may include either genes involved in common resistance mechanisms or clusters of resistance genes to different pathogens in *Brassica* species.

2.7.8.4 Clubroot

QTL analysis can indicate the number and effects of the major genetic factors controlling quantitative resistance. In several cases, few loci control the majority of the genetic variation.

Landry et al. (1992) identified two chromosomal regions responsible for most of variation for the resistance to race 2 of *Plasmodiophora brassicae* (Woronin) in *Brassica oleracea*, one locus accounting for 58% of the genetic variance.

2.8 Comparative mapping analysis

Genomes from distantly related plant species show considerable DNA homology despite differences in chromosome number and genome size (Bonierbale et al. 1988; O'Brien et al. 1988; Tanksley et al. 1988). It is therefore a pressing question to establish whether gene order is also conserved between the related species. This problem was first addressed when molecular markers, such as RFLP, were applied to plant genome mapping. It became evident that complementary DNA probes could be cross-mapped to provide anchors that allowed genomes to be compared in related species (Bonierbale et al. 1988; O'Brien et al. 1988; Tanksley et al. 1988; Whitkus et al. 1992; Zhang and Womak 1992; Ahn and Tansley 1993).

2.8.1 Concepts, terms and rational of comparative mapping

Some definitions of terms used to describe the arrangement of homologous genes between genomes are: homology segment: -one pair of homologous genes; conserved synteny: -two or more pairs of homologous genes located on the same chromosome, regardless of order; conserved linkage or collinearity: -at least two genes that show the same order without unrelated intervening genes, i.e. both synteny and gene order are conserved.

Conserved gene order, together with sequence similarity could be a valuable tool for defining functional relationships among genes in different species. If recombination has not

broken up the local order of genes between species, ancestral homologous relationships are conserved and can be revealed. However, during evolutionary processes, recombination, translocation, inversions, transpositions and other less common chromosome rearrangements disrupt ancestral linkage and synteny. By comparing the location of homologous genes (segments) in different species, it is possible to determine whether a particular chromosome segment has been conserved or disrupted during evolution.

With the advent of DNA based markers in the past two decades, a remarkable observation has been the conservation of chromosomal segments despite large differences in genome sizes between species (Moore et al. 1993). Comparative genetic linkage maps based on a common set of markers allow direct identification of homologous loci and collinear chromosomal segments. This approach has proved successful in revealing widespread collinearity (Bonierbale et al. 1988; O'Brien et al. 1988; Tanksley et al. 1988; Whitkus et al. 1992; Zhang and Womak 1992; Ahn and Tansley 1993; Prince et al. 1992). For example, in animals, evidence for conservation has been found among certain species of fish as well as between fish and mammals (Trower et al. 1996; O'Brien et al. 1999).

2.8.2 Comparative mapping in plants

Plants typically have complex eukaryotic genomes that can be substantially larger than the human genome. Investigations of comparative mapping have taken place for many plant species including several grasses (Gale and Devos 1998; Bennetzen et al. 1998), tomato and potato (Tanksley et al. 1992), maize, rice and wheat (Ahn et al. 1993; Ahn and Tanksley 1993), sorghum and maize (Whitkus et al. 1992), pea and lentil (Weeden et al. 1992), oat and

wheat (van Deynze et al. 1995a; van Deynze et al. 1995b), pepper and tomato (Tanksley et al. 1988), *Arabidopsis* and *Brassica* (Lagercrantz et al. 1996; Kowalski et al. 1994; Cavell et al. 1998; Robert et al. 1998; Sadowski et al. 1998) and within *Brassica* genera (Kiania and Quiros 1992; Truco and Quiros 1994; Truco et al. 1996).

2.8.3 Implications and use of information of comparative mapping

Comparative genetic linkage maps, based on common sets of markers, has allowed direct identification of collinear chromosome segments and conserved linkages in animal and plant kingdoms. The identification of collinear regions will enable genetic information and molecular resources to be shuttled between the species (genera). When loci controlling similar traits are mapped to equivalent regions, this will suggest that they represent homologous genes. Meanwhile, during evolution, chromosome rearrangement invariably will occur, disrupting some, but not all ancestral linkages. This scenario of conserved and rearranged segments in the related genomes, which could be illustrated by comparison of the chromosomal location of homologous loci, provides an efficient tool to elucidate the evolutionary process by which current genome organization arose (Moore et al. 1993).

2.8.3.1 Genomic relationship between *Brassica* and *Arabidopsis*

Arabidopsis, a member of the crucifer family (closely related to *Brassica*), has become an important model species for the study of nearly all aspects of plant biology, because of its relatively small genome (approximately 130 Mb) with about 10% repetitive DNA sequence. Through comparative mapping, collinearity between *Arabidopsis* and *Brassica* species

(divergence time was assumed 10~35 million years) (Muller 1981; Lagercrantz 1998), has been shown to be strong. Lagercrantz et al. (1996) reported fine scale genome collinearity between a 1.5 Mb interval of *Arabidopsis* chromosome 5 and three collinear homologous regions in *B. nigra* when comparative mapping around the *CO* locus was pursued between two species. Similarly, the mapping of QTLs controlling flowering time in *B. napus* has identified genomic regions containing conserved synteny with the *Arabidopsis CO* gene (Keith 1995). Osborn et al. (1997) compared the map positions of flowering time QTL detected in *B. rapa* and *B. napus* to the map positions of flowering time genes in *Arabidopsis thaliana*. Homologous genes control flowering time in both *B. rapa* and *B. napus* and these genes also show homology to the flowering time genes identified in *Arabidopsis*. Genetic and physical mapping in *B. napus* of DNA markers derived from a segment (approximately 30 cM) of *A. thaliana* chromosome 4 revealed duplicated homologous segments in the *B. napus* genome (Cavell et al. 1998). Robert et al. (1998) identified genomic regions in *B. napus* that contained homologues of the *CO* gene controlling flowering time in *Arabidopsis*. Four genes, located on linkage groups, N10 and N19, highly similar to each other and to the *Arabidopsis CO* gene, were isolated.

Comparative genetic mapping of the *Brassica* and *A. thaliana* genomes, assuming that the level of collinearity exists over large parts of, or even the whole, genome, will make it possible to determine which part of the *A. thaliana* genome is homologous to any specific interval of the *Brassica* genomes and the degree of synteny and/or internal inversion, deletion, etc. which occurs.

2.8.3.2 Resistance gene organization

Plant disease resistance genes frequently reside in tightly linked clusters. This was first established by classical genetic techniques (Pryor 1987). Molecular analyses are now beginning to uncover the complexity of these loci, and their structure and evolutionary development (Parniske et al. 1997; Song et al. 1997). In flax, five loci confer resistance to the rust fungus *Melampsora lini*. Among them, the *L* and *M* loci have been extensively studied. The *L* locus consists of a single gene with 13 distinct alleles. In contrast, the *M* locus is a complex locus that is composed of an array of linked genes (Anderson et al. 1997).

In maize, a group of genes conferring resistance to the common rust fungus *Puccinia sorghi*, are clustered in a small region on the short arm of chromosome 10 (Hulbert and Bennetzen 1991). Genetic analyses have identified 14 pathogen specificities within the *Rp1* complex. Most of the genetic reassortment events at the *Rp1* complex are associated with flanking marker exchanges (Hulbert and Bennetzen 1991; Sudupak et al. 1993). It has been postulated that unequal cross-over via meiotic mispairing between different genes may be the major way in which novel resistance specificities are generated and new combinations of parental resistance genes are created (Hammond-Kosack and Jones 1997, Richter et al. 1995). It is becoming increasingly apparent that such clusters are both common and complex in plant genomes (Hammond-Kosack and Jones 1997).

2.8.3.3 *Arabidopsis* resistance gene *RPS2* in *Brassica* species

Sadowski et al. (1998) used a cluster of six genes (approximately 30 kb) in *A. thaliana* to determine the possible organization in the *B. nigra* genome. This *Arabidopsis* chromosomal

complex was found to be duplicated and conserved in the *B. nigra* genome with gene number at different levels. Among the six genes, *RPS2* (resistance against *Pseudomonas syringae*), a single-copy in the complex was detected in *B. nigra*.

2.8.3.4 Blackleg resistance gene organization in *Brassica* species

Molecular markers have facilitated mapping of resistance genes to *L. maculans* in *Brassica* species. Closely linked markers can be useful for MAS of the breeding programs. Moreover, comparative mapping is an aid to understanding the organization of resistance genes in *Brassica* species. Plieske et al. (1998) compared the three B genome resistance genes derived from *B. nigra*, *B. juncea* and *B. carinata* in a uniform genetic background. By using a common set of RFLP probes, the arrangement and distances of closely linked RFLP probes on linkage groups of three resistance sources were similar to each other, and to the resistance linkage group of a *Brassica napus* cv. Major in which a resistance gene (*LEM1*) has been mapped (Ferreira et al. 1995). This synteny implies that these *Brassica* species share a conserved region in which resistance genes are located.

2.8.3.5 Resistance gene evolution

Resistance to many diseases in plants is often determined by individual members of families of genes, each member conferring resistance to a specific pathotype (race) of the pathogen, specificity has often shown to be conferred by a gene-for-gene interaction. Molecular data have supported this hypothesis (Tang et al. 1996). Many resistance genes have now been cloned, and their structure and potential function have been reviewed

(Hammond-Kosack and Jones 1997). There are a large number of sequences with similarity to resistance genes in plant genomes. Leister et al. (1998) used conserved domains in a major class (nucleotide binding site plus leucine-rich repeat) (NBS-LRR) of dicotyledonous resistance genes to amplify and isolate related gene fragments via PCR from the monocotyledonous species, rice and barley. Comparative mapping of these genes in rice and barley showed linkage to genetically characterized R genes and revealed the existence of mixed clusters in resistance loci. The R genes specifying disease resistance and their homologues are abundant and highly rearranged among the plant kingdom (Leister et al. 1998; Meyers et al. 1999). Mapping of disease resistance gene homologues across rice, barley, and foxtail millet has already led to the conclusion that these genes may be evolving faster than most (Leister et al. 1998). This is also consistent with the report by Botella et al. (1997) who estimated that a substantial portion of the *Arabidopsis* genome, perhaps 5%, is concerned with disease resistance.

2.8.3.6 *Brassica* genome evolution

Within the primary *Brassica* species, the genomes are highly duplicated, indicating that even these primary diploid species are actually secondary polyploids. The second cycle of amphidiploidy has resulted in the three amphidiploid *Brassica* species, *B. napus*, *B. juncea* and *B. carinata*. Because of their origin, the genomes of these species are expected to have conserved chromosome segments and extensive duplications, which have led to further rearrangements by homologous recombination (Prakash and Hinata 1980; Attia and Röbbelen 1986).

Comparative mapping has unraveled extensive internal chromosome segment duplication within the A (Song et al. 1991; Chyi et al. 1992), B (Lagercrantz and Lydiate 1995) and C genomes (Slocum et al. 1990). Common RFLP markers have uncovered not only the conserved chromosome segments within the A, B, and C genomes, but also showed that although the three genomes are related to each other, they have undergone extensive structural changes after divergence. Thus, the duplicated chromosome segments have formed part of different syntenic groups in the A, B and C genomes, as a result of extensive chromosomal rearrangements. In addition, there has been strong influx of translocations, transposition, aneuploidy and homologous recombination further reshaping these genomes (McGrath and Quiros 1991; Chyi et al. 1992; Teutonico and Osborn 1994; Parkin et al. 1995; Sharpe et al. 1995; Cheung et al. 1997; Lagercrantz and Lydiate 1996).

CHAPTER 3

GENETICS OF RESISTANCE TO *LEPTOSPHAERIA MACULANS* IN *BRASSICA NAPUS*

3.1 Abstract

Inheritance of resistance to *Leptosphaeria maculans* was studied in *Brassica napus* accessions, RB87-62 and DH88-752. The two resistant accessions and a susceptible *B. napus* cultivar Westar were crossed in a complete diallel and their progenies were evaluated for disease reaction by cotyledon and stem inoculation with *L. maculans*, isolate P186-12 (PG2). For crosses between Westar and either resistant parent, the segregation of F_2 populations fit a 3:1 (resistant:susceptible) model both for cotyledon and adult plant reaction, indicating a single dominant gene. Analysis of the relationship between the cotyledon and adult plant resistance showed that genes controlling these two traits were different but linked. These results were confirmed by segregation data from F_3 families. Segregation in the cross between the two resistant lines demonstrated that cotyledon and adult plant resistance from the two different sources were non-allelic and independently assorting.

Key words *Brassica napus* · *Leptosphaeria maculans* ··Genetics · Resistance

3.2 Introduction

Blackleg, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not., is a serious disease of many *Brassica* species and causes extensive yield losses in Canada, Europe and Australia (Rimmer and van den Berg 1992; Gugel and Petrie 1992). The disease is most effectively controlled by the use of resistant cultivars. Either monogenic or polygenic models of resistance have been reported in *B. napus* (Delwiche 1980; Cargeeg and Thurling 1980; Sawatsky 1989). *Brassica* species containing the B genome, i.e. the diploid species *B. nigra* (BB, 2n=16) and the two derived amphidiploids, *B. juncea* (AABB, 2n=36) and *B. carinata* (BBCC, 2n=34) are highly resistant to *L. maculans* and resistance is effective throughout the life of the plant (Rimmer and van den Berg 1992). The resistance is thought to be controlled by genes located on the B genome (Roy 1984; Sacristán and Gerdemann 1986; Chèvre et al. 1996; Chèvre et al. 1997). Among the three B genome species, *B. juncea* shows complete resistance to *L. maculans* both at cotyledon and adult plant stages. Transfer of the B genome resistance to *B. napus* through interspecific crosses has been attempted (Roy 1978; Sacristán and Gerdemann 1986; Struss et al. 1991).

L. maculans can infect all parts of susceptible plants throughout their life cycle. Two types of resistance, cotyledon resistance and adult plant resistance, referring to the plant growth stage when the resistance to *L. maculans* is expressed, have been characterized. However, the relationship between the degree of cotyledon lesion development and that of subsequent crown canker development has not been determined. Some researchers have indicated that there is a correlation between cotyledon and adult plant resistance (Newman and Bailey 1987; McNabb et al. 1993; Bansal et al. 1994). Recent studies have suggested that cotyledon

resistance and adult plant resistance could be distinct traits genetically (Ferreira et al. 1995; Pang and Halloran 1996b; Ballinger and Salisbury 1996). Since genetic information on the relationship between cotyledon resistance and adult plant resistance is rather limited, the objective of this research was to determine the genetic control of resistance to *L. maculans* at both cotyledon and adult plant stages in two *B. napus* accessions (RB87-62 and DH88-752), and to investigate the relationship between the two sources of resistance.

3.3 Materials and Methods

3.3.1 Plant materials

RB87-62 is a *B. napus* accession with the pedigree of Chikuzen*2/Zephyr × Bronowski. DH88-752, a doubled haploid *B. napus* accession, is derived from the *B. napus* breeding line 79N047-59, that was thought to be a putative *B. napus-B. juncea* recombinant line, developed by NN Roy, and kindly provided by GC Buzza of Pacific Seeds, Toowoomba, Australia. Westar is a susceptible *B. napus* cultivar.

Seeds from RB87-62, DH88-752 and Westar were selfed three times and used in the initial crosses. Five individual plants from each of the two resistant accessions were crossed with five individual plants of Westar. Plants of the two resistant accessions were also crossed with each other to determine whether the genes involved in resistance in the two resistance sources were allelic. F_1 and F_2 populations were produced for all crosses, and F_3 families were derived from randomly selected F_2 plants for crosses of Westar × RB87-62 and Westar × DH88-752.

3.3.2 Pathogen isolate

One isolate P186-12 of *L. maculans*, belonging to pathogenicity group 2 (PG2) (Koch et al. 1991), originating from Manitoba, Canada and was used in this study. This isolate is considered to be representative of the predominant aggressive pathotypes occuring in western Canada. Pycnidiospore inoculum was prepared from pycnidia collected from 10-day-old cultures grown in Petri plates at 22°C on V8 agar medium under continuous cool-white fluorescent light with a 12 h light/12 h dark photoperiod. Plates were flooded with sterile water and the surface scraped with a sterile glass slide to release pycnidiospores. The pycnidiospore suspension was filtered through a double-layer of cheesecloth and adjusted to a concentration of 1×10^7 spores/ml. Pycnidiospore suspensions were stored in 1.5-ml microtubes, frozen at -20°C, and thawed just before inoculations.

3.3.3 Cotyledon inoculation and disease reaction evaluation

To evaluate the disease reaction of individual plants, seeds were planted separately in small pots (Koral flats, Koral Co., Bramalea, Ontario) filled with a soil-less potting mixture (Metromix, W.R. Grace & Co. Ltd., Ajax, Ontario). All plants were maintained in a growth chamber at 20°C/16°C (day/night) with a 16-h photoperiod. Seven days after seeding, cotyledons were wounded with a sterile needle and a 10 µl droplet of the pycnidiospore suspension was pipetted onto the wound. Inoculated cotyledons were allowed to dry at least 12 h before the next application of watering. Twelve days after inoculation, the disease reaction on cotyledons was rated using a 0 to 9 scale (Williams and Delwiche1980). Disease rating of 0-6 was defined as resistant, 7-9 as susceptible. After rating, infected cotyledons

were removed and the plants were transplanted into 20 cm pots containing soilless mixture and subsequently maintained in a greenhouse at 22°C/16°C with normal daylight, supplemented to 16 h with fluorescent light. These plants were used for assessment of adult plant reaction by stem inoculation.

3.3.4 Stem inoculation and disease reaction evaluation

Plants were inoculated 4-5 weeks after seeding. A 10 µl droplet of a pycnidiospore suspension was injected into the stem between the cotyledon and the first node with a 24-gauge needle connected to a repeatable pipette. After 9 weeks, the adult plant disease reaction of each plant was rated using a scale of 0 to 5 (van den Berg et al. 1993) based on the discoloration of a cross section around the inoculation site at the crown. Disease ratings of 0-2 were defined as resistant, 3-5 as susceptible.

For linkage analysis based on disease reaction of individual plants, cotyledon and adult plant resistance of individual plants were assigned the following binomials (C, A), (C, a), (c, A) and (c, a) to indicate resistance (C, A) or susceptibility (c, a) at the cotyledon and adult plant stages respectively.

3.3.5 Statistical analysis

The Chi-square test was used to test the goodness of fit of segregation ratios for all data (Steel and Torrie 1980). The validity of pooling F_2 population from the same cross was tested with the Chi-square test for homogeneity.

3.4 Results

3.4.1 Westar × RB 87-62

The distribution of disease reactions of F_2 plants at cotyledon and adult plant stages in five separate crosses is shown in Figure 3.1. When classified in discrete resistant or susceptible categories, the F_2 plants segregated 3:1 (R:S) for both cotyledon (Table 3.1) and adult plant stages (Table 3.2), indicating that resistance at the cotyledon and adult plant stages was controlled by a single dominant gene respectively. Although most of the F_2 plants showed the consistent disease reactions (resistant or susceptible) on cotyledon and at adult plant stage, some plants expressed opposite reactions. Subsequently, resistance at cotyledon and adult plant stages for individual plants were combined to test for independence or linkage between the two traits, and the F_2 population did not segregate in a 9 (C, A): 3 (C, a): 3 (c, A): 1(c, a) ratio (Table 3.3). This result is supportive of two linked genes, one controlling cotyledon resistance and one controlling adult plant resistance. Furthermore, disease reactions for both cotyledon and adult plant stages of F_3 families derived from some F_2 plants were tested, some recombinant genotypes were obtained, such as Family 5 showing heterozygous resistance on cotyledon and homozygous resistance at adult plant. This is consistent with the hypothesis of two different genes controlling these two traits. Segregation data of F_3 families are shown in Table 3.4 and the corresponding genotypes of F_2 plants are proposed.

3.4.2 Westar × DH 88-752

The distribution of disease reactions of F_2 plants at cotyledon and adult plant stages in five

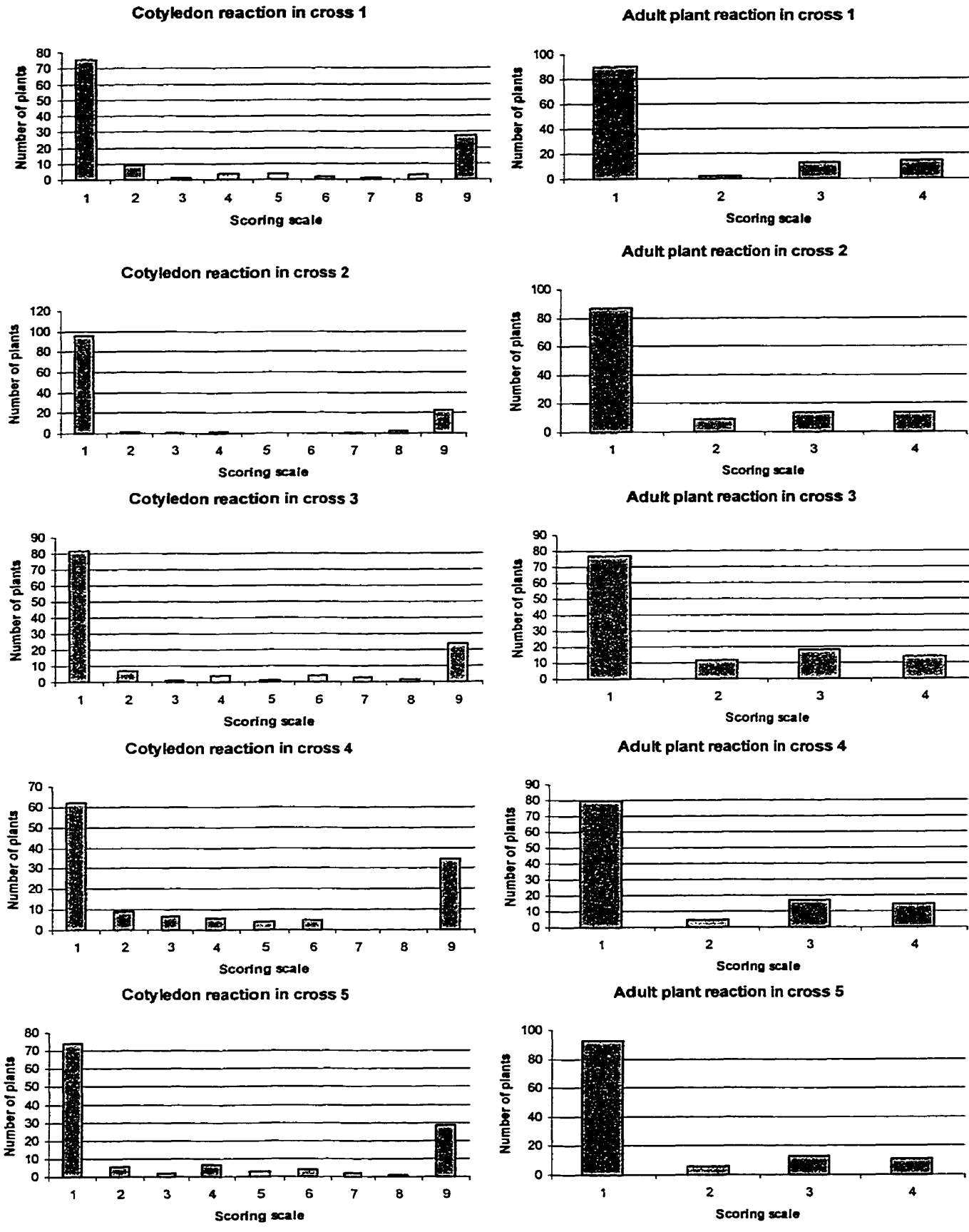


Figure 3.1. Distribution of cotyledon and adult plant reaction of F_2 plants in Westar × RB87-62

Table 3.1. Segregation of F_2 populations for cotyledon resistance in Westar × RB87-62

	Cotyledon reaction			χ^2	
Crosses	R	S	Total	3:1(R:S)	*P*
1	90	31	121	0.025	0.70-0.90
2	95	30	125	0.067	0.70-0.90
3	95	27	122	0.536	0.30-0.50
4	90	38	128	1.500	0.20-0.30
5	95	31	126	0.011	0.90-0.95
Total	465	157	622	2.139	
Pooled				0.019	0.70-0.90
Homogeneity (df=4)				2.120	0.70-0.90

Table 3.2. Segregation of F_2 populations for adult plant resistance in Westar × RB87-62

	Adult plant reaction			χ^2	
Crosses	R	S	Total	3:1(R:S)	*P*
1	93	28	121	0.223	0.50-0.70
2	98	27	125	0.771	0.30-0.50
3	91	31	122	0.011	0.90-0.95
4	96	32	128	0	
5	102	24	126	2.381	0.05-0.10
Total	480	142	622	3.386	
Pooled				1.563	0.20-0.30
Homogeneity (df=4)				1.823	0.70-0.90

Table 3.3. Tests of independence of cotyledon and adult plant reactions in F_2 populations in Westar × RB87-62

	Host reaction[a]					$\chi^2_{(df=3)}$	
Crosses	CA	Ca	cA	ca	Total	9:3:3:1	*P*
1	84	6	9	22	121	51.826	<0.001
2	85	10	13	17	125	26.250	<0.001
3	80	15	11	16	122	19.959	<0.001
4	81	9	15	23	128	44.025	<0.001
5	88	7	14	17	126	26.434	<0.001

[a]CA=cotyledon resistant, adult plant resistant; Ca=cotyledon resistant, adult plant susceptible; cA=cotyledon susceptible, adult plant resistant; ca=cotyledon susceptible, adult plant susceptible.

Table 3.4. Segregation of F_3 families for cotyledon and adult plant resistance in Westar × RB87-62

	Host reaction[a]					Expected ratio on cotyledon reaction			Expected ratio on adult plant reaction			Expected
F_3 families	CA	Ca	cA	ca	Total	R:S	χ^2	*P*	R:S	χ^2	*P*	F_2 genotype[b]
1	103	1	0	0	104	1:0			1:0			CCAA
2	102	0	2	0	104	1:0			1:0			CCAA
3	57	7	10	18	92	3:1	1.45	0.20-0.30	3:1	0.23	0.50-0.70	CcAa
4	0	0	56	27	83	0:1			3:1	2.51	0.10-0.20	ccAa
5	71	0	28	2	101	3:1	1.19	0.20-0.30	1:0			CcAA
6	0	0	0	102	102	0:1			0:1			ccaa
7	0	0	0	104	104	0:1			0:1			ccaa

[a]CA=cotyledon resistant, adult plant resistant; Ca=cotyledon resistant, adult plant susceptible; cA=cotyledon susceptible, adult plant resistant; ca=cotyledon susceptible, adult plant susceptible.

[b]CC=homozygous cotyledon resistant, Cc=heterozygous cotyledon resistant, cc=homozygous cotyledon susceptible, AA= homozygous adult plant resistant, Aa=heterozygous adult plant resistant, aa=homozygous adult plant susceptible.

separate crosses is shown in Figure 3.2. When classified in discrete resistant or susceptible categories, the F_2 plants segregated 3:1 (R:S) for both cotyledon (Table 3.5) and adult plant stages (Table 3.6), indicating that resistance at the cotyledon and adult plant stages were controlled by single dominant genes respectively. Subsequently, cotyledon resistance and adult plant resistance of individual plants were combined to test for independence or linkage between the two characters, and the F_2 populations did not segregate in a 9 (C, A): 3 (C, a): 3 (c, A): 1(c, a) ratio (Table 3.7). This result supports the hypothesis of two linked genes controlling cotyledon and adult plant resistance respectively. Disease reactions for cotyledon and adult plant stages of F_3 families derived from randomly chosen F_2 plants were tested, some recombinant genotypes were obtained, such as Family 6 showing homozygous resistance on cotyledon and heterozygous at adult plant stage. This provides more evidence that supports the proposed model of two different genes controlling these two traits. Segregation data of the F_3 families are shown in Table 3.8 and the corresponding genotypes of F_2 plants are proposed.

3.4.3 RB 87-62 × DH88-752

The F_2 progeny segregated for both cotyledon and adult plant resistance in a ratio of 15:1 (R:S) (Table 3.9). This result suggests two different dominant loci for both cotyledon and adult plant resistance in RB87-62 and DH88-752 and indicates that the two resistant parents have resistance genes that are non-allelic and independently assorting.

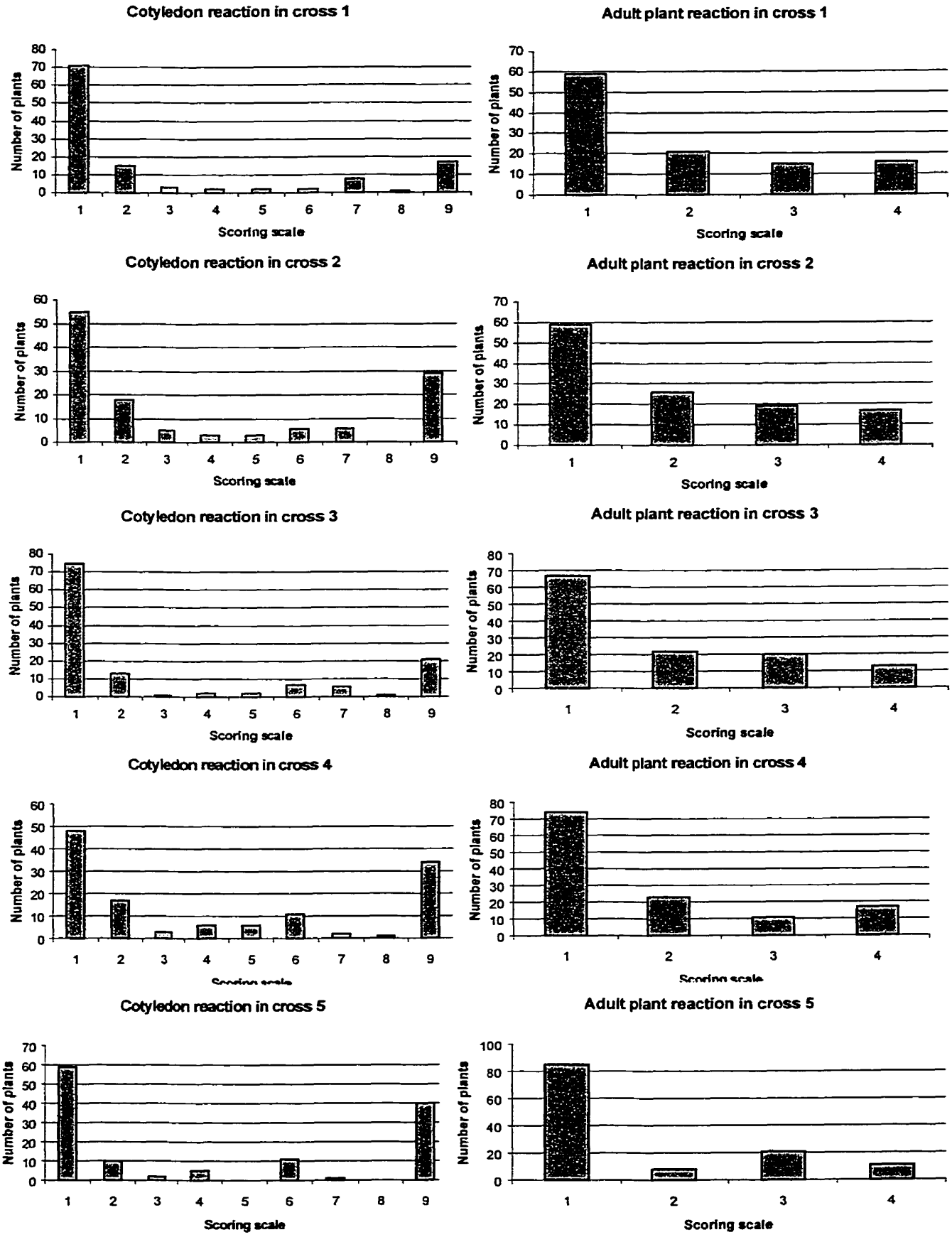


Figure 3.2. Distribution of cotyledon and adult plant reaction of F_2 plants in Westar × DH88-752

Table 3.5. Segregation of F_2 populations for cotyledon resistance in Westar × DH88-752

	Cotyledon reaction			χ^2	
Crosses	R	S	Total	3:1(R:S)	*P*
1	85	22	107	1.124	0.20-0.30
2	88	34	122	0.535	0.30-0.50
3	89	27	116	0.184	0.50-0.70
4	91	34	125	0.323	0.50-0.70
5	86	39	125	2.563	0.10-0.20
Total	439	156	595	4.729	
Pooled				0.471	0.30-0.50
Homogeneity (df=4)				4.258	0.30-0.50

Table 3.6. Segregation of F_2 populations for adult plant resistance in Westar × DH88-752

	Adult plant reaction			χ^2	
Crosses	R	S	Total	3:1(R:S)	*P*
1	83	24	107	0.377	0.50-0.70
2	90	32	122	0.098	0.70-0.90
3	84	32	116	0.414	0.50-0.70
4	99	26	125	1.176	0.20-0.30
5	93	32	125	0.024	0.70-0.90
Total	449	146	595	2.089	
Pooled				0.067	0.70-0.90
Homogeneity (df=4)				2.022	0.70-0.90

Table 3.7. Tests of independence of cotyledon and adult plant reactions in F_2 populations in Westar × DH88-752

	Host reaction[a]					$\chi^2_{(df=3)}$	
Crosses	CA	Ca	cA	ca	Total	9:3:3:1	*P*
1	76	9	7	15	107	29.091	<0.001
2	75	13	15	19	122	24.547	<0.001
3	73	16	11	16	116	18.314	<0.001
4	84	7	15	19	125	33.251	<0.001
5	81	5	12	27	125	68.805	<0.001

[a]CA=cotyledon resistant, adult plant resistant; Ca=cotyledon resistant, adult plant susceptible; cA=cotyledon susceptible, adult plant resistant; ca=cotyledon susceptible, adult plant susceptible.

Table 3.8. Segregation of F_3 families for cotyledon and adult plant resistance in Westar × DH88-752

	Host reaction[a]					Expected ratio on cotyledon reaction			Expected ratio on adult plant reaction			Expected
F_3 families	CA	Ca	cA	ca	Total	R:S	χ^2	*P*	R:S	χ^2	*P*	F_2 genotype[b]
1	103	0	1	0	104	1:0			1:0			CCAA
2	74	27	0	3	104	1:0			3:1	0.82	0.30-0.50	CCAa
3	0	0	0	104	104	0:1			0:1			ccaa
4	0	0	0	104	104	0:1			0:1			ccaa
5	61	12	8	19	100	3:1	0.21	0.50-0.70	3:1	1.92	0.10-0.20	CcAa
6	49	12	0	1	62	1:0			3:1	0.54	0.30-0.50	CCAa

[a]CA=cotyledon resistant, adult plant resistant; Ca=cotyledon resistant, adult plant susceptible; cA=cotyledon susceptible, adult plant resistant; ca=cotyledon susceptible, adult plant susceptible.
[b]CC=homozygous cotyledon resistant, Cc=heterozygous cotyledon resistant, cc=homozygous cotyledon susceptible, AA= homozygous adult plant resistant, Aa=heterozygous adult plant resistant, aa=homozygous adult plant susceptible.

Table 3.9. Segregation of F_2 populations for resistance in RB87-62 × DH88-752

				χ^2	
Host reaction	R	S	Total	15:1 (R:S)	*P*
Cotyledon reaction	253	17	270	0.002	0.95-1.00
Adult plant reaction	222	8	230	3.016	0.05-0.10

3.5 Discussion

In this study, genetic analyses of resistance to *L. maculans* isolate Pl86-12 (PG2) in RB87-62 and DH88-752 showed that cotyledon and adult plant resistance were controlled by single genes respectively in both accessions. These results suggest different *B. napus* accessions have different combinations of genes which confer resistance to *L. maculans* in *B. napus*. The fact that cotyledon resistance and adult plant resistance were controlled by single but different genes in RB87-62 and DH88-752 is in agreement with the result of Sawatsky (1989), who tested disease reactions of the progeny from two French spring rape lines (R83-14 and R83-17) crossed to a susceptible cv. Regent. When inoculated with a Manitoba isolate Pl85-10 (PG2) of *L. maculans*, segregation data of the F_2 plants indicated that cotyledon resistance was controlled by a recessive gene and adult plant resistance was governed by two dominant genes. Analysis of cotyledon and adult plant resistance in *B. napus* by Ferreira et al. (1995), using a DH population derived from Major × Stellar mapped resistance genes to a *L. maculans* isolate, PHW1245 (PG2). A single major locus (*LEM1*) controlling cotyledon resistance was mapped on linkage group (LG) 6. Putative QTL for adult plant resistance were identified in LG 6 also and on LG 17. In LG 6, the peak LOD score was in the chromosomal interval adjacent to the interval containing *LEM1*. This implied that the cotyledon resistance and adult plant resistance to *L. maculans* isolate PHW1245 in cv. Major are linked.

Bansal et al. (1994) used DH lines containing resistance derived from a cross of *B. napus* cv. Shiralee with a susceptible breeding line, to study correlation between cotyledon and adult plant resistance in greenhouse tests. For most lines, disease severity data were highly correlated. However, a few DH lines were susceptible on the cotyledon but moderately

resistant at the adult plant stage. Given that cotyledon and adult plant resistance are linked, as shown in the present study, most DH lines, developed from a single F_1 plant through microspore culture, should show consistent disease reactions (resistant or susceptible) at both cotyledon and adult plant stages. Only a few DH lines (the number depending on the distance between the two genes) would show inconsistent reactions (resistant on cotyledon and susceptible at adult plant stage, or vice versa) due to crossover occurring during chromosome pairing.

Analysis of the cross between the two resistant parents (RB87-62 × DH88-752) showed that the F_2 population segregated 15:1 (R:S) for both cotyledon and adult plant resistance. This result further supports a single gene model controlling cotyledon and adult plant resistance respectively in the two accessions and also indicates that the two pairs of resistance genes are non-allelic and independently assorting. Therefore, the cotyledon and adult plant resistance genes from these sources reside either on different chromosomes or sufficiently apart on the same chromosome to behave independently. This implies that there are different regions in the *B. napus* genome where resistance genes to *L. maculans* are located. Comparison of linkage maps of *B. napus* cv. Major (Ferreira et al. 1995) and cv. Shiralee (Mayerhofer et al. 1997) demonstrates that resistance genes in these two cultivars were located on linkage group (LG) 6 (Ferreira et al. 1995), also referred to as linkage group N7 (Parkin et al. 1995). Based on the presence of two consensus RFLP markers, the resistance locus (*LmR1*) in cv. Shiralee was mapped to a different location of LG6 from that of *LEM1*, resistance locus in cv. Major. In this study, cotyledon resistance and adult plant resistance were two distinct but linked traits. Selection for cotyledon resistance alone will

only be useful if loci controlling these two traits are tightly linked. As adult plant resistance to *L. maculans* is the major component of resistance required in *Brassica* breeding programs, selection for resistance needs to be based on both cotyledon resistance and adult plant resistance to isolates of *L. maculans* that are representative of the pathogen population in the field. Development of molecular markers linked with resistance genes will help the selection process in which the two accessions are used as sources of resistance.

CHAPTER 4

COMPARATIVE MAPPING OF RESISTANCE TO *LEPTOSPHAERIA MACULANS* IN *BRASSICA NAPUS*

4.1 Abstract

Doubled haploid (DH) lines, developed from single F_1 plants of crosses between the susceptible cultivar Westar and three resistant accessions, RB87-62, DH88-752 or Crésor, were employed for comparative mapping of cotyledon and adult plant resistance to *Leptosphaeria maculans*. The cotyledon and adult plant resistance genes were mapped 6.1 cM apart in RB87-62 and 10.0 cM apart in DH88-752 respectively. In cv. Crésor, only the adult plant resistance gene was mapped. Although resistance genes carried by the three accessions were derived from different sources, comparison of the arrangement and map distances of closely linked RAPD, AFLP and RFLP markers on the three resistance linkage maps demonstrated that there is a conserved chromosomal region where the resistance genes are located. The conserved regions covered 41.4 cM in RB87-62, 36.5 cM in DH88-752 and at least 32.0 cM in Crésor.

Key words *Brassica* species ··*Leptosphaeria maculans* · Comparative mapping · Resistance gene cluster · Conserved chromosomal region

4.2 Introduction

Disease resistance genes frequently occur in clusters in plant genomes. Two typical arrangements of these clusters may exist. The flax *L* locus contains at least 13 allelic rust resistance specificities, and the more complex *M* locus exists as a tandem array of at least seven genes (Islam and Shepherd 1991). In lettuce, genetic studies on resistance to downy mildew (*Bremia lactucae*) have identified 15 dominant resistant genes (*Dm* genes), most of which were mapped to three major clusters with the largest cluster containing at least 10 *Dm* resistance genes (Pryor and Ellis 1993). At these complex resistance loci, unequal cross-over via meiotic mispairing between different genes is currently thought to be a major mechanism by which novel resistance specificities are generated and new combinations of parental resistance genes are created (Hammond-Kosack and Jones 1997; Michelmore and Meyers 1998; Richter and Ronald 2000). Therefore, the identification of a resistance gene cluster that existed prior to species divergence or geographic separation after species formation (Geffroy et al. 1999) would provide an effective tool to elucidate the process of resistance gene evolution at the molecular level.

Blackleg, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. (Anamorph *Phoma lingam* (Tode:Fr) Desmaz.), is a major disease of *Brassica* species, especially to *B. napus* and *B. rapa* (Rimmer and van den Berg 1992). However, *Brassica* species containing the B genome i.e. *B. nigra* (BB, 2n=16), *B. carinata* (BBCC, 2n=34) and *B. juncea* (AABB, 2n=36), show high levels of resistance (Roy 1978; Sacristán and Gerdemann 1986; Sjödin and Glimelius 1988). Resistance genes from B genome species have been successfully

introduced into *B. napus* by interspecific hybridization (Struss et al. 1991; Chèvre et al. 1996).

A number of resistance genes (loci) conferring resistance to different isolates of *L. maculans* have been mapped in *B. napus* (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997). Similarly, three potential B genome resistances derived from *B. nigra*, *B. juncea* and *B. carinata* have been characterized through interspecific crosses with *B. napus* (Zhu et al. 1993; Plieske et al. 1998). However, most of these linkage maps were constructed independently using different sets of probes, as well as different pathogen isolates and methods of disease assessment. This hindered comparison of relationships of these linkage groups to identify molecular homology and collinearity in regions where the resistance genes are located. Comparative genetic mapping using common DNA markers has identified varying degrees of conserved synteny between the genomes of *Brassica* species at the molecular level and provides an efficient tool to elucidate evolutionary processes by which current genome organization within the Brassicaceae arose (Parkin et al. 1995; Lagercrantz and Lydiate 1996).

Genetic analysis of resistance to *L. maculans* in two *B. napus* accessions, RB87-62 and DH88-752, showed that cotyledon resistance and adult plant resistance are controlled by single dominant genes respectively and that cotyledon and adult plant resistance genes are linked (Chapter 3). In Crésor, a single major locus controlling adult plant resistance was reported by Dion et al. (1995). In the present study, comparative mapping using DH populations was employed to map resistance genes in these three resistant accessions, and to identify possible homologous relationships among the chromosomal intervals carrying

resistance genes. Our results provide evidence that there is a conserved chromosomal region in which the cotyledon and adult plant resistance genes are located in these *Brassica napus* accessions.

4.3 Materials and methods

4.3.1 Plant material

A susceptible *B. napus* cv. Westar was crossed with each of the three resistant accessions, RB87-62 (a *B. napus* breeding line with the pedigree of Chikuzen*2/Zephyr × Bronowski), DH88-752, a doubled haploid *B. napus* line, derived from 79N047-59, a putative *B. napus-B. juncea* recombinant line developed by NN Roy and kindly provided by GC Buzza of Pacific Seeds, Toowoomba, Australia and Crésor, a *B. napus* cultivar with the pedigree of Canbra × Cresus*3. Single F_1 plants from each cross were used to develop doubled haploid (DH) lines, following the microspore culture method of Coventry et al. (1988). For the cross of Westar × Crésor, DH lines were developed by Dion et al. (1995).

4.3.2 Disease reaction of doubled haploid lines

Disease evaluations of DH populations developed from Westar × RB87-62 and Westar × DH88-752, were conducted on cotyledons and at adult plant stages after inoculation with a single isolate of *L. maculans*, Pl86-12, belonging to pathogenicity group 2 (PG2). The methods of inoculation and disease rating were as previously described (Chapter 3). Sixteen plants from each DH line were tested, and means of disease severity were calculated. Evaluation of disease reaction of the DH lines derived from Westar × Crésor was made at

the adult plant stage in Agriculture and Agri-Food Canada, Saskatoon Research Center disease nurseries at Saskatoon, Saskatchewan (Dion et al., 1995). For cotyledon resistance evaluation, disease ratings of 0-6.0 were defined as resistant, 6.1-9 as susceptible; for adult plant resistance, the disease ratings of 0-2.5 were defined as resistant, 2.6-5.0 as susceptible.

4.3.3 Leaf sample preparation

Leaves from 5-6 week-old plants were harvested from each DH line and their parents in liquid nitrogen, lyophilized, ground to a fine powder and stored at -20°C.

4.3.4 RAPD analysis

DNA was extracted from 30-50 mg of dry tissue in a 1.5-ml microtube by adding 1 ml of 95 °C extraction buffer (0.1 M Tris-HCl (pH 8.0); 10 mM EDTA, 1 M KCl) and incubating at 95 °C for 10 min with occasional vortexing. The homogenate was centrifuged to remove cell debris, and the supernatant was treated with RNase. DNA was precipitated with isopropanol, washed with 70% ethanol, resuspended in water, reprecipitated with 70% ethanol and resuspended in 60 µl of water. This procedure typically yielded 20-30 µg of DNA. For RAPD analysis, all DNA samples were diluted to 2.5 ng/µl. Bulked segregant analysis (BSA) (Michelmore et al. 1991) compared DNA of the parents with DNA from a resistant and a susceptible bulked line. The bulked DNA was prepared by combining equal amounts of DNA from each of ten resistant or ten susceptible lines based on cotyledon reaction. In total, 625 UBC decamer random primers (UBC, Vancouver, Canada) were screened using bulked segregant analysis. Each PCR reaction was carried out in a 20 µl

reaction volume containing 10 ng template DNA, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM primer, and 1 U of *Taq* DNA polymerase (BRL, Mississauga, Ont.). The cycle protocol was 95°C for 1.5 min, followed by 35 cycles of 95°C for 20 s, 36°C for 1 min, (ramp 1°C/s), 72°C for 1.5 min and a final 72°C for 7 min. The reaction products were analyzed on 2.0% (W/V) agarose gel in 1 × TAE by electrophoresis at 106 V for 3 h. Gels were stained with ethidium bromide and photographed on a digital gel documentation system (Stratagene).

4.3.5 AFLP analysis

DNA was extracted using DNeasy Plant Mini Kit (Qiagen) following the instruction of the manufacturer. For AFLP analysis, 250 ng DNA from each DH line was digested with *Eco*RI and *Mse*I according to the procedure of Vos et al. (1995). BSA was employed to identify informative markers, and these were then mapped in the whole population. Thirty combinations of AFLP primer pairs (*Eco*RI + *Mse*I) were screened.

4.3.6 RFLP analysis

DNA was extracted basically as described by Sharp et al. (1988) except that Kirby mix (Covey and Hull 1981) was used for initial extraction of freeze-dried tissue. For RFLP analysis, 15 μg DNA from each DH line was digested with *Eco*RI or *Hin*dIII. Digested DNA was separated on 0.8% agarose gels and blotted on Hybond-N^+ nylon membrane (Amersham). *Brassica* RFLP probes (genomic or cDNA clones) were kindly supplied by TC Osborn (University of Wisconsin, USA). Hybridization probes were prepared by PCR or gel

isolation of inserts and were labelled with ^{32}P-dCTP. Hybridization of DNA blots with radioactively labeled RFLP probes was performed according to Sharpe et al. (1995) with minor modification.

4.3.7 Linkage analysis

The RAPD, AFLP and RFLP data from DH lines were analyzed with MAPMAKER/EXP3.0 (Lander et al.1987). The grouping and genetic linkage maps were generated using a minimum LOD (log of the odds ratio of linkage vs. no linkage) score of 3.0 and a maximum recombination fraction of 0.3 was used to group loci. Map distances were converted to centiMorgan using the Kosambi function (Kosambi 1949).

4.4 Results

4.4.1 Genetic analysis of resistance in DH lines

Previous genetic analyses of F_2 and F_3 populations in the crosses of Westar with either RB87-62 or DH88-752 established a single dominant gene model for both cotyledon and adult plant resistance to *L. maculans* in RB87-62 and DH88-752, with two linked genes controlling cotyledon and adult plant resistance respectively in each accession (Chapter 3). A total of 122 DH lines from Westar × RB87-62 and 153 DH lines from Westar × DH88-752 and their parents were tested for cotyledon and adult plant resistance by inoculation with one isolate Pl86-12 (PG2) of *L. maculans* in a growth chamber or greenhouse. The resistant parents, RB87-62 and DH88-752, showed high levels of resistance both at the cotyledon and adult plant stages. Frequency distributions of disease evaluations of DH lines from the two

populations are shown in Figure 4.1. For both cotyledon and adult plant resistance of the DH lines to *L. maculans*, neither of two DH populations showed 1:1 (R:S) segregation ratios (Chi-square test, $P<0.01$). Segregation of DH lines in both populations was biased towards susceptibility. Nevertheless, there were seven recombinant lines showing opposite disease reactions at cotyledon and adult plant stage in DH population derived from Westar × RB87-62 and fifteen recombinant lines in DH population derived from Westar × DH88-752. Of 122 DH lines from Westar × RB87-62, 20 showed cotyledon resistance and 23 were resistant at the adult plant stage. Of 153 DH lines from Westar × DH88-752, 26 showed cotyledon resistance and 37 were resistant at the adult plant stage (Table 4.1).

Table 4.1 Disease reaction of DH lines derived from Westar × RB87-62 and Westar × DH88-752

	Host reaction				
Population	CA	Ca	cA	ca	Total
Westar × RB87-62	18	2	5	97	122
Westar × DH88-752	24	2	13	114	153

4.4.2 Resistance linkage map in RB87-62

Based on BSA analysis, there were six decamer primers and two AFLP primer combinations that amplified polymorphisms between the resistant and susceptible parents and the bulked DH samples (Table 4.2). A cotyledon resistance gene (CLmRB) and an adult plant resistance gene (ALmRB) were mapped on the same linkage group. The distance between the two genes was 6.1 cM. Two RAPD markers, RAPD1 (Figure 4.2) and RAPD2 (Figure 4.3), which cosegregated in this population, were the closest markers to CLmRB (0.8

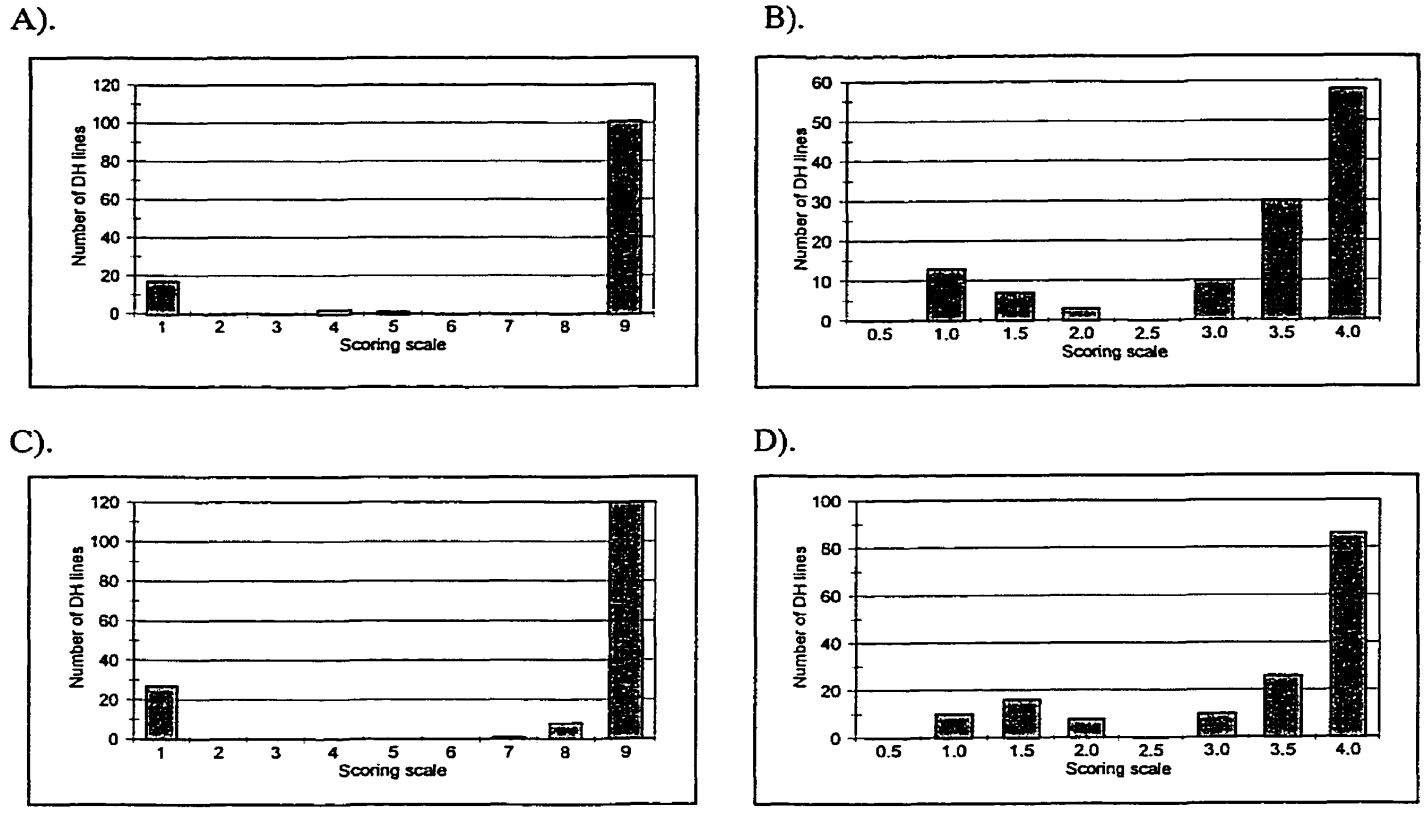


Figure 4.1. Distribution of cotyledon and adult plant reaction in DH lines derived from Westar × RB87-62 and Westar × DH88-752. A and B, cotyledon and adult plant reaction respectively in DH lines derived from Westar × RB87-62. C and D, cotyledon and adult plant reaction respectively in DH lines derived from Westar × DH88-752.

Table 4.2 Molecular markers identified in three DH populations

Marker	Primer/probe	Size(bp)	Allele
RAPD127	UBC127	1080	RB87-62, DH88-752 and Crésor
RAPD237	UBC237	1650	DH88-752
RAPD239	UBC239	365	Westar
RAPD275	UBC275	1100	DH88-752
RAPD282	UBC282	430	Westar
RAPD393	UBC393	1950	DH88-752
RAPD654	UBC654	915	Westar
RAPD682	UBC682	760	RB87-62
AFLP1	E-AAG/M-CTA	280	Westar
AFLP2	E-AAG/M-CTT	180	Westar
AFLP3	E-AAG/M-CTA	240	DH88-752
AFLP4	E-AAG/M-CTT	220	Westar
RFLP1	Wg8g3[a]		Westar

[a] Ferreira et al. 1995

Figure 4.2. Amplification profiles of the RAPD1 marker in three DH populations derived from Westar × RB87-62 (A), Westar × DH88-752 (B) and Westar × Crésor (C). M molecular-weight marker; P1 RB87-62 (A), DH88-752 (B) and Crésor (C); P2 Westar in A, B and C; R cotyledon resistant DH lines in A and B, adult plant resistant DH lines in C; S cotyledon susceptible DH lines in A and B, adult plant susceptible DH lines in C. Markers are indicated by arrows.

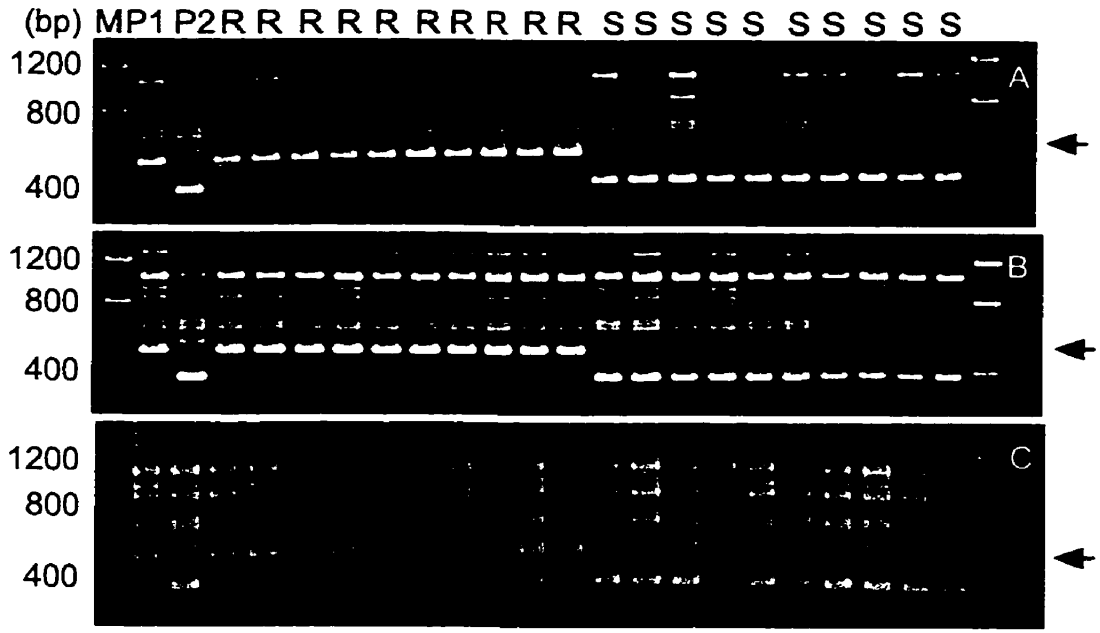
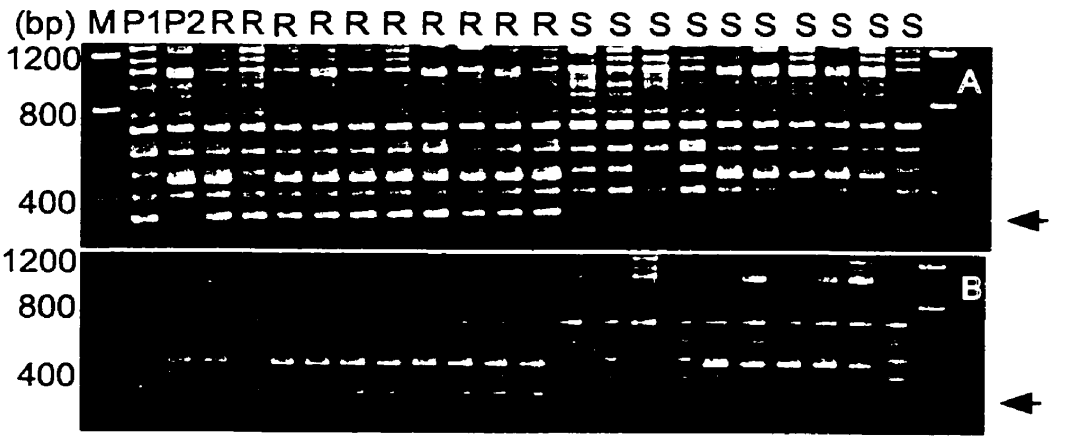

(bp) MP1 P2R R R R R R R R R R S S S S S S S S S S
1200
800
400
A
1200
800
400
B
1200
800
400
C

Figure 4.3. Amplification profiles of the RAPD2 marker in two DH populations derived from Westar × RB87-62 (A) and Westar × Crésor (B). M molecular-weight marker; P1 RB87-62 (A) and Crésor (B); P2 Westar in A and B; R cotyledon resistant DH lines in A and adult plant resistant DH lines in B; S cotyledon susceptible DH lines in A and adult plant susceptible DH lines in B. Markers are indicated by arrows.


(bp) M P1 P2 R R R R R R R R R R S S S S S S S S S S
1200
800
400
A
1200
800
400
B

cM) and to ALmRB (5.3 cM). Two AFLP markers, AFLP1 and AFLP2, and one RFLP marker (RFLP1) (Figure 4.4) were also mapped on the linkage group (Figure 4.5).

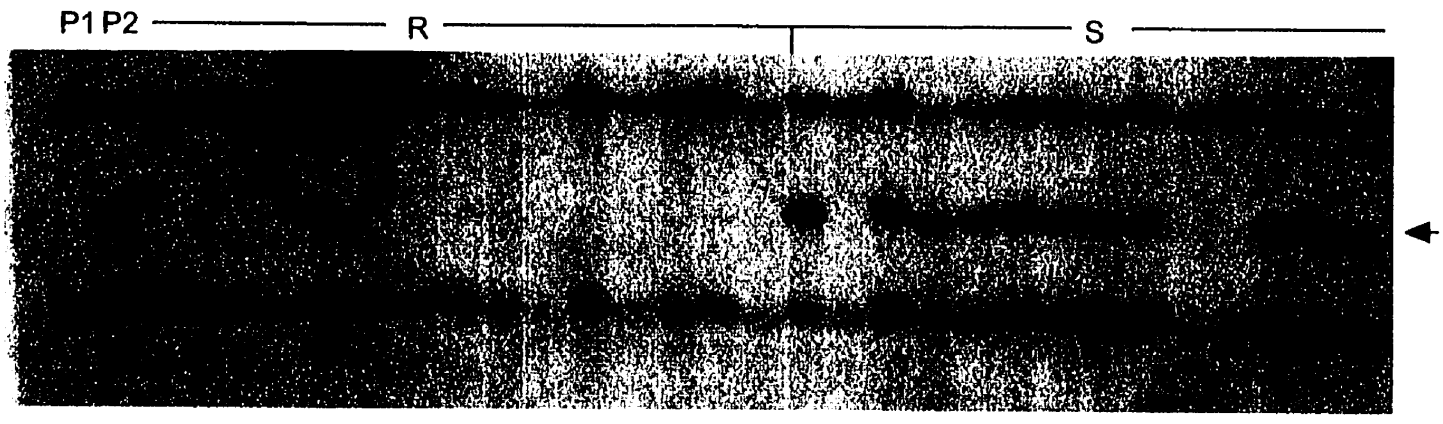
4.4.3 Resistance linkage map in DH88-752

Seven decamer primers and two AFLP primer combinations were polymorphic between the resistant and susceptible parents and the bulked DH samples (Table 4.2). A cotyledon resistance gene (CLmDH) and an adult plant resistance gene (ALmDH) were mapped to the same linkage group. The distance between the two resistance genes was 10.0 cM. Marker RAPD1 cosegregated with CLmDH. The next closest marker, RAPD654, was 0.7 cM to CLmDH and 9.3 cM to ALmDH. Two AFLP markers, AFLP3 (Figure 4.6) and AFLP4 were also mapped in this interval (Figure 4.5).

4.4.4 Resistance linkage map in Crésor

One dominant gene (BLR) controlling adult plant resistance to *L. maculans* was previously characterized and mapped with a set of RFLP probes in Crésor (Dion et al. 1997). Cotyledon resistance was not tested by the authors. In the present study, 96 resistant and 96 susceptible DH lines from the same DH population and their parents were used for mapping the adult plant resistance gene. Three decamer primers amplified polymorphisms between the resistant and susceptible parents and the bulked DH samples. The adult plant resistance gene (BLR) was flanked by two RAPD markers, RAPD1 (Figure 4.2) and RAPD2 (Figure 4.3) with distances of 0.8 cM and 1.6 cM respectively (Figure 4.5).

Figure 4.4. Hybridization profile of RFLP1 marker in DH population derived from Westar × RB87-62. P1 RB87-62; P2 Westar; R cotyledon resistant DH lines; S cotyledon susceptible DH lines. Marker is indicated by an arrow.


P1 P2
R
S

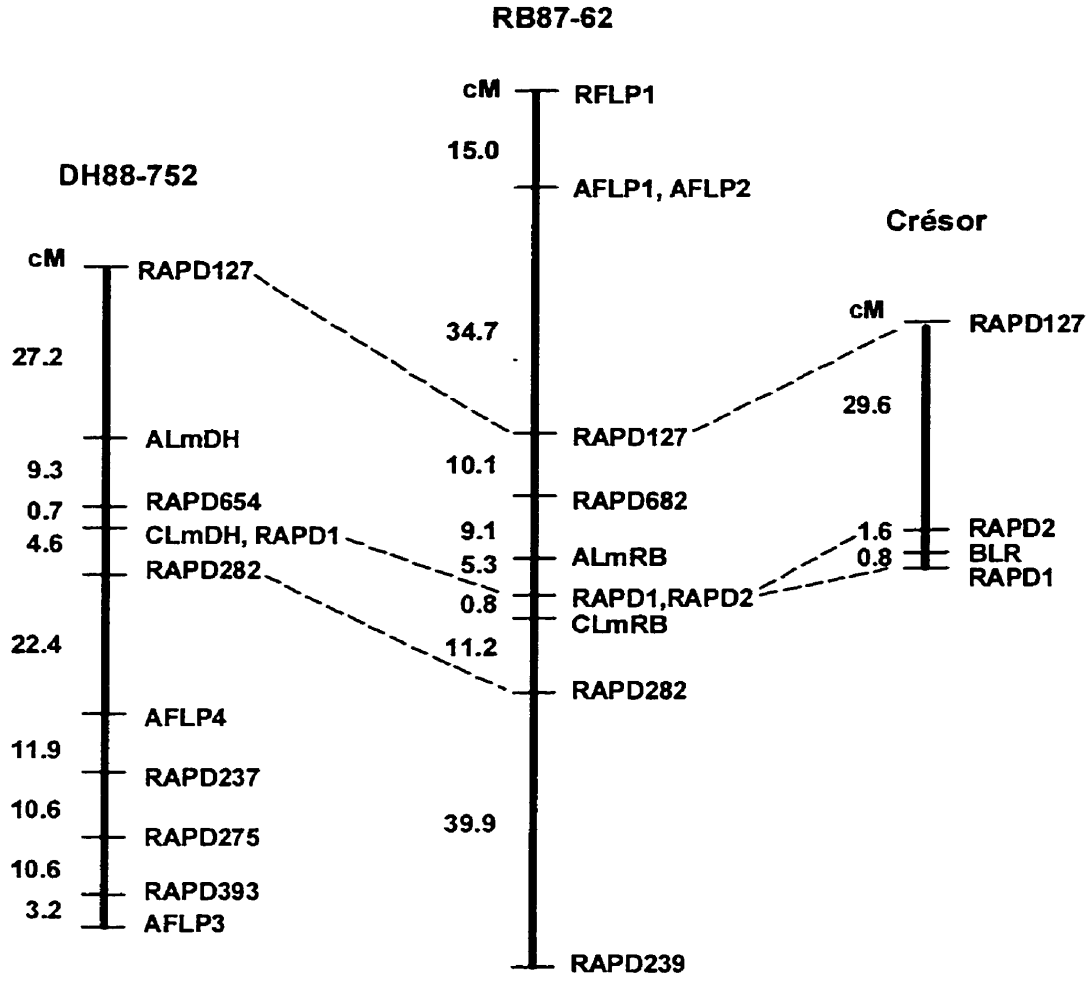
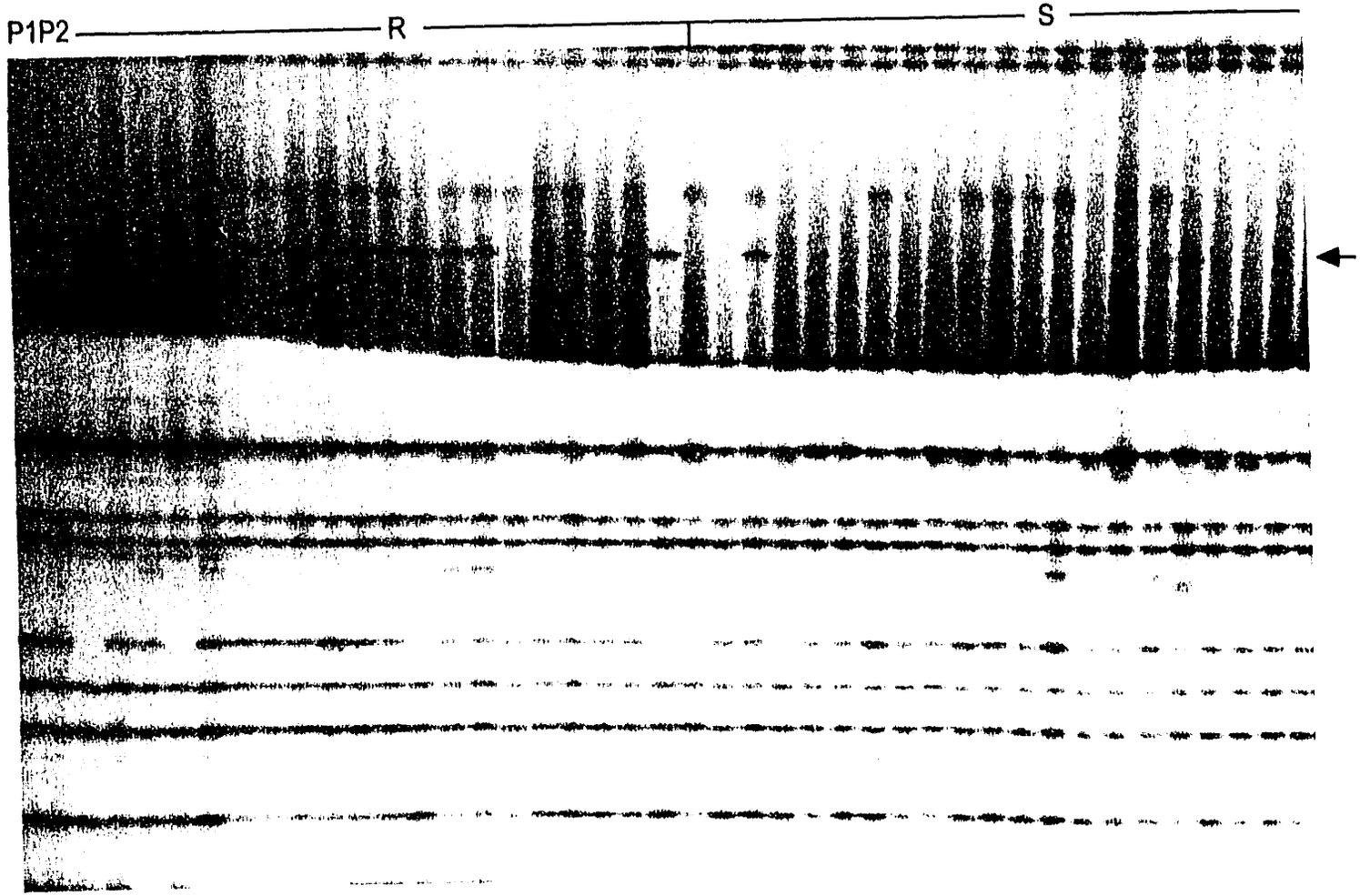


Figure 4.5. Conserved linkage of loci controlling resistance to *L. maculans* in DH88-752, RB87-62 and Crésor. Loci connected with a dash line are detected by the same DNA marker.

Figure 4.6. Amplification profile of the AFLP3 marker in DH lines derived from Westar × DH88-752. M molecular-weight marker; P1 DH88-752; P2 Westar; R cotyledon resistant DH lines; S cotyledon susceptible DH lines. Marker is indicated by an arrow.


P1P2
R
S

4.4.5 Comparative analysis of the resistance linkage maps in RB87-62, DH88-752 and Crésor

There were three consensus RAPD markers, RAPD127 (Figure 4.7), RAPD1 (Figure 4.2) and RAPD282 (Figure 4.8), between the resistance linkage maps of RB87-62 and DH88-752, which flanked the cotyledon and adult plant resistance genes. This region from RAPD127 to RAPD282 covered 41.4 cM in the RB87-62 linkage group and 36.5 cM in the DH88-752 linkage group. In RB87-62, two markers, RAPD1 and RAPD2 cosegregated and were the closest markers to both the cotyledon resistance gene (0.8 cM) and the adult plant resistance gene (5.3 cM). In DH88-752, only RAPD1 was polymorphic and cosegregated with the cotyledon resistance gene. Another closely linked marker, RAPD654, was 0.7 cM from the cotyledon resistance gene and 9.3 cM from the adult plant resistance gene. In Crésor, although RAPD282 was not polymorphic, there was a similar chromosomal region, from RAPD127 to RAPD1, encompassing the adult plant resistance gene (BLR) to those in the linkage maps of RB87-62 and DH88-752. The comparison of consensus markers in Crésor with those in RB87-62 and DH88-752 revealed that the conserved region covered at least 32.0 cM in Crésor.

Figure 4.7. Amplification profile of the RAPD127 marker in three DH populations derived from Westar × RB87-62 (A), Westar × DH88-752 (B) and Westar × Crésor (C). M molecular-weight marker; P1 RB87-62 (A), DH88-752 (B) and Crésor (C); P2 Westar in A, B and C; R cotyledon resistant DH lines in A and B, adult plant resistant DH lines in C; S cotyledon susceptible DH lines in A and B, adult plant susceptible DH lines in C. Markers are indicated by arrows.

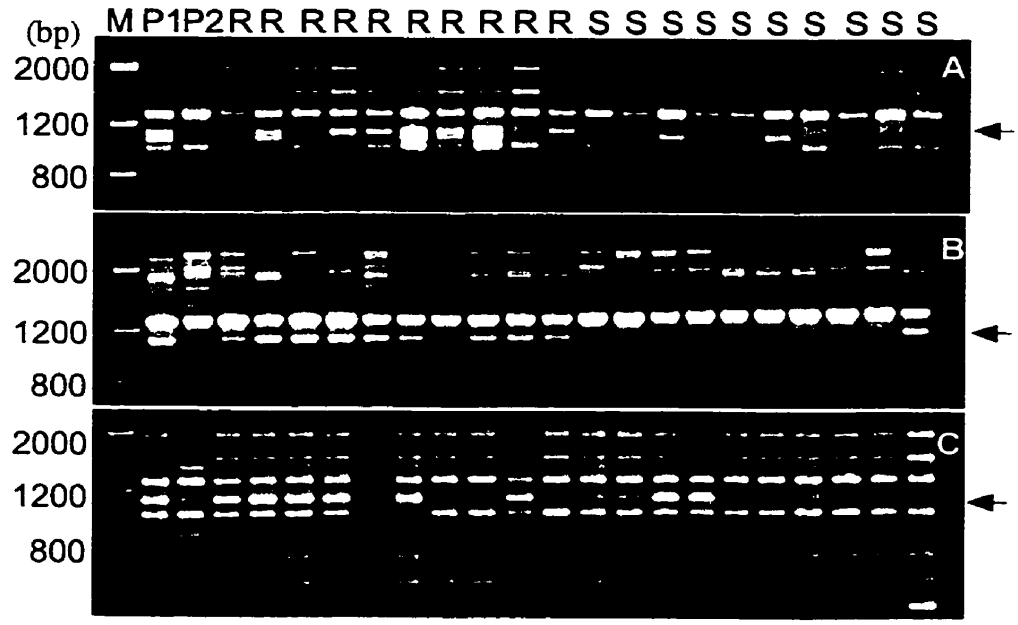
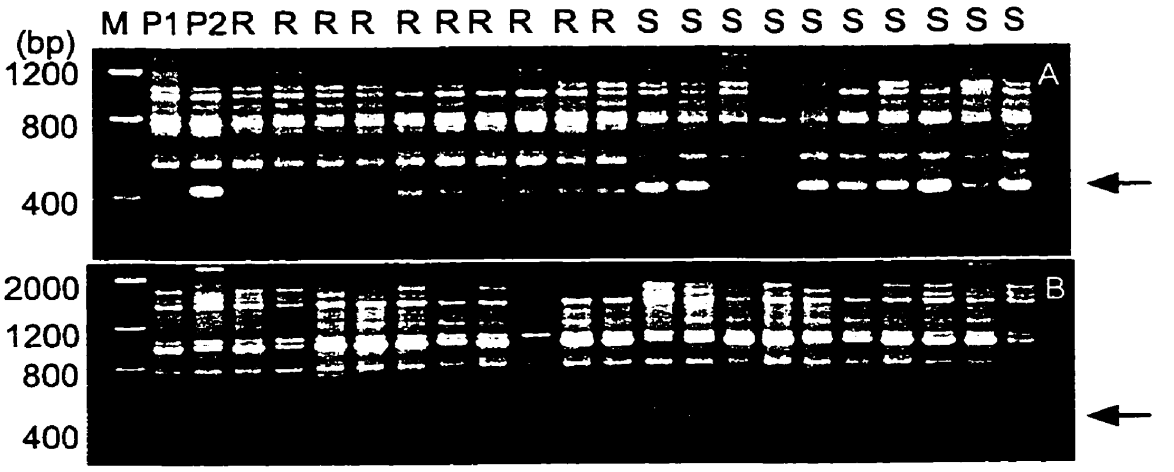

(bp)
M P1 P2 R R R R R R R R R S S S S S S S S S
2000
1200
800
A
2000
1200
800
B
2000
1200
800
C

Figure 4.8. Amplification profile of the RAPD282 marker in two DH populations derived from Westar × RB87-62 (A), Westar × DH88-752 (B). M molecular-weight marker; P1 RB87-62 (A) and DH88-752 (B); P2 Westar in A and B; R cotyledon resistant DH lines; S cotyledon susceptible DH lines. Markers are indicated by arrows.


M P1 P2 R R R R R R R R R R S S S S S S S S S S
(bp)
1200
800
400
A
2000
1200
800
400
B

4.5 Discussion

Disease evaluation of the two DH populations derived from Westar × RB87-62 and Westar × DH88-752 showed that cotyledon resistance was associated with adult plant resistance in most lines, however, a few lines (recombinants) showed opposite reactions for these two types of resistance. These results are in agreement with the genetic studies that demonstrated that two separate but linked dominant genes control cotyledon and adult plant resistance in RB87-62 and DH88-752 (Chapter 3). Comparisons of recombination frequency between cotyledon resistance gene and adult plant resistance gene in F_2 population and DH population derived from Westar × RB87-62 and Westar × DH88-752 are shown in Table 4.3 and Table 4.4 respectively. In both crosses, the recombination frequency in F_2 population was higher than that in DH population. This could be explained by the fact that the cotyledon or adult plant disease reaction of a few individual F_2 plants was incorrectly scored, especially for those plants showing intermediate disease reactions. This mischaracterization could increase the number of recombinants. DH lines were previously used for mapping of resistance genes in *Brassica napus* to *L. maculans* (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997), where disease reaction evaluations of individual DH lines could be repeated, allowing for accurate disease phenotypic data for resistance gene mapping. Segregation ratios of disease reaction deviated significantly from the expected 1:1 (R:S) ratio (Chi-square test, $P<0.01$) in both DH populations. Segregation distortion in a DH population for a single dominant gene for resistance to *L. maculans* was also reported by Mayerhofer et al. (1997) who used a DH population to map a resistance gene to *L. maculans* in *B. napus* cv. Shiralee. Disease evaluation and resistance mapping in DH lines derived from Westar × Crésor

Table 4.3 Comparison of recombination frequency between cotyledon resistance gene and adult plant resistance gene in F_2 populations and DH populations derived from Westar × RB87-62

	Host reaction					
Population	CA	Ca	cA	ca	Total	Recombination frequency
Cross1	84	6	9	22	121	12.3%
Cross2	85	10	13	17	125	18.4%
Cross3	80	15	11	16	122	21.3%
Cross4	81	9	15	23	128	18.7%
Cross5	88	7	14	17	126	16.6%
Subtotal	418	47	62	95	622	17.5%
DH line	18	2	5	97	122	5.7%
Map distance						6.1 cM

Table 4.4 Comparison of recombination frequency between cotyledon resistance gene and adult plant resistance gene in F_2 populations and DH populations derived from Westar × DH88-752

	Host reaction					
Population	CA	Ca	cA	ca	Total	Recombination frequency
Cross1	76	9	7	15	107	14.9%
Cross2	75	13	15	19	122	22.9%
Cross3	73	16	11	16	116	23.2%
Cross4	84	7	15	19	125	17.6%
Cross5	81	5	12	27	125	13.6%
Subtotal	389	50	60	96	595	18.4%
DH line	24	2	13	114	153	9.8%
Map distance						10.0 cM

showed one dominant gene (*LmFr1*) controlling adult plant resistance in Crésor (Dion et al. 1995).

Comparative mapping of resistance to *L. maculans* in RB87-62, DH88-752 and Crésor indicated that resistance genes in these *B. napus* accessions were mapped to a chromosomal region which covered 41.4 cM in RB87-62, 36.5 cM in DH88-752 and at least 32.0 cM in cv. Crésor. The resistance in these accessions were derived from quite different sources. The resistance in RB87-62 was derived from a *B. napus* cv. Chikuzen from Japan; in Crésor, the resistance was from a European source (Cresus). Resistance in DH 88-752, developed through interspecific hybridization between *B. napus* and *B. juncea* (Roy 1984), is assumed to be from *B. juncea*, although the pedigree of the resistant parent is unknown. Plieske et al. (1998) compared the three B genome resistance genes derived from *B. nigra*, *B. juncea* or *B. carinata* in a nearly uniform genetic background. All three species showed monogenic inheritance, and had similar arrangement and map distances of closely linked RFLP markers flanking the resistance genes despite independent origins. These results suggest that resistance genes to *L. maculans* in the three *Brassica* species may be located in a conserved region.

The linkage of cotyledon and adult plant resistance genes to one isolate (P186-12) of *L. maculans* was first established in RB87-62 and DH88-752 in the genetic study (Chapter 3). Map distances between cotyledon and adult plant resistance genes were 6.1 cM in RB87-62 and 10.0 cM in DH88-752 (Figure 4.2) while cotyledon reaction to *L. maculans* was not tested in cultivar Crésor. Dixelius and Wahlberg (1999) recently reported that cotyledon resistance and adult-leaf resistance loci among the three B genome *Brassica* species were

located on three linkage groups. Many plant resistance genes reside at complex loci. This structure may facilitate the rate of resistance gene diversification (Pryor and Ellis 1993). The maize *Rp1* (Sudupak et al. 1993), the flax *M* (Anderson et al. 1997), the lettuce *Dm3* region (Anderson et al. 1996), the tomato *Cf-4/9* (Parniske et al. 1997), and the *Arabidopsis RPP* loci (Botella et al. 1997), all consist of genetically linked resistance specificities. Recent molecular analyses of plant resistance genes and the structure of resistance gene clusters have postulated the evolutionary processes of resistance specificities in plant species (Meyers et al. 1998; Geffroy et al. 1999; Michelmore and Meyers 1999). Cloning of blackleg resistance genes and elucidating genomic structure of different resistance loci at the molecular level will enable us better to understand the evolutionary relationships of resistance genes in *Brassica* species.

Based on the map locations of a common set of RFLP markers, a resistant locus (*LEM1*) in cv. Major (Ferreira et al. 1995) and a resistance gene (*LmR1*) in cv. Shiralee (Mayerhofer et al. 1997) had been mapped to different positions on linkage group (LG) 6 in the *B. napus* genome. These results demonstrate that there are different locations in the *B. napus* genome in which resistance genes to *L. maculans* could be incorporated. Studies on the genetic relationship of resistance between RB87-62 and DH88-752 showed that resistance in these two accessions are non-allelic and showed independent assortment (Chapter 3). Therefore, the regions encompassing the resistance genes should be sufficiently separate from each other on the same chromosome or reside on different chromosomes. However, when the resistance linkage maps were developed for RB87-62 and DH88-752, the two resistance linkage groups shared one RAPD allele (RAPD282) from Westar. This was conflict with the

result from genetic analysis which showed the two resistance sources were independently assorting. It was known that in cv. Westar, there is a translocation region between N7 and N16 (IAP Parkin personal communication), the influence of this translocation region on chromosome pairing and recombination though crossover in this region, needs to be elucidated, since Westar has been frequently used as a susceptible parent in many crosses. One RFLP marker, Wg8g3, mapped on linkage group six (or N7) (Ferreira et al. 1995) was also mapped on the resistance linkage group of RB87-62. To clarify the locations of these resistance genes, more RFLP markers from linkage group 6 need to be tested in the resistance linkage groups of RB87-62 and DH88-752. In cv. Crésor, adult plant resistance gene (BLR) was mapped on N7 (IAP Parkin personal communication). This indicates that N7 is a major linkage group in which resistance genes to *L. maculans* in *B. napus* are located.

In DH88-752, resistance was assumed to be derived from *B. juncea* through interspecific hybridization. Consensus markers identified in this study could be used to detect genomic DNA of the parent of DH88-752 to determine whether the same conserved region is present. If present, it would allow us to estimate the size of the same conserved region in *B. juncea*. However, the initial accession used for the interspecific cross was not available. Other *B. juncea* accessions perhaps could be substituted for this purpose, but this would depend on the presence of intraspecific polymorphism between different *B. juncea* accessions. This assumption was addressed by Barret et al. (1998), who used RAPD specific markers from *B. juncea* as probes and localized them on a genetic map of *B. napus*. Based on the observation of the disappearance of *B. napus* markers in recombinant lines, they estimated the minimal size of the substituted *B. napus* fragment to be 39 cM. This size is very

comparable with our results that showed the resistance intervals to be 39 to 41 cM in RB87-62 and DH88-752 and at least 32.0 cM in Crésor. It is worth pointing out that within the resistance region, RAPD2 marker, which co-segregated with RAPD1 in RB87-62, and was closely linked with adult plant resistance gene in Crésor (0.8 cM), was not polymorphic in DH88-752 in which RAPD1 cosegregated with the cotyledon resistance gene.

Comparative analysis of the *Brassica* A, B and C genomes has shown a striking conservation of genome content (McGrath and Quiros 1991; Chyi et al. 1991; Teutonico and Osborn 1994; Parkin et al. 1995; Sharpe et al. 1995; Cheung et al. 1997; Lagercrantz and Lydiate 1996). This is reflected by both different copy numbers of chromosomal segments and rearrangements in the *Brassica* genomes (Lagercrantz et al.1996). Diploid *Brassica* genomes are thought to have evolved from an ancestor with 5 or 6 chromosomes (Röbbelen 1960; Prakash and Hinata 1980; Lagercrantz et al. 1996). Subsequently, duplications and rearrangements have taken place in the present genomes. This was confirmed by the fact that a large number of genomic segments in the *Brassica* genomes are generally present in several copies when tested with RFLP probes (Lagercrantz and Lydiate 1996). The genes derived from duplications may perform similar functions throughout their evolution. It is tempting to speculate that the genes conferring resistance to *L. maculans* in these *B. napus* accessions arose from a common ancestral origin. Not only does the resistance to *L. maculans* in the B genomes of *Brassica* have a conserved region (Dixelius and Wahlberg 1999), it seems likely that the resistance in *B. napus* shares a conserved region with that in *B. juncea*, possibly even with all three B genomes of *Brassica*. The molecular basis and origin of *RPM1* (resistance to *Pseudomonas syringae* pv. *maculicola*) bacterial resistance gene in *A. thaliana* and *B.*

napus has been explored (Grant et al. 1998). The *Brassica RPM1* region is syntenic with that in resistant *Arabidopsis* accessions. This synteny implies that the functional *RPM1* alleles in the two related species were derived from a common progenitor. Knowledge of the relationship and the degree of synteny among *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC) has important potential consequence for developing cultivars with disease resistance. Further studies will allow us to elucidate the evolutionary processes of *Brassica* genomes and to transfer agronomically important traits (such as disease resistance) from one species to another more effectively.

CHAPTER 5

DEVELOPMENT OF A SCAR MARKER FOR SELECTION OF RESISTANCE TO *LEPTOSPHAERIA MACULANS* IN *BRASSICA NAPUS*

5.1 Abstract

Blackleg, caused by *Leptosphaeria maculans*, is a serious disease of *Brassica napus*. This disease can be effectively and economically controlled by the use of resistant cultivars. RAPD1, a previously identified RAPD marker, was closely linked with cotyledon and adult plant resistance in two *B. napus* accessions, RB87-62 and DH88-752. In this study, we reported that RAPD1 was converted to a sequence characterized amplified region (SCAR) marker by sequencing the targeted RAPD products. The SCAR marker was tested in two doubled haploid (DH) populations, developed from Westar × RB87-62 and Westar × DH88-752, and the F_2 plants, developed from RB87-62 × DH88-752. This marker amplified the locus corresponding to RAPD1 in all plants tested in these populations and could provide an efficient and reliable screening tool for selection of blackleg resistant plants in breeding programs.

Key words Resistance · *Leptosphaeria maculans* · SCAR · *Brassica napus*

5.2 Introduction

Molecular markers have become fundamental tools in plant genetics. One of their applications in plant breeding programs is marker-assisted selection (MAS). This can considerably facilitate selection for traits linked to a marker. DNA-based markers such as RAPD, combined with bulked segregant analysis (Michelmore et al. 1991), have been widely used for identifying markers associated with various agronomically important traits. However, the short random primers (10 bp) used in RAPD analysis usually anneal with multiple sites in different regions of the exploited genome. Thus, they may amplify a number of loci unassociated with the traits of interest. The unrelated fragments present in typical RAPD profiles can impede the accuracy of scoring the targeted fragments. To overcome these problems and improve the utility of RAPD markers in marker-assisted selection, more specific primers can be developed from sequencing the targeted RAPD fragments. When the targeted RAPD fragments are cloned and sequenced, longer primers (15-25 bp) can be designed and a PCR amplification reaction can be carried out under high stringency. These more specific primers generate a sequence characterized amplified region (SCAR) (Paran and Michelmore 1993), which can be particularly useful to follow the inheritance of defined regions of the genome (loci controlling important traits such as disease resistance). Such markers have been developed for downy mildew resistance in lettuce (Paran and Michelmore 1993), anthracnose resistance in common bean (Adam-Blondon et al. 1994), stem rust resistance in barley (Penner et al. 1995), and *Fusarium* wilt resistance in melon (Wechter and Dean 1998).

Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not., is a serious disease of *Brassica napus* in Australia, Europe and western Canada (Rimmer and van den Berg 1992; Gugel and Petrie 1992). Although disease management by cultural practices and chemical means is often inadequate, effective control can be obtained using resistant cultivars. Therefore, breeding for resistance, through incorporating resistance genes into desirable breeding lines, has been a major component in *B. napus* breeding programs. Development of molecular markers closely linked with blackleg resistance genes will greatly facilitate the selection for resistance and assist in pyramiding resistance genes.

Cotyledon and adult plant resistance are controlled by different but linked genes in two *B. napus* accessions, RB87-62 and DH88-752 (Chapter 3) and corresponding resistance linkage maps were developed for these accessions (Chapter 4). One RAPD marker, RAPD1, was closely linked to the cotyledon resistance gene (0.8 cM) and to the adult plant resistance gene (5.3 cM) in RB87-62. RAPD1 also cosegregated with the cotyledon resistance gene and was linked to the adult plant resistance gene (10.0 cM) in DH88-752. In the present paper, the development of a SCAR marker from the RAPD1 marker and its application in selection for blackleg resistance in two DH populations and F_2 plants are described.

5.3 Materials and Methods

5.3.1 Plant material

A susceptible *B. napus* cultivar Westar was crossed with each of two *B. napus* resistant accessions, RB87-62 (pedigree of Chikuzen*2/Zephyr × Bronowski) and DH88-752 (a

doubled haploid line, derived from 79N047-59, which was a putative *B. napus-B. juncea* recombinant line developed by NN Roy and kindly provided by GC Buzza of Pacific Seeds, Toowoomba, Australia). Single F_1 plants from the two crosses were used to develop doubled haploid (DH) populations, following the microspore culture method of Coventry et al. (1988). F_2 plants were derived from the cross of RB87-62 × DH88-752.

5.3.2 Disease evaluation

Disease evaluation of DH lines, F_2 plants and other additional resistant *Brassica* accessions was carried out to determine cotyledon and adult plant reactions to one isolate of *L. maculans*, PI86-12 (belonging to PG2). The methods of inoculation and disease rating were as previously described (Chapter 3; Chapter 4).

5.3.3 DNA extraction

Leaves from each DH line and the F_2 population were harvested from 5-6 week-old plants and stored at -80°C, lyophilized and subsequently ground to a fine powder. DNA was extracted from 30-50 mg of dry tissue in a 1.5 ml microtube by adding 1 ml of 95°C extraction buffer (0.1 M Tris-HCl (pH8.0); 10 mM EDTA, 1 M KCl) and incubating at 95°C for 10 min with occasional vortexing. The homogenate was centrifuged to remove cell debris, and the supernatant was treated with RNase. DNA was precipitated with isopropanol, washed with 70% ethanol, resuspended in water, reprecipitated with 70% ethanol and resuspended in 60 µl of water.

5.3.4 RAPD analysis

Each PCR reaction was carried out in 20 µl reaction volumes containing 10 ng template DNA, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM primer, and 1 U of *Taq* DNA polymerase (BRL, Mississauga, Ont.). The cycle protocol was 95°C for 1.5 min, followed by 35 cycles of 95°C for 20 s, 36°C for 1 min, (ramp 1° C/s), 72°C for 1.5 min and a final 72°C for 7 min. The reaction products were separated on 2.0% (W/V) agarose gel in 1 × TAE by electrophoresis at 106 V for 3 hrs. Gels were stained in ethidium bromide and photographed on a digital gel documentation system (Stratagene).

5.3.5 Cloning and sequencing of RAPD fragments

The polymorphic RAPD1 fragments were removed from the agarose gel by excising the corresponding DNA bands and purified using the Qiagen PCR gel-extraction kit. The purified fragment was cloned into the pGEM-T ^{R} plasmid vector (Promega) following the manufacturer's instructions and the resulting ligation mixture was used to transform the electrocompetent *Escherichia coli* strain DH5α. The cells were plated on LB-Amp plates containing 40 µl Xgal (20 mg/ml) and 4 µl IPTG (200 mg/ml) and colonies were grown overnight at 37°C. White colonies were identified and transferred to a grid plate and to sterile water. Cells in water were lysed by heating tubes for 10 min at 95°C. A colony PCR was performed using the M13 primers to amplify the insert. The M13 PCR reaction consisted of 50 mM KCL, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each of M13 forward and reverse primers, 1 U of *Taq* DNA polymerase (BRL, Mississauga,

Ont.) and 10 µl lysed product in a 20 µl volume. The amplification protocol was 2 min at 95°C, 25 cycles of (30 s at 95°C, 45 s at 50°C, 1.5 min at 72°C), and 7 min at 72°C. Colonies with the correct insert size were identified and grown overnight at 37°C in LB broth. The plasmid was purified and the RAPD products were then sequenced by dye-deoxy terminator cycle sequencing™.

5.3.6 SCAR design and analysis

Based on sequences of each cloned amplification product, oligonucleotide primer pairs were synthesized. These new primers were designed to include the complete or partial original 10 bp random primer plus the next 8-14 adjacent internal bases. Amplification of genomic DNA with SCAR primers was conducted in 20 µl solutions containing 10 ng template DNA, 50 mM KCl, 0.2 mM of each dNTP, 1 U of *Taq* DNA polymerase (BRL, Mississauga, Ont), 1.5 mM $MgCl_2$ and 0.1 µM of each of forward and reverse primers. The cycle protocol was 95°C for 1.5 min, followed by 25 cycles of 95°C for 20 s, 60°C for 45 s, 72°C for 2 min and final 72°C for 7 min. Reaction products were separated on 2% (W/V) agarose gel in 1 × TAE by eletrophoresis at 106 V for 3 h. Gels were stained in ethidium bromide and photographed on a digital gel documentation system (Stratagene).

5.4 Results

5.4.1 Conversion of the RAPD marker into a SCAR marker

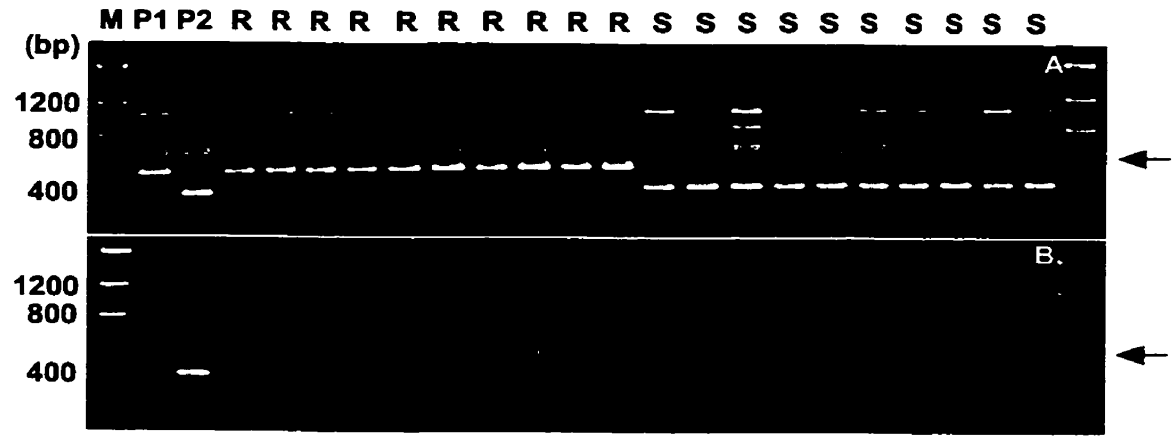
The amplification products of the RAPD1 primer from resistant parents, RB87-62 and

DH88-752, were chosen for cloning and sequencing. The corresponding fragments were first removed from the gels and amplified with 10 bp random primer to confirm that the correct bands were selected and no other untargeted products were present. RAPD1 fragments from RB87-62 and DH88-752 were then cloned and sequenced. Based on sequence information, the sizes of the fragment of RAPD1 in RB87-62 and DH88-752 were 489 bp and 490 bp respectively and sequences shared high similarity (99%) between the two sources. Specific primers were designed to include the complete or partial original decamer primers plus 9-15 adjacent internal bases.

5.4.2 Amplification of SCAR marker in DH population of Westar × RB87-62

100 DH lines derived from the cross between Westar × RB87-62 were tested with both the RAPD1 and the SCAR primers. Using the SCAR primers, the same polymorphic fragment as in the RAPD1 profile was detected. In all tested DH lines, the presence or absence of the targeted RAPD1 fragment amplified with the SCAR primers was consistent with those amplified with the RAPD1 primer. This indicated that the SCAR marker segregated as the original RAPD1 fragment in resistant DH lines as well as in the recombinant lines (this marker was about 0.8 cM apart from the cotyledon resistance gene, Chapter 4). Comparison of RAPD1 and SCAR profiles in resistant parent RB87-62, susceptible parent Westar, 10 resistant DH lines and 10 susceptible DH lines is shown in Figure 5.1. The specific primers designed for the sequence of the RAPD1 fragment amplified the same fragment in RB87-62 and 10 resistant DH lines. No fragment of this size was amplified in Westar and 10 susceptible DH lines. In addition, the SCAR primers

Figure 5.1. Comparison of RAPD (A) and SCAR (B) profiles in DH lines derived from Westar × RB87-62. M molecular-weight marker; P1 RB87-62; P2 Westar; R cotyledon resistant DH lines; S cotyledon susceptible DH lines. Marker is indicated by arrows.

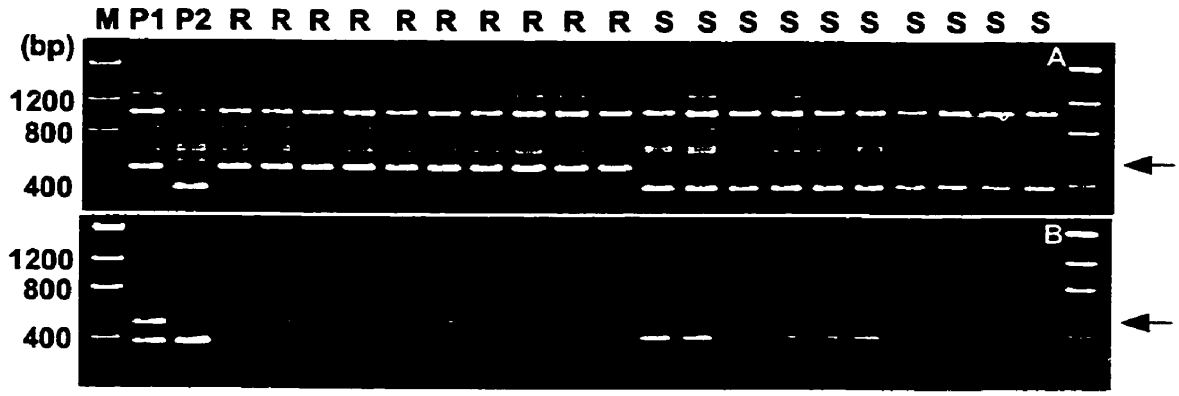

M P1 P2 R R R R R R R R R R S S S S S S S S S
(bp)
1200
800
400
A
1200
800
400
B.

amplified a second fragment of 380 bp in the two parents and all tested DH lines. The corresponding fragment was also amplified with the RAPD1 primer. This implies that these two fragments shared similar sequences at 5'- and 3'- ends (i.e. forward and reverse primers). If sequence size of the specific primers were extended (more specific to upper fragment), the lower fragment could perhaps be eliminated. However, this lower fragment fortuitously serves as a useful control indicating the DNA from all samples was amplified.

5.4.3 Amplification of SCAR marker in DH population of Westar × DH88-752

120 DH lines derived from the cross between Westar× DH88-752 were tested with both the RAPD1 and the SCAR primers. Using the SCAR primers, the same polymorphic fragment as in the RAPD1 profile was amplified. In all tested DH lines, the presence or absence of the targeted RAPD1 fragments amplified with the SCAR primers was consistent with those amplified with the RAPD primer. This fragment cosegregated with the cotyledon resistance as did RAPD1 in DH88-752 and no recombinant DH lines were observed. This result confirmed that the same locus was amplified by these two kinds of markers. Comparison of the RAPD and SCAR profiles in resistant parent DH88-752, susceptible parent Westar, 10 resistant DH lines and 10 susceptible DH lines is shown in Figure 5.2. Specific primers designed for the sequence of RAPD1 fragment amplified the same fragment in DH88-752 and 10 resistant DH lines. No fragment of this size was produced in Westar and 10 susceptible DH lines. Similar to RB87-62, the SCAR primers amplified a second fragment in all tested samples and the corresponding fragment was

Figure 5.2. Comparison of RAPD (A) and SCAR (B) profiles in DH lines derived from Westar × DH88-752. M molecular-weight marker; P1 DH88-752; P2 Westar; R cotyledon resistant DH lines; S cotyledon susceptible DH lines. Marker is indicated by arrows.


M P1 P2 R R R R R R R R R R S S S S S S S S S
(bp)
1200
800
400
A
1200
800
400
B

also amplified by the RAPD1 primer.

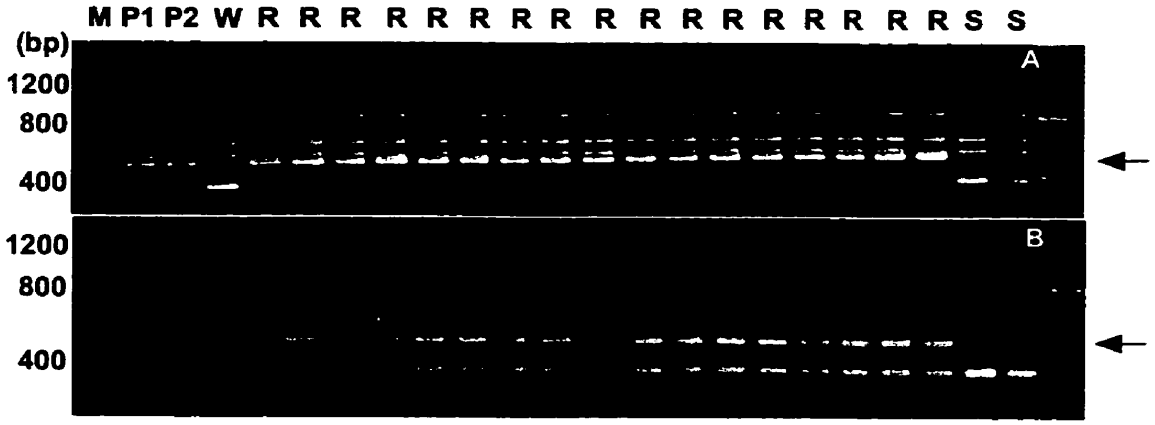
5.4.4 Comparison of RAPD and SCAR markers in F_2 plants of RB87-62 × DH88-752

96 F_2 plants of the cross of RB87-62 × DH88-752 were tested with both RAPD1 and SCAR primers. Presence or absence of the targeted RAPD1 fragments amplified with the SCAR primers was consistent with those amplified with the RAPD primer. Comparison of the RAPD1 and SCAR profiles in parents RB87-62 and DH88-752, 17 resistant F_2 plants and 2 susceptible F_2 plants is shown in Figure 5.3. Corresponding polymorphic RAPD1 marker and SCAR marker were amplified in all resistant F_2 plants from the cross of RB87-62 × DH88-752, but no fragment was present in susceptible F_2 plants. Consistently, the 380 bp fragment was detected in all tested samples, which indicated that DNA from all samples was amplified.

5.4.5 Amplification of SCAR marker in other cultivars/accessions of *Brassica* species

Four cultivars of *B. napus*, eight cultivars/accessions of *B. juncea* and one ecotype of *B. rapa* were tested for cotyledon resistance and adult plant resistance inoculated with isolate P186-12 of *L. maculans*, and DNA from these accessions was tested for amplification of the SCAR marker. In most cultivars/accessions, resistance reactions were consistent with presence of the SCAR marker, except *B. juncea* accession UM3132 which showed a susceptible reaction both on cotyledon and at adult plant stage with the presence of the SCAR marker, and *B. sylvestris*, a *B rapa* ecotype, which showed a resistant reaction both on cotyledon and at adult plant stage with the absence of the

Figure 5.3. Comparison of RAPD (A) and SCAR (B) profiles in F_2 plants developed from RB87-62 × DH88-752. M molecular-weight marker; P1 RB87-62; P2 DH88-752; W Westar; R cotyledon resistant F_2 plants; S cotyledon susceptible F_2 plants. Markers are indicated by arrows.


M P1 P2 W R R R R R R R R R R R R R R R S S
(bp)
1200
800
400
A
1200
800
400
B

SCAR marker. Disease reaction and amplification of the SCAR marker in these *Brassica* cultivars/accessions are shown in Table 5.1.

5.5 Discussion

A number of resistance genes (loci) conferring resistance to different isolates of *L. maculans* have been identified and characterized in *B. napus* (Ferreira et al. 1995; Dion et al. 1995; Mayerhofer et al. 1997). In cv. Major, the closest marker to the cotyledon resistance locus, *LEM1*, was wg2a3b (RFLP marker) with a distance of 1.0 cM (Ferreira et al. 1995). In cv. Shiralee, the distance from the blackleg resistance locus, *LmR1*, to the closest RAPD marker was 4.8 cM, and all markers lay on the same side of the cotyledon resistance gene (Mayerhofer et al. 1997). In cv. Crésor, the closest marker cDNA011 (RFLP marker) was about 5 cM from the adult plant resistance gene, *LmFr1* (Dion et al. 1995). Due to the type of markers or the loose linkage relationship with the resistance genes, these markers are not suitable for large scale resistance selection. In this study, one SCAR marker was developed through sequencing a targeted RAPD product and designing specific primers for its amplification. The primers were tested in two DH populations from Westar × RB87-62 and Westar × DH88-752 and in F_2 plants derived from RB87-62 × DH88-752. In all tests, the presence of the target fragments using the SCAR primers was consistent with those using the RAPD primer, which indicates that the same locus was amplified by these primers. The SCAR primers amplified a second fragment of 380 bp in both resistant and susceptible plants in the SCAR amplification profile. It is probable that these two fragments share similar sequences at 5'- and 3'- ends

Table 5.1. Cultivar/accession of *Brassica* species, disease reaction to *L. maculans* isolate P186-12 and presence/absence of SCAR marker.

Accession [c]	Species	Cotyledon reaction	Adult plant reaction	SCAR marker
Glacier	*B. napus*	R	R	+
Quinta	*B. napus*	R	R	+
Oscar	*B. napus*	R	R	+
Legend	*B. napus*	S	S	-
AC Vulcan	*B. juncea*	R	R	+
Cutlass	*B. juncea*	R	R	+
UM3132[a]	*B. juncea*	S	S	+
UM3323[a]	*B. juncea*	R	R	+
UM3021[a]	*B. juncea*	R	R	+
UM3043[a]	*B. juncea*	R	R	+
J90-4253[b]	*B. juncea*	R	R	+
J92-223[b]	*B. juncea*	R	R	+
B. sylvestris	*B. rapa*	R	R	-

[a] University of Manitoba accessions (Keri 1991)
[b] Canola quality *B. juncea* accessions (Love et al. 1991)
[c] +=presence, -=absence

(forward and reverse primers). A corresponding fragment was also amplified with the RAPD1 primer. If the length of the specific primers were extended (more specific to upper fragment), the lower fragment could perhaps be eliminated. However, this lower fragment fortuitously serves as a useful control indicating that DNA from all samples (resistant or susceptible plants) was properly amplified. Therefore, it serves to avoid cases in which resistant lines would possibly be scored as susceptible if their DNA samples were not amplified for some reason or had been damaged or degraded.

The fragment of RAPD1 was successfully converted into a SCAR marker and the SCAR primers effectively amplified the targeted fragment. This SCAR marker could be very useful for accurate selection of plants at early developmental stages in segregating populations in breeding programs where these resistant accessions are being used. In the *Brassica-L. maculans* pathosystem, different sources of resistance, genetic backgrounds and environmental conditions can influence the resistance reaction to *L. maculans* (McGee and Petrie 1979; Badawy et al. 1992; Plieske et al. 1998; Zhu and Rimmer in preparation). This was reflected by the fact that some sources of resistance e.g., Crésor, show segregating disease reactions under different environment conditions. Moreover, even within a DH line (theoretically homozygous for all alleles), individual plants may show different disease reactions to *L. maculans* (resistant or susceptible) (Mayerhofer et al. 1997; Zhu and Rimmer in preparation). Thus, it is difficult to select appropriate plants for blackleg resistance in a segregating population using conventional disease testing and the use of molecular markers would be advantageous. Incorporation of resistance genes into advanced breeding lines may provide a long-term solution to this disease. In fact,

only a few sources of resistance have been widely used in breeding programs (Rimmer and van den Berg 1992; Salisbury et al. 1995). Therefore, the development of molecular markers tagging these commonly used resistance genes will facilitate future breeding through marker-assisted selection. In addition, the SCAR marker was also amplified in some other *Brassica* cultivars/accessions. The relationship between resistance genes in these materials and the presence of SCAR marker needs to be characterized in the future.

CHAPTER 6

GENERAL DISCUSSION

There can be little doubt that oilseed *Brassica* crops are destined to play an ever-increasing role in supplying the world's food, feed, and industrial needs in the future (Downey and Rimmer 1993). The economic importance of *Brassica* necessitates improvement of agronomic characteristics and has prompted extensive investigation into their genome structure and evolutionary relationships. Meanwhile, expansion and intensification of oilseed *Brassica* acreages in Australia, Europe, and North American has been accompanied by potential outbreak of blackleg, which has caused significant yield loss on these crops (Rimmer and van den Berg 1992; Downey and Rimmer 1993). Since the introduction of the resistant *B. napus* cultivar Jet Neuf in 1977 (Gladders and Musa 1979), resistant cultivars have been a major factor in controlling blackleg (Rimmer and van den Berg 1992; Gugel and Petrie 1992; Salisbury et al. 1995).

Through genetic analysis of reaction to *L. maculans* in *Brassica* species, both monogenic and polygenic models to explain heritability of resistance have been reported (Rimmer and van den Berg 1992; Downey and Rimmer 1993; Pang and Harollan 1996b, 1996c; Zhu et al. 1993; Keri et al. 1997; Plieske et al. 1998, Pilet et al. 1998). Genetic analysis in this study showed that single dominant genes confer resistance on cotyledon and at the adult plant stage respectively in both RB87-62 and DH88-752. In Crésor, a single gene for adult plant resistance in a derived DH population was reported by Dion et al. (1995). These results demonstrate that different genetic control of resistance to *L.*

maculans exists in *Brassica* species. In the B genome of *Brassica*, three chromosomes were shown to carry blackleg resistance genes in *B. nigra* and *B. carinata* through development of addition lines or introgression of resistance into *B. napus*. Two chromosomes carrying resistance genes were reported in *B. juncea* and a possible third gene was located on uncharacterized groups (Struss et al. 1996; Zhu et al. 1993; Chèvre et al. 1997). It is of particular interest that B genome resistance genes confer a high level of resistance to *L. maculans* and that the resistance can be conferred by a single gene (Plieske et al. 1997).

A large collection of resistant germplasm has been characterized in *Brassica* species, however, information on the inheritance of resistance is rather limited (Rimmer and van den Berg 1992). To develop and maintain adequate resistance to *L. maculans* in *B. napus*, characterization and genetic studies of new sources of resistance needs to be conducted. Breeding for resistance in *B. napus* to *L. maculans* will benefit from improved knowledge of the number and model of action of resistance genes in different accessions of *Brassica* species.

Although some researchers observed a correlation between cotyledon and adult plant resistance (Newman and Bailey 1987; McNabb et al. 1993; Bansal et al. 1994), more recent studies suggest that cotyledon resistance and adult plant resistance are two distinct traits and under different genetic control (Ferreira et al. 1995; Pang and Halloran 1996b; Ballinger and Salisbury 1996). Our research showed that cotyledon resistance and adult plant resistance were controlled by separate but linked genes in both RB87-62 and DH88-752. This would explain why previous studies found a correlation of resistance at

cotyledon and adult plant stages. This relationship was confirmed by constructing linkage maps of resistance with molecular markers in DH populations. Distances between the two resistance genes were 6.1 cM in RB87-62 and 10.0 cM in DH88-752. In blackleg resistance breeding programs, given the relationship between cotyledon and adult plant resistance as distinct but linked traits, selection for cotyledon resistance will be useful as a marker for adult plant resistance when the loci controlling the two traits are sufficiently linked. Cotyledon screening was used to identify resistance in some resistance sources and to characterize the pathogenicity types of isolates of *L. maculans* using a set of differential cultivars (Koch et al 1991). However, if adult plant resistance is the major component of field resistance in oilseed crop production, selection for resistance needs to be based on both cotyledon resistance and adult plant resistance to the mixture of pathotypes that are representative of the pathogen population in the field (Salisbury et al. 1995).

In the *Brassica-L. maculans* pathosystem, different sources of resistance, genetic backgrounds and environmental conditions can influence the resistance reaction to *L. maculans* (McGee and Petrie 1978; Badawy et al. 1992; Ferreira et al. 1995; Dion et al.1995; Plieske et al. 1998). This is well illustrated by the complexity of resistance factors in plants tested under different conditions. In cv. Major, Ferreira et al. (1995) reported that a single major locus (*LEM1*), in addition to four other genomic regions, was associated with cotyledon and adult plant resistance to PHW1245 (a PG2 isolate). Two genomic regions, different from those identified in cotyledon and adult plant tests, were involved in field resistance tested in western Canada. This suggests that different

resistance genes could be operating at different developmental stages of plants, under varied environmental conditions or in response to different virulence of pathogen populations (Ferreira et al. 1995; Dion et al. 1995). Even within a DH line (theoretically homozygous for all alleles), individual plants may show different disease reactions to *L. maculans* (resistant or susceptible) (Mayerhofer et al. 1997). Thus, it is difficult to select appropriate plants for blackleg resistance in a segregating population in breeding programs. Using DNA-based markers, introgression of resistance genes can be easily followed without screening with the pathogen and irrespective of environmental conditions. In this study, a SCAR marker was developed through sequencing the targeted RAPD product. SCAR specific primers have been designed and tested in two DH populations developed from Westar × RB87-62 and Westar × DH88-752 respectively, and in F_2 plants derived from RB87-62 × DH88-752. The presence of the SCAR marker was consistent with the primary RAPD marker in all tests. Thus, the SCAR marker will be useful for selecting resistant plants in segregating populations where these two resistance materials are used as sources of resistance.

There is some evidence for the loss of field resistance in Australia to *L. maculans* in canola cultivars such as Maluka after years of cultivation (Salisbury et al. 1995). Therefore, we need to discover new sources of resistance to broaden the genetic base for resistance to *L. maculans* in *B. napus* (Rimmer and van den Berg 1992; Salisbury et al. 1995). The source of most resistant cultivars in Europe was the cultivar Jet Neuf (Renard et al. 1983). Resistance in Jet Neuf was reported to be only partial and expressed as adult plant resistance under French field conditions. It is worth noting that Jet Neuf has a

pedigree of R9 × Primor/R9*3. Primor is a low erucic acid derivative from cv. Major in which two genomic regions were associated with field resistance in western Canada (Ferreira et al.). Pilet et al. (1998) mapped 13 genomic regions in cv. Darmor (with the pedigree of Jet Neuf × Bronowski/Jet Neuf*2) that were associated with blackleg resistance under French field conditions. This implies that different number of genes or their locations can be mapped in the genome depending on different virulence of the pathogen populations or environmental conditions. Some other sources of *B. napus* resistance were also considered to be polygenically inherited (Thompson 1983; Pang 1992). Development of molecular markers and construction of resistance linkage maps will enable resistance quantitative trait loci (QTL) to be characterized and localized. Markers will help progeny selection in cases where polygenic resistance is used as resistance sources.

Through comparative mapping of resistance in RB87-62, DH88-752 and Crésor, the arrangement and map distances of closely linked RAPD, AFLP and RFLP markers indicates that there is a conserved region carrying resistance genes which covered 41.1 cM in RB87-62, 36.5 cM in DH88-752 and at least 32.3 cM in cv. Crésor. Plieske et al. (1998) compared the three B genome resistant species, i.e. *B. nigra*, *B. juncea* and *B. carinata*. The arrangement and distances of four closely linked RFLP markers were similar to in the three species those on linkage group 6 of *B. napus*. For resistance to *L. maculans* in *B. napus*, linkage between cotyledon resistance and adult plant resistance was first established in RB87-62 and DH88-752 through genetic analysis and further confirmed by resistance mapping in DH populations. The map distances between the

cotyledon and adult plant resistance genes were 6.1 cM in RB87-62 and 10.0 cM in DH88-752. For resistance associated with the three *Brassica* B genome species, single sub-regions located on linkage groups 2, 5 and 8 were involved in cotyledon resistance and/or adult leaf resistance in *B. nigra*, *B. juncea* and *B. carinata* (Dixelius and Wahlberg 1999). One PG2 isolate (P186-12) of *L. macualns* was used in this research to identify the conserved region carrying resistance genes in three *B. napus* accessions. These accessions, challenged with different pathotypes of the pathogen, can be analyzed for the location of other resistance genes in the future. Keri (1999) recently reported that in cv. Quinta, cotyledon resistance to a PG2 isolate was linked with the resistance to a PG3 isolate with a recombination frequency (RF) of 7.0-9.5%. Many plant disease resistance genes are reported to exist as clusters in plant genomes (Pryor 1987; Parniske et al. 1997; Hammond-Kosak and Jones 1997). Molecular analyses are now beginning to uncover the complexity of these loci and their structure and evolutionary relationships (Parniske et al. 1997; Song et al. 1997). Cloning of blackleg resistance genes and elucidating their genomic structure at the molecular level will help us better understand resistance evolutionary process in *Brassica* species.

The cross of RB87-62 × DH88-752 segregated 15:1 (R:S) for resistant and susceptible reactions for both cotyledon and adult plant reactions in the F_2 population. This indicates that the pairs of resistance genes in each parent are non-allelic and independently assorting. Therefore, the resistance genes from the two sources are located either on different chromosomes or sufficiently apart on the same chromosome to segregate independently. Through a comparison of the resistance linkage maps of cv. Major

(Ferreira et al. 1995) and cv. Shiralee (Mayerhofer et al. 1997), the resistance genes were located on linkage group (LG) 6 (Ferreira et al. 1995). Based on the presence of two consensus RFLP markers, the resistance locus (*LmR1*) in cv. Shiralee, when compared with *LEM1* in cv. Major, mapped to a different location on LG6 assuming two loci are on the same chromosome. A RFLP marker on linkage group 6 in Major (Ferreira et al. 1995), wg8g3, also mapped on the resistance linkage group in RB87-62. In Crésor, the resistance locus has been mapped on linkage group 6 (Parkin IAP and Lydiate DJ personal communication). These results demonstrate that linkage group 6 in the *B. napus* genome is a important location of resistance genes to *L. maculans*.

The *Brassica* B genome is also an important source of resistance to *L. maculans*. Intensive efforts to transfer B genome resistance to *B. napus* have been made (Roy 1978; Sacristán and Gerdemann 1986; Struss et al. 1991). Molecular mapping of B genome resistance has been done in the three *Brassica* B genome species. Due to the arbitrary and independent designation of various linkage groups, the location of resistance genes in these three species is easily confused. Markers linked to resistance to *L. maculans* were located on chromosome B8 in *B. juncea* (Chèvre et al. 1997). Chromosome B4 was previously described as carrying a resistance gene in *B. nigra* (Chèvre et al. 1996). Dixelius and Wahlberg (1999) mapped cotyledon and adult-leaf resistance on linkage groups 2, 5, and 8 in the three B genome *Brassica* species. Struss et al. (1996) proposed consensus synteny groups of the three B genome species and adult plant resistance to *L. maculans* was located on groups 1, 3, and 4. This confusion makes comparison of location of resistance genes in these species impossible. Different parental materials as

resistance gene donors and different types of markers and mapping populations have been used in the different studies. Thus, whether the number of resistance genes is constant within the three B genome species is unknown. Consequently, there is a need to standardize genetic maps of *Brassica* species and align existing linkage groups in each species. Alignment of four independent maps has been recently conducted in *B. oleracea* (Hu et al. 1998). This allows better understanding and utilization of mapping information by the *Brassica* research community. Alignment of B genome maps will serve in gaining further insight into the genome structure and genome evolution of these species.

Comparative analyses of *Brassica* A, B and C genomes have shown a striking conservation of genome content (McGrath and Quiros 1991; Chyi et al. 1991; Teutonico and Osborn 1994; Parkin et al. 1995; Sharpe et al. 1995; Cheung et al. 1997; Lagercrantz and Lydiate 1996). Species within the Brassicaceae (Cruciferae) exhibit a continuous range of haploid chromosome number from 5 to 15, excluding a large number of known polyploid species with higher chromosome numbers (Lagercrantz 1998). It has been proposed that the present diploid species in the genus *Brassica* were derived from smaller ancestral genomes of n=5 (Catcheside 1934) or n=6 (Sikka 1940; Röbbelen1960). An increase in chromosome number may have occurred by duplication, which provided the opportunity for homologous recombination to take place. This is supported by the findings of Lagercrantz and Lydiate (1996) that there are a series of triplicated regions in different *Brassica* genomes. Because of their common origin, these genomes are expected to have conserved chromosomal segments including those regions carrying disease resistance genes. This could explain the similar arrangement and map distance of

molecular markers flanking the genes that govern resistance to *L. maculans* identified in this research and in other *Brassica* species (Plieske et al.1998).

Arabidopsis has been widely used as a model plant for studies in all aspects of plant biology. Through comparative mapping of *Brassica* and *A. thaliana* genomes, collinearity between large portions of the two genomes has been observed (Osborn et al. 1997; Lagercrantz et al. 1996; Scheffler et al. 1997; Cavell et al. 1998; Kowalski et al 1994; Robert et al. 1998), such as flowering time loci. It is an interesting question whether genes conferring disease resistance are also conserved between the two genomes. Sadowski and Quiros (1998) reported that the *Arabidopsis* chromosomal complex containing *RPS2* (the gene conferring resistance to *Pseudomonas syringae* strains carrying *avrRpt2* gene) was duplicated and conserved in the *Brassica nigra* genome. The origin of *RPM1* gene (conferring resistance to *P. syringae* strains carrying *avrB* or *avrRpm1* genes) in *A. thaliana* and *B. napus* has also been explored (Grant et al. 1998). The *Brassica RPM1* locus is syntenic with *RPM1* from resistant *Arabidopsis*. This synteny suggests that the functional *RPM1* allele in the two related species was derived from a common progenitor. These results imply that it is possible to determine which portion of the *A. thaliana* genome is homologous to any specific interval of the *B. napus* genome. Further analysis can be carried out in the *Brassica* genomes to determine their organization and micro-collinearity compared with the *A. thaliana* genome. Using well characterized gene complexes of known physical size from *A. thaliana*, it will be probable to determine the level of collinearity between *Brassica* and *A. thaliana* over the whole genome. Once comparative mapping of *Brassica* and *A. thaliana* genomes has

been completed, the established collinearity will facilitate efficient utilization of physically mapped clones and sequence data from *A. thaliana* to assist in genetic mapping and gene isolation in *Brassica* crops. The *A. thaliana* whole genome sequence will be completed by the end of 2000 (Meinke et al. 1998).

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Appendix

Table 8.1. Disease severity classes – Cotyledon test

Class	Description
0	Pinprick only, no blackening
1	Limited blackening around wound; lesions diameter 1-1.5 mm
2	Blackening with some chlorosis around wound; lesion diameter 1-1.5 mm
3	Blackening around wound; lesion diameter 1.5-3 mm
4	Large (3-5 mm) lesion, irregular darkening
5	Tan-centered lesion, variable in size; sharp black margin
6	Greyish-green tissue collapse, sharp black margin; lesion size variable
7	Greyish-green tissue collapse, sharp margin; lesion size variable
8	Spreading tissue collapse, few pycnidia; lesion diameter greater than 5 mm
9	Spreading tissue collapse, many pycnidia; lesion diameter greater than 5 mm

(Williams and Delwiche 1980)

Table 8.2. Disease severity classes – Crown lesions on adult plants

Class	% Girdling
0	None
1	<25
2	26-50
3	51-75
4	76-100
5	Dead plant

(van den Berg et al. 1993)